Demonstrating the Concepts of Mutagenesis and Genetic Screening Using the Fungal System *Coprinus cinereus*

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Introduction

Coprinus cinereus, basidiomycete fungus, is an excellent model system to use for the studies of meiosis because the process is naturally synchronous. *C. cinereus* can grow as a monokaryon or a dikaryon. As a monokaryon, it can reproduce asexually through the production of asexual spores called oidia. Two compatible haploid strains can mate (fuse) to form a dikaryon. Under the appropriate conditions triggered by a light/dark cycle, the dikaryon can be induced to form a fruiting body or mushroom. Within the cap of this mushroom, meiosis occurs to produce haploid spores. These spores will germinate to form the next generation of haploid individuals.

Mating is controlled by two mating type genes, A and B. In order to mate, two haploid strains must have different alleles at both loci. Mutations in both of these genes gave rise to a strain that is self-compatible or can mate with itself. This strain, *AmutBmut* (Swamy et al., 1984), is utilized in screens for mutations that affect the processes of mushroom formation and meiosis. In this laboratory exercise, we will harvest the asexual spores (oidia) from cultures of the *AmutBmut* strain. The oidia will be mutagenized using UV radiation. Surviving mutants will be capable of self-mating, eliminating the need for backcrossing the mutation into a compatible strain. These mutants will be screened for the ability to undergo meiosis and produce spores. Potential meiotic mutants will produce white mushrooms (no spores) compared to those survivors that produce black mushrooms (spores), indicating the capacity to undergo meiosis. This exercise demonstrates the process of creating random mutants and the necessity of a specific genetic screen to identify only those mutants with meiotic defects.

Student Outline

First week

1. To harvest oidia from the *Coprinus* cultures growing on Petri plates, add 5 ml of sterile H_2O to each plate. Using a flame sterilized spatula to gently scrape the surface of the culture to release the oidia. Remove liquid using a 1ml pipette. Filter through a sterile 10 ml syringe with a glass wool

plug into a 15 ml conical tube (blue cover). Wash each plate with an additional 2 ml of sterile water, and filter the wash through the syringe into the tube. Pellet the sample by placing the tube in a clinical centrifuge and spin for 5 minutes.

2. Remove supernatant by pouring it into a clean beaker, carefully as to not disturb the pellet. Resuspend each pellet in 1 ml sterile water and pool samples to a single tube. Wash the tubes with 1 ml sterile water. Combine the wash to the tube containing the concentrated oidia. Repellet as in step 1.

3. Resuspend pellet in 1 ml or less of sterile water, depending on the size of your pellet. Check with your instructor BEFORE you resuspend your pellet to get some advice on the volume to use.

4. Using a hemocytometer, count oidia and calculate your cell density per ml of solution. Use a small aliquot of your total sample to make a 1:100 and 1:1000 dilutions to count cells.

5. You will need a working concentration of 5×10^7 cells /ml. Adjust your sample to that concentration. The majority of this sample will be transferred to a small sterile Petri dish for irradiation. Keep an aliquot of the unirradiated sample as a control. Check with your instructor for specific volumes. You will perform a dilution series on this control. The dilutions and plating can occur during the mutagenesis.

Note: To make a 10-fold serial dilution, add 9 ml of water to a series of test tubes. Label tubes!!

a. Add 1 ml of sample to the first test tube (Tube 1). Mix. You have diluted your original sample 10 fold. This is a 1:10 dilution.

b. Now remove 1 ml from Tube 1 and add it to Tube 2. You have diluted your original sample 100 fold. This is a 1:100 dilution.

c. Continue this process to make as many dilutions as needed.

d. To determine cell numbers or viability, you will plate 0.1 ml aliquots from each of these dilutions onto plates. Your goal is to find a dilution that enables you to count the number of individual colonies easily. Based on the data collected, you can calculate the cell concentration from the original sample. You will report your data using the units of CFU or colony forming units.

**It is important to keep track of all your dilutions. The dilution factor includes not only the series dilution, but also the proportion of sample plated.

6. Each group will irradiate their sample for a different amount of time. In other words, each sample will be exposed to a different dose of UV radiation. Plates should be swirled every 10 minutes. Be sure to wear gloves and eye protection before opening the light box.

Group A	20 minutes	Group C	40 minutes	Group E	60 minutes
Group B	30 minutes	Group D	50 minutes		

Not all the samples can be irradiated at the same time. Your instructor will tell you the order in which the groups will irradiate their samples.

Note: While your sample is being irradiated, you should set up the dilution series for your control and label all your plates. Be prepared to complete your serial dilution for your experiment and spread your plates when your irradiation period is completed. Your instructor will demonstrate plating technique and serial dilutions during the irradiation period.

7. After irradiation, dilute your sample 1:10. This is tube 1. Remove 1 ml for the next dilution. Plate 1 ml per plate of the remainder of this dilution.

8. Dilute 1 ml from tube 1, 1:10. This is tube 2. This is a 1:100 dilution of your original sample. Remove 1 ml for the next dilution. Plate 9 plates, each with 1 ml of sample with the remainder of the dilution.

9. Dilute the 1 ml from tube 3, 1:10. This is a 1:1000 dilution of your original sample. Remove 1 ml for the next dilution. Plate 9 plates, each with 1 ml of sample with the remainder of the dilution.

Note: Your plates will be very wet. Keep them upright on your bench overnight. Tomorrow they will be put in an incubator. Make sure all your plates are labeled.

10. Tomorrow your plates will be transferred to the 37^{o}C incubator. It is your responsibility to check your plates on a regular basis. As colonies arise, you will need to transfer the cultures to new plates. You can put 10 individuals per plate (demonstrated by instructor). Incubate those plates for two days, being careful that the isolates do not grow into each other.

IT IS VERY IMPORTANT THAT YOU KEEP EVERYTHING LABELED IN A CLEAR AND ORDERLY FASHION.

Second week

11. Evaluate serial dilutions.

To determine the concentration of your original sample:

(Number of colonies counted) (Dilution factor) = Number of cells in original solution

For example, you plated 1 ml of your 1:1000 dilution and counted 50 CFU on one plate and 60 CFU on another. The average number of colonies counted would be 55 CFU.

Your dilution factor would be 1000 (from your dilution series) x 10 (from the % of your sample represented on a single plate because you plated 1/10 {1 ml} of the total in that dilution {10 ml}).

(55) (10 000) = 550 000 CFU/ml or 5.5 x 10^5 CFU/ml

For your laboratory, you will want to calculate the % kill of your mutagenesis. Using this information, how would you determine the % kill for your mutagenesis?

12. You will inoculate fruiting tubes to screen for meiotic mutants for each of the individual isolates. Incubate your fruiting tubes at 37^{o}C until the cultures are confluent, and then transfer your tubes to the light shelves in lab. To induce mushroom formation, your cultures need to be exposed to a 16 hr day/8 hr night light cycle at room temperature (approx. 25^{o}C). Your plates that contain your collection of mutants can be stored in the refrigerator once they have been used to inoculate fruiting tubes. Monitor your tubes. When mushrooms are produced, score isolates for the presence

(black) or absence (white) of spores in the mushroom cap. Any white mushrooms indicate possible meiotic mutants.

Instructor's Notes

The goals for the laboratory exercise are as follows:

1. To conduct a UV mutagenesis using a fungal system.
2. To utilize serial dilutions to measure viability and survival.
3. To screen the survivors of the mutagenesis for meiotic defects.
4. To reinforce good sterile technique.

This system has been used as a teaching tool in an upper level advanced genetics course to provide students with a practical experience with mutagenesis and genetic screens. This course meets six hours a week with three one hour lecture periods and one three hour lab period. The course is typical taken by junior and senior Biology and Biochemistry majors. The enrollment ranges from 16-20 students.

This laboratory exercise is preceded by a series of class lectures on mutations. These lectures deal with the types of mutations, causes of mutations and the role of DNA repair preventing mutations. The analysis of specific mutants to understand a biological process is discussed throughout the course. The exercise illustrates the process involved in both creating mutants and in screening the survivors for a specific phenotype. This exercise aids students in their understanding of the role mutation plays in genetic analysis and the approach required in identifying a specific class of mutants. It is only until mutants with that specific phenotype are identified, that characterization of a specific process, in this case, meiosis, can begin.

Students have the opportunity to create mutations using UV radiation. The advantage of UV radiation over other mutagens is that it is not toxic and students can work with it using proper precautions (gloves and eye protection) with minimal risks to themselves. A relatively simple set up consisting of a UV bulb housed in a protective wooden box is required.

Students can follow the fate of their mutagenized cells. They determine the kill rate by comparing the number of colony forming units of the mutagenized cells to that of their untreated cells. This procedure serves as a control for their experiment. It allows them to monitor the effectiveness of their work by calculating % viability of the untreated cells and % survival of the UV mutagenized cells. This experiment provides an opportunity to incorporate use of serial dilutions, a technique students previously have used in other courses. It requires students to have good mastery of sterile technique. In preparation for this exercise, an earlier laboratory exercise that focuses on serial dilutions and sterile technique is recommended.

Finally, students are able to screen the survivors of the mutagenesis for meiotic mutants. The experience gives them a sense for the numbers of isolates that may need to be screened to find a mutant in a specific process. It reinforces that mutations are random and the processes that create mutation cannot do so in a directed way. Mutagenesis increases the rate at which mutations occur, but the process does not have any sort of specificity. The necessity of a screen is demonstrated when students realize the limitations of creating random mutants.

The other equipment required for this laboratory include a clinical centrifuge for pelleting oidia and microscopes and hemocytometers for cell counting. Other materials for harvesting and plating oidia are standard for most laboratories that teach microbiology. Oidia harvesting,

mutagenesis and plating can be completed in one three hour laboratory period. Additional time is needed to evaluate viability, isolate and screen the survivors.

C. cinereus isolated are grown on YMG (Yeast Maltose Glucose) agar medium. The recipe for one liter is 4 g Yeast Extract, 10 g Malt Extract, 4 g Dextrose and 12 g Agar. Bring to one liter with distilled water, autoclave and pour plates. To make fruiting tubes, heat the medium before autoclaving to completely dissolve the agar. Pour medium into clean test tubes. Fill to between one quarter and one third of the total volume of the test tube. Cover test tubes and autoclave. After autoclaving, lean racks of test tubes on their side to create slants and allow the medium to cool.

AmutBmut culture is available upon request from the author (ramesh@roanoke.edu). If, during the course of conducting this exercise, your class does discover any white mushrooms, I would greatly appreciate it if you could send a culture of the mutant(s) to me. This experiment is linked to my research interests and I would be very interested in further characterizing any potential meiotic mutants that were generated through this mutagenesis.

AmutBmut C.cinereus cultures for harvesting oidia need to be confluent and require exposure to light for the oidia to mature. Cultures can be grown at 37^{o}C until confluent. They should then be transferred to a **constant** light source for several (4-5) days before being harvested. This step can be performed at room temperature. The instructor may need to experiment with the length of time depending on temperature. To induce fruiting, cultures require a 16 hr day, 8 hr night cycle at 25^{o}C or room temperature. DO NOT put confluent plates for oidia production under the day night cycle because they will also produce fruiting bodies.

The definitive step of this experiment occurs when students have determined their oidia concentrations prior to mutagenesis. Students' cell concentration can vary widely depending on the quality of the cultures and their technique. The amount of cells they have collected will determine how they set up their serial dilutions and the number of plates they will spread. The instructor needs to be very flexible at the time to develop variations in the dilution procedure that will enable each student group to use the cells they have collected. Alternatively, the instructor could decide to pool all the oidia collected to one common pool from which each group is given an aliquot to ensure consistency in experimental procedure. However, if one group has poor sterile technique, this approach could negatively impact the experiment for the entire class.

Acknowledgements

We would like to thank Dr. Pat Pukkila for providing the *C. cinereus AmutBmut* strain, Dr. Mimi Zolan for suggesting the idea for a teaching lab (inoculating the spore, so to speak), research students Sarah Brooks and Sam Sliwa for their assistance, and the Roanoke College Biology 380 students from Spring 2006 and Spring 2007 semesters. The development of this laboratory exercise was in part supported by a Curriculum Grant from Roanoke College to MAR.

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About the Authors

Marilee is an Assistant Professor of Biology at Roanoke College. She teaches courses in General Biology, Genetics, Molecular Biology and Genomics. She has also been involved with a program at Roanoke College to teach writing across the curriculum. Her research interests are in the area of fungal genetics. She earned her B.S. from the University of Wisconsin at Stevens Point and her Ph.D. from Indiana University studying meiosis in *Coprinus cinereus*. She has been assisted in developing this exercise by Nicole, one of her undergraduate research students. Nicole plans to graduate with a double major in Biology and Psychology in December 2007. She is planning to apply to medical school in the spring.