Dispersal of fungal spores by gravity currents

UCLA Math REU 2013

Lisa Yamada & Junius Santoso
8/9/2013
Abstract

Spore is an asexual reproduction unit of fungi, also often found in plant kingdom. The physics behind spore dispersal of the Basidiomycete fungi or more known as mushroom will be the main focus of our study in this paper. We hypothesize that the spore dispersal is driven by the gravity current under the mushroom cap due to the density difference between the spore air laden and ambient air. Here we show that the gravity current is present when the gap under the mushroom cap is small i.e. \( \leq 12 \text{ mm} \). We validated that the dispersal length is inversely proportional to the shorter dimension of the mushroom cap and directly proportional to the gap under the mushroom cap. Furthermore, our results demonstrate that there are other possible contributors beside gravity current that help the spore dispersal. We anticipate our work to be a starting point for more study in finding the other possible driving force of the spore dispersal. This is important as we might be able to understand why mushrooms excel at spore dispersal. Two possible future works are: looking at the temperature gradient of the air under and surrounding the mushroom cap, and looking at the role of water evaporation from the mushroom cap.

Dispersal of fungal spores

Spore dispersal plays a vital role in the survival of the Basidiomycete fungi or more known as mushroom because they are completely immobile. Wind is widely known to be the main driving force of spore dispersal of Ascomycete fungi as the aerial hyphae grows upward thus completely leaving them exposed to wind. In contrast, Basidiomycete fungi cannot rely on the wind as they often do not grow tall enough to avoid the boundary layer of still air next to the ground or under the mushroom cap. Furthermore, they also often grow on top of each other thus only leaving small gaps under the cap. These two features are the main obstacles causing the spores fail to disperse further from its parent fruiting body. In this paper, we will take a look at how does Basidiomycete overcome these obstacles with a hypothesis that gravity current is the driving force for the spore dispersal.

Gravity Current

Gravity current is a horizontal spreading in a gravitational field, due to density differences. The fundamental idea is that heavy things have the tendency to spread. This concept may be easier to comprehend with an example of honey. If honey is poured onto a surface, its natural response is to flow outwards and spread out. This phenomenon is exactly what is witnessed in spore dispersal of mushrooms. Spore laden air underneath the mushroom cap is much denser than the fresh air surrounding it. Therefore, the spore laden air drifts outside of the mushroom cap, carrying the spores along with it. Below is a derivation of how far a spore could travel, with respect to key factors, such as \( h \) (the height of the mushroom cap) and \( L \) (the distance along which the gravity current occurs).

Assuming the density of the surrounding air to be negligible, the estimation of the density difference between the air underneath the mushroom cap and the surrounding air is shown below.

\[
\Delta \rho = \frac{m_s \cdot q}{u_s},
\]
where $\Delta \rho = \text{density difference between spore laden air and fresh air}, m_s = \text{mass of spore}, q = \text{spore release flux}, \text{and } u_s = \text{sedimentation speed}$.

Next, the pressure gradient of the mushroom cap is studied. By force balance between the push of the spore laden air and the resisting, frictional force, the pressure gradient was set to be proportional to the viscous drag, as shown below.

$$\frac{\Delta \rho}{L} = \frac{\Delta \rho gh}{L} \sim \frac{\mu u}{h^2},$$

where $\frac{\Delta \rho}{L} = \frac{\Delta \rho gh}{L} = \text{pressure gradient of mushroom cap}, \mu = \text{Viscosity of air}, u = \text{horizontal spreading speed}, \text{and } h = \text{gap between the mushroom cap and the ground}$.

The time for a spore to sediment is also related to the vertical speed and distance for it to travel and the horizontal spreading speed and distance it could travel.

$$t_{\text{sedimentation}} = \frac{h}{u_s} = \frac{L_{\text{spread}}}{u},$$

where $t_{\text{sedimentation}} = \text{time for the spore to sediment}, \text{and}$

Figure 1 (on left): Theoretical diagram of spores driven by gravity current and fresh air entering from the other side.

 derivation is possible.

$$L_{\text{spread}} = \frac{u h}{u_s} \sim \frac{\Delta \rho gh^4}{u_s \mu L} \sim \frac{m_s \ast q \ast g \ast h^4}{u_s^2 \ast \mu \ast L}$$ Equation (1)

Using force balance between the gravitational force and the drag force in the vertical direction of the spore, the sedimentation speed ($u_s$) is defined as the following:

$$u_s = \frac{m_s \ast g}{6\pi R \mu},$$

where $R = \text{volumetric radius of spore}$.
The value for R can be obtained using the following equation:

$$\frac{4}{3} \pi a^2 b = \frac{4}{3} \pi R^3$$

$$R = (a^2 b)^{\frac{1}{3}}$$

where a and b are defined in Figure 2.

Figure 2: Measurements of an ellipsoid

Three experiments are organized to test the concept of gravity current: dispersal kernel test, gravity current test, and laser imaging test. The dispersal kernel test measures the density of spores as a function of distance away from the mushroom cap. In this test, several different dimensions of mushroom caps and heights were tested (varying L and h, respectively). In the gravity current test, the distance in which the spores traveled away from the mushroom cap was measured for various sizes of the mushroom caps, height, and species of mushrooms (varying L, h, m, and R). According to Equation (1), the gap between the mushroom cap and the ground is significant in the dispersal of spores, due to the power of four. In the gravity current test, the height was widely varied from 3mm to 48mm. The results of the two experiments will be compared with Equation (1) to check the consistency. Finally, the laser imaging test made it possible to see the movement of spores as they are dispersed from the mushroom. The videos made from this experiment will be used to confirm the idea of gravity current and some other factors that affect the dispersal of spores.
Materials and Methods

1. Dispersal Kernel (Spore density measurement)

A. Sample preparation

A spore print sample is obtained by leaving a mushroom cap of certain dimension (i.e. 1 by 4 cm, 2 by 4 cm) on top of two different media: petri dish or transparency paper, for about 2-3 hours. A petri dish is used when the gap under the mushroom cap is 6 mm otherwise transparency paper is used as the storing medium. All storing media are cleaned using 70% ethanol before every experiment to make sure that no foreign object will be captured during the imaging process. 4 sharpie marks are then placed on top of the media to indicate each corners of the mushroom cap. The 6mm gap is achieved by using two cylindrical tubes placed on the short edges of the mushroom providing the support while the 12mm gap is achieved by using two posts made out of cardboard.

Once the storing medium is prepared we can then place the mushroom cap which comes from carefully selected mushroom that produces noticeable amount of spores. The mushroom cap needs to be aligned properly with the 4 dot marks placed on the medium. Last but not the least, a damp check (Calcium Chloride) wrapped with the cheese cloth is added to prevent water droplets forming on the surface of the storing media.

B. Spore print imaging

The image acquisition is started by aligning the two lower dots marking (representing the short edge of the mushroom cap) on the petri dish/transparency with the tape that is oriented horizontally on the microscope stage. Furthermore the right edge of the petri dish/transparency paper is aligned with the tape that is vertically oriented on the microscope stage. This is to make sure that we have a consistent set up. After the sample is properly placed on the stage, we then proceeds to locate and record the x- y coordinates of the 4 dot markings on the petri dish/transparency. These coordinates are important because they will be used in determining the location of the mushroom cap. Next we have to find the range of y values at which a continuous spore print can be found. This range is then divided into 6 transects and then pictures are taken across those transects with an increment of 2mm starting from right to left until we cannot see spores anymore. For each transect, we will need to determine the x coordinate of the right edge at which the spore can first be found. All those coordinates are recorded in an excel file that will also contain the corresponding image names.

C. Image Processing

There are three main methods implemented in the image processing part of the experiment. First, the image is passed into a band pass filter to remove both the pixel and background noise (i.e. uneven illumination). The filtered image is then converted to a binary image with the threshold value of 0.2. This value is chosen after applying several different threshold values to the test images that have low spores density (spores can easily distinguishable) and high spore density (spores cluster together forming clumps of spores). The threshold value of 0.2 is able to make most parts of the spore clumps to appear
in the image while still maintaining the overall circular shape of the spores that are not clumped together. After that, the binary images are then turned into labeled images, meaning that the connected pixels are grouped together and given a label. This allows us to count the number of spores in each photo. Additional steps are required when we are working with the images that contain high density spores region as the labeling process in our algorithm will identify a clump of spores as one single spore which is not what we want. In order to solve this problem, we decided to divide all the grouped pixels that make up the clumps by 40, where the number 40 represents the average number of pixels contained in a single spore.

2. Gravity Current Test

For this experiment, the spore print of mushrooms caps raised to multiple heights (3mm, 6mm, 12mm, 24mm, 36mm, and 48mm) were studied. To produce a reliable spore print, an enclosed space was necessary so that the spores are only driven by gravity currents or other effects that mushrooms utilize for spore dispersal, uninfluenced by the surrounding air flow in the lab. What we used to achieve this were Corning petridish (2.5 centimeters tall) for gaps up to 12mm and Tupperware containers (10 centimeters tall) for larger gaps greater than 12mm. The containers should have a large enough surface so that the spores dispersed by the mushroom cap will not reach the sides of the containers, but only the bottom of the container. In our case, the “bottom” of the container applied to the bottom of the petridish and the lid of the Tupperware containers since we used the Tupperwares upside down. Before starting the experiment, the containers were thoroughly washed by 70% ethanol and Type II water so that the experiment were not be affected by spore residue from previous experiments.

To prepare these containers, transparencies should be cut up so that it covered the entire surface that the mushroom will be placed. Since the data will be analyzed from the pictures of spore prints deposited on the transparencies, the transparencies should be cleaned using a glass cleaner so no marks would cause uncertainties to the spore print. The transparencies were taped down to the bottom of each container. Then, a desiccant (“Damp Check” or CaCl$_2$ wrapped around by cheesecloth) was placed into each Tupperware to prevent water droplets to form on the spore print.

Next, the mushrooms that were producing many spores were selected and cut into various shapes, and their lengths and widths were recorded. In our experiments, we used both Oyster and Shiitake mushrooms. Then, they were raised to their appropriate heights. Mushrooms were raised to 3mm using cut pieces of straw; 6mm and 12mm using cut applicator sticks and white tack; and 24mm, 36mm, and 48mm using a thread to suspend them.
from a cardboard “fake” ceiling, as shown in Figure 5. After the mushroom caps were raised to their proper heights, the boundaries of the mushroom caps were marked using an X-acto knife. Then, the containers were closed and left for four hours.

After four hours, the containers were gently opened, so that the mushrooms were not disturbed. Then, the X-acto knife markings were checked to see if they still accurately outlined the mushroom caps. If they were not, the mushroom caps were traced again on the same transparency, and during the analysis, the outer X-acto knife marking was considered. The mushroom caps were then removed, and pictures of spore prints were taken very carefully. These pictures play a major role in our data analysis, so proper lighting and high-quality camera are advised. Also, there should always be something in the picture in order to know the scaling. For example, we used petridish with grids, where each side of the square was equal to 2cm.

Later, each transparency was cut into small strips to fit a test tube, which was properly labeled. If no spores were visible in the outer parts of the transparency, it may be trimmed down. During this process, the scissors, gloves, and workspace should be cleaned with 70\% ethanol, in between transparencies so that spores from the previous transparency will not be passed on to the next transparency. Afterwards, 5mL of Type II water was pipetted into the test tube and mixed by a vortexer for at least 10 seconds, in order to fully wash off the spores from the transparency. Then, 20µL of the sample was pipetted onto each grid of the hemacytometer, and the cover slip was gently placed on top.

As shown in Figure 6, each grid of the hemacytometer is made up of 25 medium squares (5x5), and each medium square is made up of 16 small squares (4x4). Depending on the number of spores on the grid, the spores were counted from the entire grid, six medium squares, four medium squares, or six small squares. For accuracy, at least thirty spores are advised to be counted, no matter which method was chosen. The number and size of squares, in which the spores were counted, were recorded. To make the counting process easier, a counter was used.

Using the volume of the squares that was used to count the spores on the hemacytometer, the total number of spores deposited on the transparency was calculated. Each small square has a volume of 0.00025mm³. Below are equations to calculate the total number of spores dispersed by the mushroom cap in all methods:
Afterwards, the pictures of the spore print were analyzed on an image processing package called Fiji. The picture to be analyzed is dragged into Fiji, which automatically reopens the picture in Fiji. By clicking “straight” to draw a line along an object with known dimensions, and clicking “Set Scale” under the “Analyze” tab, the scale was set. Then, the shortest line from the spore that traveled furthest was drawn to the boundary of the mushroom cap. To determine the length of the line, “Ctrl” and “M” was pressed at the same time. By doing so, the results box automatically appears with the desired length.

**Figure 5 (on right):**  
*An example of a spore print.*  
The yellow line indicates the distance the spores traveled, measured by Fiji.

Using the spore count, the dimensions of the mushroom caps, duration of the experiment, and total number of spores dispersed, the spore release flux was calculated by the equation below.

\[
\text{Spore Release Flux (}\frac{\text{spores}}{\text{cm}^2 \cdot \text{s}}\text{)} = \frac{\text{Total Number of Spores Dispersed by Mushroom Cap}}{\text{Surface Area} \cdot \text{Duration of Experiment}}
\]
The accepted value for the acceleration due to gravity (9.8 m/s\(^2\)), viscosity of air (1.83E-5 Pa*s), and the mass and volumetric radius of the spore for the corresponding species of mushroom was used. The right side of Equation (1) was then calculated. Finally, the horizontal distance that the spore traveled (\(L_{\text{spread}}\)) was plotted as a function of the right hand side of the equation reproduced below.

\[
L_{\text{spread}} \sim \frac{m_s q g h^4}{u_s^2 \mu L}
\]

where
- \(\rho = \text{density difference between spore laden air and fresh air}\)
- \(m_s = \text{mass of spore}\)
- \(q = \text{spore release flux}\)
- \(u_s = \text{sedimentation speed}\)
- \(\rho = \text{pressure difference between spore laden air and fresh air}\)
- \(\mu = \text{viscosity of air}\)
- \(u = \text{horizontal spreading speed}\)
- \(R = \text{volumetric radius of spore}\)

Results

1. Dispersal Kernel

Figure 6 - 9 below depict the spore distribution under the mushroom cap and at a distance from the edge of the cap. The vertical axis in the graph represents the spore proportion while the horizontal axis represents the distance across the spore print along the short edge of the mushroom. The right and left edge of the 1 cm mushroom cap is indicated on the vertical axis as 0 and -10 while for the 2 cm mushroom cap they are indicated as 0 and -20. Each graph is obtained from interpolating and averaging the data from 3 – 6 different spore print samples with the same gap and mushroom cap size. All the graphs show a nice bell shape characteristic where the spore proportion is denser under the cap and it decreases as the distance away from the cap increases. This makes sense because the spores right under the cap will just fall straight down due to the gravity while the spores near the edges mushroom cap will be carried away by the gravity current as we hypothesized before.
Figure 6: Graph of average spore proportion as a function of distance away from the mushroom cap for 1 by 4 cm mushroom cap and 6mm gap.

Figure 7: Graph of average spore proportion as a function of distance away from the mushroom cap for 2 by 4 cm mushroom cap and 6mm gap.
Figure 8: Graph of average spore proportion as a function of distance away from the mushroom cap for 2 by 4 cm mushroom cap and 6mm gap.

Figure 9: Graph of average spore proportion as a function of distance away from the mushroom cap for various sizes of mushroom cap and gap underneath the cap.

From figure 6 and 7 we can see there is an interesting finding that the dispersal distance decreases from about 20 mm to 10 mm. This result validates the equation (1) that is previously explained where the dispersal distance ($L_{\text{spread}}$) is inversely proportional to the length of the mushroom cap ($L$). From figure 7 and 8 we can observe another interesting finding that the dispersal distance increases as the gap size increases from 6mm to 12 mm. This once again validates the equation (1) where the dispersal distance ($L_{\text{spread}}$) is directly proportional to the gap height ($h$). However, the result does not quite follow the relationship described in the equation as the dispersal distance only increases with a factor of 2 while
the equation predicts that it should increases with a factor of 16. We believe this indicates that there are other possible factors causing the spores to take off and possible deposited on the wall of the petri dishes/container instead of being deposited on the storing medium. Further discussion regarding this will be discussed more in the next section.

2. Gravity Current Test

Below is a plot of $L_{\text{spread}}$ as a function of the right hand side of Equation (1), when the gap between the petri dish and the mushroom cap is less than 12mm.

Figure 10: Spreading of Spores of mushroom caps raised no more than 12mm

Below is a plot of $L_{\text{spread}}$ as a function of the right hand side of Equation (1), when the gap between the Tupperware and the mushroom cap is less than 12mm.

Graph 11: Spreading of Spores of mushroom caps raised higher than 12mm
Blue data points ($h \leq 12$mm) and red data points ($h > 12$mm)
Equation (1) explains how far a spore entrained by gravity current travels. Figure 10 displays the relationship between $L_{\text{spread}}$ and the right hand side of the equation when the gap between the mushroom cap and the petri dish is less than 12mm. The experimental data points from Figure 10 collapse onto one curve, $y = 5E-12x + 0.481$. For the benefit of the uninitiated, a $R^2$ value of 0.3587 is completely acceptable in the field of biology, in which no organism behaves the same way. Each mushroom has their quirks that cannot be summed up in a simple equation. In more technical terms, not all spores may have the same exact size and mass as it has been considered. The linear fit represented on the graph is an attempt to estimate the prefactor, which is apparently $5E-12$. The collapse of the experimental data indicates that the spreading of the spores is driven by gravity current.

Figure 11 is the same graph, showing the same relationship between $L_{\text{spread}}$ and the right hand side of Equation (1); however, data for mushroom caps with heights greater than 12mm were newly added as red data points. It is evident that the red data points are extremely scattered and no longer have a linear fit, as the blue data points (heights ≤ 12mm) did. From this observation, it can be concluded that the spores for large gaps (> 12mm) are no longer driven by gravity current but by some other effect.

The spore prints obtained from mushroom caps with large gaps also suggest that there are some other effects that dominate the spreading of spores.

Figure 12: Spore Print Examples
A and B: Gravity Current (h ≤ 12mm)
C and D: Other Effects (h > 12 mm)
The orange markings on Figure 12 signify the boundaries of the mushroom cap during the four hours that it was dispersing spores. Figure 12A and 12B represent a continuous, steady flow of spores presented in gravity current. However, Figure 12C and 12D displays a spore pattern that cannot be described by gravity current. This wave like pattern has been witnessed in multiple samples with gaps larger than 12mm. The pictures below are captured by a high-speed camera during laser imaging tests. A Class 3B laser has been used to create a laser sheet and illuminate the spores that are dispersed by the mushrooms.

![Figure 13: Spores driven by Gravity Current](image1.jpg) ![Figure 14: Spores driven by Some Other Effect](image2.jpg)

Figure 13 illustrates a nice, uniform gravity current, carrying away the spores. Nonetheless, Figure 14 shows that the spores are not steadily spreading out and depositing to the ground. Some other flow other than gravity current is taking into effect, swirling the spores in an upward direction. The picture on the left is a mushroom cap raised no more than 1cm, whereas the picture on the right is a mushroom cap raised to 1.5cm.

**Conclusions and Future works**

From these findings we conclude that gravity current is observed when the gap under the mushroom cap is small which in our case is smaller or equal than 12 mm. Furthermore, we hypothesize that there are other possible contributors that help the spore dispersal of Basidiomycete mushrooms e.g. convective current caused by temperature gradient. Thus, in the future we might want to monitor the temperature below and around the mushroom cap to determine the possible role of temperature gradient as another driving force of spore dispersal. Furthermore, we can also look at the role of water evaporation from the mushroom cap as one of the other possible contributor that helps the spore dispersal. The role of water evaporation from the mushroom cap in the dispersal of spores should also be studied.


Dispersal of spores by aerial hyphae

YUXI LIN
Department of Mathematics, University of California Los Angeles
Applied and Computational Mathematics REU Program Summer 2013

ABSTRACT

The filamentous fungus, Neurospora crassa, reproduces by using aerial hyphae to disperse asexual spores. The hyphal length varies in different environments. The specific factors driving the fungal hyphal length are still unclear. Here we show that the length of the aerial hyphae scales with the thickness of the surface boundary layer. We show that the length of the aerial hyphae increases with decreasing rate of evaporation by measuring the length of the hyphae in a range of humidities. Then we explore how wind speed affects the rate of evaporation by measuring the weight loss of agar dishes in a wind tunnel while varying the wind speed. We found that the rate of evaporation is directly proportional to wind speed. Our results demonstrate how Neurospora crassa, and other similar fungi, is able to disperse spores despite gravity and the surface boundary layer. We envision our exploration of the fungal reproduction by spores to be a step towards a deeper understanding of the pathogenic fungi that have caused extinctions of many species.

INTRODUCTION

Many extinctions around the world are caused by fungi, yet relatively little is known about them. A type of non-pathogenic, filamentous fungi, Neurospora crassa, grows in sugar cane fields after a burning for harvesting. The fungus reproduces by dispersing asexual spores from its aerial hyphae, which grow to varied lengths in different environments. When the hyphae grow too tall, they collapse due to gravity, but if the hyphae are too short, the spores are stuck in the surface boundary layer, which is a region close to any surface where there is no wind flow. See figure (1). The surface boundary layer thickness is defined by the equation of the Blasius boundary layer. See equation (1). The hypothesis is that the length of the aerial hyphae scales with the thickness of the surface boundary layer. The hypothesis is tested by two major experiments -- one exploring the relationship between evaporation rate and hyphal length and one examining the relationship between wind speed and evaporation rate.
METHOD

The relationship between evaporation rate and hyphal length was examined by varying the humidity of the environment. Humidity was changed by adding calcium chloride or water. Pristine agar dishes were inoculated, and the cultures were left in a temperature and humidity controlled incubator to mature until they were ready to grow aerial hyphae (around 15 hours). Then the dishes were transferred to a range of humidities (40%--90%) by adding various amounts of calcium chloride or water to bottom of tupperwares (30g CaCl₂, 15g CaCl₂, 10g CaCl₂, 5g CaCl₂, 0g CaCl₂, wet filter paper, 10mL water, 50mL water, and 100mL water). The agar dishes with the matured colonies were taped to the lids of the tupperwares and left upside-down until they start conidiation (around a day) because at dry conditions, aerial hyphae growth almost stops at condition [1]. Finally, the lengths of the aerial hyphae were measured by taking a picture of the dish and using FIJI.

The relationship between wind speed and evaporation rate was tested by leaving two agar dishes on top of cylindrical tubes (r. 0.5cm h. 3.5cm) inside the wind tunnel. The dishes were first weighed after the condensation had evaporated. Then the dishes were weighed and the wind speeds were changed, using the dial from the fan, in intervals of an hour. The results were plotted using Matlab.

RESULTS

Data from the evaporation rate vs. hyphal length experiment show that the length of the hyphae increases as the evaporation rate decreases and vice versa. At the highest evaporation rate (driest environment examined -- 30g of calcium chloride), the hyphal length was 1.66mm, while at the lowest evaporation rate (most humid environment examined -- 100mL of water), the hyphal length was 30.71mm. When the data was plotted on a log-log graph, the slope was about -1.

Data from the wind tunnel experiment suggest that the evaporation rate and wind speed are directly proportional -- the highest evaporation rate (0.012g/min) was at the highest wind speed tested (66.52cm/s), and the lowest evaporation rate of 0.0028g/min was at the lowest speed tested (6.62cm/s). The data also show a clear trend when plotted on the log-log graph -- the slope was around ½. See figure 2.

DISCUSSION

According to the Blasius boundary layer thickness equation, the thickness is proportional to approximately the inverse of the square root of the wind speed. Since the results from the experiments
show that the evaporation rate is proportional to the square root of the wind speed and that evaporation rate is inversely proportional to the hyphal length, the equation shows that the hyphal length is directly related to the thickness of the boundary layer. The direct relationship between the boundary layer thickness and hyphal length is significant because it indicates that the fungus has a way of detecting whether or not its aerial hyphae have grown past the surface boundary layer -- by measuring the rate of evaporation at the surface of its hyphae through their turgor pressure.

Although the overall data supported the hypothesis, there were three data points in the evaporation rate vs hyphal length that seemed to stray further than others from the trend. An area of possible error was the timing of the transfers and the measurements because the length is the integral part of the data. The cultures must be transferred to their environments after they have matured enough to grow aerial hyphae so the environment will have minimal effect on the fungal mycelia. Also, when the measurements were taken, although it was believed that all hyphal growth had stopped, growth could have just been greatly stunted to a rate that was unnoticeable in a short period of time.

In conclusion, the experiments supported the hypothesis that the hyphal length scales with the thickness of the surface boundary layer. The outcomes of these experiments may help further studies in the mechanisms of spore dispersal as well as in deeper understandings of some of the pathogenic fungi that have caused extinctions.

![Fig. 1: Neurospora crassa aerial hyphae collapsed due to gravity.](image-url)
Fig. 2: (Left) Log-log plot of evaporation rate vs hyphal length. Line shown is at a slope of -1. (Right) Log-log plot of wind speed vs evaporation rate. Line shown is at a slope of $\frac{1}{2}$.

REFERENCE

Nuclear dynamics in one dimensional growth in *Neurospora crassa* colonies

THOMAS CURRAN
*Department of Mathematics, University of California Los Angeles*
*Applied and Computational Mathematics REU Program Summer 2013*

The goal was to see how starting with different ratios of red and green labeled nuclei affects the genetic composition of the colony as it grows along a race tube. *Neurospora crassa* is made up of fine threads called hyphae. These hyphae are multinucleate and there are no cell walls that separate the hyphae into individual cells. They propagate through tip extension, and they can branch and fuse together. This branching and fusing creates a complicated network, and pressure gradients drive the nuclei to flow from the interior of the colony to the growing hyphal tips, which promote genetic mixing. In addition, hyphae form conidia, which are asexual spores that contain multiple nuclei and are utilized for dispersal. Genetic diversity is considered an advantage on the population level because it increases the chances of that population’s survival. But genetic diversity within an individual, known as chimerism, is not tolerated by most species. In these species, starting a colony with genetically distinct nuclei with result in sectoring, where one of the nucleotypes becomes fixed in one part of the colony, and the other nucleotype is not present. However, in *N. crassa*, genetic mixing maintains genetic diversity within an individual so the distinct nuclei will be found throughout the entire organism. This can increase the organism’s virulence and allow the organism to better adapt to environmental change.

The experimental set up was that a race tube with 29 holes separated by 2cm was inoculated with a solution containing spores that had either GFP or DsRed labeled histones in their nuclei. Five different proportions were used for the starting solution: 50G/50R, 25G/75R, 75G/25R, 90G/10R, 10G/90R. To make these solutions, spores were isolated from cultures through vortexing and filter tips. Once the spores were put in solution, a 20 μL sample was placed on a hemocytometer, and the number of spores were counted in five 0.0025 mm² squares. This was done for both the GFP strain and the DsRed strain, and then using these counts the different solutions were prepared. In addition to these proportions, one race tube was also inoculated with only GFP labeled nuclei. Because the GFP manages to stay only in the nuclei, whereas the DsRed protein can diffuse throughout the entire spore, the GFP only race tube was inoculated as a control. From this GFP only race tube, the histogram of pk, the probability a spore has k nuclei, was calculated at various lengths along the race tube. Once the race tubes were inoculated, they were placed in an incubator for 2-3 weeks so they could grow. Then, spores were collected at various sites along the race tube and placed under the microscope where images were taken. For the GFP only race tube, two pictures were taken: one with only the transmitted light, and one with green fluorescent light. These images were then analyzed to find the number of nuclei in each spore, which was then compiled into a vector where the jth entry represents the number of spores counted that had j nuclei. For the other race tubes, three images were taken: transmitted light, green fluorescence, and red fluorescence. These images were then analyzed to label each spore as being homokaryotic red, homokaryotic green, or heterokaryotic.
Image analysis using Matlab was a key component to my experiments. The first problem arose when trying to analyze the transmitted light images, which often probed difficult and converting them to binary images was messy and inaccurate. I tried using various settings in the microscope until I found a setting for the light path that gave clear contour lines around the spores so converting the image to binary was an accurate representation of what was a spore and what wasn’t. In the process of converting the images into binary images I used thresholding based on Otsu’s method. After that, I had to clean the image of anything smaller than 50 pixels, which was too small to be a spore. Finally, I computed the convex hull of every object left in my binary image. This was necessary to take care of irregularities in spores that would cause them to lose their shape in once converted to a binary image. Once I was able to detect spores, I moved on to analyzing the fluorescent images. In many instances, it was difficult to make out individual nuclei in the images by hand, which made it difficult to check the accuracy of my code’s ability to detect nuclei. After trying but failing to use thresholding based on Otsu’s method to detect individual nuclei, I ended up using the watershed method. Watershed worked quite well, but had a problem with over-counting some nuclei, particularly in areas where nuclei appeared to overlap. To solve this problem of over-counting, I used hmin, which eliminated minima that were less than a certain level less than their surroundings. So in other words, it wasn’t enough for a point to be a local minima, it had to be a local minima by at least a certain amount. To analyze the other race tubes, I used the same methods to locate the spores, and once the spores were located I found their pixel locations. I then compared the pixel locations of the spores with the pixel locations from the binary fluorescent images. If any pixel was yes in the spore and both fluorescent images, the spore was counted as mixed, and if it was yes in the spore image and only one of the fluorescent images, then it was counted as homokaryotic.

The first set of race tubes I analyzed were inoculated with 50-50 ratios and one GFP. After I found the homokaryotic and heterokaryotic probabilities, those probabilities were then used to find the probability that a nuclei was green. To do this, we assumed that the probability a spore was homokaryotic followed a binomial distribution with n trials, where n was the number of nuclei in the spore. However, in our model, n was not a constant, instead it was a variable with different probabilities that we had computed by analyzing the GFP race tube. Also, in order for a spore to be homokaryotic, n must equal k, which means that every nuclei chosen must be the same color. We then solved the inverse problem where we knew the probability it was homokaryotic and solved for q, the probability that a spore was green. We similarly solved for p, where p was the probability that a spore was red.

\[ p_g = \sum_k p_k \cdot q^k \quad p_r = \sum_k p_k \cdot p^k \]

However, it was quickly apparent that there were problems with our results, as the figure on the right shows. The first problem was that q and p did not sum to one. But what was more concerning was that these proportions were not inversely related as we expected. It would seem that if the probability a nuclei was green decreased from one hole to the next then the probability a nuclei is red would increase. However, this is not what our data showed. As the graph shows, there are numerous instances where p and q both increase or both decrease from one hole to the next.

At first, we assumed that the problem was that we were not counting enough spores. I recollected data from a few holes that seemed the most problematic. With the combined data, I had
over 700 spores at these holes. I randomized the order in which the spores were counted and labeled and then took subsets of this data. After a couple of repetitions of this, it appeared that the homokaryotic and heterokaryotic probabilities were converging at around 400 spores, but in my first round of data collection, I was only counting about 200 spores per hole. I went back and analyzed my new set of data, however, the same two problems of not having p and q sum to one and p and q not being inversely related still occurred. Next, we thought something might be wrong with our sampling procedure, and maybe the spores we were collecting were coming from a single hyphae and were not representative of the entire colony. We tried to vary our sampling procedure, and going into the colony five times, and then sampling five regions from one of those samples, yet neither of these methods led to a change in our data.

This led us to believe that something was wrong with our model. One hypothesis was that p and q were not constants, but instead where random variables. If this was the case, we would likely see over dispersion, or a greater number of homokaryotic spores and a smaller number of heterokaryotic spores than what our final results of p and q would predict. We tested this hypothesis by plugging in our calculated values of p into the Binomial model to find the predicted number of heterokaryotic spores, and did the same thing again with our calculated values of q.

\[ p_m = \sum_k p_k \left(1 - q^k - (1 - q)^k\right) \]

In both instances, the predicted percentage of heterokaryotic spores was much higher than the observed value. After searching through some literature, we came across the beta-binomial model, which had been used to describe examples of overdispersion, and one example used the beta-binomial model to explain the overdispersion of genders in families, where all girl or all boy children occurred more frequently than what the population would suggest.

\[ p(x=x) = \binom{n}{k} \frac{B(k + \alpha, n - k + \beta)}{B(\alpha, \beta)} \]

Where \( B \) is the beta function defined as:

\[ B(x, y) = \frac{\Gamma(x) \Gamma(y)}{\Gamma(x+y)} = \frac{(x-1)!(y-1)!}{(x+y-1)!} \]

In the beta-binomial model, q is a random variable that is taken from the beta distribution and then passed into the binomial model. We then had two equations involving the beta distribution and the homokaryotic probabilities we had previously calculated, and from these two equations we were able to solve for the parameters alpha and beta.

\[ p_g = \sum_k p_k \frac{B(\alpha + k, \beta)}{B(\alpha, \beta)} \]

\[ p_r = \sum_k p_k \frac{B(\alpha + k + \beta)}{B(\alpha, \beta)} \]
From these parameters we are then able to calculate the mean, which is our value for q, and also allows us to solve for rho, which is the pairwise correlation between n Bernoulli trials, and is also known as the over dispersion parameter. The over dispersion parameter ranges from 0 to 1, where 0 represents extremely large values of alpha and beta, which means that q is constant and there is no correlation between trials so the system follows the binomial distribution. When the over dispersion parameter equals one, that means the values of alpha and beta are very small, which indicates a high correlation between trials. Our experimental data fit this model and we got good results.

\[ q = \frac{\alpha}{\alpha + \beta} \quad \rho = \frac{1}{1 + \alpha + \beta} \]

We first applied our data from the 50-50 race tubes to this model, and we got a little fluctuations in the beginning of the race tube, but then the proportions of nuclei remained relatively stable at 60% green and 40% red, and we expected the fluctuations to be due to the fact that we did not sample enough spores. Next we analyzed our second batch of race tubes, however, due to contamination and stress on the colony due to the agar in the race tube drying out, we only felt confident analyzing the 10G/90R, 75G/25R, and GFP race tubes. The second GFP race tube we analyzed had the same general shape of histogram for pk, so it validated our previous data and we feel confident in our experimental measurement of pk. The 10G/90R race tube had values of .95 for p and .05 for q, and these values remained stable throughout the length of the race tube. The 75G/25R race tube had values of .7 for q and .3 for p, and these values had very small fluctuations throughout the race tube. These values were close to our expected values based on the solution used for inoculation. The fact that the values of p and q remained stable as the colony grew in one dimension indicates that there is macro mixing taking place on the level of the organism. The organism manages genetic diversity through mixing to ensure that it remains a chimera and the proportions of genetically different nuclei remains the same. Below are graphs of our results, where the green lines represent q and red lines represent p, and they are plotted versus the length of the race tube.

However, we also looked at the pairwise correlation, or the over-dispersion parameter, as the colony grew. The over-dispersion parameter varied greatly at each hole that was sampled, and it also varied greatly from one race tube to the next. We could not determine any pattern to these fluctuations, and they appeared to be random. The over-dispersion parameter represents mixing on the level of individual hyphae where the spores form. A value of zero means that there is perfect mixing at the hyphael level, and a value of one means that no mixing is taking place at the hyphael level. The figure below shows our data. We could not find any pattern to the mixing on the level of individual hyphae, which in a way supports earlier research on the subject. There are research papers that say that individual hyphae are great at mixing and there are research papers that say they are poor at
mixing, and the scientific community has not settled this debate. Our results suggest that in fact, both conclusions are correct, and that hyphae can be either good or bad at mixing, apparently at random.

There are several avenues of possibility to continue with this research. The first comes from the result stated above where we could not find a pattern to the over-dispersion parameter, which means that we cannot yet explain mixing on the hyphael level. Right now it appears to be random. One way we could try to understand the mixing on the hyphael level is through collecting spores from individual hyphae, and would require a much more meticulous sampling procedure than what was previously used. This would allow us to track the progress of mixing on the individual hyphae, rather than the entire colony, and we could determine if the over-dispersion parameter continues to fluctuate randomly, or if there is an underlying biological explanation.

The next possible avenue of continuing exploration was stumbled upon by accident. When we went to analyze the second batch of race tubes, we found that the 50/50 race tube showed obvious signs of contamination. The contamination was discovered after I had already taken images with the microscope, so I went ahead and analyzed the data. For this race tube, the nuclear proportions were diverging away from their starting points, and after hole 15, the proportions stabilized, and most of the contamination occurred around or before hole 15. This led us to the hypothesis that the fungus does not maintain genetic diversity well through mixing when under stress. One way we could test this hypothesis is by somehow adding stress to the race tube environment, either through adding peroxide to the agar, or by adding less sugar to the agar.

The third avenue of to take would be to work with a chimera where the two nuclei had fitness difference. In the experiments we ran, there was no intrinsic fitness difference between the two nucleotypes. However, this is not necessarily indicative of chimerism found in nature, where often the chimerism arises due to a mutation. There would be two possible expected outcomes to running the race tube experiment where the two nuclei used in inoculation had a fitness difference. Either the fitness difference would have no effect on the proportions, which would remain stable as the race tube grew, or the fitter nuclei would eventually become fixed in the colony. We would possibly expect the first result because the hyphae are multinucleate, so theoretically as long as one of the more fit nuclei were present, the entire organism would experience the fitness benefit. It would be interesting if we saw the second result, because that would mean that the fungus could either purposely select the fitter nuclei when producing spores, or that having a higher proportion of the fitter nuclei is beneficial to the organism.
REFERENCES


Nuclear dynamics of two-dimensional growing *Neurospora crassa* colonies

MATTHEW MOLINARE  
*Department of Mathematics, University of California Los Angeles*  
*Applied and Computational Mathematics REU Program Summer 2013*

---

I. INTRODUCTION

The ability of filamentous fungi to maintain internal genetic variation contributes to their morphological diversity and ecological success as pathogens. The focus of my research is the dynamics of nuclear mixing in two dimensions growth of the non-pathogenic *Neurospora crassa*. Our model organism is composed of branching filaments called hyphae that freely fuse to form an interconnected mycelial network. Genetically different nuclei arise from somatic mutations and are transferred laterally in a contiguous cytoplasm through pores in cell walls called septa. The mean flow of nuclei within the mycelium is toward the colony’s edge; nucleotypes become better mixed with growth due to the lateral gene transfer along pathways of varying velocities and the take up of heterogeneous histones from the cytoplasm surrounding the nuclei. Accumulations of mutations via the mixing of nuclei results in intraorganismic genetic diversity; in colonies where fusion is inhibited and the flow of nuclei is restricted to a small number of hyphae, diversity is lost \(^1\). My studies examine the feasibility of measuring the width of transverse mixing interfaces.

II. METHOD

Aerial hyphae containing nuclei expressing either GFP or DsRed-labeled hH1 histones are extracted from a slant tube using an applicator stick and suspended in deionized water. These protein labels do not confer selective advantages on either strain of *N. crassa*; therefore, our assumption is that each strain was equally fit in the exploitation of agar resource. Hyphae are filtered out from the suspension to isolate the spores. A 3 microliter droplet of either spore suspension is then used inoculate Petri dishes containing sucrose-agar media. We create our two-dimensional heterokaryotic colonies by dissecting out blocks of agar housing DsRed and GFP growing hyphal tips and placing them beside a larger block of pristine agar. We dissect out blocks of agar containing only the growing hyphal tips to control for the age of the colony, the hypothesis being that the tip region does not know its own age; no information regarding the true age of the colony is transmitted to the heterokaryotic colony.
The two-dimensional colony is then placed in the incubator at 25 degrees Celsius and monitored over a 48 to 72 hour period for the emergence of the characteristic orange color indicating the formation of the spore-producing structures, a process known as conidiation. At this point, the colony is removed and vertical transects are dissected out at horizontal distances separated by powers of two from the origin of growth (see fig.1).

![Diagram of experiment setup.](image1)

We isolate the spores from each region along a transect by suspending slices of agar containing the conidiophores in deionized water. The suspension is filtered and centrifuged, allowing us to remove excess fluid and obtain a concentrated spore solution. 20 microliters of solution are then pipetted onto a slide and imaged under the motorized fluorescence microscope. Approximately 100 to 200 spores are imaged at each region using separate EGFP and DsRed fluorescent channels. The EGFP and DsRed-channel images are processed individually using a program in MATLAB that counts the relative proportions of GFP, DsRed, and mixed spores. A control, GFP-only two-dimensional colony was used to develop a histogram for the probability that a spore has \( k \) number of nuclei. Using this information and information about the relative nucleotype proportions, a directed beta-binomial distribution model enabled us to extract the probability that nucleus is either GFP or DsRed.

This data can be used to determine the width of the mixing interface \( w(x) \) as a function of parallel position \( x \) (see fig.2). The random walk diffusion model tells us that the average squared displacement is a measure of width of the mixing interface of GFP and DsRed nuclei. The gradient of a log-log plot of \( w(x) \) vs. \( x \) gives the diffusion parameter \( \alpha \). Width is expected to be proportional to the square root of parallel distance if diffusion of the mixing interface occurs (\( \alpha = 0.5 \)).

![Yellow area represents the theoretical mixing interface. Genetic mixedness is expected to increase with the square root of parallel distance. Experiment is designed to yield diffusion parameter \( \alpha \).](image2)
III. RESULTS

It was observed in the first experimental trial that DsRed had a significantly greater nucleotypic representation than GFP. There was also a low degree of micromixing, in that the relative proportion of heterokaryotic spores was much less than the relative proportion of homokaryotic GFP and DsRed spores. The mixing interface was located at the far GFP-edge of the colony. The leading explanation for this behavior was that the initial DsRed population became sooner established on the agar block than the GFP population. As a control, we performed tests to measure the growth rate of each strain; both strains were plated out separately on Petri dishes, and the edge of the colonies where traced by hand every two hours. This data revealed that the GFP and DsRed colonies had the same rate of growth. The protocol for dissecting out hyphal tips from the Petri dish was altered to control for the behavior of the tips at the contact surface (the edge of the large pristine agar block). In order to synchronize the stages of growth at the contact surface, we increased the size of the dissections to allow for 3 millimeters of pristine agar at the end of each growing tip region. Additionally, to control for instantaneous biomass, we matched concentrations of GFP and DsRed spore suspensions used to inoculate the Petri dishes; a subsequent series of dilutions were made to both strains at 90%, 75%, 50%, and 25% of the matched concentration. Petri dishes containing 3% agar media were inoculated using 4 microliter droplets of the suspensions, placed in the incubator at 25 degrees Celsius, and monitored for mycelial growth during a 30 hour period. Preliminary tests were run to assay the initial concentrations of the GFP and DsRed strains that yielded colonies with similar hyphal tip density. However, the tests revealed no correlation between inoculum concentration and hyphal tip density. This may have been due to stochastic environmental factors such as differences in humidity caused by condensation on the lid of the Petri dish. It was decided to match GFP and DsRed colonies that grew out to 3 centimeters in radial length in the same period of time.

The experiment was replicated using the preceding protocol. Despite controlling for continual growth rate, a second trial revealed the probability a nuclei has the DsRed-label increases with parallel distance from the origin. Also, the mixing interface appeared to be aligned with the transverse axis. (see fig.3b). A third trial, inoculated with slurry of aerial hyphae, reveals GFP-nucleotype dominance. The mixing zone is represented by a spike located on the DsRed side of the colony (see fig.4b). These results contrast those of previous trials, which revealed DsRed-nucleotype dominance. This confirms our assumption that neither strain is intrinsically more fit; differences in nucleotypic proportions are due to experimental variation.

**Fig.3a:** Genotype probability at transect at 28mm. No DsRed-GFP convergence.

**Fig.3b:** Visualization of spore probability using Delaunay triangulation of data points in 3-D space. Mixing interface lies along transverse axis.
Colonies founded by two initially unmixed populations result in the fixation of one genotype across two-dimensional spatial growth. The mixing interface is unpredictable, which may be due to stochastic environmental factors such as variation in humidity or temperature. It is possible that *N. crassa* is not adapted for transverse mixing, or the methods we are using do not enable us to measuring mixing in the transverse direction. We did not account for sampling bias in the method used to remove aerial hypha from the slant tubes. Hyphae residing nearest the opening of the slant tubes may have a selective advantage in continual growth rate than those residing near the bottom. It is useful to consider that reduced population size and genetic diversity at the front of expanding populations results in the spatial segregation of organisms of different allelic types. The method by which we inoculate strains of *N. crassa* results in semi-circular fronts; the increase of front length with time may lead to further segregation. Sectoring may occur if the growth rate of a particular strain is higher than that of the rest of the colony because competition exists between populations for resources on a constricted media. Data on nuclear mixing from one-dimensional race tube experiments suggests genetic differences are maintained in one direction of growth when the colony is initially mixed. Strain proportions as a low as 10% in a given population are maintained along the length of the race tube. This suggests a possible difference between maintaining variation within one mixed population and maintaining variation between two unmixed populations. Our observations may reflect how diversity within an individual behaves differently than diversity between individuals with regards to genetic mixing. We may obtain more favorable results by beginning a colony with two mixed population placed side-by-side on a pristine agar block. These would consist of 90%/10% and 10%/90% DsRed/GFP suspensions. We will perform these tests with the hypothesis that genetic differences are maintained along the interface during initial stages of growth until mixing on the scale of the entire two-dimensional colony occurs.

**IV. CONCLUSION**

Colonies founded by two initially unmixed populations result in the fixation of one genotype across two-dimensional spatial growth. The mixing interface is unpredictable, which may be due to stochastic environmental factors such as variation in humidity or temperature. It is possible that *N. crassa* is not adapted for transverse mixing, or the methods we are using do not enable us to measuring mixing in the transverse direction. We did not account for sampling bias in the method used to remove aerial hypha from the slant tubes. Hyphae residing nearest the opening of the slant tubes may have a selective advantage in continual growth rate than those residing near the bottom. It is useful to consider that reduced population size and genetic diversity at the front of expanding populations results in the spatial segregation of organisms of different allelic types. The method by which we inoculate strains of *N. crassa* results in semi-circular fronts; the increase of front length with time may lead to further segregation. Sectoring may occur if the growth rate of a particular strain is higher than that of the rest of the colony because competition exists between populations for resources on a constricted media. Data on nuclear mixing from one-dimensional race tube experiments suggests genetic differences are maintained in one direction of growth when the colony is initially mixed. Strain proportions as a low as 10% in a given population are maintained along the length of the race tube. This suggests a possible difference between maintaining variation within one mixed population and maintaining variation between two unmixed populations. Our observations may reflect how diversity within an individual behaves differently than diversity between individuals with regards to genetic mixing. We may obtain more favorable results by beginning a colony with two mixed population placed side-by-side on a pristine agar block. These would consist of 90%/10% and 10%/90% DsRed/GFP suspensions. We will perform these tests with the hypothesis that genetic differences are maintained along the interface during initial stages of growth until mixing on the scale of the entire two-dimensional colony occurs.

**Fig. 4a:** Genotype probability at transect at 28mm. GFP dominance.

**Fig. 4b:** Visualization of spore probability using Delaunay triangulation of data points in 3-D space. Sharp mixing interface at (28mm, -14mm).
V. REFERENCES

