

A Developmental Study of *Pilobolus*  
Comparatively Using Light and SEM Photography

An Honors Thesis (HONRS 499)

By

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### **Abstract**

This study was done for the purpose of examining and documenting the development of the fungus *Pilobolus*, specifically its asexual reproductive structures. These structures, the sporangia, were comparatively photographed at several stages in their growth under both light and scanning electron microscopy (SEM). Although this part of its life cycle is generally known, no one has ever done a comparative developmental study or photographed in detail these structures. Therefore, after completion, the information gathered will be presented at the fall meeting of the Indiana Academy and submitted for publication. The information gathered will ultimately be used in conjunction with DNA analysis and other such data collected by others for the purpose of writing a new monograph for the entire family. A monograph gives detailed accounts of similarities and differences between similar species so that uniform classifications can be achieved. The existing monograph for these organisms is strictly morphological.

### **Acknowledgments**

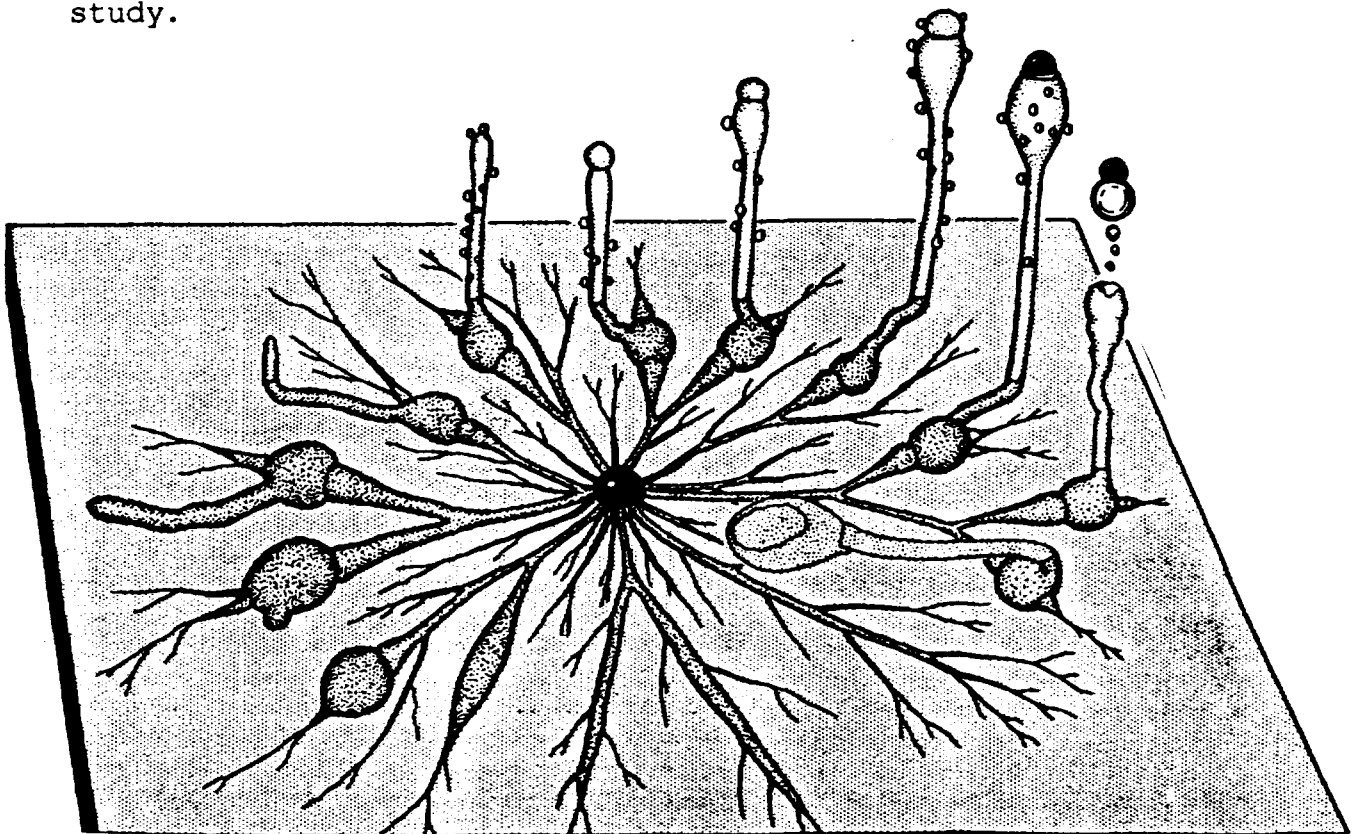
I would like to thank Dr. Ruch, my thesis advisor for being willing to work with me on this and helping me develop a specific project idea. Also, a big thank you to Mark Robbins for teaching me many of the procedures I needed including how to use the SEM, for keeping me supplied with fresh plates, and for always doing his best to answer my questions. Next, I would like to thank Beth for returning the photograph scanner of the SEM to working condition. Finally, I need to thank Dr. Michael Foos for supplying us with some of his *Pilobolus* samples, information, and knowledge, and for allowing me to use the equipment at Indiana University East for the light photography portion of the study.

### Background Information

The fungus I used is a member of the division Zygomycota. It is classified in the family Mucorales, which contains three genera. These are *Pilobolus*, *Pilaira*, and *Utharomyces* (Hu et al., 1989). All results and photographs given in this project were specimens of the species *Pilobolus crystallinus*. This genus is a known native of China (Hu et al., 1989), although it has been reported as growing in the eastern and midwestern United States, and more recently Grand Canyon National Park (Foos, 1991).

Though the name zygomycete is derived from structures present during its sexual reproduction (a zygospore), this particular genus (*Pilobolus*) more often reproduces asexually. In the wild, *Pilobolus* grows mainly on the dung of animals such as cows and horses. Its mycelia spread horizontally, forming a network within the dung to absorb nutrients (Campbell, 1993). For asexual reproduction, it grows a long upright hyphae, called a sporangiophore (Hu et al., 1989), with a dark-colored cap at its tip known as the sporangium. These sporangia each contain hundreds of spores. When the structure is fully grown and the spores are developed, an area just beneath the sporangium begins

expanding as it fills with protoplasm (Page, 1962). This sub-sporangial swelling increases steadily - up to a volume of 0.08 cubic millimeters (Page, 1964) - until the pressure is so great that the packet of spores is shot off of the hyphae with a squirt of cytoplasm (Campbell, 1993). The force of the sporangium being shot off is so great that the sporangiophore recoils and collapses. In fact, its muzzle velocity has been measured at 16 meters per second (Page, 1964). This entire process (shown in the drawing below) is the portion of the *Pilobolus* life cycle which I was trying to characterize further through this comparative light/SEM study.



Picture from Science, Vol. 138, page 1239

The spores themselves are not released at this point (Webster, 1980). Upon landing, the entire sporangium will attach itself to whatever it has come in contact, this usually being a blade of grass. The sporangium must then be eaten along with the grass by a grazing animal and pass through its digestive tract in order to release the spores themselves. The life cycle is then complete once the spores are excreted in the dung of the animal.

One of the unique features of *Pilobolus* is that it does not disperse its spores completely at random. It has the ability to "aim" this reproductive explosion.

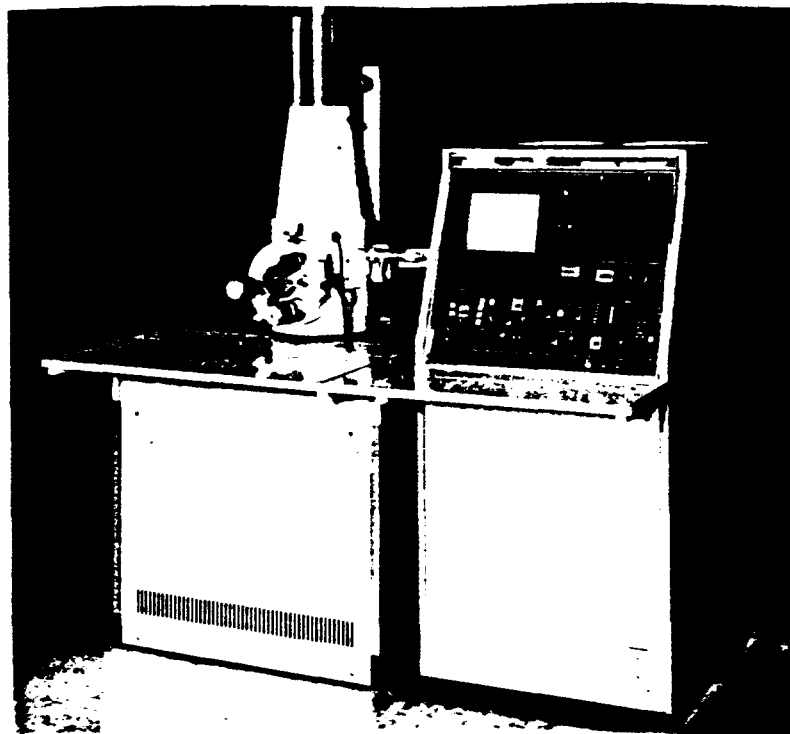
*Pilobolus* displays a positive phototropism, meaning it responds to light by bending its upright sporangiophore toward the light source and shoots off the sporangia mid-morning (Webster, 1980). It is not fully understood how this is accomplished. There is some evidence that the photoreceptor is either a carotenoid or a flavin (Page, 1962), but no specific identification of the molecule or mechanism has been attained. It is known that light with wavelengths that fall near 450 nm (in the blue range) is most effective in stimulating a response (Ellis, 1996). Also, light is important in different ways for different stages of growth. For instance, growth of sporangiophores is enhanced by light, but they will form in cultures grown

in darkness. Conversely, development of sporangia is completely dependent on light. If grown in darkness, *Pilobolus* sporangiophores will never form a sporangium (Ellis, 1996).

There are a few reasons why a phototropic response would be beneficial to the species. First, directing the sporangia toward the sun would make it very probable that grass would be growing in that area. Grass is, of course, the means by which the sporangia is taken into the digestive tract of an animal. Secondly, timing the explosion for mid-morning would mean the sun was in a position in the sky as to put the angle of trajectory at approximately 45 degrees. This would maximize the distance that the sporangium could travel before hitting the ground. The sporangia of *Pilobolus* can be carried by the shot of cytoplasm up to 2 meters from the dung where it was growing (Campbell, 1993). This is also beneficial since animals will not graze too near a pile of their dung, an area known as the zone of repugnance.

I would also like to present some background information concerning SEM or Scanning Electron Microscopy. This type of microscopy is used to examine bulk specimens, particularly the fine details of their surfaces as it produces three-dimensional images. Electrons from a

cathode speed toward the specimen surface. As they pass through the vacuum column, they are focused into a narrow beam by multiple electromagnetic lenses (Hayat, 1978). When the electrons hit the specimen, there is a release of secondary and backscattered electrons as well as several types of radiation (Reimer, 1998). The intensity of these electron signals at any given point is determined by the shape and composition of the specimen. The strength of the signal collected from a certain part of the specimen will therefore create a corresponding brightness on the viewing screen (Wells, 1974). The beam of electrons repeatedly scans the surface of the specimen, producing the image in a point-by-point fashion (Hayat, 1978).



Picture found in Hayat, 1978, page 10



## Materials and Methods

### I. Growth and Fixation of the Fungus

The *Pilobolus* isolate I worked with was first grown on rabbit dung agar, and then transferred to a plate of PSM (Pilobolus Synthesis Medium) (Page, 1962). After allowing it to grow on this agar through much of its life cycle, sections of the agar, which contained hyphae, were cut out and used to inoculate ten smaller plates. Small plates were used to increase the efficiency of diffusion of the fixative through the agar (see below).

Daily for about three weeks, I monitored these small plates for growth. During this time, I fixed (stopped the growth of) each plate at different stages in the sporangia development. To do this, I used a cork bore to punch a small hole in the agar in a place with little growth. After removal of the piece of agar, the hole could then be filled with the fixative. I used a 25% gluteraldehyde solution (diluted in distilled water) which diffused through the agar of the plate and up through the sporangia. A few days were allowed for this process.

This fixative (in theory) preserved the structures as they were at that point in time and also protected them against natural or chemical breakdown of the tissues

throughout the rest of the investigation. Therefore, once the gluteraldehyde fixative was applied to a small plate, nothing on that entire plate would continue to grow or start to decay. The plates were also flooded with a small amount of 5% gluteraldehyde solution to prevent them from drying out. This was most effective when the plates were flooded the day after initial gluteraldehyde fixation by diffusion. If liquid had been allowed to sweep across the surface of the plate before the vertical structures were held chemically in place, they would have been knocked flat, as a blade of grass might be by a sudden flow of water.

Before each time that I used the fixative, I first had to purify it with charcoal. Over time, the aldehyde structures in this solution will naturally convert to their acid form. Mixing the gluteraldehyde with charcoal reverses this process and changes the molecular structure back to the active aldehyde form. The gluteraldehyde and charcoal were mixed together for several minutes and then filtered through a Buchner funnel twice to remove all of the charcoal. It could now be applied to the plates to fix them.

## II. Light Microscopy

Once I obtained fixed samples of the different stages of development, I took them to Indiana University East in Richmond, Indiana. There, with the help of Dr. Michael Foos, I was able to take light photographs of each of my specimens at various magnifications ranging from 5X-30X. I used a inverted microscope (the Olympus SZ60 Stereo zoom) to which a digital camera (Javelin analog camera) was attached. This camera fed the image through an ATI TV Player 6.0 to a Gateway E4200 computer where the pictures could be saved onto a Zip disk and later edited and printed out.

## III. Scanning Electron Microscopy

With the light microscopy portion of the study finished, I returned to Ball State University to begin preparing the samples for the scanning electron microscope. A block of agar bearing the exact hyphae and sporangium that had been photographed under the dissecting microscope was carefully cut out and processed according to the following procedure. They were soaked in distilled water for 15 minutes (to remove any excess fixative), then placed in 1% osmium ( $\text{OsO}_4$ ) for 90 minutes. Osmium is an extremely

strong, fast fixative. Again, the specimens were washed in distilled water for 15 minutes.

The next step, in general, was to dehydrate, or remove all the water from the cells. This is done gradually by a series of increasing concentrations of ethyl alcohol. This process prevents shrinkage or distortion of the cells when one kind of solvent must be replaced by another (Ruch, 1998). I used concentrations of 35%, 50%, 70%, 80%, 95%, and 100% ethanol, in that order. The specimens were left in each of the above concentrations for 30 minutes, and they were placed in two changes of fresh 100% ethanol. When my schedule made it necessary, I stopped the procedure at the 70% ethanol stage. Specimens can be safely stored overnight in this concentration.

After the second change of 100% alcohol, a few minutes were allowed for the excess ethanol to evaporate. Next, they were treated with two changes of hexamethyldisilazane for one hour each. This is another drying agent which removes all of the alcohol from the specimens.

Finally, the blocks of agar were carefully placed right side up onto SEM object discs, or stubs. To make the blocks stay on the stubs, a sticky tab is pressed onto the metal disc surface. When the paper is peeled off, it

deposits a fine layer of adhesive onto which the specimen is placed.

The last step of processing is to coat the specimens with an extremely thin layer of gold-palladium. This substance, in essence, gives the electrons a more electron-dense surface off which to bounce. As an alternative, carbon can be used as the coating in this step as well. With either substance, the thin layer is applied in a fully automated machine called a sputter-coater. Under vacuum, atoms of gold-palladium are released from a ring around the outside of the chamber and physically thrown toward the specimen sitting in the center of the chamber (Hayat, 1978). In this way, the specimens are bombarded from all directions by atoms of gold-palladium, giving it the desired thin coating.

At last, the sporangial structures were ready to be observed under SEM. The object discs were placed in the specimen chamber of the Cambridge Model S90 Scanning Electron Microscope located in the basement of Cooper Life. The chamber was placed under vacuum. When a satisfactory image was achieved on the screen, the contrast was adjusted for photography and the SEM photograph was taken with a Model 545 Polaroid photo-recorder. The Polaroid Positive-Negative 4X5 Instant Sheet film was given 25-30 seconds to

develop. The two halves of the special film were at that time pulled apart to reveal both the positive and negative images. The positive was covered with the "coater" substance that was provided with the box of instant sheet film. This served to protect the print from scratching or fading. The negative was washed thoroughly in warm water to remove excess developer and hung up until it was completely dry. This process was repeated for each SEM picture presented in the results section of this paper.

## Problems and Obstacles

As occurs with most every research project, unexpected difficulties and delays arose. Many research scientists find this to be especially true when working with living organisms.

The first problem was the extreme unpredictability of the original isolate (9401) of *Pilobolus* with which I was working. Difficulty in maintaining *Pilobolus* on artificial growth medium is a universal problem and is mentioned by Hu in his 1989 paper. Speculation is that one of the missing elements in growth medium is iron, which *Pilobolus* seems to need in a specially chelated form (Page, 1962). Soon after my study was initiated, we discovered that after being grown and transferred onto new plates of PSM several times, the isolate lost its ability to grow and reproduce. Those plates which grew mycelia under the surface, never produced vertical hyphae structures or sporangia. Consequently, two new isolates of the same species (ATCC 36186 and 9810) were chosen and transferred onto plates. *Pilobolus* isolates can differ in many ways, the most common of which is the shape of the sporangium. The original isolate to have been studied possessed very unique sporangia. They were polygonal and had small round indentations giving them a

resemblance to a soccer ball. The 36186 and 9810 isolates had sporangia with the more typical smooth spherical shape. These alternate isolates grew more readily and produced asexual reproductive structures.

In an attempt to further ensure successful growth, I also tried using an enriched PSMY media (PSM supplemented with yeast extract). Those growing on the enriched medium grew vertical hyphae in approximately 10 days while these structures took a full two weeks to develop on PSM plates. Nevertheless, both types of media exhibited equal numbers and characteristics of sporangia-bearing hyphae. Of course, the media on which they grew best was rabbit dung, as it was the natural growth medium of wild *Pilobolus*. However, this condition made study of any structures beneath the surface nearly impossible. Therefore, it was only used when obtaining some of the specimens in the latter stages of the life cycle.

Another problem discovered during the progression of the project was the fragility of the structures I was attempting to preserve and photograph. In fact, *Pilobolus* has been noted to be particularly difficult to maintain in this manner (Hu et al., 1989). Originally, the plates were not flooded with 5% gluteraldehyde at the time of fixation. Under these conditions, it was discovered that a few days



after fixing the structures, those that had exhibited a fair amount of sub-sporangial swelling collapsed in on themselves.

I realized after consulting Hayat's book, *Introduction to Biological Scanning Electron Microscopy* (1978), that this was most likely due to the evaporation that was being allowed to take place. Hayat reports that when many soft biological specimens are air dried, the effects are often "flattening, shrinkage," or "collapsing of the specimen." In contrast, bringing the specimens through a series of ethanol solutions of increasing concentration (as described in materials and methods above), caused an average of only 10% shrinkage (Hayat, 1978). Therefore, the problem was remedied by keeping the structures submerged in liquid fixative until light photography was completed and we were ready to prepare them for SEM.

Unfortunately, this does not mean that every specimen I prepared and photographed remained precisely as they were in life at the moment gluteraldehyde fixative was applied to the plate. It is impossible to perform chemical manipulations and processes such as those described in the Materials and Methods without some deformation of the specimen. These alterations in the appearance or makeup of

the specimen due to preparatory methods are known as artifacts.

The goal when developing a protocol for a certain specimen is to choose the methods that will produce minimal artifacts. Yet, artifacts can be introduced at virtually every stage of processing a specimen for SEM. The dangers of air drying a specimen have already been discussed. Chemical dehydration (for instance, with ethanol) is preferred over air but also produces some shrinkage of cells. Sputter-coating can also produce artifacts, though this is rare and only occurs when contamination is present in the vacuum chamber. If the specimen is not dried completely, water vapors can disrupt the sputter-coating process. Other vapors may alter the final color of the specimen so that it appears to have blue or red spots instead of being solid gold (Hayat, 1978). Finally, artifacts can form while the specimen is actually being viewed in the scanning electron microscope. The direct beam of electrons can sometimes damage a specimen and result in lines or cracks on the gold-palladium coating (Hayat, 1978).

Many of these artifacts are subtle and do not cause false conclusions. The most important thing to consider in this matter is that the artifacts themselves are not a

problem. As long as you realize they exist, and you can distinguish between the real data and the aberrations in the specimen surface caused by imperfect processing methods, there should not be any substantial problem in interpretation of the image.

On the whole, doing this project has taught me something that every person who thinks they want to be a scientist should realize. Research can often present some interesting problems. When you read a published paper, the final results are there, and they make perfect sense in conjuncture with the methods the authors use. What you do not read in that publication is how they tried a slightly different methodology at first, but discovered it did not give useful results.

Any study starts with a proposed plan of action. Usually, however, that first plan of action may not work. Neither might the second, third, or tenth. Two of the most important personality traits of a research scientist are patience and perseverance. Without these traits, frustration will take hold and probably result in most investigations being given up on and dropped.

One last observation I have made during the course of this project is that the scientific community would be much more efficient if we would publish our negative results as

we do the positive ones. Understandably, if you do not make any discoveries or real progress in the understanding of a particular topic, the journals and such will not find your work that interesting and will not publish it. On the other hand, it would be of everyone's interest to be able to learn from the mistakes of others. Without any references that might point out a method that does not work on a certain organism (for example saving a collection of *Pilobolus* specimens and allowing them to become dry), many other labs often waste valuable time repeating failures. If these things could be made known, we may find the negative discoveries to be just as important to the advancement of science as the positive ones.

## **Results and Discussion**

Several observations were made while watching this fungus grow. Here, each observation will be discussed, then directly followed by the pictures which best exhibit that characteristic. Magnifications used are indicated within the outlined box to the right of each picture. Notice that only light microscopy pictures are included for the stages of growth that did not have any structures growing above the surface of the medium. This is due to the nature of SEM, which produces magnified images of the specimen's surface. Therefore, nothing inside the agar could have been seen with Scanning Electron Microscopy.

The trophocysts of *Pilobolus* are most often circular, however, some are much more elongated. This is evident upon comparison of the trophocysts in Figures 1 and 2. The string-like mycelium can also be seen in the surrounding medium. Trophocysts are dark yellow in color and often have one thicker hyphae on each side of them which taper off to thin, threadlike structures (Figure 1). One hypha eventually grows vertically from the center of the trophocyst and will become the sporangiophore (Figures 2 and 3).

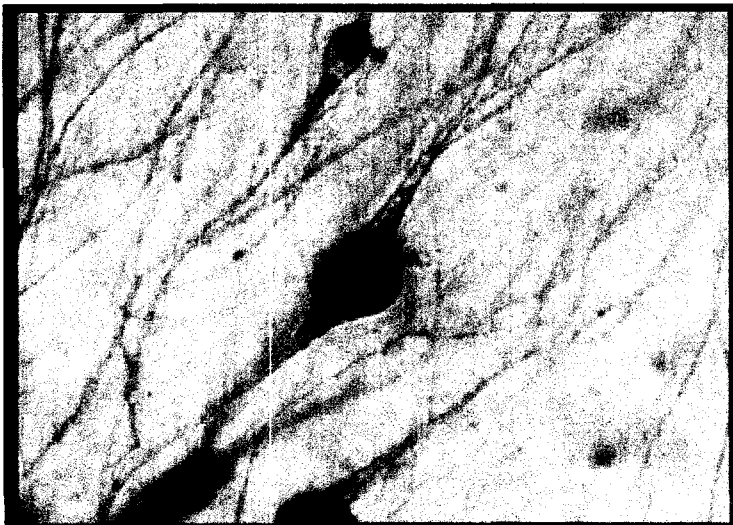


Figure 1 –  
A circular trophocyst  
of *Pilobolus*  
130X



Figure 2 –  
The hypha destined to  
become the sporangiophore  
is beginning to bulge out  
from the trophocyst and grow  
toward the light source.  
180X

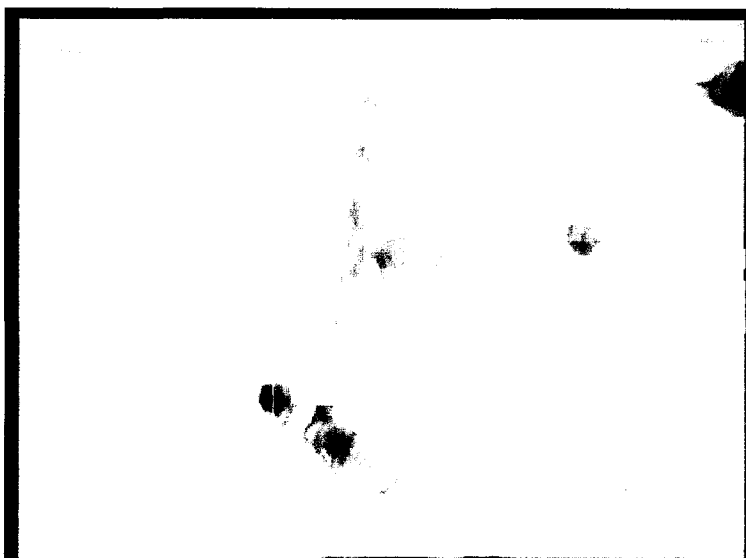


Figure 3 –  
Here, the hyphae is  
extending vertically, and  
the tip is nearing the  
surface. Other trophocysts  
and mycelium can be seen  
in the medium.  
100X

Once the structure breaks through the surface, the sporangiophore elongates. Sporangiophore length is almost completely unpredictable, but may be influenced by the presence or absence of light. Studies have shown that the sporangiophore of *Pilobolus crystallinus* will grow very long if kept in darkness (Page, 1962). As all of my samples were exposed to light in an identical manner, the inconstancy in sporangiophore length seen here is simply natural variation.



Figure 4 –  
The growing sporangiophore has emerged out of the medium into the air (the left portion of the picture is above the surface). The point at which it exits the agar is noticeable due to the glare of light coming off the point of contact.  
130X



Figure 5 –  
This entire structure is above the surface of the growth medium. It has not yet begun to form a sporangium.  
250X

Once the sporangiophore is fully elongated, a yellow cap appears at its tip. This is the immature sporangium. Notice that the sporangiophore still maintains a constant diameter at this point, all the way to where it contacts the sporangium (Figure 6). Another noteworthy observation that can be seen in this photograph is that the trophocysts begin to disappear at this point. It is thought that material from the trophocyst moves up the sporangiophore. It is unclear whether this material forms part of the sporangium itself or contributes to sub-sporangial swelling.

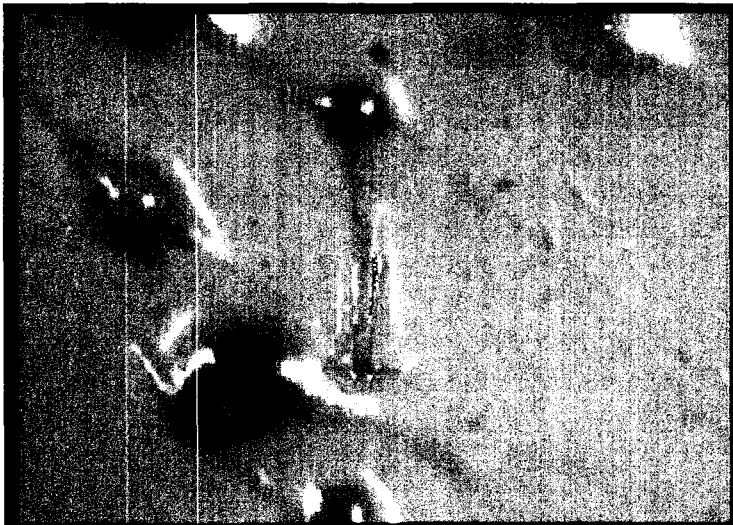


Figure 6 –  
The centered structure is a fully formed vertical sporangiophore. The yellow cap on the top will develop into the sporangium.  
120X

Of course, the most intriguing aspect of *Pilobolus* development is that of the sub-sporangial swelling. Figures 7 through 13 show the swelling as it increases. Small bubbles of liquid are often seen attached to the



sides of the sporangium, swelling, and columella (see figures 7 and 9). This is most likely condensation of moisture in the air surrounding the structures, as they generally grow best in very humid conditions.



Figure 7 –  
This sporangiophore, although shorter than that of Figure 6, is beginning to exhibit slight sub-sporangial swelling. Bubbles of condensation are attached to its sides. 200X



Figure 8 –  
At this point, the sub-sporangial swelling appears only as a widening of the columella just below the yellow sporangium. 250X

The swelling increases, taking on the appearance of a large bubble. As the sporangium matures, it changes from the yellow color to black. This appears to start at the tip of the sporangium and move downward to the base. Figures 10 and 11 display sporangia midway through this color change. In figure 12, the sporangium is black and completely mature. The sub-sporangial swelling has reached its peak in Figure 13 it is prepared to rupture and propel its sporangium a distance of up to two meters.

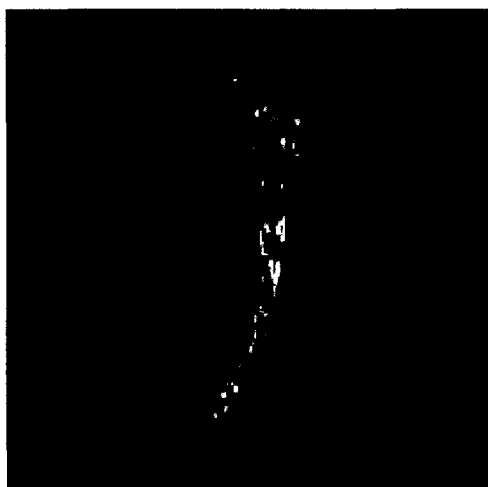


Figure 9 –  
Note increased  
swelling and  
condensation near  
the sporangium.  
70X

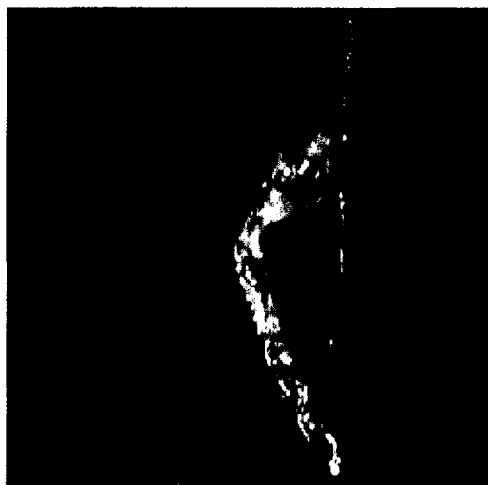
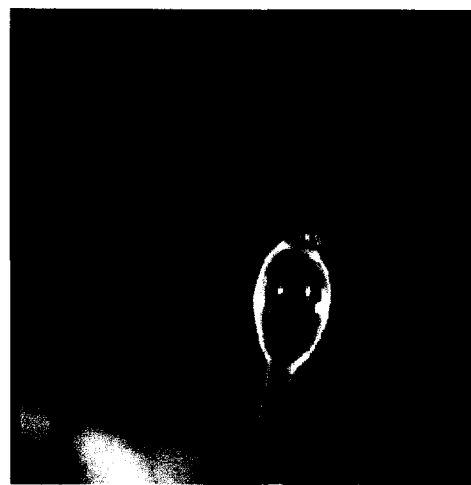


Figure 10 (left)  
and 11 (right) –  
The sporangia  
are now black  
at the tips but  
retain yellow  
bases.  
280X and  
150X,  
respectively



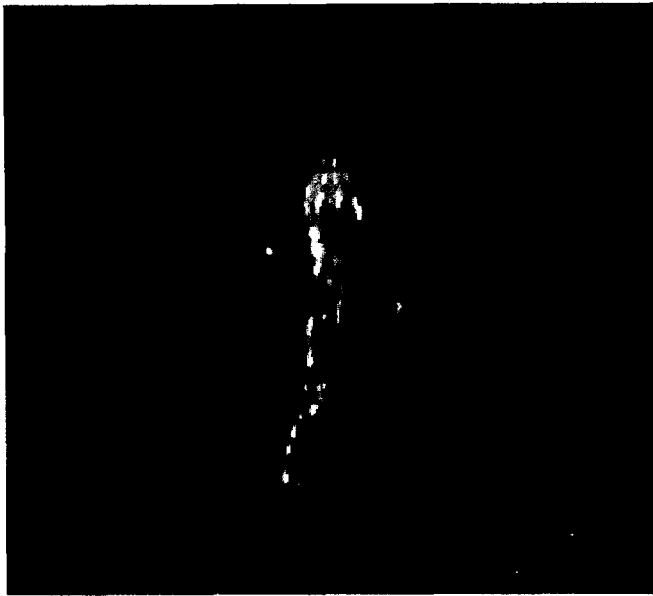


Figure 12 –  
Mature sporangium  
100X

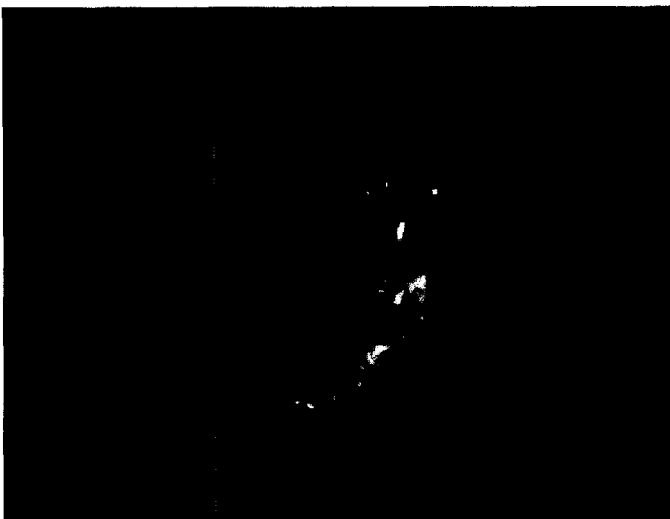


Figure 13 –  
The sub-sporangial  
swelling becomes  
incredibly large,  
making the structures  
appear top-heavy.  
130X

After the sporangium has been discharged, the remaining structure collapses onto itself from the force of the discharge (as discussed in the background information). Figures 14 and 15 show sporangiophores which have very recently released their sporangia. Here, the

sporangiophore is clearly crumpled and twisted, lying flat on the surface of the growth medium. Interestingly, the sub-sporangial swelling has not yet completely flattened. Sporangia presumably are forced off of the sporangiophore by an expulsion of a liquid substance (Page, 1964). It is still possible to see the small hole left at the top of the swelling where this liquid apparently exited.



Figure 14 - 150X  
Discharged sporangium

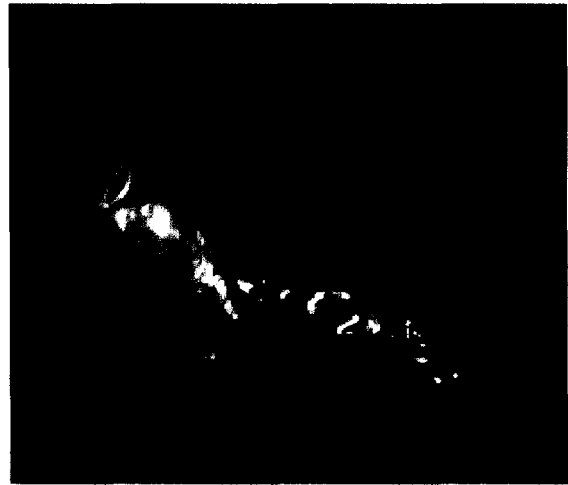


Figure 15 - 150X  
Discharged sporangium

Figure 16 is a photograph of one sporangium that has been discharged and has landed on the plate at some distance from its now collapsed sporangiophore. As noted earlier, it is not a single spore, but a package of spores which must pass through the digestive tract of a grazing animal before the individual spores will be released and able to grow new mycelium. Normally, sporangia do not land

on the agar, as this one has. They are shot off with such force in the direction of their light source that they hit the lid of the petri dish and are usually found stuck to it.

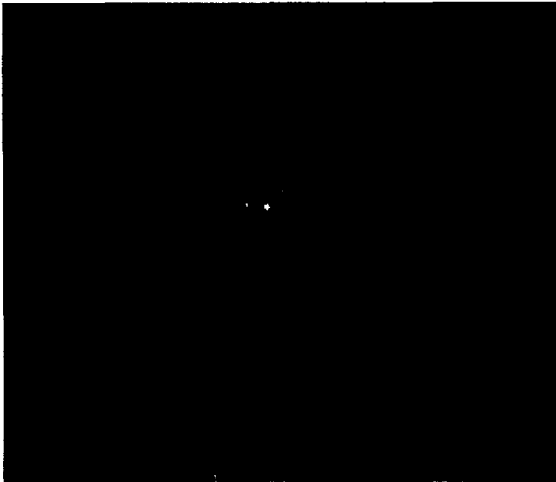


Figure 16 –  
A whole sporangium.  
It contains many  
individual spores.  
150X

This is the conclusion of the light microscopy results. Next, I chose structures representative of each of the stages of development seen with light microscopy and processed them for examination by scanning electron microscopy. SEM allows for more detailed observations of the surface of the structures. The three-dimensional images obtained are shown on the following pages. Also shown is a small light photograph of the same growth stage so that comparisons can easily be made.

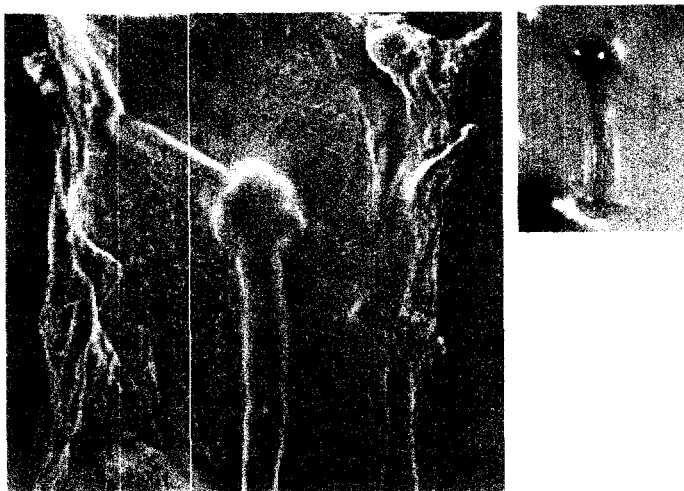


Figure 17 –  
No sub-  
sporangial  
swelling.  
590X

Both of these pictures are the exact same sporangiophore. The black and white photo is the image captured by SEM at a magnification of 590X. As you can see, the structure is now lying flat on the agar, as is true of all the SEM photographs. This occurs when liquids must be poured over the agar during processing, knocking them over, as explained on page 10. It does not appear to alter their appearance other than the fact that they are no longer standing vertically. Figure 17 shows a sporangiophore with an immature sporangium, but no sub-sporangial swelling. A detail noticeable with electron microscopy, but not able to be seen under any sort of light microscope is that the sporangium seems to have another structure coming up around it and cupping the actual globe containing the spores. This newly discovered structure seems to hold the sporangium as it matures. It is not seen in any of the

specimens taken in later stages of the life cycle, suggesting it is only a transient structure and disappears as the sporangium grows. Also notice in Figure 17 that none of the trophocysts or mycelium present in the light photograph can be seen with SEM.

Figures 18 and 19 exhibit sporangiophores with increasing amounts of sub-sporangial swelling. Characteristics made obvious by the SEM include how far separated the sporangium appears to be from the swelling itself. This is especially clear in Figure 18. Unfortunately, these figures demonstrate one of the problems of using electron microscopy to study structures such as these. The chemical drying necessary for a subject to be viewed under scanning electron microscopy collapsed the swelling, and even the columella of the sporangiophore, as they are primarily composed of liquid. When all liquid was removed from the specimens, this apparently released the pressure inside the structure and allowed it to deflate. This is an example of an artifact which is known to be an artifact and therefore can be interpreted correctly to avoid forming false conclusions.



Figure 18 –  
Moderate sub-sporangial  
swelling. The structure is  
deflated, but a small “neck”  
is clearly all that connects  
the sporangium to the  
swelling sporangiophore.  
133X

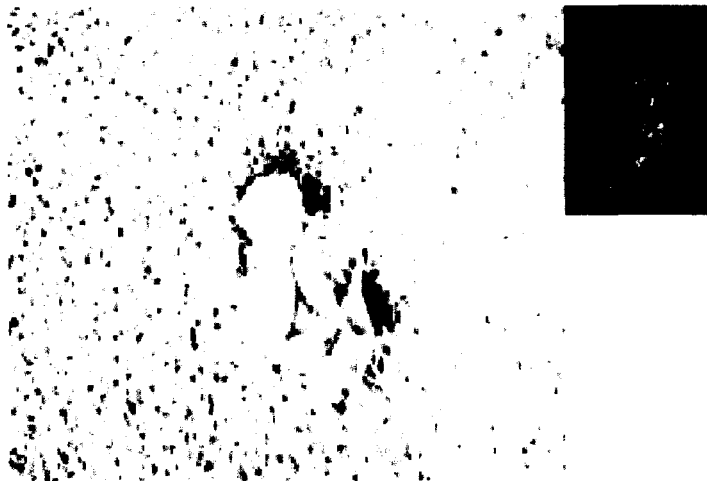


Figure 19 –  
Large sub-sporangial  
swelling. The “neck”  
begins to break down.  
131X

As the sporangium matures and sub-sporangial swelling increases, enaymes begin to degrade the walls physically connecting these structures. This is in preparation for the burst of energy and cytoplasm that will propel the sporangium off of its sporangiophore at the time of discharge. This discharge would not be as effective in attaining a long range of flight if a great deal of the available force had to be used to simply tear away



connective tissue holding the sporangium. The break down of this tissue has probably begun in Figure 19. However, it is much more visible in Figure 20. There, the area just beneath the sporangium no longer seems smooth, but rather appears ragged, as something in the process of being degraded.



Figure 20 – Moderately swelled sporangiophore with mature sporangium. It is showing some degradation of connective tissue beneath the sporangium. 133X

The last two photographs taken under SEM showed something that seemed peculiar – the absence of any changes brought on by chemical processing. This was true of both sporangiophore specimens that had already shot off their sporangia, but held their swelled shape. Even after processing these structures for SEM, although the crumpled columella flattened out more, the swelling primarily retained its bowl-like shape. I cannot explain why, once the sporangium has been released, the swelling apparently does not lose pressure and collapse when fixed and dried

chemically. This is a subject for future studies of this fungus.



Figure 21 –  
Note that in this case,  
only an indentation is  
evident, not total  
collapse and shrinkage.  
681X



Figure 22 –  
This specimen was  
preserved almost  
perfectly as can be seen  
when compared to the  
small picture taken  
before SEM processing.  
657X

Although it is difficult to see on this computer-scanned image, the original photograph of Figure 22 clearly indicates that what appears as a simple hole at small magnifications is actually a sort of plate. A hole is indeed present at the center of this plate, but the opening is only about half of the diameter of the entire plate. A

ring around the outside of this plate shows where the  
connective tissue was previously present. It has a rough,  
irregular appearance due to fragments of broken tissue.  
What resembles a thin cord is wrapped around the  
circumference of the "plate." This is the dominating ring  
that can be seen in both the SEM and light photographs.

## Conclusions

The purpose of this study was to gather information on the asexual development of the fungal genus *Pilobolus*. This information could be useful for classification purposes. Current methods of identification for Zygomycetes are based almost solely on visible morphology. This makes classification guidelines incomplete and somewhat vague. The information presented in this paper can now be combined with DNA analysis (currently being performed by colleagues) to better characterize this group of organisms.

The growth and maturation of the reproductive structures, namely the sporangiophore and sporangium, was followed closely. Many observations were made concerning the details of this reproductive life cycle. Material appeared to exit the trophocyst as time went on, the sporangia were observed to change color midway through their development, and great variation was noted in the size (or height) of the sporangiophore. The shape of the sporangium, while remaining constant for all individuals of an isolate, varied slightly within the genus and therefore should no longer be considered a reliable basis for identification.

The progression of growth, from the formation of the trophocyst through the discharge of the sporangium, was documented in a series of photographs. When viewed in sequence, these provide a fairly continuous account of the asexual life cycle of *Pilobolus*.

Several of the specimens, preserved at distinct developmental stages, were also viewed using scanning electron microscopy. This technique enabled me to examine the surfaces and shapes of these structures in great detail. Aspects of the sporangiophore not seen under light microscopy became noticeable under SEM. Of particular interest were the details seen at the point of contact between the sporangium and the swelling columella just below it. The sporangium was actually separated from the sub-sporangial swelling by a band of tissue a few micrometers wide. Also, degradation of this structure during the fungi's late stages of development occurred, not as a weakening of individual cells. Instead, it looks as though it may be done on a larger scale with destruction of large patches of tissue. This would give the area a crude patchy look, like that observed. All of these aspects were also recorded by way of photographs.

This investigation's use of both light and electron microscopy served another purpose. At several points in

the above discussions, concern has been expressed over the problem of artifacts arising in specimens as a result of chemical manipulations or preservation techniques. When multiple approaches are used to study an organism, the results can then be compared and these collaborative results are much more likely to produce valid conclusions. This is because the results obtained by one method can be checked against the results obtained by the other. This way, it becomes plain which observations are in fact valid and which are artifacts of one of the methods used. In this way, using two or three methods of investigation reduces the likelihood that artifacts will be wrongly interpreted as new discoveries.

I believe that all of the observations presented in this paper are indeed valid. However, these observations alone are not enough to distinguish the genus *Pilobolus* from the other two genre of its family, *Pilaira* and *Utharomyces*. For a new monograph (scientific classification guide) to be written, studies comparable to this one also need to be performed on each of these other two genre. A single study only identifies characteristics of one genus that may or may not distinguish it from the other genera. However, when put together, the three

investigations might clarify differences between each genus.

The eventual goal of our laboratory is to compose a complete new monograph for this fungal family. With the current guides, there is often much debate when classifying these species. While we are not investigating differences between species of fungi, we are attempting to draw clearer lines between the genera. Hopefully, this study, in detailing the asexual development of *Pilobolus*, will be useful as a small part of that process.

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The preceding paper was written specifically as an Honors Thesis. The following pages are what I will be submitting for publication.

## ABSTRACT

This study was done for the purpose of examining and documenting the development of the fungus *Pilobolus*, specifically its asexual reproductive structures. These structures, the sporangia, were comparatively photographed at several stages in their growth under both light and scanning electron microscopy (SEM). Although this part of its life cycle is generally known, no one has ever done a comparative developmental study or photographed in detail these structures. The information gathered will ultimately be used in conjunction with DNA analysis and other such data collected by others for the purpose of writing a new monograph for the entire family. The existing monograph for these organisms is strictly morphological.

## INTRODUCTION

*Pilobolus* grows mainly on the dung of animals such as cows and horses. For asexual reproduction, it grows a long sporangiophore with a sporangium bulge at the top(1). The swelling of the sporangium increases overnight until mid-morning when it is violently shot off in the direction of the sun(2). Upon landing, the entire sporangium will then attach itself to a blade of grass. This projection method can fire the sporangium as far as two meters(3), placing it

can fire the sporangium as far as two meters(3), placing it far enough away from the dung itself to be eaten by a grazing animal(4). Passage through the animal's digestive tract is what releases the spores from the sporangium, and the life cycle is completed when the spores are excreted in the dung of the animal. Although this life cycle is generally known in the scientific community, *Pilobolus* development has never been photographed or formally studied.

For this study, the fungus was grown on agar and fixed several different developmental stages. They were then photographed under light microscopy and Scanning Electron Microscope (SEM). With both light microscope and scanning electron microscope photographs of several different stages in the growth and swelling of the sproangiophore, an exact account of the asexual phase of *Pilobolus* development could then be reported.

#### MATERIALS AND METHODS

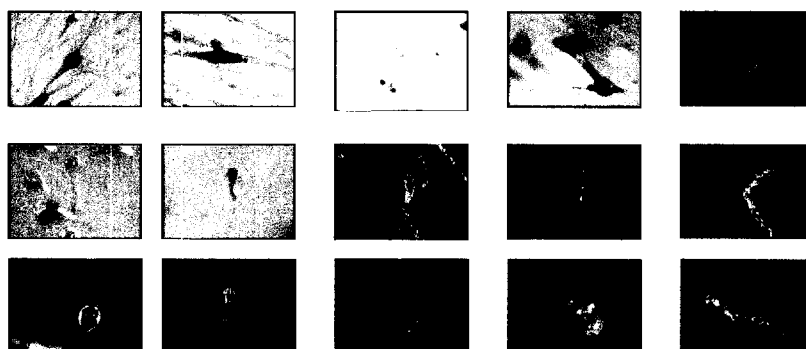
**Isolates** Specimens were obtained from Dr. Michael Foos of Indiana University East, Richmond, IN. They were grown on dung agar and fixed by diffusion of 25% gluteraldehyde through the growth medium.

**Light Microscopy** Fixed specimens were examined with an Olympus SZ90 Stereo zoom microscope. Images were viewed with an ATI TV Player 6.0 and captured on a Gateway E4200 computer.

**SEM** Chosen structures were dried by ethanol series and hexamethyldisilazane. They were coated with gold-palladium and examined with a Cambridge Model S90 Scanning Electron Microscope(5). Photographs were captured on Polaroid 4X5 Instant Sheet Film.

#### RESULTS/DISCUSSION

Figure 1: Photographic results of all light microscopy (150X-300X)



The top row shows formation of the trophocyst and vertical growth of the hyphae destined to become the sporangiophore. In the fourth and fifth pictures, the sporangiophore has emerged from the medium and is growing toward the light source. The second row demonstrates

development of the sporangium as it appears on top of the sporangiophore, begins a yellow color, and then gradually turns black from the tip to the base of the sporangium. Finally, the bottom row exhibits different stages of sub-sporangial swelling. The final two pictures (bottom right) are sporangiophores that have very recently discharged their sporangia and have collapsed onto the growth media.

It is clear now that sporangiophore length varies greatly within a species and is not useful for identification purposes. Likewise, trophocyst shape varies from spherical to quite oblong. Also, the color of the sporangia change as they mature, so this too cannot be considered a tool for classification.

Figure 2: Photographic results of SEM (150X-650X)



A couple of observations were made upon viewing the specimens under SEM that could not be seen at lower magnifications. The sporangium seems to have another structure cupping around it and holding the actual globe containing the spores when it is first forming the sporangium. It is only a transient structure and disappears

as the sporangium matures. A "neck"-like structure is then seen (second photograph from the left) separating the sporangium from the sub-sporangial swelling. This is presumably made of connective tissue that secures the sporangium.

The enzymatic breakdown of this tissue can be seen in the fourth photograph. It seems to occur in large patches, giving the "neck" structure a ragged appearance. Examination of sporangiophores that no longer have a sporangium attached reveals that remains of such tissue is present in a ring with the center being hollow. It is not known whether the structure is always hollow or if the middle portion simply degrades completely, allowing fluid to burst from the swelling and propel the sporangium off the parent structure.

#### CONCLUSIONS

Current methods of identification for Zygomycetes are based almost solely on visible morphology. This makes classification guidelines incomplete and somewhat vague. The information presented in this paper can now be combined with DNA analysis (currently being performed by colleagues) to better characterize this group of organisms.

The progression of growth, from the formation of the trophocyst through the discharge of the sporangium, was documented in a series of photographs. When viewed in sequence, these provide a fairly continuous account of the asexual life cycle of *Pilobolus*.

Several of the specimens, preserved at distinct developmental stages, were also viewed using scanning electron microscopy. This technique enabled me to examine the surfaces and shapes of these structures in great detail. Aspects of the sporangiophore not seen under light microscopy became noticeable under SEM.

The eventual goal of our laboratory is to compose a complete new monograph for this fungal family. With the current guides, there is often much debate when classifying these species. While we are not investigating differences between species of fungi, we are attempting to draw clearer lines between the genera.

Unfortunately, these observations alone are not enough to distinguish the genus *Pilobolus* from the other two genera of its family, *Pilaira* and *Utharomyces*. For a new monograph to be written, studies comparable to this one also need to be performed on each of these other two genera.

## ACKNOWLEDGEMENTS

I would like to thank Dr. Michael Foos for providing the isolates and some of the equipment needed for this study. Also, Mark Robbins for technical assistance, and Dr. Don Ruch for supporting the work in a variety of ways.

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