EXPERIMENTAL STUDY OF GROWTH PARAMETERS OF PHYCOMYCES BLAKESLEEANUS DURING PHOTOTROPIC AND AVOIDANCE RESPONSES

by

REVATHI PRIYANKA MOHAN

B.E., VISVESVARAYA INSTITUTE OF TECHNOLOGY, INDIA, 2017

A thesis submitted to the

Faculty of the Graduate School of the

University of Colorado in partial fulfillment

of the requirement for the degree of

Master of Science

Department of Mechanical Engineering

2019

This thesis entitled:

Experimental study of growth parameters of Phycomyces blakesleeanus during phototropic and avoidance responses

written by Revathi Priyanka Mohan

has been approved for the Department of Mechanical Engineering

_____

Prof. Franck Vernerey

_____

Prof. Joseph K.E. Ortega

_____

Prof. Virginia Ferguson

Date_____

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

Mohan, Revathi Priyanka (M.S., Mechanical Engineering)

Experimental study of growth parameters of Phycomyces blakesleeanus during phototropic and avoidance responses

Thesis directed by Prof. Franck Vernerey

Phycomyces blakesleeanus depicts helical growth during steady state as well as under the influence of stimuli in the form of light, barrier, wind, etc. Previously, researchers have studied helical growth of Phycomyces under steady state growth and light response, however no attempts have been made to study the elongation rate, rotation rate and bend rate during phototropic and avoidance responses. The research conducted here involves experimentally determining growth parameters that occur as a response to applied stimuli. The elongation rate as well as rotation rate seem to have increased after the application of stimulus. This increase in elongation and rotation rates is hypothesized to be the influx of a biomolecule to the region of the growth zone that is subjected to the stimulus resulting in breakage of load bearing bonds on the cell wall. Through this research one can better explain cell wall expansion models and account for expansion as a result of stimuli.

Acknowledgement

This thesis would not have been possible without the advice and supervision of Prof Franck Vernerey who guided me through each stage of my research. I would also like to thank Prof Joseph K.E. Ortega, whose mentorship in setting up and performing the experiments was monumental in completing this thesis.

I would like to express my gratitude to the members of the Soft Matter Mechanics Lab: Dr. Eduard Benet, Shankar L.S., Tong Shen, Abhishek Das and Guillaume Lostec who supported me along the way, helping me with construction of experimental setup, image and data analysis.

Lastly, I would like to thank my family, friends and fiancé Hari for standing by me and showering me with immense love, faith and support.

Table of Contents

Chapter

List of Figures

Chapter 1

Introduction

Researchers have extensively studied Phycomyces Blakesleeanus because of their growth responses to light, stretch, barrier, wind and other stimuli. Among the five stages of growth that the Phycomyces depicts stage IV b depicts the most constant growth zone length making it the ideal stage in which experiments can be conducted. When a stage IV b sporangiophore is placed between two light sources equidistant from it, the sporangiophore will show a transient increase in its growth rate this is the light growth response [1] [2] , if one of the light sources is turned off then the sporangiophore begins to bend towards this light source this is the phototropic response. If a stage IV b sporangiophore is placed parallelly in the proximity of a barrier for example a microscope cover slip and within 1 mm of the stalk it begins to grow away from it after a period of 3 minutes, this is termed as the avoidance response [1] [3]. Although an extensive number of studies have been conducted on these responses not much is known on the effect these responses have on the growth rate of the sporangiophore.

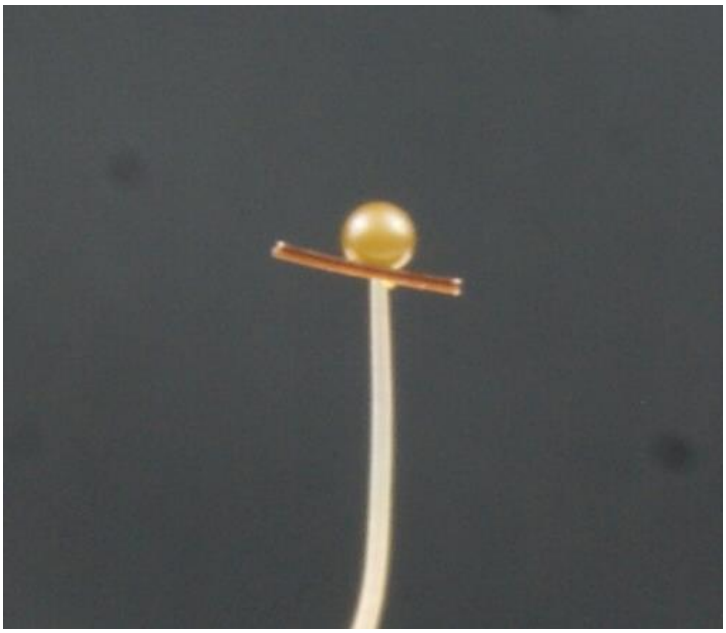

Figure 1: Stage IV b Phycomyces Blakesleeanus sporangiophore with a marker below the sporangium. Image courtesy: Revathi Priyanka Mohan

Stage IV b sporangiophores depict clockwise rotation about its axis and along the growth zone, this results in a left-handed helix and researchers termed this as ‘helical growth’ [4]. A spec of starch or any small particle placed on the sporangium will show left-handed helix formation with time. R measures the steepness of this helical growth and is calculated as rotation rate ÷ elongation rate at every instant of time. Obviously, if R is large the helix is flat, while R is small the helix is steep. During steady growth R is dependent of elongation [5], however no studies have been conducted on R and its behavior during sensory responses.

This thesis focuses on the phototropic and avoidance response and their effect on the rotation rate, elongation rate and helical growth steepness (R) of the sporangiophore. Upon receiving and reviewing the results of these conducted experiments one can better understand helical growth of sporangiophores and facilitate expansion of cell wall expansion models and fibril reorientation slippage mechanism theory.

Chapter 2

Literature review

This section contains a detailed study of the lifecycle of Phycomyces blakesleeanus, starting from its germination to mycelial growth followed by an elaborate description of the stages of growth of the sporangiophore. Phycomyces blakesleeanus sporangiophore shows a variety of responses across all five stages of its growth. Previously, a number of studies have been made on the responses to better understand how they impact the growth, essentially the elongation rate of the sporangiophore.

2.1. Life cycle of Phycomyces blakesleeanus

Phycomyces has two life cycles: a sexual one and a vegetative one. The sexual cycle has spores resistant to unfavorable conditions while the vegetative cycle has spores designed for efficient dispersal. The figure below depicts both its vegetative, as well as sexual life cycle. The fungus is commonly found in the wild, and domesticated areas growing almost anywhere from a decaying wasp nest to dead wood, and dung. The following sections discuss its spore germination, mycelial growth and stages of development [1].

2.1.1. Spore germination and Mycelial growth

The vegetative spores of Phycomyces blakesleeanus are ellipsoid, immotile cells surrounded by a thick wall in sizes ranging from 8 by 5 µm [6]. When the spores are under suitable conditions of growth (presence of salt, carbon and nitrogen sources) along with heat shock treatment(temperatures of 48 to 53 C [1] [7]) 90% of them activate. Spores can also be activated using chemicals like acetic acid, pyridine acetaldehyde, pyruvic acid and different natural extracts [8] [9]. The above stated methods are used after spores exhibit dormancy despite heat shock

treatment. Sometimes, multiple heat shock treatments may harm spores resulting in irreversible dormancy.

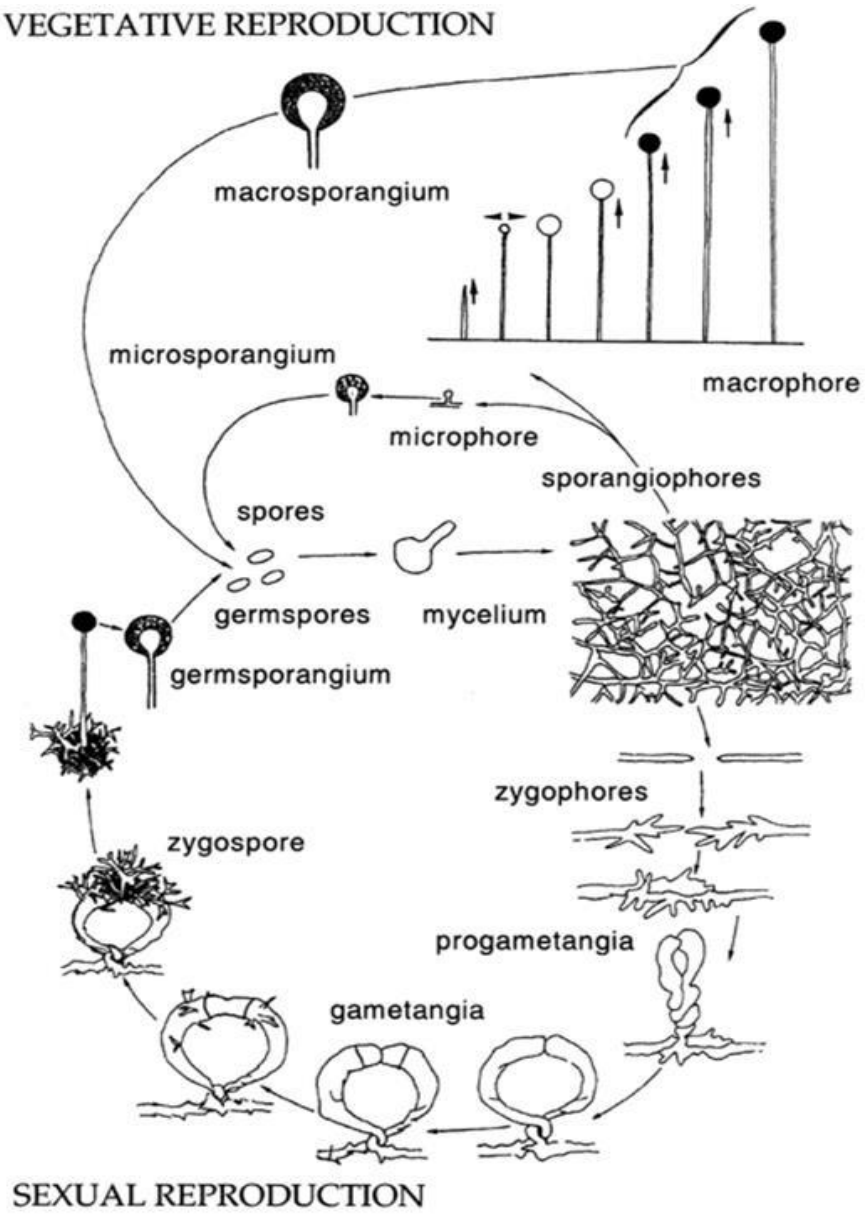

Figure 2: The life cycle of Phycomyces Blakesleeanus [3]

Once activated, the spores germinate, they begin to swell as many vacuoles take shape and coalesce to form one large vacuole followed by the emergence of germ tubes to form the hyphae. The hyphae, when placed in culture mediums made of potato dextrose agar branches out and the mycelium begins to grow.

Mycelium tends to spread out radially from the spore site and takes about two to three days until sporangiophores emerge. To ascertain good mycelial growth an optimum pH and temperature of 4.0 and 15-25° C respectively needs to be maintained [10].

2.1.2. Sporangiophore: Stages of development

The sporangiophores are large single celled and are comparable to tiny vertical cylinders. Its growth and development have been divided into five stages, to better understand the transition from one stage to the next. Sporangiophores grow up to 12 cm at the end of their fifth stage of development with varied growth speeds at each stage. They elongate and also show a peculiar spiraling about their axes which is further discussed below. Every sporangiophore has a growth zone in which all growth or wall extensibility occurs [11]. The length and position of this growth zone varies with each stage described below:

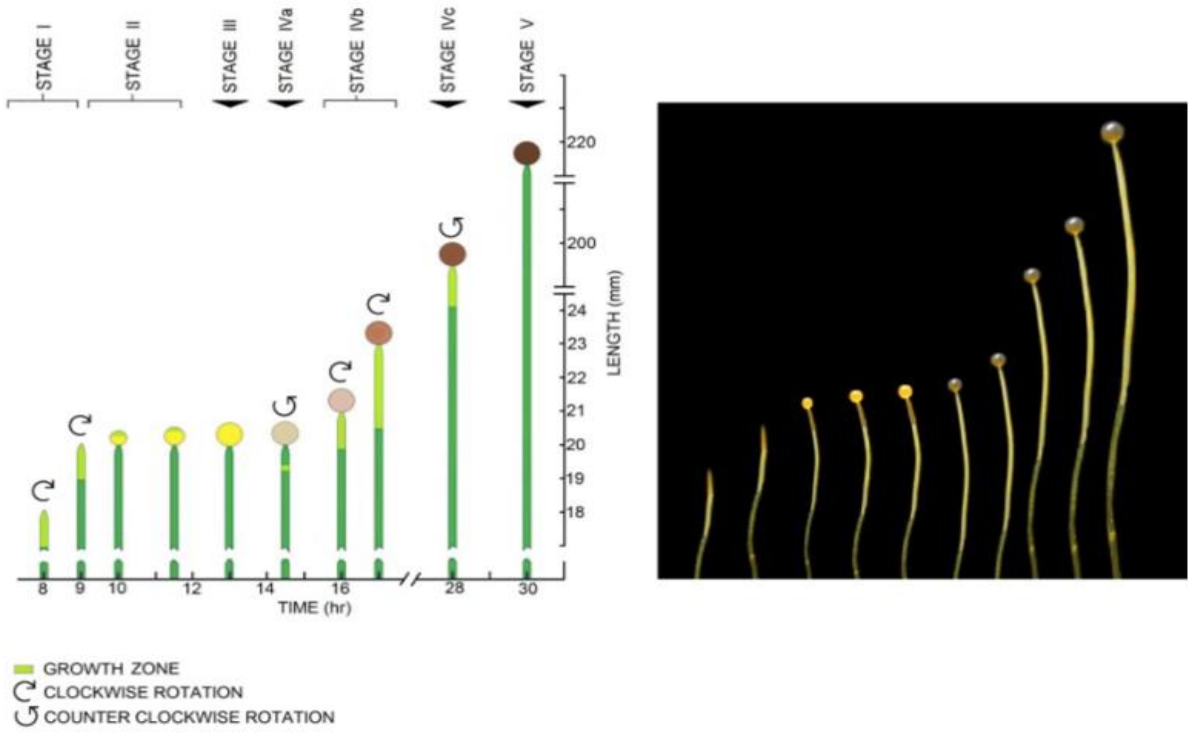

Figure 3: The plot on the left depicts each of the five stage with the duration, growth zone length and direction of rotation. The image on the right depicts a live sporangiophore across the five stages [12]

Stage I: Tiny aerial hyphae (sporangiophores) emerge and begin to grow vertically. The sporangia or head has not appeared yet. The growth zone begins at the tip and is 1mm long [13]. The cell wall extends at the rate of (5-20 µm/min) and demonstrates a left handed or clock-wise spiraling when viewed from above [5]. Stage I sporangiophores grow up to a length of ~3 cm with a diameter of 0.15-0.2 mm [5]. This stage lasts about 8 to 9 hours.

Stage II: The second stage of the sporangiophore development constitutes the formation of the sporangium or the spherical head. This stage marks the pause of both elongation and rotation of the sporangiophore. As time progresses a bright yellow sporangium rich in carotenoid begins to form [11]. This sporangium continues to grow for three hours and depicts the beginning of spore formation.

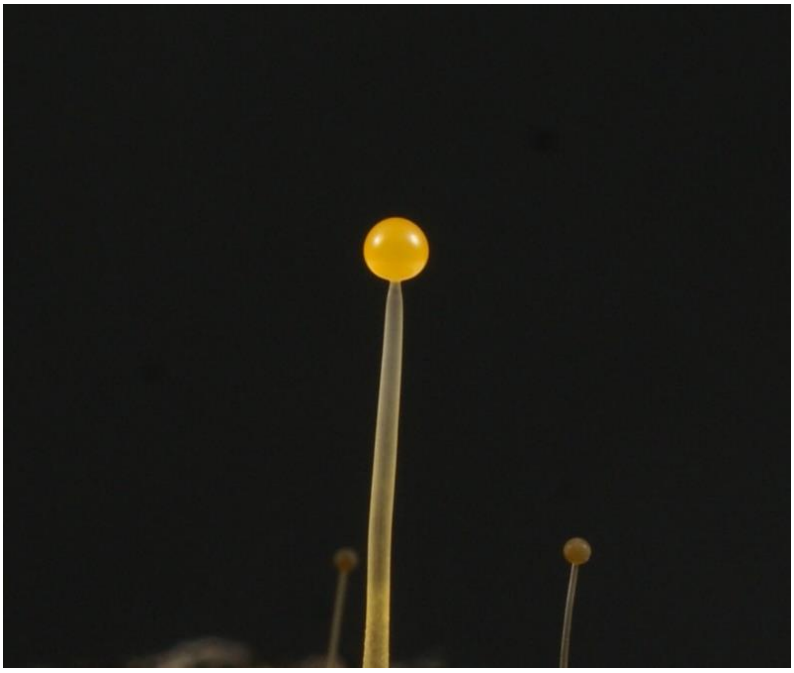

Figure 4: Stage II sporangiophore with a carotenoid rich sporangium. Image courtesy: Revathi Priyanka Mohan

Stage III: This stage shows no clear growth, both elongation as well as spherical growth freeze. One can assume that spore formation occurs in this stage in the swollen sporangium formed in the previous stage.

Stage IV is divided into three sub stages to better map the growth of the sporangiophore. These stages are namely stage IV a, stage IV b and stage IV c. The highly discussed reversal in rotation of the sporangiophore occurs in this stage.

Stage IV a: This stage marks the end of dormancy of the sporangiophore with elongation and anticlockwise rotation starting to take place in a zone right below the head known as the growth zone. It is located 0.6mm below the sporangium [5]. It is recorded to last an hour before the rotation rate begins to reduce to accommodate the reversal in rotation. The sporangium begins to turn dark brown.

Stage IV b: As the rotation rate drops to zero it indicates the start of reversal in rotation, this stage shows clockwise rotation of the sporangiophore axis and lasts long enough to permit experimental studies of the sporangiophore. In this stage the sporangiophore grows up to 15-20 cm with constant elongation rate and rotation rate reaching constant values of 50 µm/min and 12 deg/min respectively [5]. During the transition of spiraling between the two stages there is an increase in elongation rate of the sporangiophore, along with an increase in length of the growth zone. If stage IV sporangiophores are picked a new crop of sporangiophores emerge the next morning [1].

Stage IV c: This stage marks another reversal of rotation to anticlockwise. These sporangiophores reach a maximum height of 15 cm with a diameter of 100µm.

Stage V: The final stage of sporangiophore growth denotes the end of the all visible growth and the death of the sporangiophore.

2.2. Responses of sporangiophore

The sporangiophore shows a number of responses to a variety of stimulus, namely, light, wind, stretch, gravity, proximity etc. It reacts to these stimuli with a positive or negative change in current growth rate. All responses to stimuli tend to occur in the growth zone of the Phycomyces (2-3 mm in length). For the purpose of this thesis, two responses have been studied in detail and the relationship between different parameters affected by this response with respect to time is also elicited.

2.2.1. Light growth response

Light is said to alter the cell growth of the Phycomyces in time and space dramatically. The sporangiophore not only detects light and responds to it by growing towards the source, with a momentary increase in light it also depicts momentary spurt in growth [14]. This is called the light

growth response. The sporangiophore also depicts phototropism which is explained in further detail in the following sections.

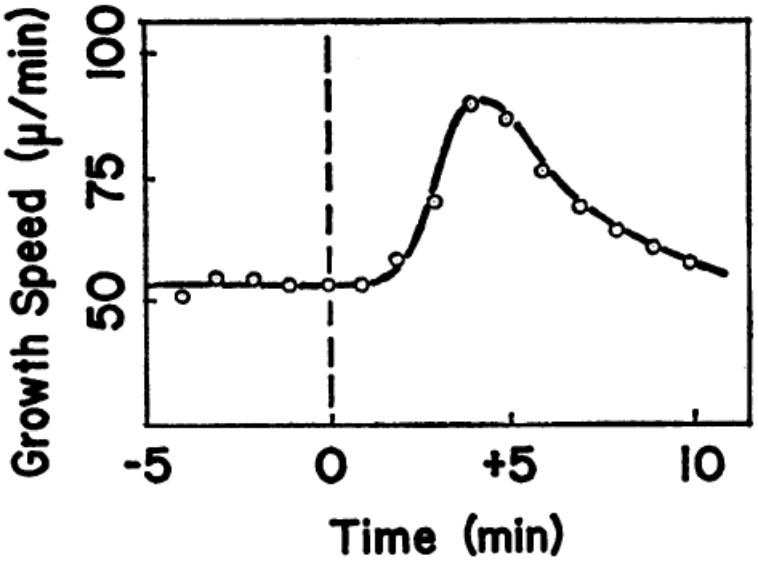

Figure 5: Light growth response seen after time = 0 mins when the incident light flux was increased ten fold [15]

Increase in growth, response to light is a phenomenon restricted to a zone right below the sporangium called the growth zone. The tip contains yellowish pigment that absorbs light ranging in the wavelengths of 230 to 500nm [16]. The sporangiophore does not require light to irradiate the entire surface of the tip, light (equally distributed) on a portion of its length can act as a stimulus and facilitate growth spurt or even bending (unequal distribution, as is the case of phototropism) as a response. Once light is shone on the stalk the cell reacts to it symmetrically, there is a delay of 4 minutes after which the sporangiophore responds with an increase in elongation rate to 50 um/min. If the light intensity is constant the growth speed begins to standardize over a period of 15 minutes [15]. This reaction of the sporangiophore is a form of adaptation. Light growth response is typically seen in stage I and IV sporangiophores. The sporangiophore's growth and adaptation is dictated by light intensity.

2.2.2. Phototropic response

When the sporangiophore is radiated unevenly (unequal light distribution) it begins to grow in the direction of the light source this is a form of light response called phototropism. The Phycomyces growth zone is comparable to a transparent cylinder. Phototropism and bending are a result of unequal distribution of light in two halves of the aforementioned cylinder. This is called lens effect; incident light focuses on the distal side of the sporangiophore causing a stimulation in on the distal wall to a greater extent as compared to the proximal side growth by attracting a biomolecule that absorbs light to promote growth in turn resulting in bending of the sporangiophore. This was proved by Shropshire by immersing a sporangiophore in media of refractive index greater than the growth zone causing the convex cylinder to act concave going by the rules of reflection [17] [18].

Figure 6: A stage IV b sporangiophore exposed to light on the left side causing it to bend towards the light source. The three images represent the increase in bend angle with time first image being clicked at t = 10 mins, second at t = 30 min and third at t = 50 min with one light being turned off at t= 10 mins. Image courtesy: Revathi Priyanka Mohan

The bending reaction of the sporangiophore to light is attributed to a pigment/photoreceptor whose chemistry is still unknown. The intensity difference between proximal and distal side causes the photoreceptors to absorb more light and react or expand more on the distal side causing phototropic curvature. When a sporangiophore is moved from steady light to unevenly distributed light, it begins to bend, this bend rate increases over time reaches a peak and begins to fall to original value. One of the goals of this thesis is to study the effect of phototropism on the relationship between the different growth parameters.

Phycomyces also depict phototropic inversion, during bending if there is sudden decrease in light intensity or complete darkness it begins to show reversal in bending in the same bending plane [19]. Phototropism is interlinked to gravitropism which dictates the extent to which a sporangiophore opposes and grows against gravity.

2.2.3. Avoidance response

Phycomyces grow away from objects which are placed close to it, this is called avoidance response. The barrier which initiates this response can be made of any material (glass, wood, plastic, etc.,) and shape. The response begins 3 minutes after the barrier is placed, the optimal distance is placed 2mm away from the sporangiophore. With reduction in distance between the sporangiophore and the barrier the bend rate increases. When the sporangiophore is placed in between two barriers (symmetrically) there is a transitory increase in growth [20]. This further proves that placing a single barrier causes an increase in growth on the side nearer to the barrier.

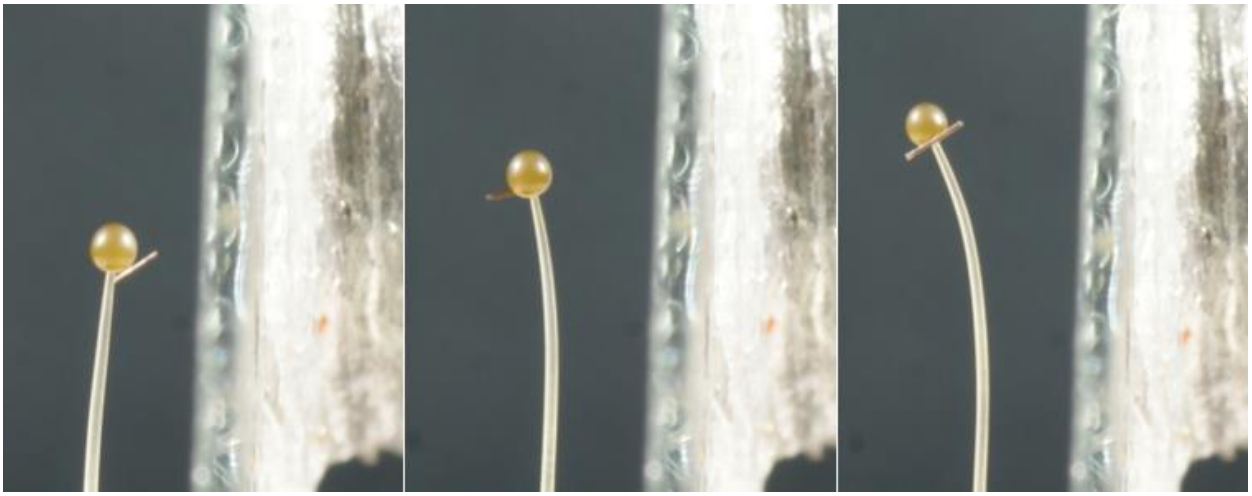

Figure 7: A stage IV b sporangiophore subjected to a barrier on the right as seen in the above series of images causing it to bend away from the barrier. This is the avoidance response. Starting from the left each image was taken at t = 10 min, t = 30 min and t = 50 min. Image courtesy: Revathi Priyanka Mohan

It has been stated that the growing zone emits a certain type of gas (at higher concentration on the proximal side to barrier) resulting in a concentration gradient which in turn is said to cause bending along with increasing the growth rate [1]. Factors such as humidity, light and the aforementioned material type and shape of the barrier do not affect the response [20].

2.2.4. Wind response

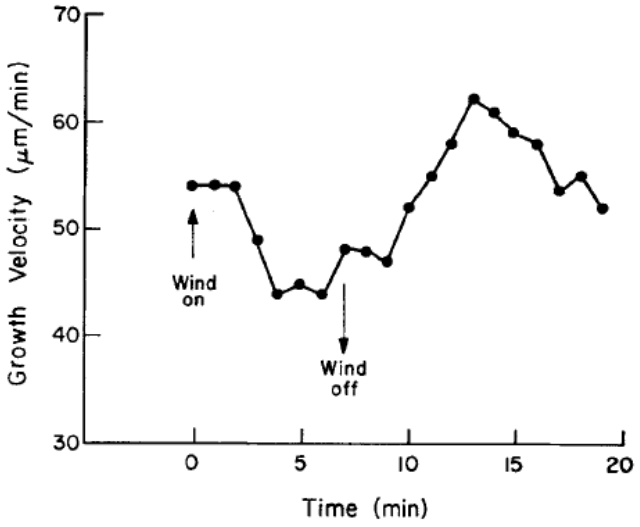

Figure 8: Growth velocity vs time of a sporangiophore subjected to the wind response [21]

When an air current of speeds as low as 5-15 cm/s is applied on the sporangiophore it depicts a transient reduction in growth rate, when the same current is now turned off the sporangiophore depicts an increase in growth rate and tends to straighten itself [21]. As the wind speed decreases the negative effect on growth also begins to decrease. If a lateral wind current is used it can also cause a tropism of around 5-6º.

2.3. Growth zone of sporangiophore

The growth zone of a sporangiophore is a part that lies exactly below the sporangium where all elongation or cell wall extension occurs. The length of this zone varies with each stage along with its response to stimulus in the form of pressure and stretch. Stages I and IV b depict largest elongation rates in the growth zone i.e. 0.3-1.5 mm/hr and 1.5-3.6 mm/hr respectively [22]. Along the length of this growth zone the elongation and cell extension decrease as one moves away from the tip.

Figure 9: The images above represent the top view of the sporangiophore with markers placed on the growth zone. The thicker marker (marker 1) is placed closer to the sporangium or the top most part of the growth zone and the thinner marker (marker 2) is placed farther below it. Starting from the left, the first image was clicked at t = 1 min, the second at t = 15 min. when the sporangiophore is subjected to uniform light placed above it. It is evident that marker 1 (placed closer to the sporangium) has rotated more as compared to marker 2 for a total duration of 15 mins. This experiment indicates that the growth zone portrays different rotation rate along its length. Image courtesy: Revathi Priyanka Mohan

It is not just elongation rate that varies along the length of the growth zone, rotation rate along with R, which is defined as rotation rate ÷ elongation rate also varies. Experimental evidence from Ortega, Harris and Gamow proved that elongation rate diminishes faster than rotation rate when plotted against increasing distance of growth zone from the tip [23]. This means rotation rate exists in the lower region in the absence of elongation rate [24]. Spiraling of the sporangiophore is attributed to these fibrils, at the bottom portion of the growth zone these fibrils are transversely oriented as the sporangiophore stretches the fibrils aid in elongation and rotation by stretching longitudinally in a diagonal fashion resulting in a left-handed spiral in the case of stage IV b. This finding laid the foundation to the Fibril reorientation mechanism theory which explained helical growth of the sporangiophore [23].

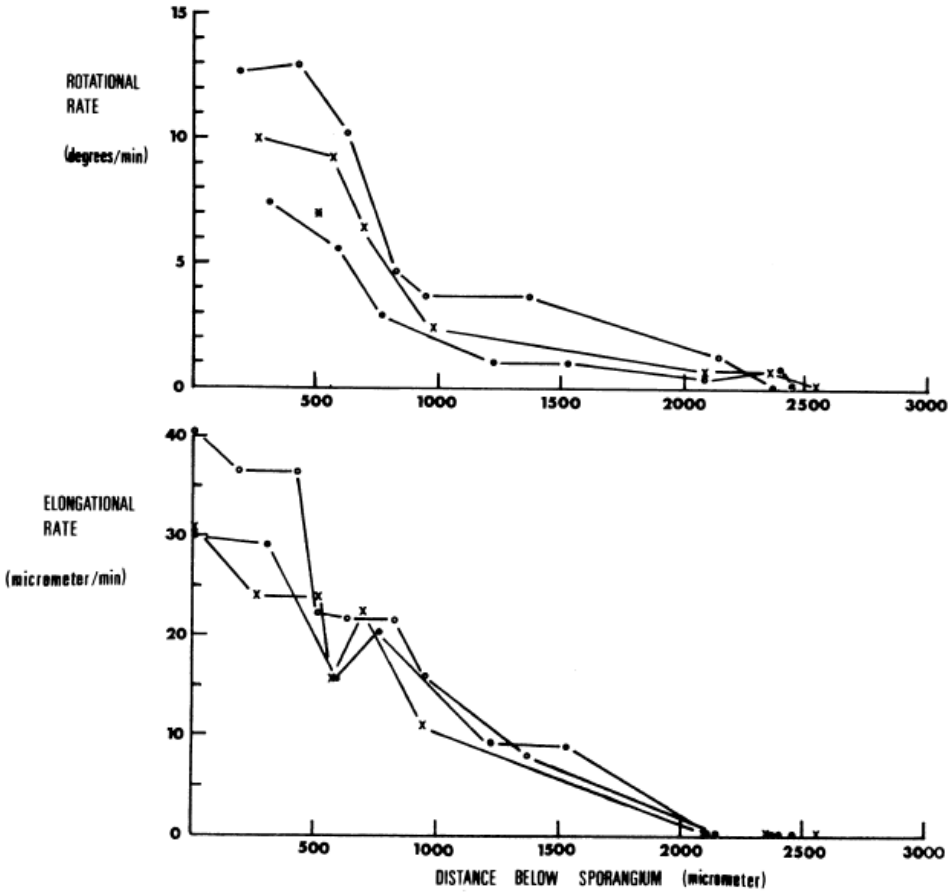

Figure 10:According to the fibril reorientation theory rotation rate exists in regions of the growth zone farthest from the sporangium in the absence of elongation rate. From the above plots it is clear that at a distance 2500 µm from the sporangium rotation rate exists in the absence of elongation rate. While at 100 – 500 µm below the sporangium only elongation rate exists. [23]

However, this theory was based on R being independent of elongation rate if proved wrong changes need to be made to the theory. It was experimentally verified that R is in fact dependent of elongation rate, meaning rotation rate and elongation rate do not share a constant relationship. This led to the postulation of the Fibril reorientation-slippage mechanism theory.

2.4. Fibril reorientation slippage theory

After the assumption that R is independent of changes in elongation rate was proved wrong to show that R decreases with increase in elongation rate, the fibril reorientation theory needed to be modified. R was tested before and during the pressure response to reveal that R is larger during pressure response than during steady elongation rate. The table below shows us the experimental results from the conducted test.

Parameter	Before Pressure response	During Pressure response
Elongation rate (µm/min)	45.9±2.6	19.0±1.5
Rotation rate (degrees/min)	13.8±0.7	6.6±0.5
R (degrees/µm)	0.31±0.02	0.35±0.01

Table 1: The above table has been extracted from the Fibril reorientation slippage theory which aims to prove that R is in fact dependent of elongation rate. [5]

If a light stimulus is applied the sporangiophore responds by forming a new growth zone region at the farther end of the growth zone from the sporangium where measurable elongation occurs in the absence of rotation these sporangiophores are fast growing sporangiophores. It can be said that the relationship between rotation rate and elongation rate is different for fast and slow growing sporangiophores.

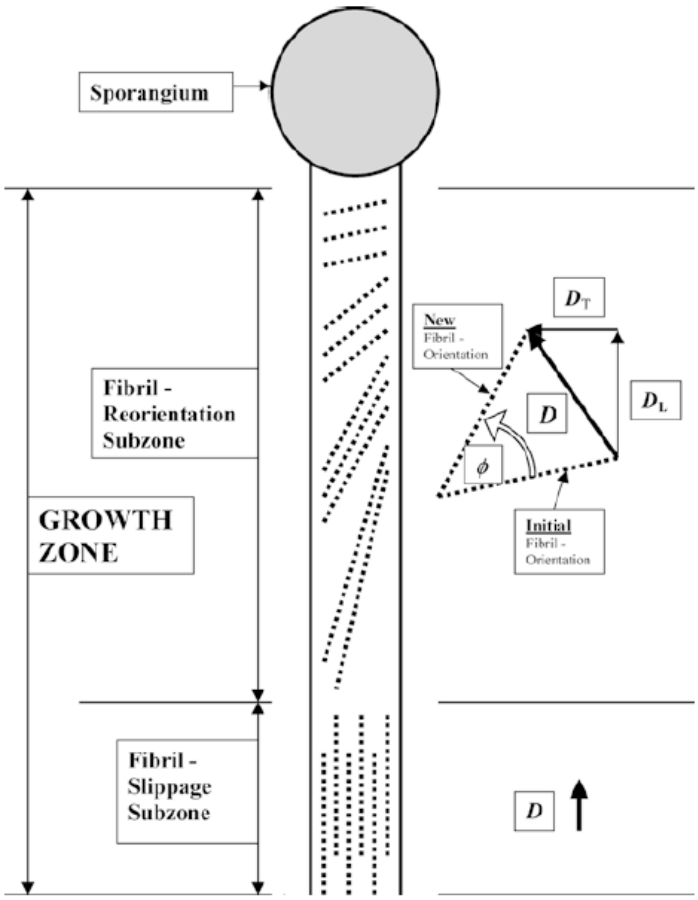

Figure 11: An illustration depicting Fibril reorientation slippage mechanism. The growth zone of a sporangiophore consists of two regions namely, the fibril reorientation zone and the fibril slippage zone. The fibril reorientation zone consists of transversely oriented fibril which cause both elongation and rotation. The fibril slippage zone consists of longitudinally oriented fibrils which result in elongation only. This is further explained the relevant section. [5] [23]

Consider the figure above, it explains the helical growth in fast growing sporangiophores. Microfibrils are represented by the dotted lines. It is seen that the growth zone is divided into two zones namely, the fibril reorientation zone and the fibril slippage zone. In the fibril reorientation zone, it can be seen that the fibrils are oriented diagonally and begin to orient longitudinally to cause both irreversible deformation (elongation) as well as rotation due to reorientation from diagonal to longitudinal position this formulates the helical growth. The fibril slippage zone exists in fast growing sporangiophores, it consists of longitudinally oriented fibrils which cause

irreversible deformation of the cell wall (elongation) it does not cause any rotation and represents the region of the growth zone farthest from the sporangium. [5]

It is safe to say that slow growing sporangiophores only possess the fibril reorientation zone while fast growing sporangiophores contain both fibril slippage and fibril reorientation zones. Therefore, one can say that R is smaller in the case of fast growing sporangiophores than slow growing sporangiophores. Fibril reorientation slippage theory predicts that R increases with distance from the sporangium [5].

Chapter 3

Scope

This thesis aims to answer the questions on impact of responses on elongation rate and rotation rate. Although many researchers have previously studied the responses of Phycomyces blakesleeanus to a variety of stimuli, no one has studied the implications of these stimuli on the growth of a sporangiophore. By understanding these growth responses one can further expand existing models of cell expansion and bending.

The fibril reorientation slippage mechanism theory successfully explained the helical growth in fast and slow growing sporangiophores and the dependence of R on elongation rate, however it does not explain the helical growth of a sporangiophore during uneven light distribution or phototropism and avoidance response. Helical growth during the aforementioned responses maybe influenced by the bending caused by these stimuli. The intention behind these studies is to measure elongation, rotation and bend of the sporangiophore overtime to accommodate changes in existing models of fungal growth.

The Vernerey group has proposed a statistical model to explain the helical growth observed in plant and fungal cells. This model explains helical growth of Phycomyces blakesleeanus using a network of tethers and fibrils. The fibrils reorient with the help of tethers resulting in helical growth. Through this model, insights on cell wall expansion and its dynamics have been achieved [25]. Through this thesis the aforementioned model can be expanded to incorporate a plant or fungal cell under the influence of a stimulus (phototropic and avoidance response) that causes bending.

Chapter 4

Materials

4.1. Culture conditions

The first batch of hyphae was purchased from Carolina Biological supply in the form of Phycomyces blakesleeanus (-), Living, Plate Culture [26], from which a small culture was cultivated on a petri dish of prepared 4% potato dextrose agar. After three to four days the sporangiophores began to grow and crowd under the lid of the petri dish, once the petri dish is filled the lid is twisted to crush the sporangium heads and release spores which will now be stuck to the inside of the lid. These spores are now stored DI water and saved for future experiments.

The medium on which all further batches were grown on was prepared from potato dextrose agar manufactured by Alpha Biosciences [27] which is a medium used for the cultivation of yeasts and molds. The approximate formula per litre includes, potato infusion 4g, dextrose 20g and agar 15g. The media was prepared in batches of ~100 ml, for which 3.9 g of the dried media was mixed in 100ml of DI water. This solution is repeatedly stirred under heat until all of the solid particles are dissolved. The solution is then autoclaved at 100º C for 25 minutes after which it is stored for future use in a beaker covered with parafilm.

Vegetative spores are inoculated in the culture medium and grown in small glass vials (capacity: 1 dram). These are vials are then placed in a box made of red plexi glass (plexi glass restricts light from passing through it). The box is temperature and humidity maintained, at 23° C and high humidity. Sporangiophores began to appear by the end of the third day. If a stage IV b sporangiophore is plucked the night before, the next day a new crop of stage IV b sporangiophore

appear. To conduct experiments, stage IV b crop of the third to fifth day was used and is required to be of at least 2-5 cm in length.

4.2. Experimental setup for Phototropic response

To conduct the phototropic response experiments a setup was constructed to house the light bulbs and camera along with the sporangiophore in its glass vial. The setup was built using red plexi-glass acrylic sheet as it is opaque and prevents entry of ambient light into the box. The dimensions of this setup were 8 x 10 x 8 in^3 (l x b x h), with a hole in the center of the front side (5 in diameter) to make room for the camera lens. The box was designed using SolidWorks and laser cut to incorporate joints facilitating better attachment of each side of the box. Acrylic cement was used to combine strongly all sides of the box. On both right and left faces of the box holes were made to house the light bulbs that were light sources to conduct these experiments.

Figure 12: The above set of images represent the setup used to conduct experiments on phototropism. Top left- TL, Top right- TR, Bottom left- BL, Bottom right- BR. The TL image depicts the setup which houses the sporangiophore and in which the experiments are conducted. It has three holes, two for light bulbs and one to allow the camera to capture front view images of the sporangiophore. The BL image shows the inside of the box with the two bulbs and a USB microscope camera which is used to capture top view images. The BR image shows the camera with a macro lens setup to capture front view images.

Light bulb specifications are: Fiet electric BPEFC40/850/LED/2 DAYLIGHT 220-16-44 E330072, 4.5W/5000K/300lm 120VAC/60Hz/33mA. A Sony DSLR-A200 with a Tamron P 90mm f/2.8 Di Macro Lens. The camera was used to capture front view images of the sporangiophore to determine change in length (elongation) and bend towards the light source (bend

angle phototropism), while a Celestron 5MP handheld digital microscope captured top view images of the sporangiophore head.

4.3. Experimental setup for Avoidance response

The same setup which was utilized to conduct the phototropism experiments was altered and reused to perform the avoidance response experiments. As previously mentioned, avoidance response occurs in the presence of a barrier, in these experiments a microscope slide cover slip was used as a barrier. The cover slip is attached to the end of a threaded bolt which can be screwed in and out of the right face, thus altering the distance between the sporangiophore and the barrier (cover slip). In these experiments both light bulbs are turned on, resulting in light growth response followed by steady growth. The same camera and digital microscopes were used to capture images of the sporangiophore as it begins to avoid or bend away from the barrier.

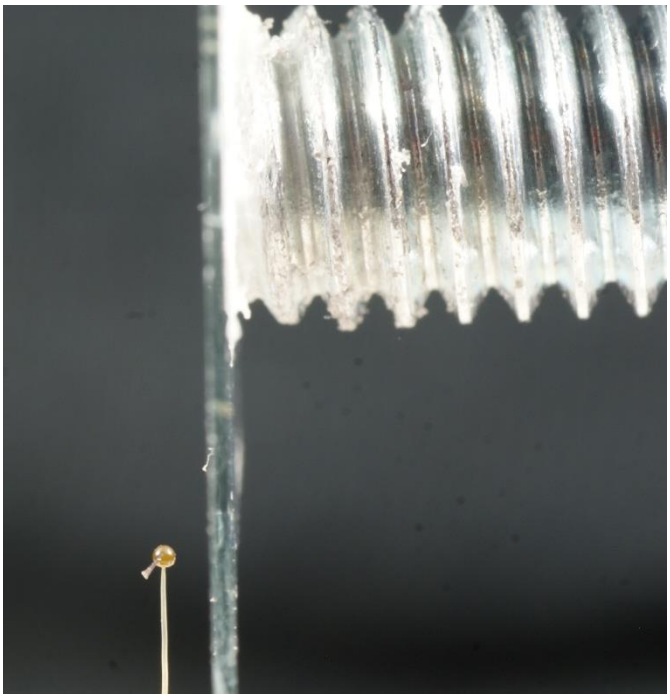

Figure 13: The above image depicts the setup used for the avoidance response. A microscopic slide attached to a threaded bolt is used as a barrier.

Chapter 5

Method

5.1. Experimental method of Phototropic response

As explained in section 2.2.2 Phototropic response is a form of response by the sporangiophore wherein when exposed to uneven light the growth zone responds by more cell expansion on the distal side than proximal side with respect to light zone, resulting in bending. This bending towards the light source has not been studied in relation to the elongation and rotation rates of the sporangiophore.

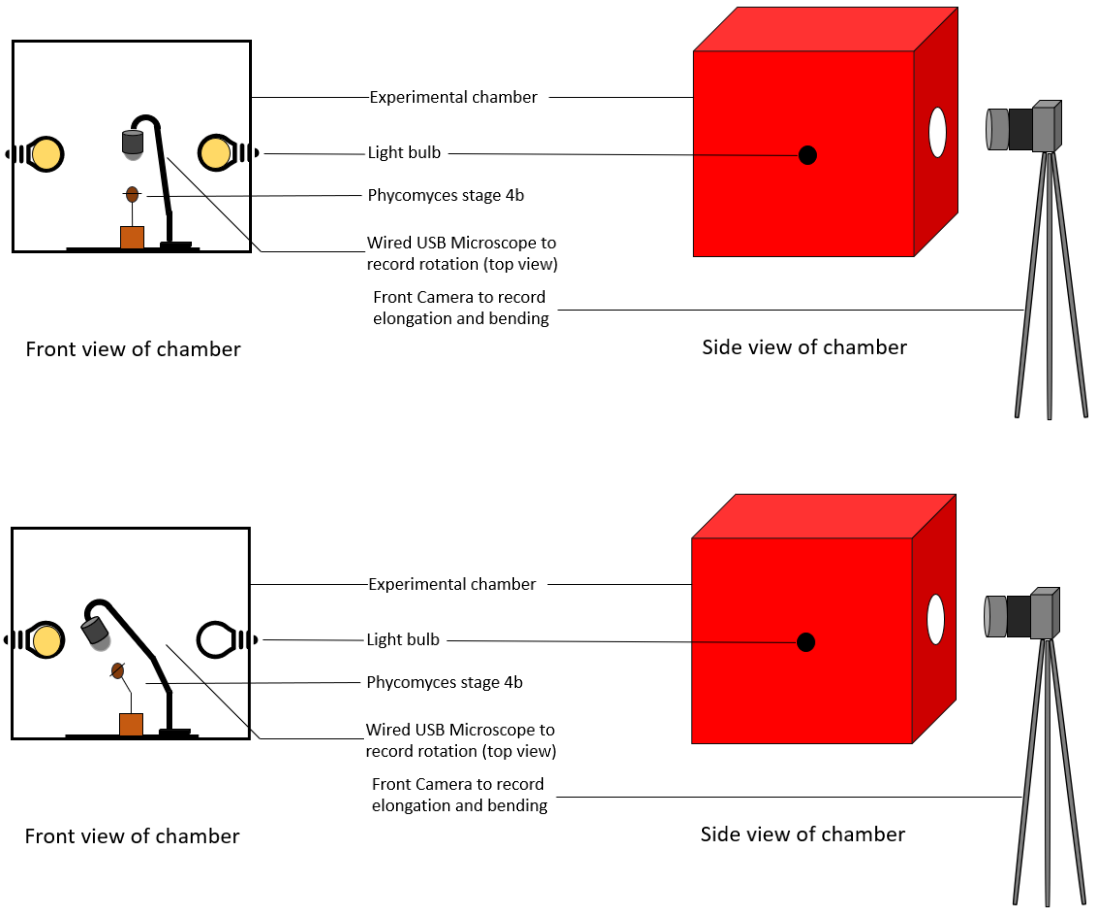

Figure 14: The diagram above depicts the experimental setup for conducting phototropic response experiments.

Once a stage IV b sporangiophore appears and is of appropriate height ~2cm, a hair of 1-2 mm length is placed on the sporangium and the sporangiophore is now placed inside the setup to be tested for phototropic response. The purpose of the hair is to act as a marker and aid in detection of rotation when viewing the sporangiophore using the top camera.

Before beginning the experiment both light bulbs are turned on along with the cameras to capture images. Each experiment is run for sixty minutes, while the cameras capture an image at every minute. The experiment begins with both light bulbs turned on for the first ten minutes during which steady growth is established. At the end of ten minutes, one light bulb is turned off, the response takes about 4 - 5 minutes to become visible to the eye. The sporangiophore begins to bend towards the light source and elongate in that direction.

With the passing of time the bend angle becomes more evident not just because it increases but also because of the increase in the elongation of the sporangiophore. The digital microscope which is installed to capture top view images has a flexible neck and can also be bent along with the sporangiophore to track the rotation rate. As the 60 mins are coming to an end the elongation, rotation and bend rate also begin to drop and steady out, this can be explained in further sections. At the end of the experiment the images are saved for further studies and interpretation using MATLAB.

5.2. Experimental method of Avoidance response

Avoidance response results in the bending away of the Phycomyces from a barrier or obstacle brought as close as a 2 mm near the growth zone. Unlike the phototropic response, the cell wall expansion is opposite i.e. The cell wall growth on the proximal side increases more than the distal side consequentially the Phycomyces bends away from the barrier.

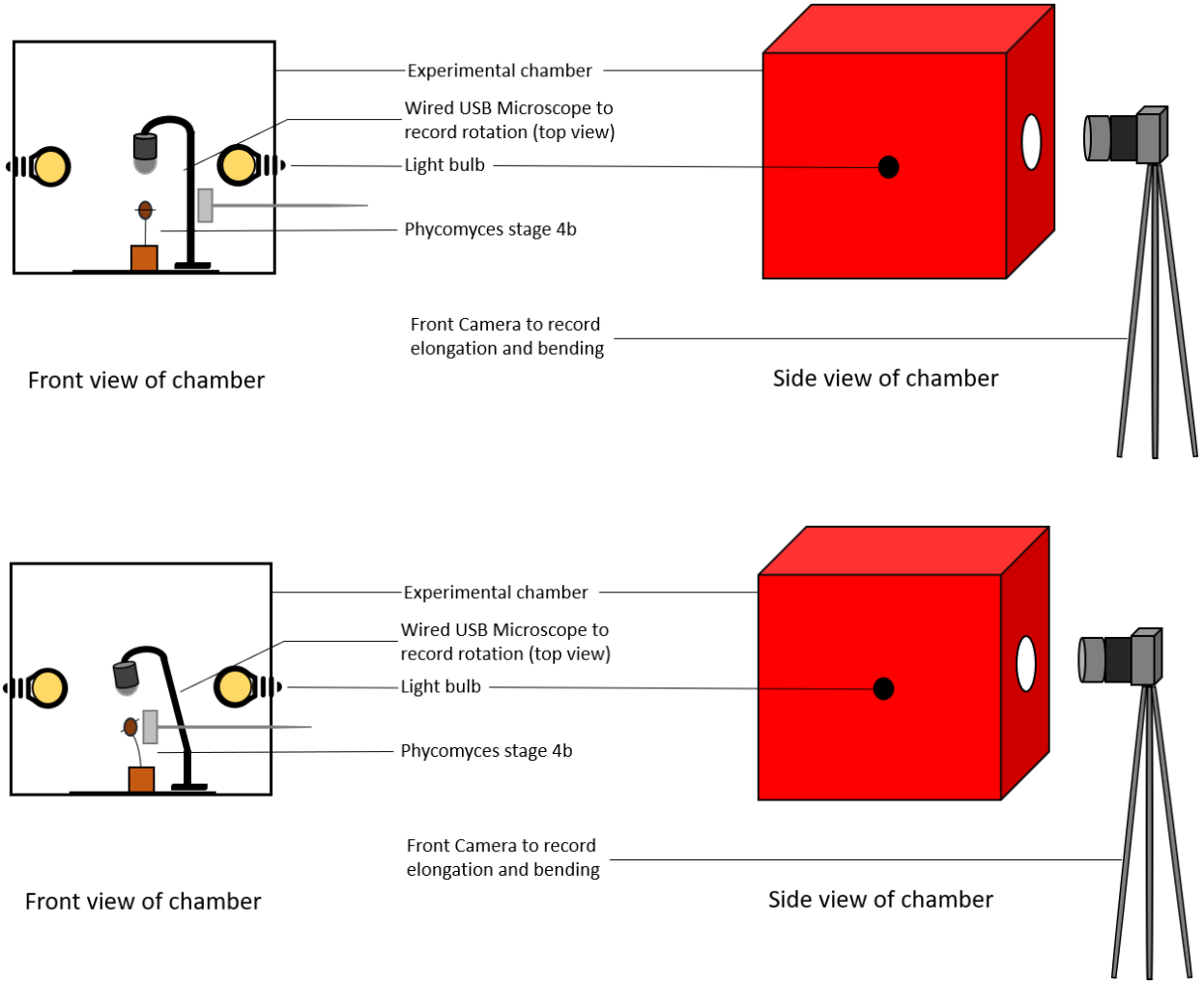

Figure 15: The diagram above depicts the experimental setup for conducting avoidance response experiments.

The experimental method of avoidance response is very similar to section 5.1 wherein the same setup of red plexiglass was utilized. The setup is modified to include a threaded bolt that has a microscope cover slip, as the bolt is screwed into the box. Once a stage IV b sporangiophore of appropriate height (2cm) is grown, it is isolated, and experiments are conducted on it. A 1~2 mm long hair is used as marker to facilitate rotation rate measurement. It is placed on the sporangium and the USB microscope is used to track changes in its orientation.

Each experiment lasts sixty minutes during which both light bulbs are on. The sporangiophore is placed inside the setup and begins to show steady growth in the first ten minutes of the experiment. After the completion of ten minutes the barrier is moved closer to the sporangiophore about 1~2mm gap is left between the sporangium and the barrier.

After the end of the third minute the sporangiophore begins to bend away from the barrier. An image is captured at every minute from the camera placed in front of the setup and the USB microscope which captures images from the top view. With the passing of time the bend angle becomes more evident and the flexible neck microscope is adjusted to capture the changes in orientation of the hair marker. As the 60 mins are coming to an end the elongation, rotation and bend rate also begin to drop and steady out, this can be explained in further sections. At the end of the experiment the images are saved for further studies and interpretation using MATLAB.

Chapter 6

Measurement

The same measurement technique is used to study both phototropic s well as avoidance response of the sporangiophore. The images are studied and analyzed using a code which runs sets of 60 images and measures changes in length and rotation of the sporangiophore. Each image is cropped, sharpened, converted to greyscale and then binarized to get the best quality black and white image. The object of interest in our case, the sporangiophore is white while the background is black. A trace boundary function was used to identify the white region of interest or sporangiophore. This trace boundary function isolates the sporangiophore from the background. The first version of the code also took into consideration the sporangium or head of the sporangiophore in the region of interest however this was soon scrapped to improve the results. The issue with version 1 of the code was that it did not exclude the hair present on the sporangium head and would also consider the hair as a part of the sporangium. This issue was overcome by eliminating the sporangium from the region of interest and only considering the stalk. Version 2 of the code tracked the top most pixel of the stalk for each image in the case of front view images. The sections below emphasize on each measurement.

6.1. Measurement of elongation rate

The images clicked using the macro lens camera emphasize on the front view of the sporangiophore to map changes in its height or in other words its elongation rate. As explained above, each image is converted to black and white to better track pixel motion. At the beginning of each experiment, a picture is clicked with a known scale to calibrate and baseline the number of pixels to that known scale. Once this is done the experiment is started and an image is taken

every minute for the next sixty minutes. With the passing of time each image shows a bending sporangiophore growing towards the light. At the end of the experiment a significantly bent sporangiophore can be seen. Each image is cropped, converted to gray scale, sharpened followed by conversion to binary. The object of interest or sporangiophore is white while the background is black. The sporangium is then negated from the region of interest by estimating its radius size and blocking it out by overlapping it with a black circle of the same radius, this way only the stalk is white in a black background.

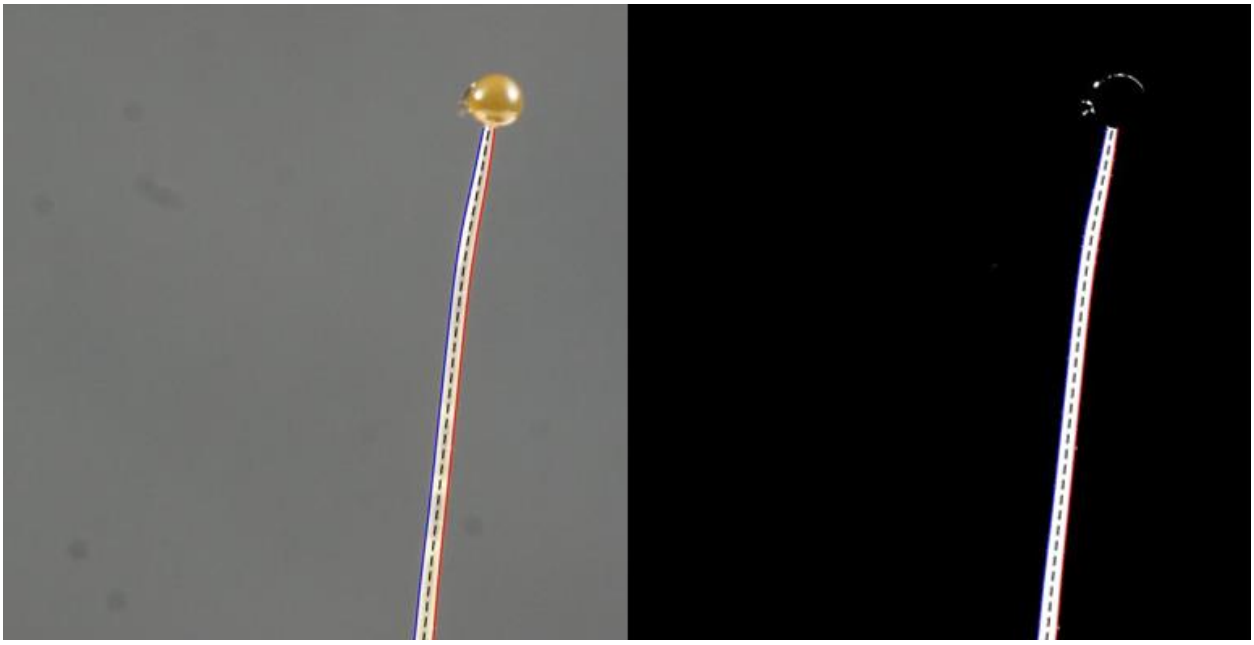

Figure 16: The set of images above depict the measurement technique to measure the elongation and bend rate of a sporangiophore. The image on the right shows us the post processed image to attain accurate measurements. Image courtesy: Revathi Priyanka Mohan

MATLAB was used to analyze the images, for each image the tip of the stalk was detected by looking for the first pixel that is white, moving from the top left corner towards the right and bottom of the image. Once the pixel is detected the Trace boundary function is used to trace the boundary of the stalk [28]. This function traces the edges of the stalk and determines the axis by

averaging out the x and y values on either end. The length of this dashed line as shown in the figure below gives us the elongation of the Phycomyces. If each stalk has a particular elongation the difference between the elongation of the stalk of that image and its previous image gives us the elongation rate for each minute.

The elongation rate is plotted against time to understand how it varies before and after the application of stimulus. From the plot, it is evident that the elongation rate gradually increases after the application of stimulus (light or barrier) and plateaus out toward the end of the experiment.

6.2. Measurement of bend rate

The images clicked by the macro lens for the elongation rate studies are re-used for bend rate studies. The bend rate studies of phototropic response and avoidance response are different in the way that for the former, the sporangiophore bends towards the stimulus while in the case of the latter, it bends away from the stimulus. This indicates that the sporangiophore can show both positive and negative responses while still showing elongation and rotation.

At the beginning of each experiment, a picture is clicked with a known scale to calibrate and baseline the number of pixels to that known scale. Each image is analyzed using a MATLAB code that first crops, converts to grayscale, sharpens, and converts to black and white the given image. The object of interest (sporangiophore) is white while the background is black. The sporangium is then negated from the region of interest by estimating its radius size and blocking it out by overlapping it with a black circle of the same radius, this way only the stalk is white in a black background. The code scans the image from the top left to the bottom right and detects the first white pixel it encounters. Once, the white pixel is detected, the trace boundary function traces the edges of the stalk and determines the axis by averaging out the x and y values on either end.

Naturally, this axis portrays the bend of the sporangiophore and the angle and the axis and the vertical gives us the bend angle.

6.3. Measurement of rotation rate

The USB microscope is used to capture top view images of the sporangium. This is done to measure the rotation rate of the sporangiophore by tracking changes in orientation of the hair (marker) that is placed on top of the sporangium. The microscope has a flexible stand which can be bent according to the bending of the sporangium. This accommodates capturing the orthogonal view of the sporangium at all points of time.

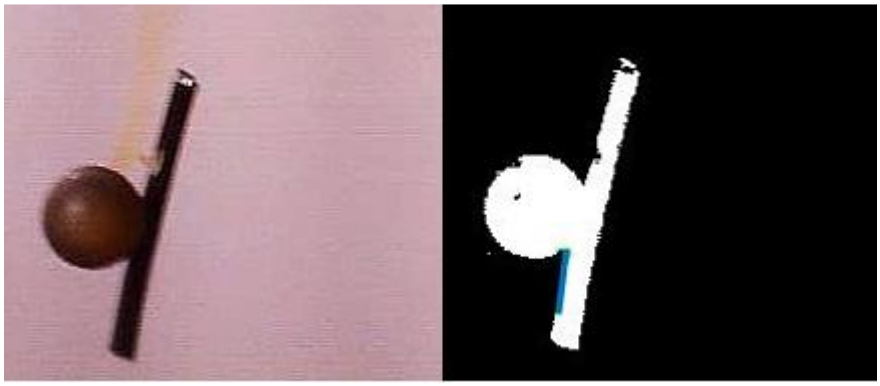

Figure 17: The series of images above depict the measurement technique to measure rotation rate using ImageJ

The application ImageJ is used to measure the rotation of the sporangium. The angle feature measures the angle between the hair and the y-axis for each image thus providing us with the angle of rotation of the sporangium over time. The difference between the angle of rotation of a particular image and its previous image gives us the rotation rate for each minute.

The rotation rate is now plotted against time for the duration (60 minutes) of the experiment. From the plot, it is evident that the rotation rate increases with time after the application of stimulus.

Towards the end of the experiment, the rotation rates plateau out indicating that the sporangiophore is beginning to get accustomed to the stimulus (steady state growth).

6.4. Measurement of R

R, helical growth steepness is defined as rotation rate over elongation rate of the sporangiophore at any instant. To understand cell wall expansion, one must understand the relationship between rotation rate and elongation rate. Larger the R, lesser the helix angle. Smaller the R, steeper the helix. For each experiment that is conducted a ratio of the recorded rotation rate and elongation rate is taken, this ratio is R. By studying R one can understand the relative change in rotation and elongation rates this way one can attribute the reaction to stimulus in the form of light, barrier, wind etc., to either one or both rates.

Chapter 7

Results

The objective of this research is to compare elongation, rotation and bend rates for both phototropic and avoidance response experiments. The results obtained in each case are plotted against the same scale to facilitate comparison. Through better understanding of these parameters current cell wall expansion models can be advanced.

7.1. Results of Phototropic response

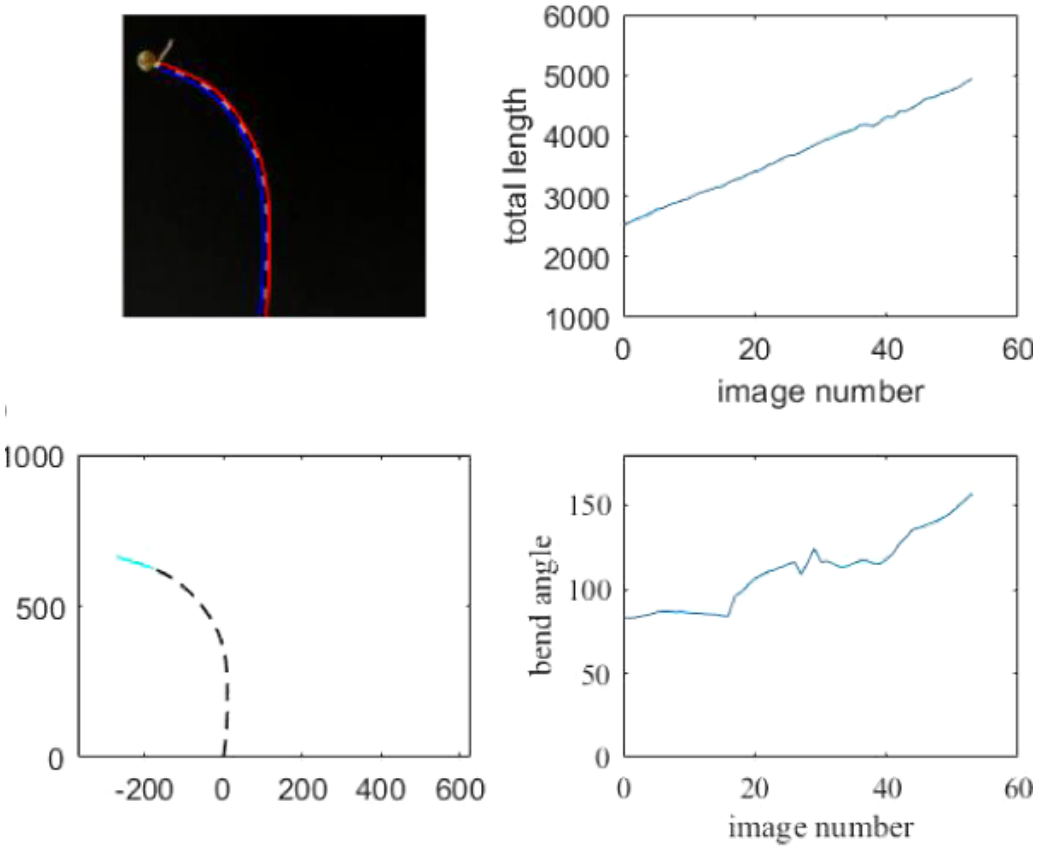

Figure 18: TL: An image of a sporangiophore undergoing phototropic response, TR: a plot of total length of sporangiophore over time (each image number also represents the nth minute at which it was captured), BL: a curve representing the stalk of the sporangiophore as it undergoes phototropic response and BR: a plot of bend angle of sporangiophore over time (each image number also represents the nth minute at which it was captured)

In the case of Phototropic response from the measurements of rotation, elongation and bend rates it is evident that the magnitude is greater than that of avoidance response. When one of the lights is turned off the sporangiophore begins to bend towards the only light source. This implies that the distal side of the sporangiophore (with respect to the light source) has greater cell wall expansion than the proximal side causing this bend. Arrows on the x axis in each of the following plots depicts the point at which stimulus was applied. The figure 18 depicts a sporangiophore undergoing phototropic response.

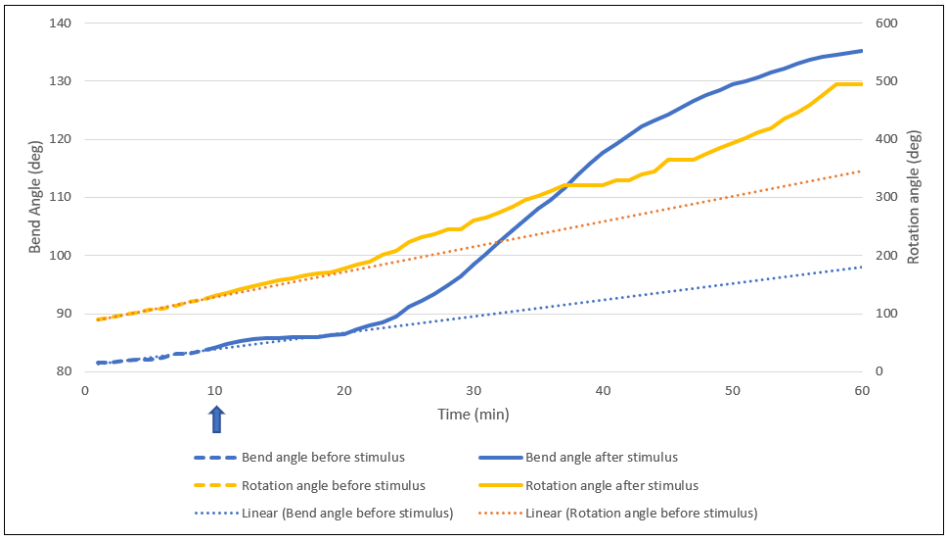

Figure 19: A plot of bend angle and rotation angle vs time for a typical phototropic response experiment, the linear trendline represents the relation that bend, and rotation angles would have followed without the application of the unequal light stimulus.

From the above plot it is evident that both the bend and rotation angles increase ~5 minutes after the application of stimulus. This behavior is typical in most sporangiophores although there is a difference in the time at which this increase manifests.

The trendline depicts the path the bend and rotation angles follow in the absence of the stimulus, typically, the bend angle increases at a greater rate than rotation angle.

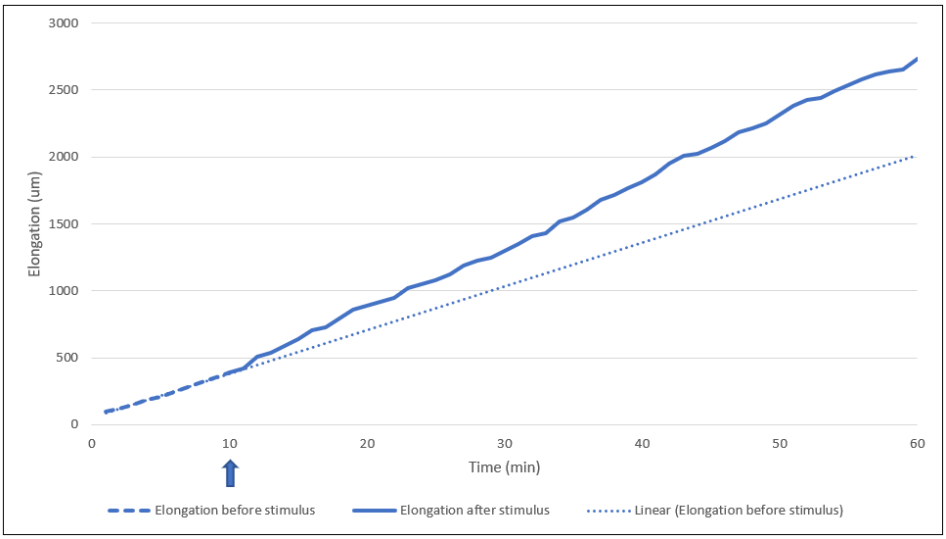

Figure 20: A plot of elongation vs time for a typical phototropic response, the linear trendline represents the relation that elongation would have followed without the application of the unequal light stimulus.

In the case of elongation, the increase begins to take place much faster than both bend and rotation angles. However, this increase is different for each sporangiophore and follows a different slope. Some sporangiophores grow at a faster rate than others. In the average elongation rate plot (which is explained in detail below) it is clear from the fluctuations how differently each sporangiophore reacts to the stimulus. However, in all cases there is an increase in elongation after the application of stimulus.

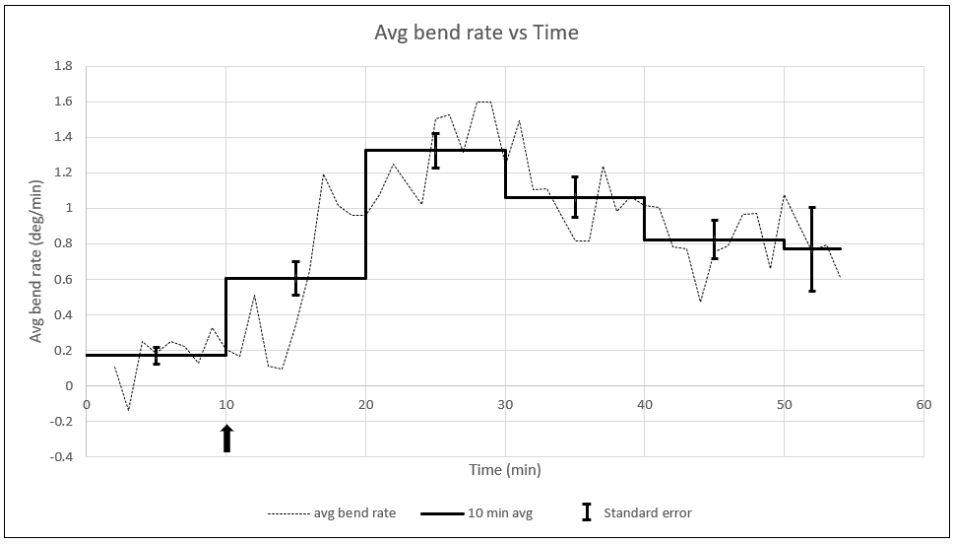

Figure 21: Average bend rate vs time for phototropic response experiments

Average rate plots are used to study the general behavior of sporangiophores before and after the application of stimulus. The bending begins 5-7 mins from when the stimulus is applied. The bend rate starts from 0°/ min gradually increases to 1°/ min at and at around 13 mins there is a linear rise to 3°/min, this lasts for about 30 mins after which the bend rate begins to fall. The fall in the bend rate signifies that the sporangiophore is now accustomed to the stimulus signifying steady growth. However, even after this fall it is still greater than it was before the stimulus was applied.

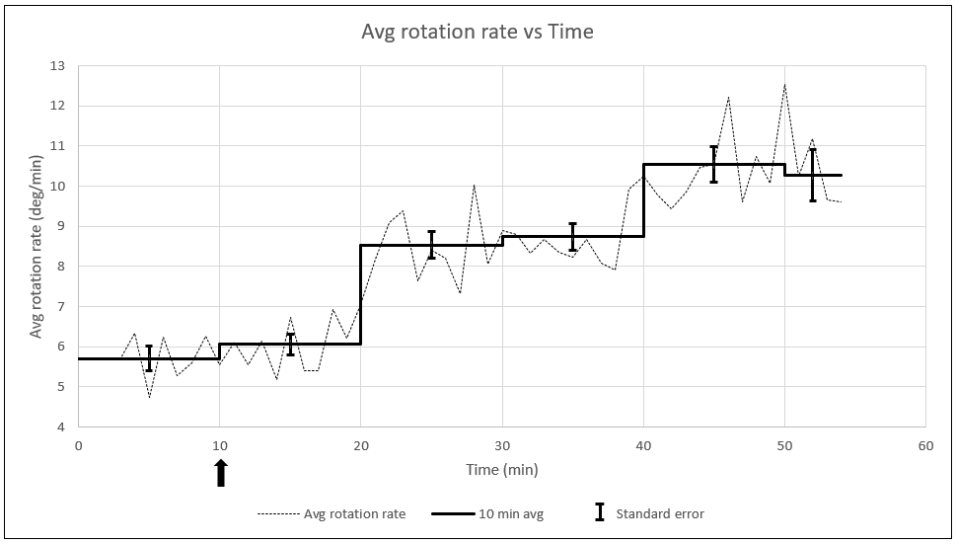

Figure 22: Average rotation rate vs time for phototropic response experiments

Before the application of light stimulus, the rotation rate is about 5- 7°/min. Around 5 minutes after the stimulus is applied there is a linear increase of rotation rate for the entire duration of the experiment to about 20°/min. The plots of bend rate and rotation rate are different in the sense that bend rate begins to fall towards the end of the 35 mins however rotation rate only rises linearly.

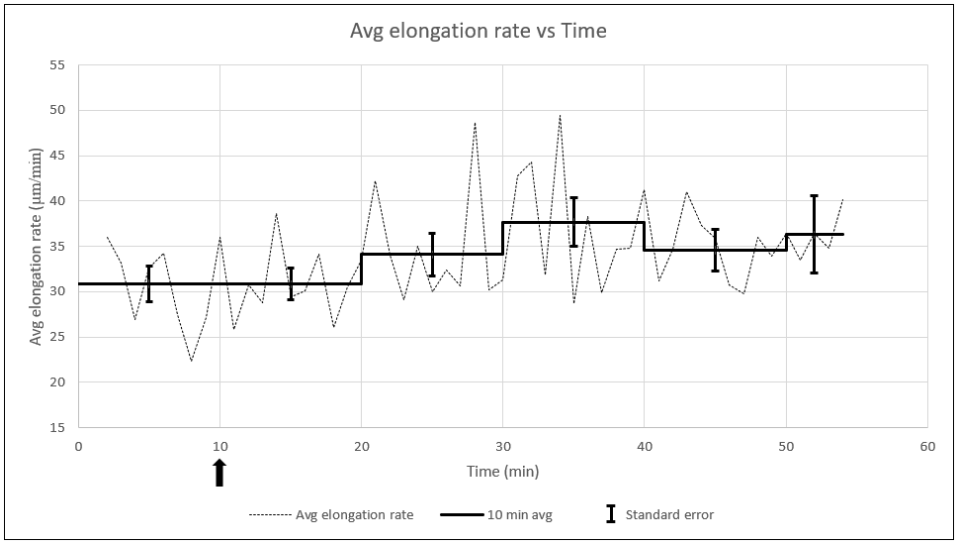

Figure 23: Average elongation rate vs time for phototropic response experiments

Similarly, the elongation rate rises linearly during the duration of the experiment resembling that of rotation rate however with a smaller slope. Due to different slopes or different behavior of each sporangiophore resulting in fluctuations the increase is not very evident.

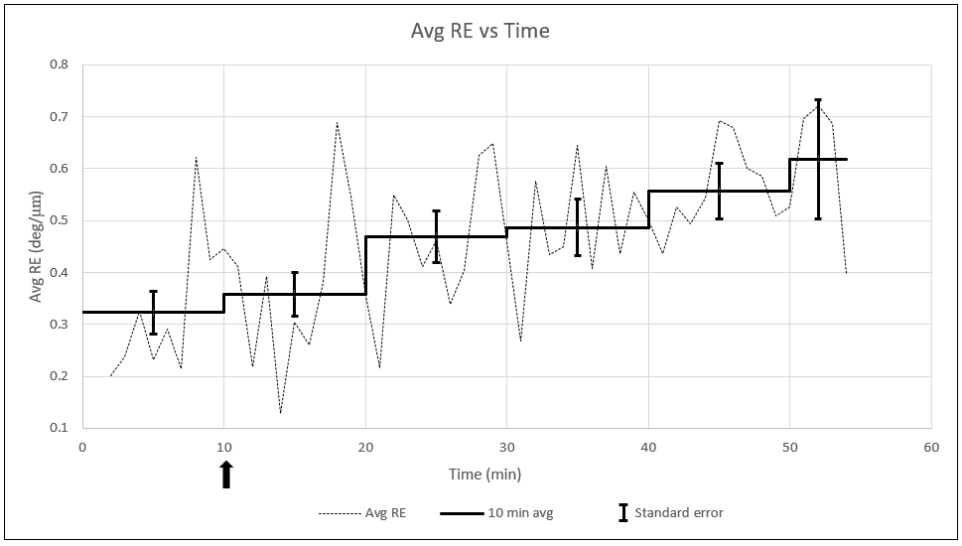

Figure 24: Avg RE vs time for phototropic response experiments

When R (a measure of helical growth steepness, rotation rate ÷ elongation rate) is plotted vs time it is seen that the plot is increases linearly but not steep. This is as expected as the increase in rotation rate is much more that that of elongation rate indicating that the helical steepness of a sporangiophore during phototropic response is flatter than usual.

7.2. Results of Avoidance response

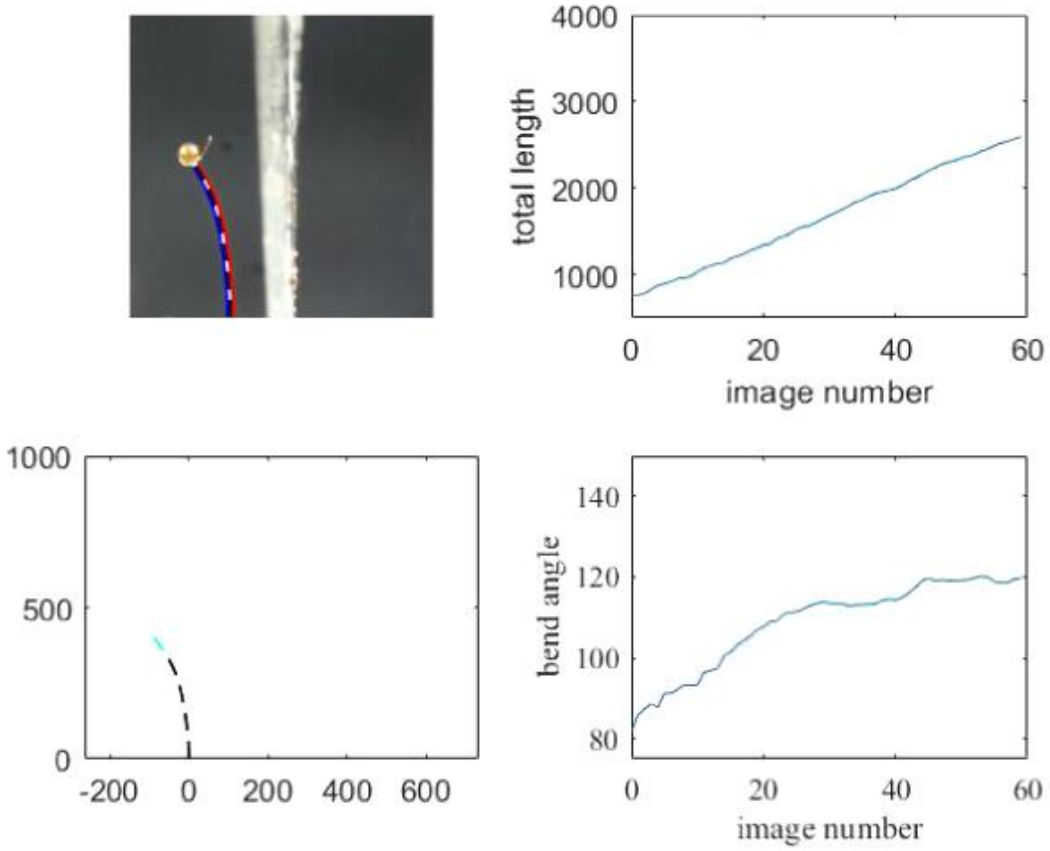

Figure 25: TL: An image of a sporangiophore undergoing avoidance response, TR: a plot of total length of sporangiophore over time (each image number also represents the nth minute at which it was captured), BL: a curve representing the stalk of the sporangiophore as it undergoes avoidance response and BR: a plot of bend angle of sporangiophore over time (each image number also represents the nth minute at which it was captured)

In the case of avoidance response, once a barrier stimulus is applied, it takes about 3 minutes for the sporangiophore to start bending away from the barrier. In the case of avoidance response, the cell wall expands more on the distal side of the sporangiophore than the proximal side with respect to the barrier causing the sporangiophore to bend away from it. Arrows on the x axis in each of the following plots depicts the point at which stimulus was applied. Figure 25 depicts a sporangiophore undergoing the avoidance response.

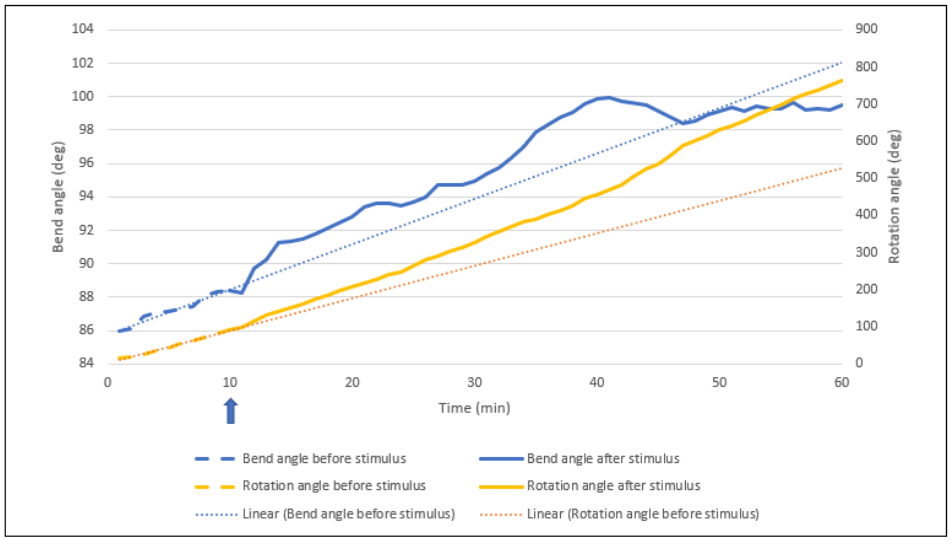

Figure 26: A plot of bend angle and rotation angle vs time for a typical avoidance response experiment, the linear trendline represents the relation that bend, and rotation angles would have followed without the application of the barrier stimulus.

From the above plot it is evident both rotation angle as well as bend angle increase after the application of stimulus, however it is seen that the bend angle begins to fall during the last ten minutes of the experiment. The trendline depicts the path bend and rotation angles follow in the absence of barrier stimulus.

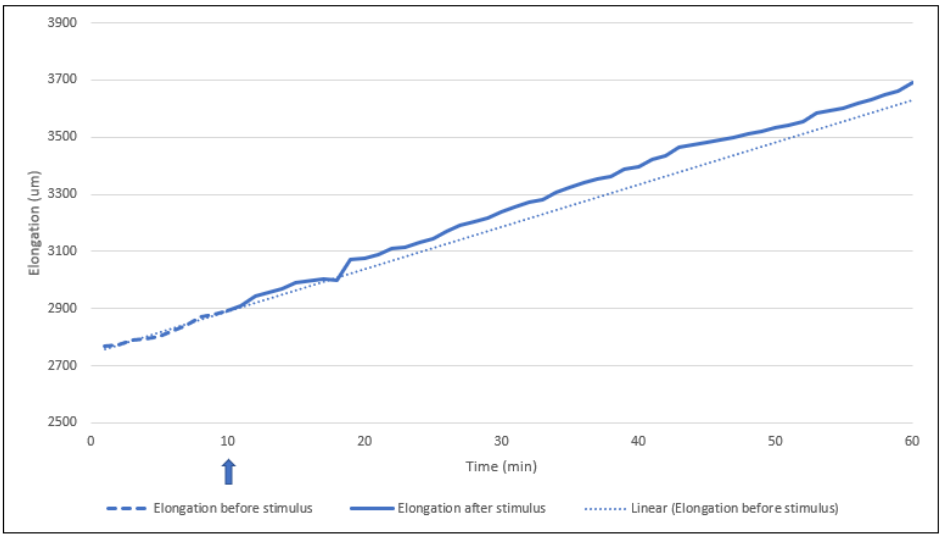

Figure 27:A plot of elongation vs time for a typical phototropic response, the linear trendline represents the relation that elongation would have followed without the application of the barrier stimulus.

There is a small increase in elongation after the application of barrier stimulus however similar to the phototropic response, each sporangiophore reacts to a different extent to the stimulus and the slopes vary.

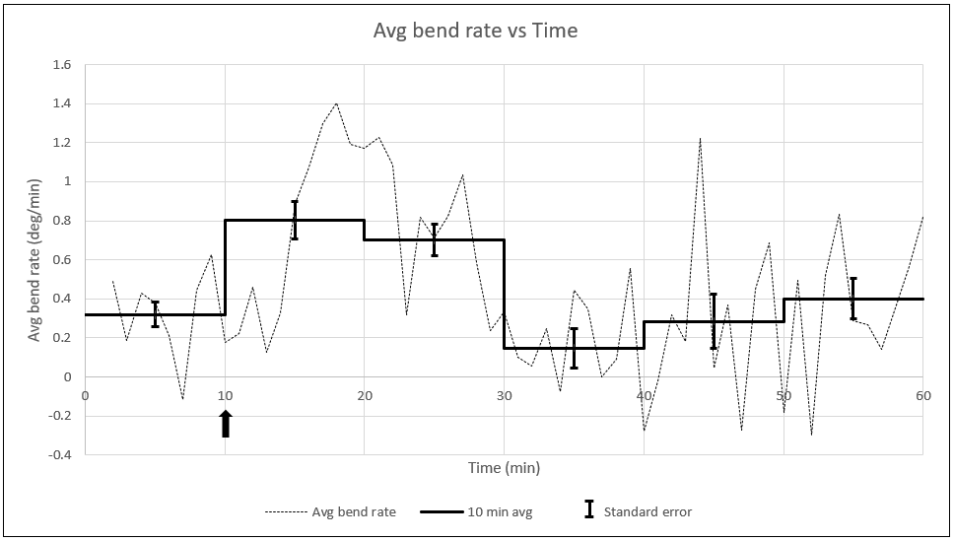

Figure 28: Average bend rate vs time for avoidance response experiments

The bend rate due to avoidance response is lesser than that shown by the sporangiophore during phototropic response. Bending begins to take place four minutes after the barrier stimulus is applied and continues to increase linearly but only for a short period of time after which it begins to fall.

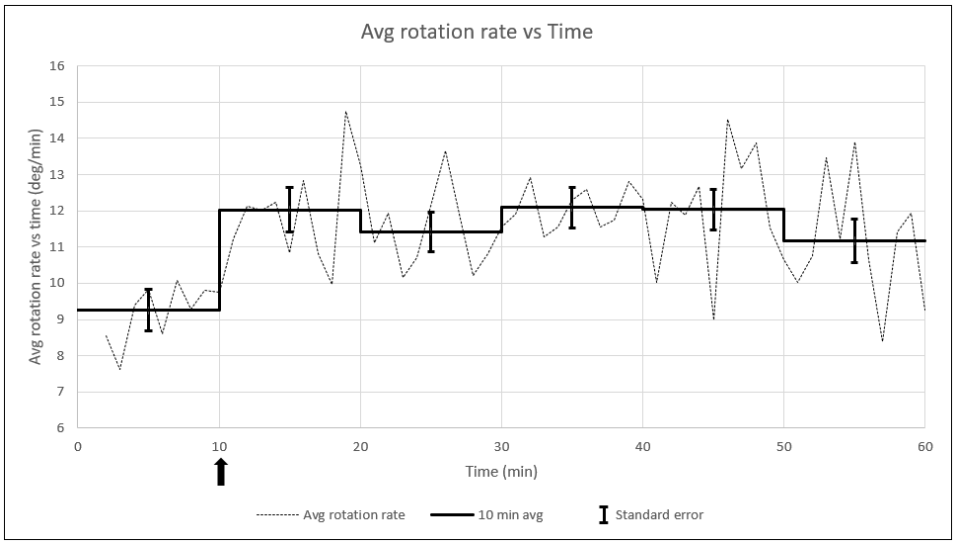

Figure 29: Average rotation rate vs time for avoidance response experiments

Similar to rotation rate during phototropic response rotation rate during avoidance response also increases linearly to ~ 20°/min during the entire duration of the experiment for typical sporangiophores.

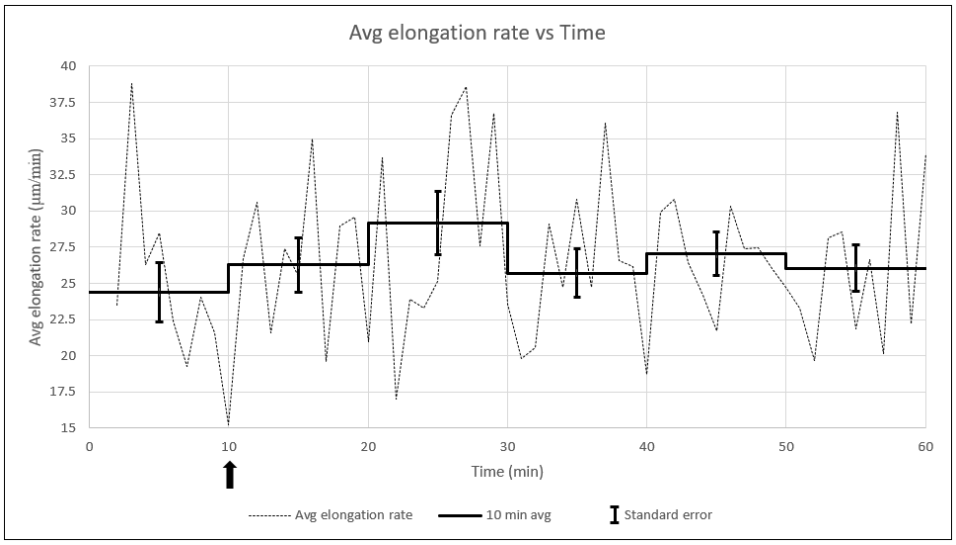

Figure 30: Average elongation rate vs time for avoidance response experiments

It is seen that there is a small linear increase in elongation rate however, this is still lesser than that seen during the phototropic responses and has much more noise of fluctuations.

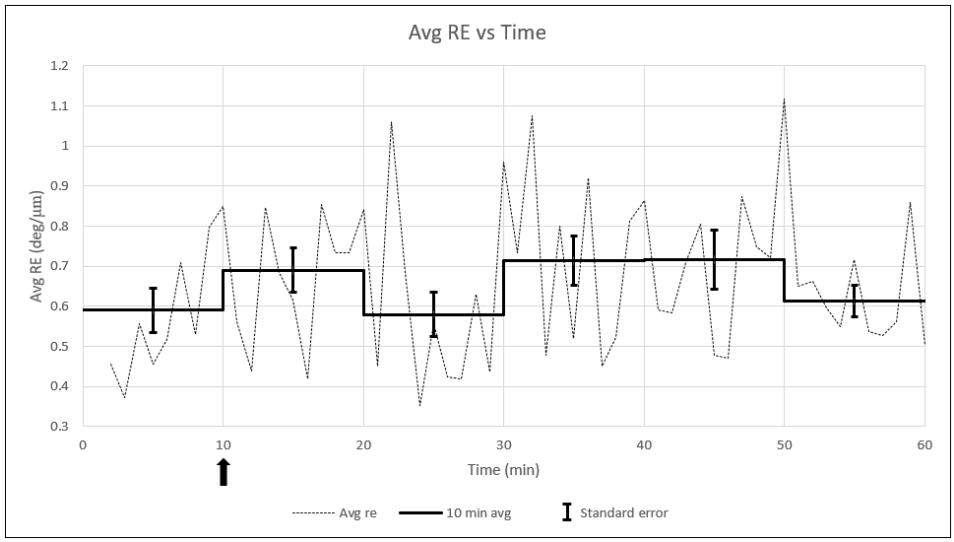

Figure 31: Average RE vs time for avoidance response experiments

R vs Time in the case of avoidance response is flatter than phototropic response meaning the steepness or helical growth is flatter during the avoidance response. The section below further explains these parameters vs time for both responses.

Chapter 8

Discussion

8.1. Conclusion

The overall objective of this thesis was to understand the effect phototropic and avoidance responses have on helical growth and bending of the sporangiophore. By obtaining this information one can better formulate local models of cell wall expansion. The fibril reorientation-slippage theory was introduced to explain measurable rotation in the absence of elongation during steady growth as well as measurable elongation in the absence of rotation during light response that exist at the bottom of the growth zone in slow and fast growing sporangiophores respectively.

It is seen that all three parameters i.e., elongation rate, bend rate as well as rotation rate have different slopes for both responses. The bend rate of phototropic response is much higher than that of avoidance response. In some cases of avoidance response, the bend rate begins to fall towards the end of the 60 min duration experiment. This can be attributed to the increase in distance between the barrier and the GZ of the sporangiophore as it bends away from it hence resulting in reduction of bend rate.

During the phototropic response the cell wall extends to a greater extent on the distal side of the sporangiophore (from the light source) causing it to bend towards the source of light. During the avoidance response the cell wall extends to a greater extent on the proximal side of the sporangiophore (from the barrier) causing it to bend away from the barrier. Although both responses are different, they occur due to asymmetric extensibility of the cell wall as a reaction to the stimuli. One can attribute this asymmetric extensibility to breakage of load bearing bonds between polymers that exist on the cell wall.

Let us consider a biomolecule that causes the breakage of load bearing bonds in turn causing wall extensibility. As known, during the light response an increase in elongation rate occurs, this is due to a higher concentration of the biomolecule in that region. When an additional stimulus is added to the existing light, such as the phototropic response, an influx of the biomolecule to a region with preexisting high concentrations of the biomolecule will occur. This will cause an increase in wall extensibility (due to light response) as well as bending (due to phototropic response) as seen in the measurements of the conducted experiments.

According to the fibril reorientation-slippage theory rotation rate is a result of reorientation of fibrils that occur due to expansion or elongation of the wall. At the start of the growth zone, right below the sporangium transverse microfibrils are generated and replenish the length of the growth zone. If these fibrils are introduced at the same rate as they are originally, the increase in rotation rate that is observed in both responses cannot be explained. To cause this increase in rotation rate there must be a significant increase in the transverse microfibrils at the tip of the growth zone. An influx of the biomolecule that causes the observed bending and increase in elongation rate due to breakage of load bearing bonds between polymers on the cell wall results in introduction of transverse fibrils at the tip of the growth zone, thereby resulting in increased rotation rate.

8.2. Future work

The research conducted above involves total growth parameters (elongation rate, rotation rate and bend rate) of the sporangiophore. The Soft Matter Mechanics Lab is currently working on studying strain rates along the growth zone of the sporangiophore. A setup has been constructed to house the sporangiophore as light growth response is conducted. A USB microscope is used that can be controlled using a computer thus leaving the entire setup unaltered.

Figure 32: The experimental setup for strain rate measurements along the growth zone

The setup is made of the same plexi glass that was used for the previous experimental setup. It is big enough to house a USB microscope on the inside along with the sporangiophore. Stage IV b sporangiophores were used for these experiments. A tube light was placed exactly above the sporangiophore, when turned on it causes the light growth response. Specs of starch are blown on to the sporangiophore. The USB microscope clearly captures these starch particles.

A MATLAB function called PIV (particle image velocimetry) is used to determine the strain rate. It tracks each starch particle and its movement to determine its elongation rate and rotation rate overtime. Preliminary studies are being conducted to study how the strain rate vary along the length of the growth zone. Future studies include determination of elongation and rotation rates along the growth zone during phototropic and avoidance responses.

References

[1] K. Bergman *et al.*, “Phycomyces.,” *Bacteriol. Rev.*, vol. 33, no. 1, pp. 99–157, Mar. 1969.

[2] E. Cerdá-Olmedo, “Phycomyces and the biology of light and color,” *FEMS Microbiol. Rev.*, vol. 25, no. 5, pp. 503–512, Dec. 2001.

[3] K.-H. Riemay, “E. Cerda-Olmedo and E. D. Lipson (Editors), Phycomyces. XIV + 430 S., 140 Abb., 22 Tab. Cold Spring Habor 1987. Cold Spring Harbor Laboratory. $ 88.00. ISBN: 0-87969-199-9,” *J. Basic Microbiol.*, vol. 28, no. 9–10, pp. 578–578, 1988.

[4] R. D. Preston, *The Physical Biology of Plant Cell Walls*. Springer US, 1974.

[5] J. E. Ortega *et al.*, “Helical growth of stage-IVb sporangiophores of Phycomyces blakesleeanus: the relationship between rotation and elongation growth rates,” *Planta*, vol. 216, no. 4, pp. 716–722, Feb. 2003.

[6] C. R. Benjamin and C. W. Hesseltine, “Studies on the Genus Phycomyces,” *Mycologia*, vol. 51, no. 6, pp. 751–771, Nov. 1959.

[7] H. Rudolph, “WEITERE UNTERSUCHUNGEN ZUR WÄRMEAKTIVIERUNG DER SPORANGIOSPOREN VON PHYCOMYCES BLAKESLEEANUS: III. Mitteilung,” *Planta*, vol. 57, no. 3, pp. 284–312, 1961.

[8] W. J. Robbins, “EFFECT OF EXTRACTS OF PHYCOMYCES UPON ITS DEVELOPMENT,” *Am. J. Bot.*, vol. 27, no. 7, pp. 559–564, Jul. 1940.

[9] W. J. Robbins, V. W. Kavanagh, and F. Kavanagh, “Growth Substances and Dormancy of Spores of Phycomyces,” *Bot. Gaz.*, vol. 104, no. 2, pp. 224–242, Dec. 1942.

[10] P. R. Burkholder and I. McVeigh, “GROWTH OF PHYCOMYCES BLAKESLEEANUS IN RELATION TO VARIED ENVIRONMENTAL CONDITIONS,” *Am. J. Bot.*, vol. 27, no. 8, pp. 634–640, Oct. 1940.

[11] E. S. Castle, “Spiral Growth and Reversal of Spiraling in Phycomyces, and Their Bearing on Primary Wall Structure,” *Am. J. Bot.*, vol. 29, no. 8, pp. 664–672, 1942.

[12] J. K. E. Ortega, J. T. Truong, C. M. Munoz, and D. G. Ramirez, “Cell Wall Loosening in the Fungus, Phycomyces blakesleeanus,” *Plants*, vol. 4, no. 1, pp. 63–84, Mar. 2015.

[13] E. S. Castle, “THE TOPOGRAPHY OF TIP GROWTH IN A PLANT CELL,” *J. Gen. Physiol.*, vol. 41, no. 5, pp. 913–926, May 1958.

[14] J. K. E. Ortega, R. I. Gamow, and C. N. Ahlquist, “Phycomyces: A Change in Mechanical Properties after a Light Stimulus,” *Plant Physiol.*, vol. 55, no. 2, pp. 333–337, Feb. 1975.

[15] E. S. Castle, “Light Responses of Phycomyces,” *Science*, vol. 154, no. 3755, pp. 1416–1420, Dec. 1966.

[16] G. M. Curry and H. E. Gruen, “ACTION SPECTRA FOR THE POSITIVE AND NEGATIVE PHOTOTROPISM OF PHYCOMYCES SPORANGIOPHORES*,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 45, no. 6, pp. 797–804, Jun. 1959.

[17] W. Shropshire, “The Lens Effect and Phototropism of Phycomyces,” *J. Gen. Physiol.*, vol. 45, no. 5, pp. 949–958, May 1962.

[18] P. Galland, “Chapter 22 Phototropism in Phycomyces,” in *Comprehensive Series in Photosciences*, vol. 1, D.-P. Häder and A. M. Breure, Eds. Elsevier, 2001, pp. 621–657.

[19] P. Galland and V. E. Russo, “Tropic reversal in Phycomyces,” *Planta*, vol. 163, no. 3, pp. 401–404, Mar. 1985.

[20] J. K. E. Ortega and R. I. Gamow, “Phycomyces: Habituation of the Light Growth Response,” *Science*, vol. 168, no. 3937, pp. 1374–1375, Jun. 1970.

[21] R. J. Cohen, Y. N. Jan, J. Matricon, and M. Delbrück, “Avoidance response, house response, and wind responses of the sporangiophore of Phycomyces,” *J. Gen. Physiol.*, vol. 66, no. 1, pp. 67–95, Jul. 1975.

[22] J. K. Ortega, M. E. Smith, and M. A. Espinosa, “Cell wall extension behavior of Phycomyces sporangiophores during the pressure response,” *Biophys. J.*, vol. 68, no. 2, pp. 702–707, 1995.

[23] “The Analysis of Spiral Growth in Phycomyces Using a Novel Optical Method | Plant Physiology.” [Online]. Available: http://www.plantphysiol.org/content/53/3/485.short. [Accessed: 16-May-2019].

[24] K. Yoshida, T. Ootaki, and J. K. E. Ortega, “Spiral growth in the radially-expanding piloboloid mutants ofPhycomyces blakesleeanus,” *Planta*, vol. 149, no. 4, pp. 370–375, Jan. 1980.

[25] “A Statistical Model of Expansive Growth in Plant and Fungal Cells: The Case of Phycomyces. - PubMed - NCBI.” [Online]. Available: https://www.ncbi.nlm.nih.gov/pubmed/30514633. [Accessed: 17-Oct-2019].

[26] “Phycomyces blakesleeanus (-), Living, Plate Culture | Carolina.com.” [Online]. Available: https://www.carolina.com/fungi/phycomyces-blakesleeanus-living-plate-culture/156183.pr?question=. [Accessed: 12-Oct-2019].

[27] “Potato Dextrose Agar (P16-109),” *Pro Lab Supply Corp*. [Online]. Available: https://prolabcorp.com/potato-dextrose-agar-p16-109/. [Accessed: 12-Oct-2019].

[28] “Trace object in binary image - MATLAB bwtraceboundary.” [Online]. Available: https://www.mathworks.com/help/images/ref/bwtraceboundary.html. [Accessed: 12-Oct-2019].