Auctus Electrica: Electrical field dependence of *Neurospora crassa* growth[1].

Dmitry Neymark[2] and Roger R. Lew, Biology Department, York University

Revision 1.60 (2015)

OBJECTIVE

To investigate the effects of external electric fields on hyphal growth of the fungus *Neurospora crassa*.

[1]

[2] RAY (Research at York) Biophysics Research Assistant. Experiments were performed 01MAY2014 through 30APR2015 in the Lew Laboratory (RRL email: planters@yorku.ca) and were funded in part by NSERC (Natural Sciences and Engineering Research Council)

INTRODUCTION

Mycelial fungi can be electrically polarized near the growing hyphal tip region. The polarization is caused by endogenous electrical fields and longitudinal electrical currents in the region of the growing tips (Gow, 1984). According to various studies, growing hyphal tip are polarized, with a tip-localized Spitzenkorper and tip-high Ca^{2+} gradients in the first 50 microns (Jackson et al. 2001, Potapova, 2014). At a larger scale, it is known that the direction of current is normally from distal regions of the hyphae into the growing tip (McGillivray and Gow 1986). What is not understood are the mechanisms establishing and maintaining polarity of hyphal tip growth.

To understand hyphal tip polarization better, I investigated the effects of electric field on the growth of *Neurospora crassa*. In various mycelial fungi, McGillivray and Gow (1986) have shown that the orientation of germling formation as well as tip growth changes in response to external electrical fields. The direction of galvanatropism varied in different fungal species. The same study also showed that strong fields induce branching in *Neurospora crassa*. In *Candida*, hyphae oriented themselves towards the cathode, but there was a decrease in polarized (directional growth) at stronger fields (Crombie et al. 1989). My previous experiments (Neymark and Lew 2014) did not find any effects of voltage fields on the direction of hyphal growth in *N. crassa*, perhaps because growth ceased before any galvanatropism could be observed.

In the second experimental attempts described here, the fungus was grown in larger Petri dishes so that the distance between the platinum electrodes was much farther. Clear growth responses to electrical fields were successfully observed prior to growth inhibition.

MATERIALS AND METHODS

Strain. *Nerurospora crassa* (wildtype 74-OR23-IVA, FGSC #2489) was used for all experiments.

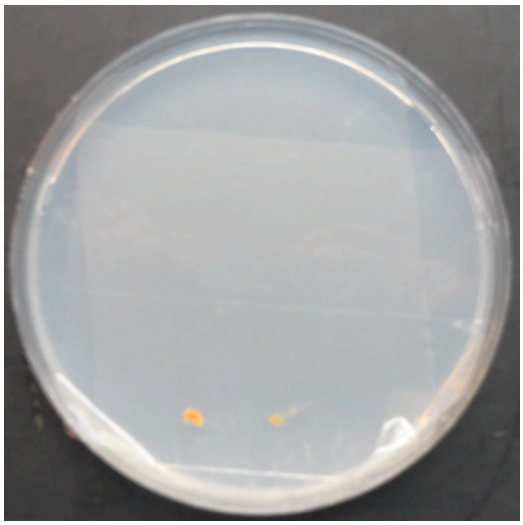

Figure 1: Petri dish with agar gel, dialysis tubing and hyphae.

Growth conditions. Stock cultures were maintained on Vogel's medium (Ion composition: (mM): K^+ (36.7), P_i (36.7), Na^+ (25.5), NH_4^+ (25), NO_3^- (25), citrate (8.5), Cl^- (1.36), Mg^{2+} (0.81), SO_4^{2-} (0.81), and Ca^{2+} (0.68); pH is 5.8 [Lew, 2007]) and 2% agar. Conidia were inoculated on large Petri dishes (diameter=143 +/-1 mm) between two layers of dialysis tubing with molecular weight cut off of 14000, on top of agar (Figure 1). Dialysis tubing was used in order to create a stable, 2-dimensional mycelial colony, and to minimize the shifting of hyphae during experiments. The Petri dishes were incubated at 28° C in the dark. They were removed from the incubator, and equilibrated at room temperature prior to the experiments.

Experimental conditions. The Petri dishes were flooded with 60 ml of low conductivity buffer solution (10 mS/cm) prior to experiments. The solution had the following composition: sucrose (6.83 gm/150ml [133 mM]), MES (0.293gm/150ml [9.1 mM]) at a pH of 5.8, KCl 10.0 mM.

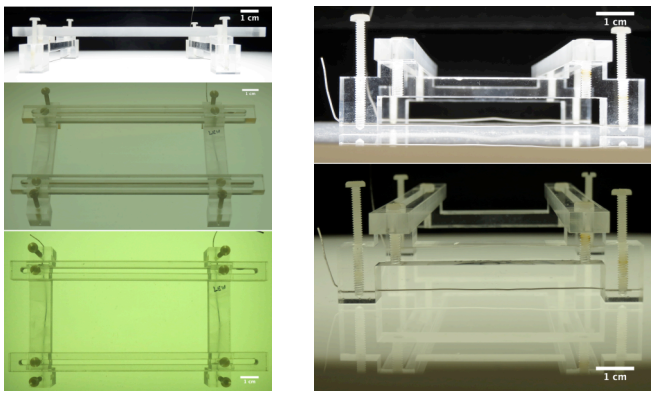

Figure 2: Apparatus used to hold platinum electrodes at fixed position inside the Petri dish.

Experimental set up. A power supply (BK Precision 1685B) was used to generate external voltage around two platinum wires (electrodes). Electrodes were positioned in a Petri dish via apparatus, specifically designed for this type of experiments (Figure 2). The distance between the electrodes was 10 cm (compared to 2.5 cm in my initial experiments). The field was 1.99 V/cm, the currents were less than 0.01 milliAmpere.

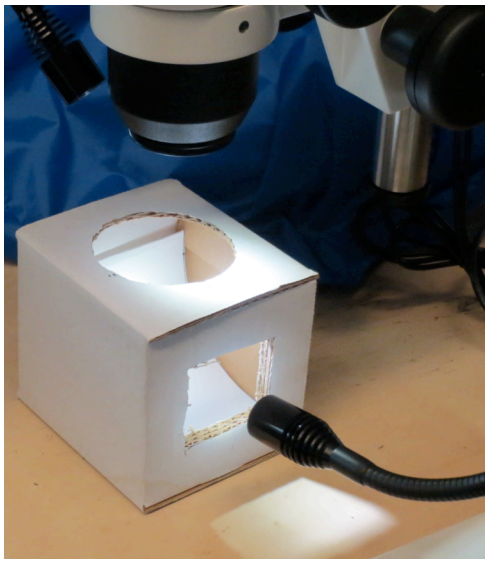

Figure 3: Lighting mount used to illuminate the Petri dish.

Depending on the experiment performed, electrodes were oriented either along the direction of hyphal growth or perpendicular to it, so four different orientations of electrodes were used (cathode to the left, right, in front, and behind). A microscope (Zeiss Stemi 2000-CS) was placed directly above the dish. In order to obtain a better quality image on the microscope a special lighting system was used (Figure 3). A piece of diffuse reflective material (paper) was placed inside the lighting mount. The paper reflector redirected the incident light coming from a lamp into the opening on the top surface of the mount, which is positioned under growing hyphae. Reflected light would enter the eyepiece directly through the colony edge, resulting in a clearer image.

Collecting Data. Images of hyphae at the colony edge were acquired every 2 minutes at ×1.6 magnification. The first 12-20 minutes of each experiment was used as a negative control (that is, no electrical field); hyphal growth in the absence of an external field was recorded during that interval. Once the field was turned on, the hyphae continued to be recorded until they: a) stopped growing (discussed later) or b) grew out of the field of view.

Analyzing hyphal orientation. Once the recording of hyphal growth was complete, the images were converted into an image stack using the program ImageJ (Rasband, 2014). Positions of hyphal tips at various time points were marked and their coordinates were measured. The coordinate values were normalized by subtracting the values of coordinates of the tip position on the first image.

$$(x_{n(normalized)} = x_n - x_0, y_{n(normalized)} = y_n - y_0) \qquad \text{eq (1 and 2).}$$

This was done in order to obtain the net growth response of the hyphae from the start of the experiment. The normalized values were then converted from pixels to microns by multiplying them by a conversion factor of 1000 microns/178 pixels. Finally the growth rate was obtained by calculating average rate of change of position over time (that is, every 2 minutes).

$$(V_{xn} = (x_n - x_{n-1})/\Delta t, V_{yn} = (y_n - y_{n-1})/\Delta t) \qquad \text{eq (3 and 4).}$$

In some cases the apparent position of the hyphae relative to the microscope lens changed due to refraction of light, rather than hyphal growth. To account for change of position of hyphae due to refraction of light, an object on the image that is expected to be nearly

stationary (such as an air bubble) was selected. The position of the object was tracked and recorded. The coordinates of the object at different points in time were normalized using equations 1 and 2, yielding the change of apparent position of the entire plate due to refraction of light. Those values were then subtracted from the normalized positions of hyphal tip. Very few trials required such correction, as effects of light refraction tend to be negligible. The growth rates of 3-4 hyphae were calculated for each experiment. The angle of growth with respect to initial growth direction was obtained using the following relation:

$$\theta_n = \arctan(V_{xn} / V_{yn}) \qquad \text{eq 5.}$$

Growth angles at various time intervals were then compared using a t-test to determine the statistical significance of any deflection in response to a electrical field.

Analyzing hyphal branching. The number of branches each hyphae formed was recorded. The data for each tip was grouped based on type of experiment (i.e. electrode orientation) and compared to controls with a χ^2 test.

Graphic representation of data. Experimental results were visualized by a positional graph (figure 4A) and vector field graphs (figure 4B, C). The positional graph was obtained by plotting the normalized x position *versus* normalized y position of the tip where x corresponds to initial growth direction and y represents deflection. Asterisks indicate positions where the growing tips branched. The vector field graph shows the velocity vectors of each hypha over the time span of the entire experiment. The x-axis represents the time. Negative x-values correspond to time intervals prior to application of the field. The y-axis represents the x-component of the growth rate (i.e. along initial growth direction). Vector arrows point in new direction of growth. The graph was plotted such that each arrow corresponds to the average of three consecutive velocity vectors, centered at the middle time point. This was done to improve the clarity of the graph and decrease the overlap between arrow vectors. Figure 4B shows the vector fields for individual hypha, while figure 4C shows the average vector field for all four hypha.

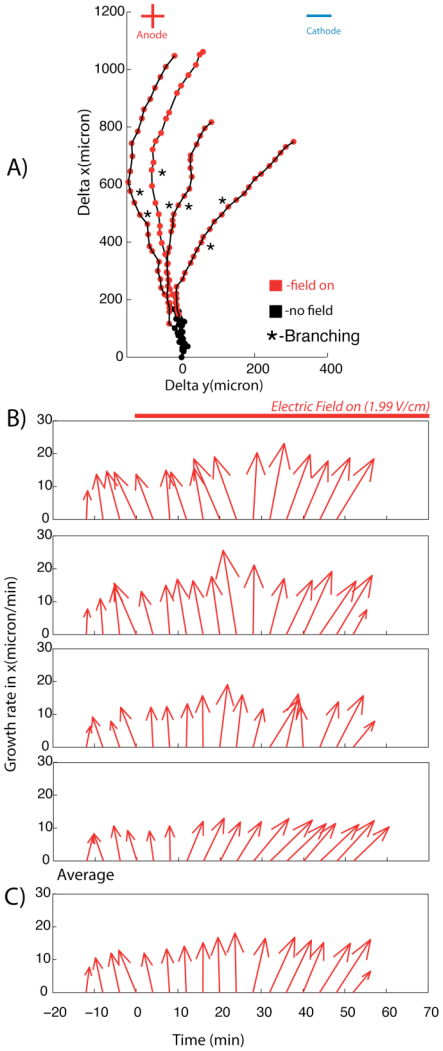

Figure 4: Experimental example. A. positioning; B. growth vectors for four hyphae; C. average growth vector.

RESULTS

Hyphal orientation. Average growth rate vectors of *N. crassa* under various electrode orientations are plotted *versus* time in figure 4. In figure 4a,b, the field was perpendicular to initial growth and caused tips to grow towards cathode in either orientation. The control was growth in the absence of an external field (figure 4e): The hyphae did not change growth direction over time. In experimental, where the cathode was nearly aligned with growing colony edge (in front of it), hyphal growth and direction was fairly constant for the first 36 minutes of exposure to voltage field, followed by reorientation in various directions and eventual alignment towards cathode. When hyphae were positioned facing anode (figure 4d), the growing tips tended to bend away from the anode and/or towards the cathode. Deflection occurred in either direction, so figure 4d understates the magnitude of the change due to averaging.

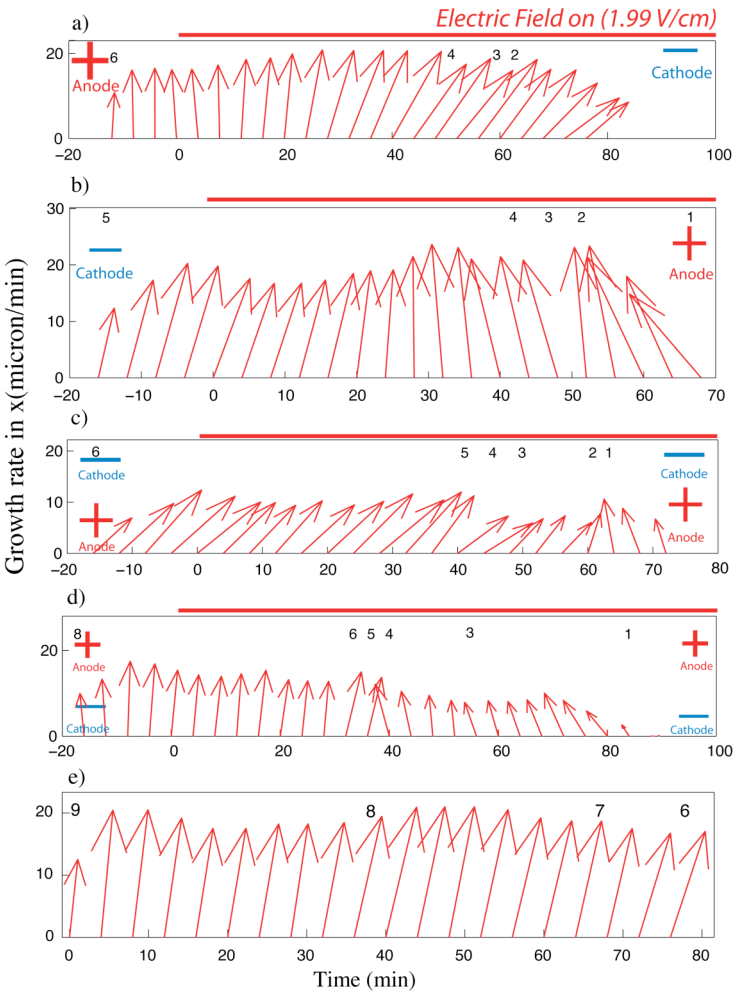

Figure 5: Average polarization of hyphae exposed to voltage fields of various orientations. Each arrow represents an average over a 4 minute interval, over all hyphae grown under identical conditions. Sample size for each point in time is indicated above. Field applied at 0 min. The control (no voltage) is shown in e).

Significance of reorientation Analysis of growth angles for the experiments with perpendicular initial orientation between hyphae and electrodes (figure 5a,b) determined that the shift in angle distribution increased in significance over time.

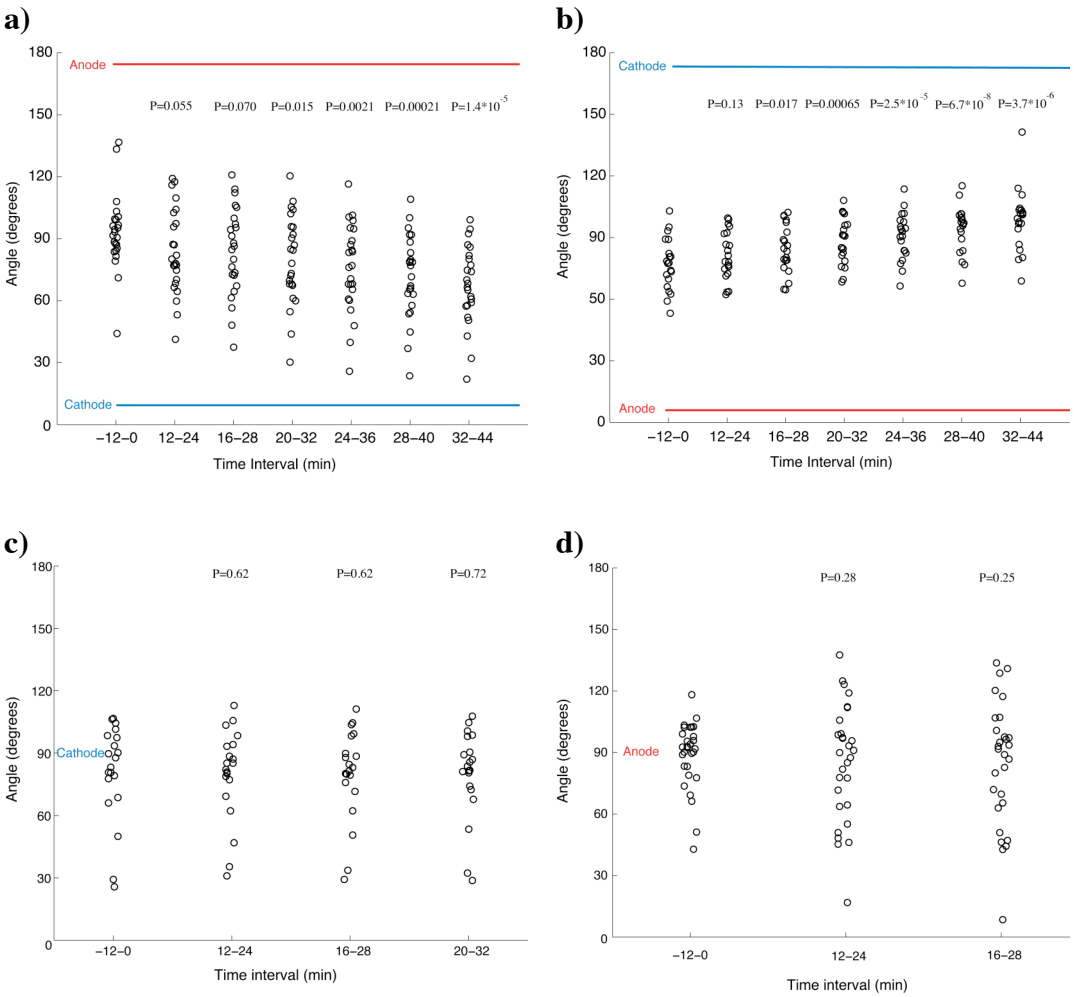

Figure 5: Angle distribution at different time intervals for various electrode orientations. Each point represents angle of growth of a single hypha averaged over corresponding time interval. P values were obtained by performing paired, two-tailed T-test between selected time interval and control (-12 to 0 min) interval.

With longer exposures to the field, there was a stronger orientation of hyphae towards cathode. When growing tips were aligned with the cathode (Figure 5c) any changes in direction of growth were found to be statistically insignificant, meaning that the field was not likely to be the source of any deflections observed (discussed later). Analysis of the growth angle of anode-oriented hyphae (Figure 5d) confirmed that cells have a tendency to bend away from anode in either direction, as evidenced by the fact that angle distribution spread apart rather than shifted in a single direction, but any differences between growth angles were not statistically significant.

Induced branching. *N. crassa* hyphae showed a tendency to branch at a higher frequency when exposed to electric fields at certain electrode orientations (figure 6). The most significant increase in the number of branches occurred when hyphae were facing the cathode initially. In trials where electrodes were aligned perpendicular to the growing edge, there was no significant changes in branching patterns for one of the orientations (cathode-right). However, with reversed electrodes (cathode-left), there was a quite a significant increase in branching. Hyphae that were oriented towards the anode showed a slight change in branching patterns that was statistically significant.

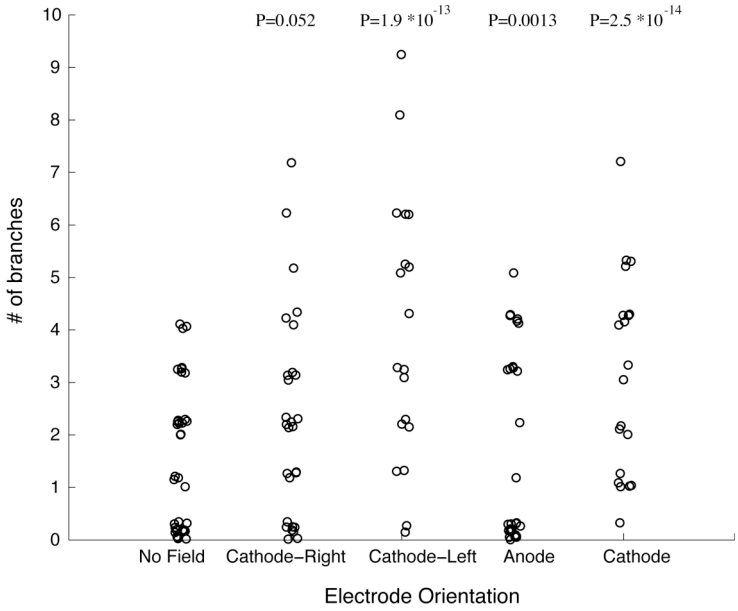

Figure 6: Branching distribution at various electrode orientations. Each point represents # of times a single hyphae branched throughout entire experiment. P values were obtained by performing χ^2 test.

Growth cessation. Prolonged exposure to voltage fields resulted in a decrease in growth rate (figure 4) followed by complete cessation of growth in all hyphae observed. The duration of field necessary to induce growth cessation varied greatly between different trials and seemed to depend on multiple variables such as distance from each electrode and orientation (discussed later).

DISCUSSION

Our results demonstrate galvanatropism of *Neurospora crassa* towards the cathode, opposite to what was observed by McGillivray and Gow (1986). The discrepancy in growth direction toward the cathode or the anode suggests that hyphal polarity is not an inherent property of the fungi itself, but is dependent on multiple external factors as well. For example, Cho et al. (1990) found that growth medium has an effect on hyphal galvanatropism. Changing from a phosphate buffer to a PIPES buffer reversed the growth direction of *Neurospora crassa* from anode- to cathode-directed. We used MES buffer (no phosphate was present). Other research revealed that *Neurospora crassa* exhibits cathodotropism at low pH values and anodotropism at higher pH (Lever et. al. 1994). We used pH 5.8.

Difference in branching patterns (figure 6) for various electrode orientations also supports the idea that behavior of cells under the influence of exogenous fields is dependent on several variables. Increased branching in the trials where colony edge was oriented towards the cathode may potentially be a consequence of cathodal galvanatropism. Katz, Goldstein and Rosenberger (1972) proposed that hyphal branching can be a means of increasing the rate of biomass flow. If this is the case, our results indicate a tendency for hyphae to increase the rate of biomass flow towards the preferred electrode. However there are several potential causes of increased branching and additional experiments are required to determine whether the proposed model is valid. The fact that branching distributions for the two experiments with electric fields oriented oppositely (cathode-right and cathode-left) were statistically different (P values of 0.052 and 1.9×10^{-13} when compared to control) suggests uncontrolled experimental factors are at play.

We observed growth cessation when a shorter electrode separation was used (2.5 cm compared to 10 cm). One possible cause is a change in pH due to red-ox reactions at the electrodes. An electrolytic cell consisting of two platinum electrodes undergoes the following reactions when placed in an aqueous solution (Shreir 1994). At the cathode:

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \qquad \text{eq 6}$$

and at the anode:

$$H_2O \rightarrow \frac{1}{2}O_2 + 2H^+ + 2e^- \qquad \text{eq 7}$$

Therefore, the local environment at the anode becomes increasingly acidic over time while the region around cathode will turn basic. The fact that electrodes span across entire Petri dish means that a significant fraction of the dish is influenced by the reduction-oxidation reactions written above. As a result, over time, several regions on the plate, located near either electrode will reach pH that is not optimal for fungal growth, potentially causing it to stop.

To determine how much pH changed, I measured pH at various times and positions across the dish. The results are summarized below (Figures 7 and 8).

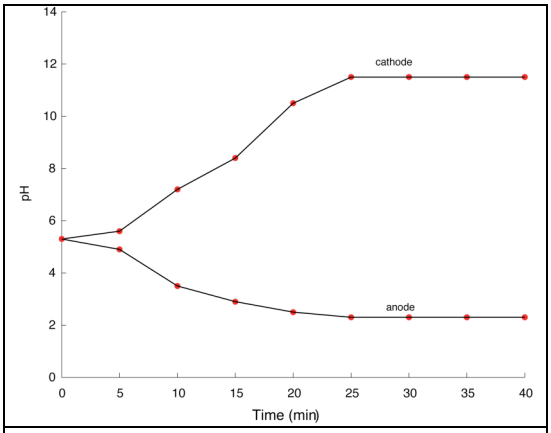

Figure 7: Time dependence of pH near electrodes. Measurements were taken 0.5 cm away from each electrode. Note the uncertainty in pH values: +/- 0.2

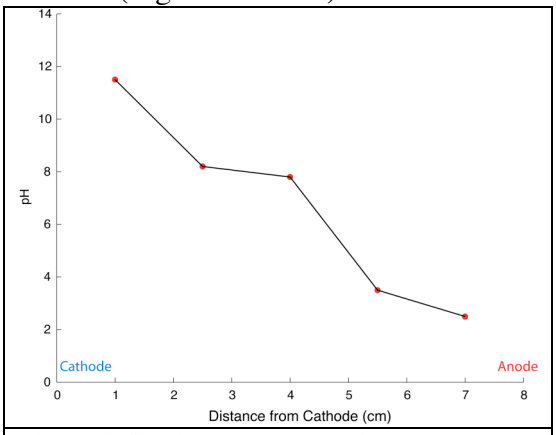

Figure 8: Spatial dependence of pH. Measurements were taken at 40 minutes after the field was generated. Note the uncertainty in pH values: +/- 0.2 and in distance:+/- 0.3 cm.

Growth ceases as early as 30 minutes during the experiments. By that time the pH values near both electrodes are extreme (about 2 at the anode and 12 at the cathode). These may inhibit growth of *Neurospora crassa*. The pH changes linearly between the two probes. (Figure 8). In the middle of the dish, pH increased by nearly 3 units over the course of 40 minutes. Thus the hyphae does not need to be located near electrodes in order to be affected by pH change.

Another factor that may play a key role in aborting hyphal growth is the production of oxygen and hydrogen gases at anode and cathode respectively. Although O_2 has not been observed to influence the growth of mycelial fungi, certain species of plants such as *Andromeda* have shown to exhibit negative oxytropism (Blasiak et al. 2001). It is a possibility that *Neurospora crassa* favours growth away from oxygen rich environment, hence deflection away from anode, due to the fact that oxygen inhibits hyphal growth. Hydrogen gas may also be the source of growth cessation.

The number of moles of OH^- and H^+ (and oxygen and hydrogen) produced per second locally can be found using the relationship:

$$\frac{n}{t} = \frac{I}{F} \qquad \text{eq 8}$$

Where F is Faraday's constant. An increase in concentration per second is therefore:

$$\frac{c}{t} = \frac{I}{FV} \qquad \text{eq 9}$$

Where V is the total volume of the solution.

In our experiments the values of currents were smaller than 0.01 mA. The average current was approximated to 0.005 mA. The volume of region, where change in ion concentration is significant was approximated to be 5 cm × 2 cm × 0.3 cm=3ml. Growth cessation started to occur approximately 1800 seconds into the experiment. Meaning the increase in ion concentration near electrodes was approximately 3.1 mM at that point. Hydrogen and hydroxide ions are in a ration of 4:1 and 2:1 with oxygen and hydrogen gases respectfully, therefore increase in concentration of oxygen gas is approximately 0.8 mM and increase in concentration of hydrogen gas is 1.6 mM. Some of the production of gasses is likely to be offset by evaporation, so the increase will be significantly less.

In future experiments, it will be imperative to avoid the confounding effect of pH gradients on the growth of the fungal hyphae in an electrical field.

REFERENCES:

Blasiak J., Mulcahy D.L., Musgrave M. E.. (2001) Oxytropism: a new twist in pollen tube orientation. Planta. 213: 318-322.

Cho C.-W., Harold F. M., **Schreures W. J. A.** (1991) Electric and ionic dimensions of apical growth in *Achlya* hyphae. Exp Mycol. 15:34-43.

Crombie T., Gow N. A. R., Gooday G. W. (1990) Influence of applied electrical fields on yeast and hyphal growth of *Candidia albicans*. J Gen Microbiol. 136:311-317.

Gow N. A. R. (1984) Transhyphal electrical currents in fungi. J Gen Microbiol. 130:3313-3318.

Jackson S.L., Morris E. J. S., Garrill A. (2001) Hyphal branching and the induction of cell polarity. in *Cell Biology of Plant and Fungal Tip Growth* (**Geitmann A., Cresti M., Heath I. B.**) NATO Science Series, IOS Press, Amsterdam-Berlin-Oxford-Tokyo-Washington. 69-79 p.

Katz D., Goldstein D., Resenberger R. F.. (1972) Model for branch initiation in *Aspergillus nidulans* based on measurements of growth parameters. J. Bacteriol. 109 (3): 1097-1100.

Lever M.C, Robertson B. E. M., Buchan A. D. B., Miller P. F. P., Gooday G.W., Gow N. A. R. (1994) pH and Ca^{2+} dependent galvanotropism on filamentous fungi: implications and mechanisms. Mycol. Res. 98 (3): 301-306.

Lew R. R. (2007) Ionic currents and ion fluxes in *Neurospora crassa* hyphae, J Exp Bot. 58(12): 3475-3481.

McGillivray A. M., Gow N. A. R. (1986) Applied electrical fields polarize the growth of mycelial fungi. J Gen Microbiol. 132:2515-2525.

Neymark D., Lew R. R. 2014. *Auctus Electrica:* Voltage field dependence of *Neurospora crassa* growth. [Internet]. Available from: http://www.yorku.ca/planters/student_reports/auctus_electrica.pdf

Potapova T.V. (2014) Structural and functional organization of growing tips of *Neurospora crassa* hyphae. Biochem. 79 (7): 753-769.

Rasband, W. S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014

Shreir L. L., Jarman R. A., Burstein G. T.. (1994) Corrosion (3rd Edition). Volume 1 Butterworth Heinemann. 78 p.

APPENDIX A-CREATING PLOTS IN MATLAB

Vector Field:

quiver(Time,ans,Vfx,Vfy,scale)

Time=x-coordinate of the vector tail. Represented by time interval over which growth occurs.
Vfx=x-component of the vector. Represents initial growth direction
Vfy= y-component of the vector. Represents direction perpendicular to initial growth
ans= x-coordinate of the vector tail. Represented by zero matrix equal in size to Time, Vfx, Vfy
scale=1x1 matrix. Makes vectors unscaled.

Scatter Plot:

```
scatter(y1,x1,S,C)
hold on
scatter(y2,x2,S2,C2)
plot(y2,x2)
scatter(y3,x3,S3,C3)
plot(y3,x3)
scatter(y4,x4,S4,C4)
plot(y4,x4)
```

Plots 4 scatter plots, one for each hypha, overlapped by a regular line plot on the same graph.
x1-4=y-coordinate in of hyphal tip position
y1-4=x-coordinate in of hyphal tip position
S1-4=Size of the markers on scatter plot, set to constant value of 30. Matrix is equal in size to x1-4 and y1-4.
C1-4=Color of markers on scatter plot

Branching Distribution:

```
scatter(x1,control,s1,c1)
hold on
scatter(x2,anode,s2,c2)
scatter(x3,rf,s3,c3)
scatter(x4,cathode,s4,c4)
scatter(x5,of,s5,c5)
```

Plots 4 scatter plots one for each treatment on the same set of axis.
x1-4=type of treatment. x-values within same treatment are constant. Different treatments separated evenly along the x-axis by an arbitrary interval.
control, anode, rf, cathode, of=# of branches each hyphea exposed to corresponding treatment has formed. Plotted on y-axis
s1-4=Size of the markers on scatter plot, set to constant value of 30. Matrix is equal in size to x1-4 and control, anode, rf, cathode, of.
c1-4=Color of markers on scatter plot

Angle Distribution:

```
scatter(x1,Af1)
hold on
scatter(x2,Af2)
scatter(x3,Af3)
scatter(x4,Af4)
scatter(x5,Af5)
scatter(x6,Af6)
scatter(x7,Af7)
```

Plots n-scatter plots corresponding to n # of intervals analyzed. Each plot is for one treatment.
x1-n=selected time intervals. x-values for the same time interval are constant. Different intervals separated evenly along the x-axis by an arbitrary interval.
Af1-n=average angle of growth over selected interval for each hyphea exposed to treatment.