Mechanical Engineering and Materials Science Independent Study

Mechanical Engineering & Materials Science

5-9-2018

Testing the Viscoelasticity of Arabidopsis Hypocotyls

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Experiment Date: Spring Semester 2018

I hereby certify that this report is my own original work.

I. ABSTRACT

The mechanical responses of hypocotyls, the embryonic stem of a plant between the cotyledons and root, are of interest because they can reveal insight into the timing and mechanosensitivity of growth hormones in plant growth and development. This independent study project aimed to develop a protocol for quantifying the viscoelastic responses of Arabidopsis hypocotyls.

II. INTRODUCTION

Hypocotyls, the embryonic stem of a plant between the cotyledons and root, extend in the early stages of development through elongation of embryonic cells. The mechanical regulation of this has not been fully characterized. A protocol for characterizing the time course and hormonal sensitivity of hypocotyl mechanics is therefore a pressing need.

The objective of this independent study was to develop such a protocol. The protocol built from several technologies available in Dr. Genin's lab. The first was a home-built stretching device that enabled gram-level forces to be measured [1-5]. The device had to be adapted to testing plants.

When hypocotyls were stretched, the force necessary to maintain their length dropped over time. This is a material behavior called viscoelasticity that is exhibited by many biological tissues [6]. Although most biological materials are nonlinear in addition to being viscoelastic [2, 6-7], the timescales over which the viscoelastic relaxation occurs are often insensitive to the degree of straining [6-10]. Interpretation of the viscoelastic tests was therefore done using linear viscoelastic spectral analysis tools [8-9]. The specific tools used are tools under development by Roger Rowe.

Although factors such as cytoskeletal disruption [11-13], mechanically activated signaling [14-16], and remodeling [17] can affect viscoelastic relaxation spectra, these were not factored into the analysis.

III. APPARATUS AND PROCEDURES

Testing the viscoelasticity of hypocotyls of Arabidopsis requires diligence and gentle hands. To begin the experiment one needs steel wire, wire cutters, Super Bonder 495 superglue

seen below in figure 1, forceps, one week old Arabidopsis plants grown in constant yellow light, tweezers, and an apparatus that can provide loads on the order of dynes.

Fig. 1 Image of Super Bonder 495 adhesive.

It is important to note that The Super Bonder 495 superglue used is required to be refrigerated when not in use. The specific type of wire is not critical to the success of the experiment; it is only important that the wire is stiff enough that it will not yield before the Arabidopsis hypocotyls do. Below in figure 2 is an image of the load cells used during this experiment:

Fig. 2 Image of four load cells without the wires and Arabidopsis hypocotyls attached.

Above in figure 2, the black casings with a metal extrusion are the load sensors and the L-shaped bars below them are the load bars. Now that all the materials have been acquired, one can begin adhering the Arabidopsis hypocotyls to the steel wires. First, remove the superglue

from the refrigerator and acquire the steel wire. Using the wire cutters, cut the wire into eight equal lengths that should correspond to the distance between the load cell sensor and bar. It is not necessary for the wire attached to the load cell to be the same length between load cells, however, the bottom wire must be all the same length. This will ensure the reaction force due to gravity from the weight of the wire will be uniform across the four load cells. Next, bend the ends of the wire using forceps and a table. Pinch the forceps onto the end of the wire so in the orientation where the wire is perpendicular to the forceps. The end of the wire should be flush with the jaws of the forceps when pinched. Bend the wire using a table and twisting motion to bend the end of the wire into a hook. Each wire should lay flat when with the hooks. This means that the hooks should bend the same direction similar to the four wires towards the top of the image seen in figure 3:

Fig. 3 Image of eight steel wires: four wires with hooks in parallel and four with hooks in a perpendicular orientation

Repeat this process until all four wires have ends with hooks. Depending on the apparatus it may be be beneficial to have half of the hooks with the hooks bent in a perpendicular direction relative to the other hook on the same wire. In this experiment, due to the orientation of the load

sensor and load bar, it was logical to have half of the wires with hooks bent in a perpendicular orientation also seen in figure 3.

Place each of the wires on an elevated surface with approximately a 5mm gap between the ends of each hook. The importance of the gap and the elevated surface is for the wire to avoid contact with anything but the Arabidopsis hypocotyl upon application of the superglue. The 5mm corresponds to the approximate length of the hypocotyl, but it is not necessary to measure this distance between the wires yet as the Arabidopsis hypocotyl will be measured prior to running the experiment. First, apply less than a drop of superglue to the end of the wire. This process can be achieved by squeezing the superglue until a bead forms at the tip of the tube and carefully placing the bead onto the end of the hook without letting the entire drop fall. The amount of glue is not as important to securing the plant as it is to the amount of time the glue will take to dry. A smaller amount of glue is beneficial as it will still hold the hypocotyl in place during the experiment, and will dry much quicker. The glue should be placed at the furthest end of the hook, not the tip of the wire. Next, take the Arabidopsis hypocotyl from the dish and carefully trim the roots and leaves off the plant. This is done to ensure that the only part of the plant being stretched is the hypocotyl. Once the plants are trimmed place the end of each hypocotyl into the super glue at the ends of each hook and let them sit for at least 20 minutes. Again, the time is dependent on the amount of superglue applied to each wire, so this amount of time can vary. It is critical to ensure the glue is dry before proceeding to the next step. Below in figure 4 is an image of the glued ends of the wires with the hypocotyls of Arabidopsis drying prior to testing.

Fig. 4 Image of ends of the hooks with superglue and hypocotyls attached

After waiting at least 20 minutes or until the glue completely dries, place one hook from each of the four assemblies onto each of the four loading sensors. Make sure that when attaching the wire and Arabidopsis assemblies to be extremely careful as the hypocotyls are very fragile. If some of the wires were not the same length, remember that it is only important that the lower wires should all be the same length so they apply the same stress on the hypocotyls initially. Now that all of the assemblies are attached to the load cells looking at the sensor readings, rotate the motor by hand to put the plant assembly and load cell in contact. This is not intended to stretch the hypocotyls, but to preset the load apparatus to the correct initial length. Look for a slight change in the sensor reading to ensure contact has been made. Once all of the assemblies have made contact with their respective load cells, wait at least 30 minutes to allow the hypocotyl to relax after being stretched by the weight of the lower wire.

While waiting for the 30 minutes to pass and the hypocotyls to relax, one should measure the initial lengths of each hypocotyl. The goal is to stretch each hypocotyl by 10% so it is important to record the initial lengths of each of the hypocotyls in a lab notebook. Furthermore, it is important to label each one with the correct load cell. To measure each hypocotyl, use metric digital calipers and measure the hypocotyls already in the load apparatus. Measure only the distance between where the hypocotyl is glued to each of the two wires it is attached to. The

software used in the experiment, Poker, requires an input of microns (μm) into the CPX file, so it is beneficial to initially measure in metric for a simple conversion.

Another task that can be accomplished during the 30-minute relaxation time is to make a new directory for the experiment and edit the CPX file for the Poker software. Poker runs the experiment and controls the motors on all of the load apparatuses. First, in DOS one should make a new directory. This can be done using the command *mkdir filename* and hitting the enter key *<enter>*. The file name can be whatever one chooses, although it is recommended to choose a name such that corresponds to the date the experiment was executed. Next create a CPX file by copying an old CPX file from one of the previous Arabidopsis hypocotyl experiments and the edit the file by typing *edit cpxname.cpx <enter>*. Now in the editor, one can edit the desired stretch lengths for each of the hypocotyls. Enter each value as 10% of the length of each corresponding hypocotyl in microns. Now that the CPX file is correct, in DOS type *Poker <enter>*. The computer will now open up the Poker software and it is time to tell Poker where to save the data when the program concludes. This is done by typing *"filename\" 'rufp def/r* *<enter>*. Now the program is ready to run by typing *@,cpxname <enter>*. After at least 45 minutes has passed one can begin the Poker software that stretches the Arabidopsis hypocotyls 10% of its initial length. Once this is completed, the program will run four programs over the course of 40 minutes and 30 seconds.

The first program is a monitoring step that lasts 5 minutes. The second program is a calibration step that brings the load bar down slightly and back up over the course of 30 seconds. Next, the most important step is the ramp step. This step lasts for 5 minutes and induces a 10% strain on each of the hypocotyls over 9.9 seconds. Then the load bar stops moving as the force sensor continues to record data. This is where the hypocotyl begins to relax. After the stretch, and the proceeding ramp monitoring, the next step continues monitoring for 30 minutes. Once the program concludes, the data is saved to a previously assigned directory on a floppy disk. The new CSV file can then be run through MATLAB to analyze.

IV. ANALYSIS

In this experiment the data in the experiment were collected via computer and analyzed using MATLAB. The software used to execute the experiment called Poker reads a CPX file created by the user communicating to the motors to apply a certain stretch. During the ramp step

lasting 5 minutes, the program reads 50 frames per second or a total of 15,000 during the duration of the step as the hypocotyls relax. For the analysis of the data MATLAB was used to clip files and scale them. The data was then scaled where the maximum force was determined and then all the data points were divided by that value. This creates a scale where the maximum is equivalent to 1 and all other points are equivalent to a percentage of the maximum. This makes it easier to analyze and compare hypocotyls that experienced different stresses. The raw data when plotted also has an x-axis of points instead of time. It is important to scale the x-axis to be in terms of time, thusly. This was done by dividing the vector by 50 as there where 15,000 points for 30 seconds.

An initial assumption that was made was that all the hypocotyls had the same diameter and therefore experienced the same stress. This assumption is likely incorrect as seen in the following results section. It would be expected that a hypocotyl that experienced the same stress would also experience the same viscoelastic response, but the three different hypocotyls clearly do not. Another source of error is the potential for the superglue to not be completely dry and allow for some slipping or deflection when undergoing stress. This will prevent the hypocotyl from being fully stretched to the amount prescribed by the CPX file. Another source of error is drafts in the room. The highly sensitive force sensors can certainly pick up on any other small drafts and movements of air from a lab worker walking by the apparatus too fast. Another source of error is the measurements made in the experiment. Although calipers are a very accurate method for measurement, the magnetic nature of the calipers made it difficult to measure the hypocotyls on the steel wires which opposed the caliper jaws. This made it extremely difficult to get an accurate measurement for each of the hypocotyls and therefore made it difficult to apply a uniform strain across the four hypocotyls.

V. RESULTS

The entirety of the data was obtained using the computer attached to the tissue stretcher using Poker and DOS. The analysis was completed by using MATLAB to clip, scale, and to use the Tikhonov Regularization Method to model each of the hypocotyl's relaxation. Clipping was the first step in scaling the data and it was required to be done twice: once before the ramp and also cutting out the ramp separately. All the figures were made using MATLAB. Below, figure 5 provides the beginning of the ramp step that was cut out. The importance of this step is not only

8

to better align different data sets for comparison purposes, but also to determine exactly when each hypocotyl started to undergo stress.
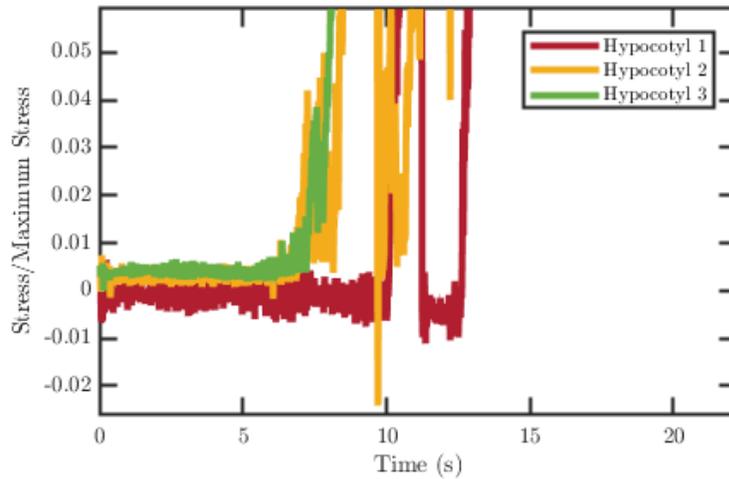

Fig. 5 Beginning section of the ramp plot used to determine when a stress was indeed applied

Next, the same idea was applied to determine when the ramp function ended and when each one of the hypocotyls began to relax. Figure 6 depicts the maximum stresses for each of the three hypocotyls:
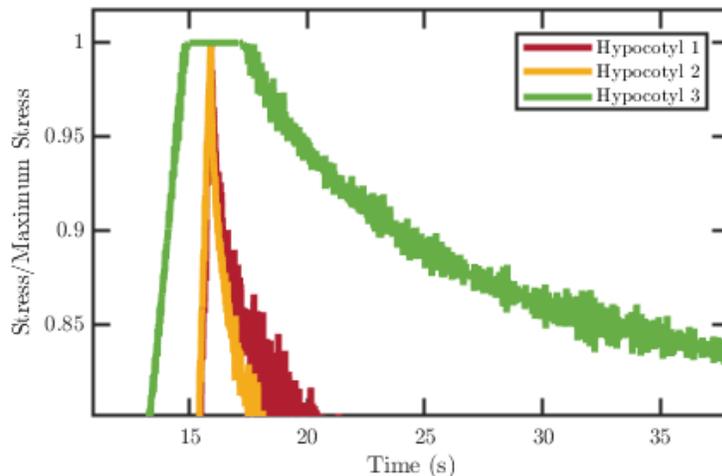

Fig. 6   Middle section of the ramp plot used to determine when the maximum stress was reached and when the hypocotyls began to relax

Note that the third hypocotyl might have exceeded the peak force of the force transducer; the plateau evident in Figure 6 is unlikely to represent a physiological response. Next, the plot was reformatted so that all of the hypocotyls were scaled in which they all had a maximum at time equal to zero seconds. This provides the best comparison between the three different hypocotyls. After the plots were made, the Tikhonov Regularization method was applied to apply a line of best fit to each of the curves seen below in figure 7:
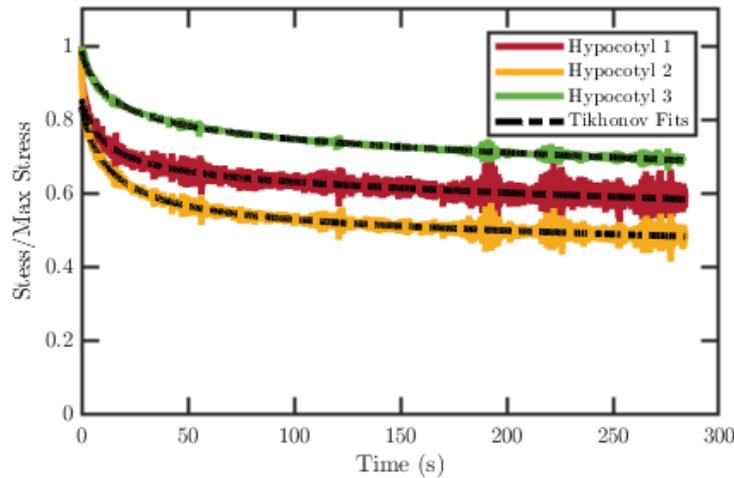

Fig. 7   Ramp plot reformatted to scale all hypocotyls with Tikhonov Fits

The next plot below represents the results of the elastic constants determined by the normal least squares method with the additional smoothing term seen in equation $%1. The three regularization parameters, λ, were 74.9372, 87.3244, and 48.8099 for hypocotyls 1, 2, and 3 respectively. Below in figure 8 are the functions determined by this method:
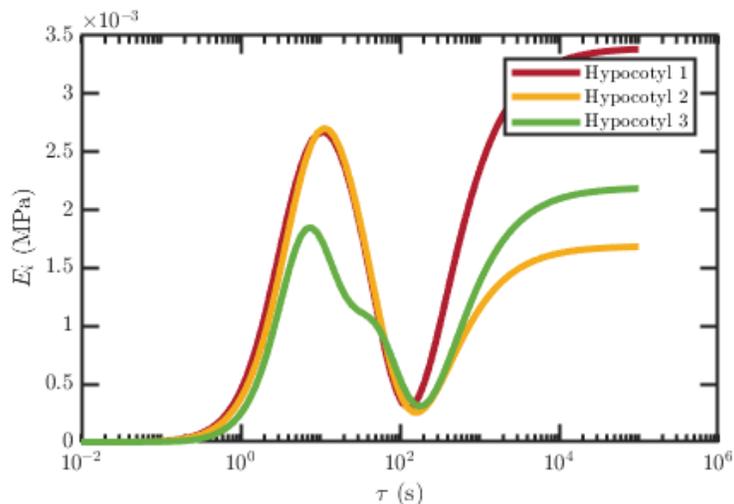
Fig. 8   **Elastic constants for the three hypocotyls**

For comparison proposes, without clipping the different data sets to determine which data points mark the beginning of the ramp and which data points marked the end of the ramp, the plot would look like the plot in figure 9 below:
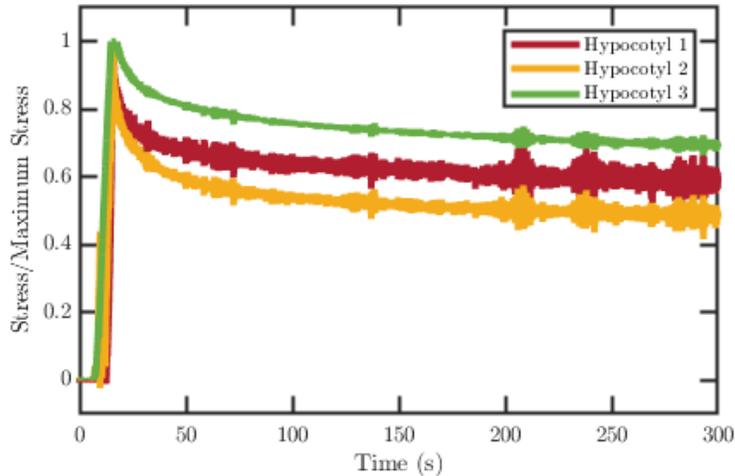
Fig. 9   **Ramp plot without clipping**

The above plot looks as if all three hypocotyls did begin relaxation at the same time, however, returning the attention back to figure 5 one can see this is not the case.

VI. DISCUSSION

First and foremost, the three hypocotyls all behaved exactly as expected and similar to other biological materials. After the initial stretch each of the hypocotyls began to relax in an intial rapid rate, but slowly began to taper off after approximately one minute. After reformatting the data and rescaling figure 7 one can analyze the three hypocotyls effectively. It would be expected for the three hypocotyls to experience the same stretch relatively, but the viscoelastic response is different for all three. Furthermore, the three hypocotyls were designed to experience the same amount of stretching, but due to difficulty in measurement the actual amount stretched was not always as accurate as hoped. This of course

This is likely due to the fact that the three different hypocotyls have different cross sectional areas. A larger cross sectional area would yield a smaller stress and would certainly have a different viscoelastic response than that of a thinner stem. One might also notice the

amount of noise present in all of the results figures barring figure $%4. Some of the more noticeable noise that can be pinpointed at approximately 140, 210, and 240 seconds are all likely to movement around the load apparatus. Walking too fast past the load cell apparatus can cause enough of a breeze to affect the force sensors. This environmental noise is most likely the case as all three hypocotyls experienced the same noise at precisely the same time.

The quality of data has room for improvements and future suggestions are provided in the conclusion. This set of data includes some noise, but the overall shape is exactly what one would expect in a viscoelastic material. The data collected is accurate as the sensors were all calibrated prior to use, however sources of error are always present and are discussed later in the analysis. In figures 5 and 6 it is easy to see that there is some delay in the system. For example, in figure 5 all three hypocotyls begin the ramp stage at different times and in figure 6, hypocotyl 3 plateaus at its maximum stress. This is quite unusual and is likely due to a system bug that was brief as it would not be expected for the hypocotyl to delay a viscoelastic response.

VII. CONCLUSION

The results found in this laboratory are an excellent start to better understanding the stress dependence of Auxin. This particular experiment acts as the benchmark for future work to be done and to determine if the stress applied by the load cells will induce a higher output of auxin within the plant cells. It would be recommended to look at the hormone response by differing the amount of stress applied to the hypocotyls. This could result in a correlation between the stress applied and the amount of auxin produced. Another improvement would be improving the measuring techniques to determine both the length of the hypocotyls and the diameter. In this experiment the area was not determined as using calipers were not appropriate because they could damage the hypocotyl, jeopardizing the integrity of the hypocotyl. Measurement could be improved by using confocal reflectance imaging to get the exact length and diameter. In fact, a test to see if the confocal reflectance imaging would be a viable solution was determined to be successful and the image taken from the confocal apparatus is seen in figure A1 in Appendix A. This would certainly improve the accuracy of measurement and therefore, result in a more accurate stress applied. Theoretically, the initial length of the hypocotyl could also be determined this way. Applying a curtain to the apparatus to reduce the risk of drafts from interfering with the experiment is also encouraged. Lastly, fabricating a set up apparatus that secures the hooks and

ensures that they are all equally separated prior to adding the superglue and hypocotyls. This should also improve the accuracy of stress applied. This experiment provides the foundation to look further into the plant hormone auxin and its response in plants.

VIII. ACKNOWLEDGMENTS

IX. REFERENCES

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X. APPENDICES

Appendix A
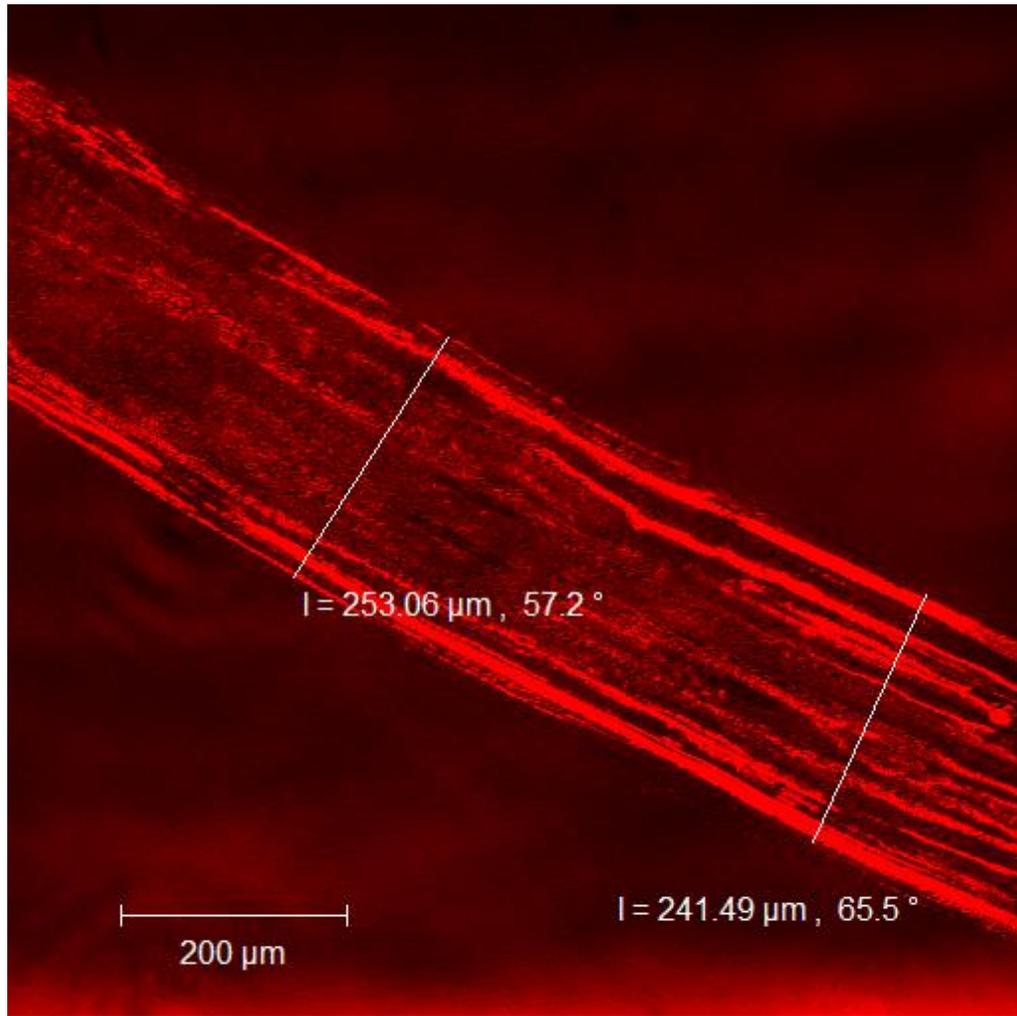
Fig. A1   **Confocal Reflectance Imaging Microscope image of hypocotyl with two measurements of diameter**