LIGHT PRODUCES CALCIUM WAVES IN NATURE

An Undergraduate Research Scholars Thesis

by

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RESEARCH COMPLIANCE CERTIFICATION

Research activities involving the use of human subjects, vertebrate animals, and/or biohazards must be reviewed and approved by the appropriate Texas A&M University regulatory research committee (i.e., IRB, IACUC, IBC) before the activity can commence. This requirement applies to activities conducted at Texas A&M and to activities conducted at non-Texas A&M facilities or institutions. In both cases, students are responsible for working with the relevant Texas A&M research compliance program to ensure and document that all Texas A&M compliance obligations are met before the study begins.

I, Sofia E. Patino Hernandez, certify that all research compliance requirements related to this Undergraduate Research Scholars thesis have been addressed with my Research Faculty Advisor prior to the collection of any data used in this final thesis submission.

This project did not require approval from the Texas A&M University Research Compliance & Biosafety office.

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ABSTRACT

Light Produces Calcium Waves in Nature

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Calcium waves that arise from high intensity 405nm blue light photostimulation in *Arabidopsis thaliana* have been observed and studied in the Griffing Laboratory to identify photoreceptors and signaling significance. This project attempts to understand when and if this high-intensity 405nm light occurs in nature by analyzing the parameters that lead to high-intensity light in real-life conditions. Our model is that the light not only has to be of a specific wavelength and photon dose, but it also needs to shine on a specific subcellular region, the ER-chloroplast nexus, to get direct stimulation and produce the calcium wave. This research measures photon dose and wavelength required for photostimulation with a microscope-based spectrometer and power meter and whether dew drops or rain drops might focus the light to a subcellular location. It also explores the varied factors that may affect the interaction of light with plant cells to create the photostimulation effect. Experiments analyze the threshold at which photostimulation triggers signaling and correlate these experiments to how sunlight could also trigger signaling in real-life conditions. Other experiments explore the effect of a circadian rhythm in the respective calcium wave. Connections between the observed wavelengths under

weather conditions and how the presence of drops increase the power of light support our hypothesis. Structures such as trichomes can also influence the focal point of the light in the plant surface and direct it to the ER-chloroplast nexus at the optimal power to create the calcium wave.

DEDICATION

Para mi mamá por amarme siempre. Para mi papá por apoyarme siempre. To my little sister for being my little best friend.

To Michael, for always being there for me and for all the happiness.

Para mi familia en México, por recordarme siempre de lo orgullosos que están de mi.

To Professor Mori, for always believing in me.

To my friends who are always present no matter the distance.

Thank you for all the love and support throughout this research process!

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Contributors

I would like to thank my faculty advisor, Dr. Lawrence Griffing, and Sara Maynard for their guidance and support throughout the course of this research.

A special thanks goes to my lab partner Kaitlin Hill for helping me by obtaining some of the data for the sunlight power and wavelength measurements.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

Finally, thanks to my friends for their encouragement and to my family for their patience and love.

The analyses depicted in *Lenses results* were conducted in part by Kaitlin Hill and Sara Maynard and these data are unpublished.

All other work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

ER	Endoplasmic Reticulum
nm	Nanometers
A. thaliana	*Arabidopsis thaliana*

1. INTRODUCTION

The perception of light by plants occurs through multiple known photoreceptors (Jones et al., 2012). The Griffing lab is using the model plant, *Arabidopsis thaliana*, to investigate the consequences of the perception of high intensity (high photon fluence or high photon dose) 405 nm light for which there is no known photoreceptor. One of the immediate responses is the production of an internal calcium wave. This project attempts to understand when and if this high-intensity 405 nm light occurs in nature by analyzing the parameters that lead to high-intensity light in real-life conditions. Our model is that the light not only has to be of the right wavelength, but it also needs to shine on a specific subcellular region, the endoplasmic reticulum (ER)-chloroplast nexus, to get direct stimulation and produce the calcium wave. The photostimulation could happen via focusing 405 nm light on the nexus, reaching a photon dose threshold at optimal wavelength.

This research measures photon dose and wavelength required for photostimulation with a microscope-based spectrometer and power meter. We are also curious about whether dew drops or rain drops might focus the light to a subcellular location. To imitate drops of water which could function as magnifiers, we constructed plastic and glass lenses. Experiments adjusting the power dose in the photostimulation and then analyzing the calcium wave will tell if the signature calcium wave we study changes in nature. These findings will help our lab further examine the dose-response curve for photostimulation and calcium wave production. We also explored under what natural conditions the photostimulation and calcium wave production can occur. Broader applications of this understanding can be applied to neurodegenerative disease and understanding the ER as part of the circulatory network of the cell.

1.1 Literature review

Considering various factors that may affect the manifestation of dew drops in nature can provide evidence for the biological significance of calcium waves. By considering sources that contain more experiments on interactions of light and plant cells more evidence can be correlated to our own experiments. The following literature review explores various factors of the interaction of light with water and plant tissues to provide background for the experiments. An introduction to calcium channels and the signature calcium wave will then lead to a discussion of how high fluence photon dosage light may manifest in nature. The properties of leaf surface microstructures and epicuticular waxes are correlated to dew drop and rain drop formation. Other light interactions within the plant tissue and their effects in the plant that determine the spatial location within the environment are utilized to further explore why light interactions may be used and create signals. Finally, the effect of a circadian rhythm on development and physiological processes throughout the day are analyzed to connect them to our experiments.

1.1.1 Photostimulation background

My work in previous semesters has examined calcium signaling in *A. thaliana* responding to light stress. Cytoplasmic waves of calcium originating from the endoplasmic reticulum act as signals in animals, and our lab is working on finding homologous signaling in plants. The goal of the project is to identify endoplasmic reticulum (ER) calcium channels, like the known glutamate receptor-like channels, that are responsible for these calcium waves. These are channels that are genetic homologs of ionotropic glutamate receptors in animals (Wudick et al., 2018). The ER and chloroplast nexus refers to the junction membrane of the organelles, and the study of the putative channels located in this region is related back to our labs study of the

ER as part of the circulatory network of the cell due to its signaling role (Griffing, 2010; Griffing et al., 2017).

In order to explain how light signals this response, it is helpful to acknowledge known light responses in plants. Focus is placed on blue-light photoreceptors, such as phototropins, which modulate certain plant growth and movement of chloroplast and stomata, cryptochromes, and other physiological responses to blue and ultraviolet light through phosphorylation cascades (Campbell, 2011; Jones et al., 2012). Other than capturing light for energy production, light responses in plants affects their development, and their sensitivity to seasonal and transient changes in light varies from species to species. Known responses involve photoreceptors with specific absorption spectra which these help plants sense changes in its environment and signal biological responses. This creates an action spectrum, which correlates the response intensity to the required wavelength for response (Campbell, 2011). The result of the specific light conditions is a signature wave of responses which communicate the environmental conditions to the plant and the needed physiological processes.

Calcium signaling is a widely used response towards stimuli with various biological significances (White & Broadley, 2003). Signals from different stressors create different calcium signatures (McAinsh & Pittman, 2009). Calcium signatures are the readout of how fast and how much the calcium increases in a particular cell location. We identify the specific calcium signature of our response towards changing parameters. Calcium signaling comes from both the plasma membrane and the endoplasmic reticulum (Carreras-Sureda et al., 2018). Our interest is the calcium wave from the ER since our lab has determined this is the response to the high fluence blue light.

The first observations of the photostimulation using a 405 nm laser used tobacco cotyledons, or seed leaves (Griffing, 2011). Protein aggregate formation and a signature calcium wave were observed upon high fluence photostimulation. Further studies in our lab determined that this calcium wave originated from the ER and the light was sensed by channels in the ER-chloroplast nexus, by observing an increase in cytoplasmic calcium and a decrease in the ER lumen calcium, which we now target for photostimulation when observing the signature calcium wave. Observations of ER-chloroplast nexus light responses can also provide insight to their association to signaling in plant cells (Choi et al., 2014; Kudla et al., 2018). Currently, various experiments in our lab are in progress to understand the biological significance of this signature calcium wave.

1.1.2 Experiments testing whether dew drops or rain drops can focus light

Many "old wives' tales" have created the belief that watering plants during high sunlight hours will harm and kill plants due to a lack of understanding of the effects of light concentration and temperature (Jones, 2010). Egri et al. (2010) investigates the scorching of plant surfaces using marbles and different leaves, focusing on the structural physiology of leaves and which types are more prone to magnification. It studied the scorching effect of the intensity and angle of light and the shape of droplets. The conclusions included three experiments with changing shapes of droplets, with the largest amount of photobleaching with rounder droplets and with the light source coming at an angle (Egri et al., 2010). These experiments occurred at the macro level, showing how photobleaching of the leaves significantly increased with large artificial drops (marble-size) and large leaves (maple leaves). We will be doing our experiments at the micro level, using small droplets of water or artificial lenses focusing light on subcellular features.

The leaf surface morphology and wettability have an effect on leaf hydrophobicity and interactions with water droplets (Masrahi, 2020). The differences in wettability on microstructures in adaxial and abaxial surfaces of the plant change dew collecting ability (Masrahi, 2020). In Figure 1.1, some of the microstructures of *A. thaliana* can be observed. Microstructures in *A. thaliana* may influence the shape of droplets and the focal point of the light reflected by the water drops. Leaf structures in 3-week-old *A. thaliana* include trichomes, or leaf hairs, which have a glassy appearance. These help by creating a barrier to prevent water loss of the plant and helps reflect light (Suo et al., 2013; Zhou et al., 2017). Trichomes can be up to 0.5mm high and rich in polysaccharides and cuticular waxes (Suo et al., 2013). I hypothesize that these trichomes play a role in holding drops and shaping them to create optimal light conditions to create a signature calcium wave.

Figure 1.1: 3-week-old A. thaliana leaf surface trichomes taken in Zeiss Axioplan 2 microscope.

Epicuticular wax can also affect light refraction (Schneider et al., 2016). The structure and formation of these waxes can influence dew drop formation and staying in the plant surface. These waxes can also form crystal structures which affect light refraction on the surface of leaves, influencing the interactions of sunlight with plant cells (Schneider et al., 2016). An

analysis of the different aliphatic molecules on leaves and their role in light refraction would be useful in creating realistic conditions to measure the change of the light in dew drops.

1.1.3 Other impacts of dew drop known effects

Light transmission through leaves and scattered light can be caused by internal reflections and refractions. The leaf internal space is composed of 40-60% air creating a larger pathlength of photons (Luk & Vogelmann, 1998). There are different models of rays passing through and interacting with parts of the leaves that can help to further understand the interactions of light with plant tissue in different species (Luk & Vogelmann, 1998). Measurements of optical parameters within plant tissues provide further evidence on how the structure of leaves affect the interactions of the sun with organelles (Roy et al., 2021; Vogelmann, 1993).

The biological significance of water droplets and their effect on leaf surface phenotypic manifestation is another important water and plant tissue interaction (Watson et al., 2014). Self-propulsion of water droplets on the leaf surface can be correlated with the resulting leaf structure due to its benefits of protection and functional efficiency for the plant (Watson et al., 2014). Dew drops serve to radiatively cool off the plant below the temperature of the surrounding air's humidity dew point. Another property of droplets is substrate emissivity which produces differences in dew water condensation and size of droplets (Trosseille et al., 2022). The surface coverage of dropwise condensation has been observed to change through time as a surface cools, so the presence of dew drops can be correlated to the temperature surrounding the plant and therefore impact the signaling capacities with light (Trosseille et al., 2022).

Another factor that can be taken into account is the differences in intensity of absorbance of high and low light leaves (Ruhle & Wild, 1979). The spatial location of leaves and resulting ability to perceive sun may affect the calcium signaling. Plant orientation and light energy

absorbance at various times of day can affect plant development (Horvath et al., 2020). Measuring maximum direct absorbance showed the spatial orientation of leaf surfaces can increase the effect of sunlight on cells (Horvath et al., 2020). Another important function of dew drops with plants is the effects on distribution of plant location. This effect can be observed when dew drops are needed as a water resource, especially in the desert (del Prado & Sancho, 2007; Gui et al., 2021; Hill et al., 2015; Yokoyama et al., 2021). A comparison of different species of leaves' optical properties could provide us with data on how different types of leaves may experience calcium wave signaling from photostimulation (Knapp & Carter, 1998). On another extreme of the environmental spectrum, tropical cloud forest leaves have differences in their processes and behavior depending on their ability to receive direct and low light from diffused sunlight through the forest canopy (Berry & Goldsmith, 2020). Light stimulation by sunflecks, which also focus light on leaves, change carbon gain and dynamic irradiance, creating different types of stress (Leakey et al., 2005). Sunflecks may alter seedling growth and survival by changing photon flux density (Leakey et al., 2005). This is a sunlight effect that manifests in a specific environment, which serves as evidence of types of stimuli for the calcium wave produced by photostimulation. Relating macro level aspects of light affecting different plant species located in different environments is useful for understanding how light signaling changes through organisms and be able to be translated into universal system signaling.

In order to better replicate the natural environment of our plants, circadian rhythms must be taken into account since light periods will be intermittent throughout the day and seasons. (Avello et al., 2019; Duren et al., 2019; Zhao et al., 2012) Circadian rhythms impact growth and development, so recreating the conditions under which a circadian rhythm is connected may impact the respective signaling and therefore the calcium wave (Khan et al., 2010). Plants adapt

towards this by utilizing receptors to sense the environmental signals and then be able to anticipate necessary internal physiological processes (Khan et al., 2010). These rhythms are established over 24-hour intervals, and we can foster them through creating growing conditions that imitate a normal daylight and night dark environment a plant would experience in nature.

The experiments detailed in this paper analyze the data obtained from power calculations and explore the thresholds that we can create in the lab and to then focus on correlating the significance to the factors previously mentioned. Next, the experiments to explore the actual effect of changing light towards *A. thaliana* first leaves and the analysis of the resulting calcium wave will be detailed. For concluding, all the factors mentioned in the literature review will be correlated to the suggested calcium wave as evidence for the biological significance.

2. METHODS

2.1 Plastic lenses

Lenses were created using Smooth-Cast™ ONYX™ Epoxy Resin and Easy-Release agent. For the dew drop procedure: a small amount E6000 adhesive was placed on mold to adhere 3/32” D borosilicate glass spheres. Alternatively, pipette tip was glued to the bottom of mold and secured in place with tape to create a hole in the filter for water droplets. Part A and Part B from the epoxy resin were mixed in a 120A:100B weigh ratio on scale in weigh boat according to needed volume. The finished mold was cured for 10 minutes.

2.2 Power measurements

StellarNet Inc StellarRAD Handheld SpectroRadiometer data was exported by generating a PDF report that includes the full front panel plus summary information. Weather conditions were recorded for sunlight readings. For power measurements, the THORLABS Optical Power and Energy Meter PM400 with 9 Pin DSUB Thorlabs C-Series Power and Energy sensor was used, and internal calibration wavelength was changed accordingly.

Zeiss Axioplan 2 microscope used was equipped with 405, 380, 360, 560/485, 560, and 485nm laser. Power measurement comparison was achieved through the use of uranium glass and the power meter. Power measurement experiments using the lenses were set up with the THORLABS power meter beneath the lenses under the Axioplan 2 microscope. Power was measured for each filter and the wavelength was adjusted in the power meter. Field shutter was used to obtain the power per data changes using the different filters.

2.3 Plant photostimulation

Transgenic *A. thaliana* seeds expressing the fluorescent calcium sensor R-GECO were surface sterilized with 70% ethanol for 3 minutes and grown in half strength MS on agar plates and wrapped with tape and grown under direct 24-hour light for 2-3 weeks. R-GECO calcium expressor seeds were obtained from Ohio State University's Arabidopsis Biological Resource Center.

Photostimulation using 3-week-old R-GECO seedlings was done by trimming primary leaves from 3-week-old R-GECO and placing them in agar plate. A drop of distilled water was placed on a glass slide and then primary leaves were placed on slide using tweezers and covered with glass coverslip. Chloroplasts in epithelial pavement cells were targeted for photostimulation, avoiding chloroplasts near guard cells due to their higher concentration of newly developed cells.

All photostimulations were done using the Texas A&M Microscopy Center's OLYMPUS FLUOVIEW FV1000 confocal microscope equipped with 405 diode SIM laser and a CFP+GFP GaAsP high sensitivity detector. Images were captured for calcium waves utilizing our lab's original procedure as stated in the original tobacco aggregates paper (Griffing, 2011): Chloroplasts were located in surface cells of trimmed primary leaves. The 405 SIM laser photostimulation was set to a raster-based circular ROI. Emitted fluorescence was detected at 543nm with 5% transmission for RFP. Multiple photostimulation trials were done on same primary leaves, however, a new chloroplast in a location further away was chosen for each trial. Videos were collected through water immersion 60x, 1.20NA objective, over 198 seconds with and a frame rate of 1.644 seconds per frame. A total of 120 frames were obtained with photostimulation happening at frame 20.

For the power adjustment experiments, initial trials were set up with original photostimulation procedure and then the SIM laser power percentages were changed to 80%, 50%, 25%, 12.5%, 10%, and 8% accordingly. For control, photostimulation of cell junctions without chloroplasts was conducted at full power and 8% power.

For the circadian rhythm experiments, two sets of R-GECO seedlings were planted in agar plates and one set was grown under the conditions described above and the other set was grown under 16-hour light in CONVIRON chamber number 3 model CMP3023 set at 21°C, 99% humidity with 11 lamps set at 30% LED light dimmer setting. Photostimulation power adjustment procedure was applied as stated above and compared against a full light seedling.

2.4 Data analysis

All photos, timelapses, and videos were taken on Zeiss Axioplan 2 and FLUOVIEW confocal microscope. Visuals and graphs were created with Microsoft Excel. All image analysis was completed using ImageJ version 2.3.051 by obtaining the mean, maximum, minimum, and medial pixel values through 120 frames. The change in brightness of the area near photostimulated chloroplast against the background change, and photostimulation of cell junctions without chloroplasts at full power was utilized to analyze the calcium wave. The pixel values were then used to quantify the observed calcium wave and the time of increase of average brightness was noted.

3. RESULTS

3.1 Signature calcium wave

Baseline calcium wave laboratory observations generally show an immediate but transient peak in calcium that streams in the cell. Figure 3.1 shows the result from typical calcium wave observation after photostimulating the ER-chloroplast nexus using a high intensity 405nm blue light. Using the R-GECO, we can observe an immediate increase in calcium through the increased brightness through videos and then analyzed the pixel value change and adjust for any background change. The calcium wave then plateaus until there is an observed recovery.

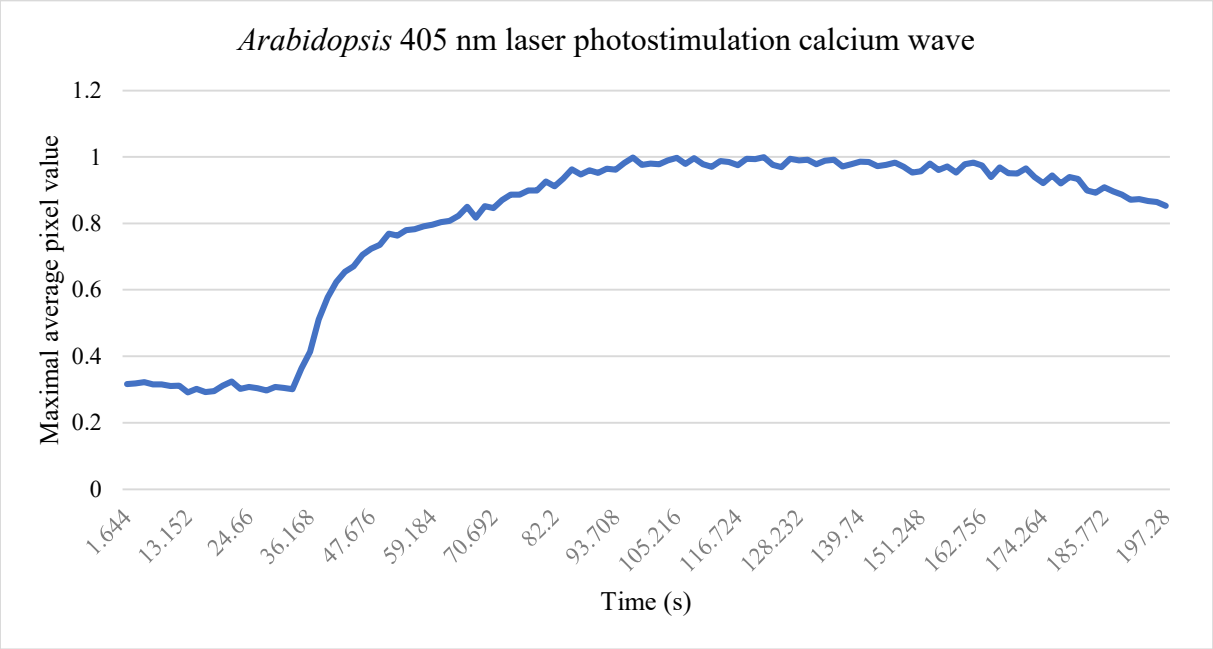

Figure 3.1: Typical calcium wave using R-GECO seedlings and regular photostimulation procedure.

3.2 Adjusted power photostimulation

Experiments began to determine the threshold of photon dose and wavelengths needed to observe a calcium wave through photostimulation at various powers, the following results can be observed in Figure 3.2.

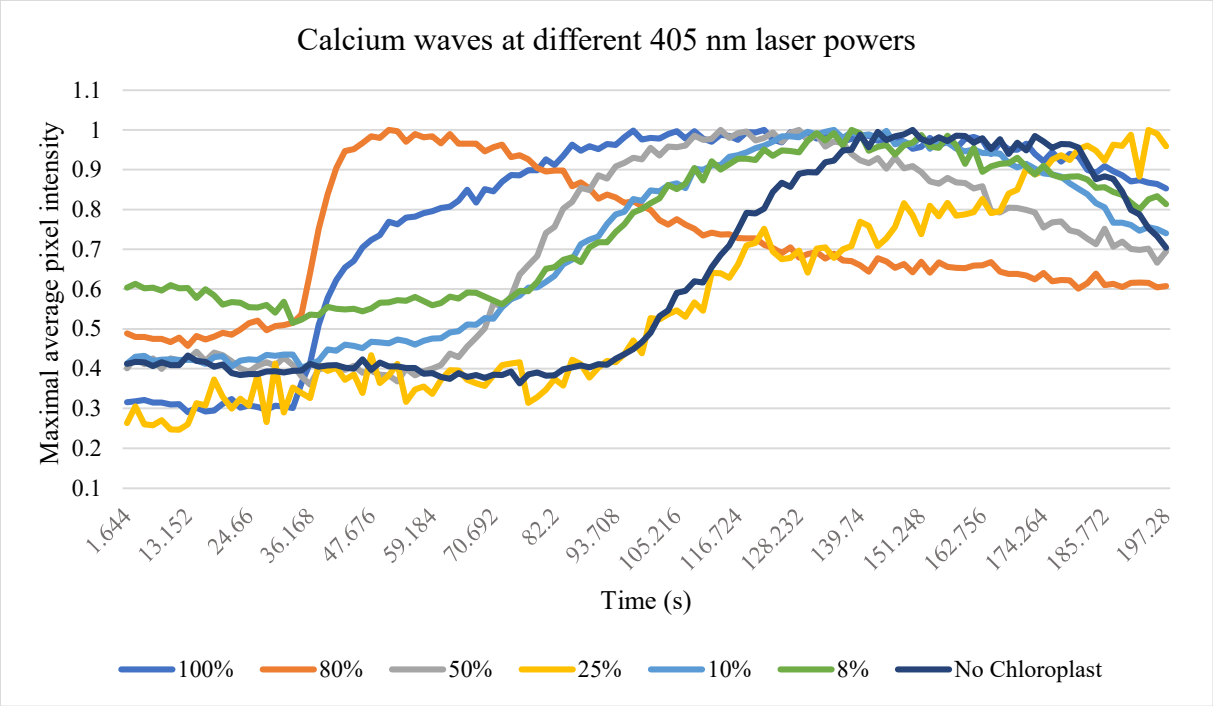

Figure 3.2: Calcium waves from 405 nm laser photostimulation at different powers.

Results showed a delayed increase in the calcium wave appearing as the photostimulation power decreases. This has been previously observed in our lab (unpublished). An important aspect of cytoplasmic calcium waves is that if the photostimulation area is not that of the ER-chloroplast nexus, there still is a calcium wave that is manifested, but it is not originated from our channels of interest and is related to another stress response. We observed this through our photostimulation of an area of cell junction without chloroplast. The signature calcium wave changed through the delay of appearance of the calcium wave while maintaining the plateau of brightness before recovery. The appearance of brightness past the 20-frame mark, or 32 second mark, with increased delay as the power of the laser diminishes. The only percentage power which does not correlate to a delay in brightness with the decrease of the power of the laser manifested in the 10% power dosage. A notable exception is the 80% power recovery, which was more immediate than the other trials and had a speedy recovery.

3.3 Lenses results

In order to further explore how light sources are affected by passing through drops, we began by obtaining the power measurements using the Axioplan 2 microscope to simultaneously use the power meter and observe the effect of drops of water and water-like materials in our laboratory. The purpose of these studies was to observe the change in power as a result of water droplets. Area was first measured utilizing a uranium glass filter. As the area of illumination decreased, the power per area increased accordingly.

The goal of utilizing various lenses to change the magnification was to replicate the dew drops that happen in nature. Calculation data included in Appendix: Power measurements Table A.1. The following results were obtained:

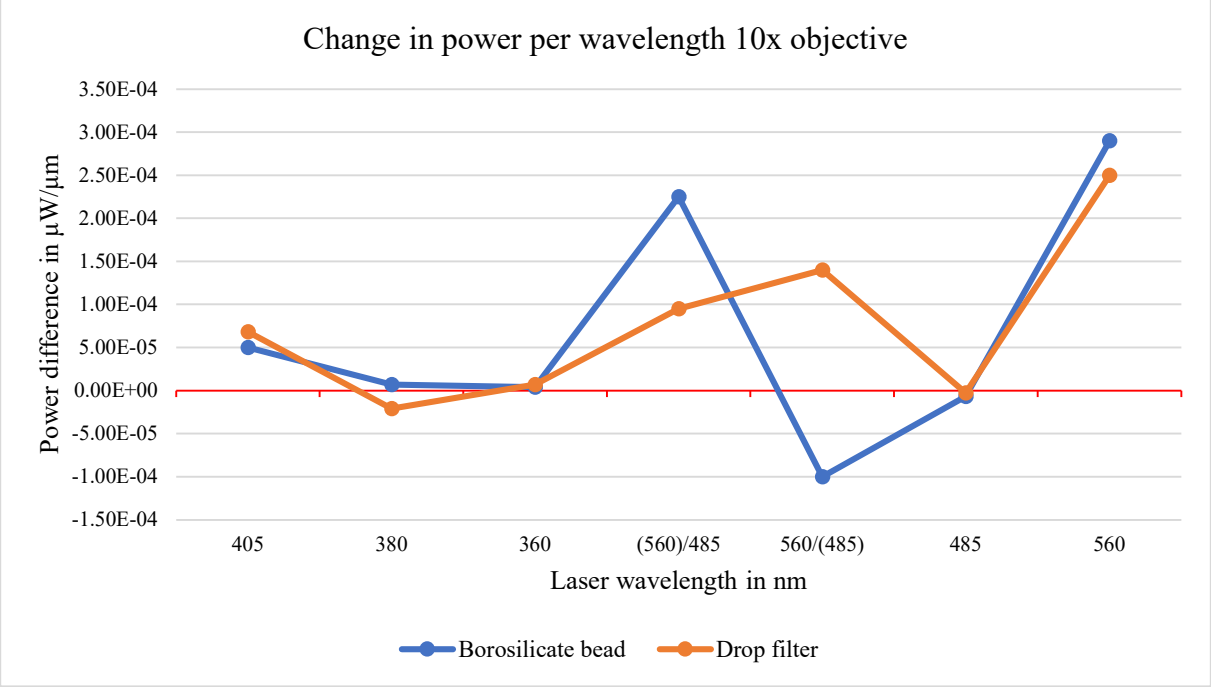

Figure 3.3: Chart showing the effect of water drops and beads on power of light sources through 10x objective.

The values in Figure 3.3 show that the power of the different wavelengths in the lasers changed in respect to the presence of the filters and drops. The drop filter increased the power of

most of the lasers, except for the 380 and 560 dichroic filter wavelength, which decreased in comparison to the borosilicate filter. The 405 nm laser power was increased at about the same rate for both filters.

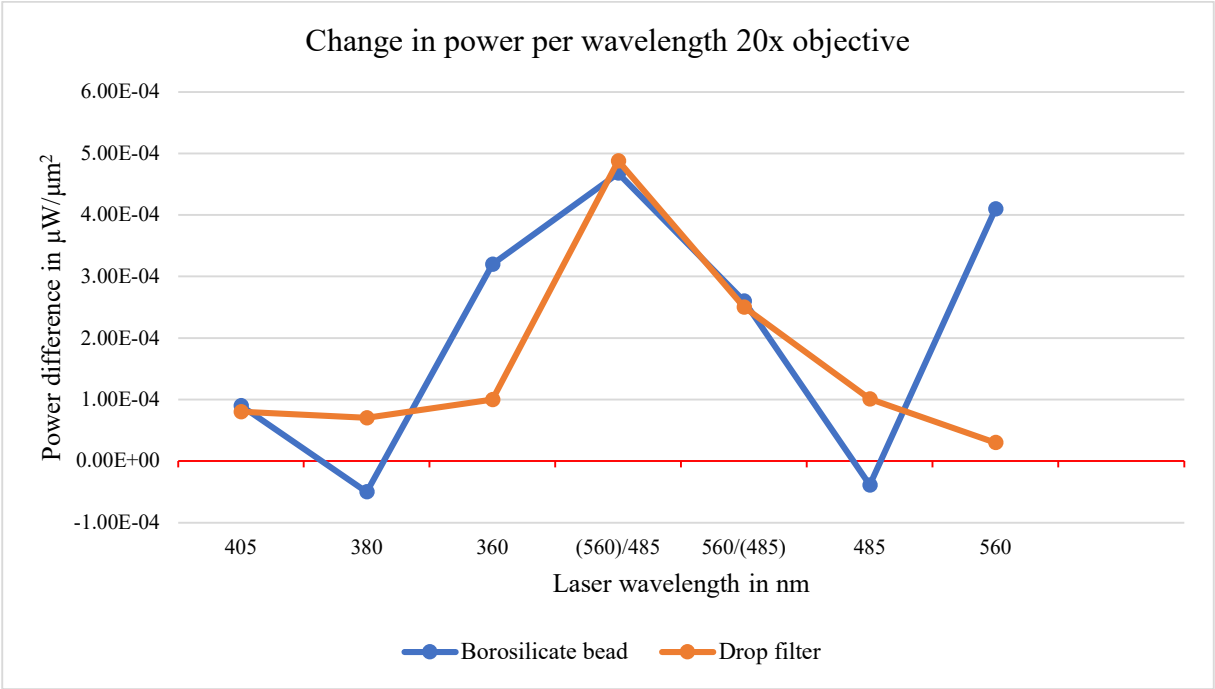

Figure 3.4: Chart showing the effect of water drops and beads on power of light sources 20x objective.

The values in Figure 3.4 show that the power of the different wavelengths in the lasers changed similarly to those from the 10x objective, with the water drop increasing all of the wavelength's powers and the borosilicate filter only decreasing at the 380 and 485 wavelengths. The 405 nm laser power was increased at a similar rate for both the filters.

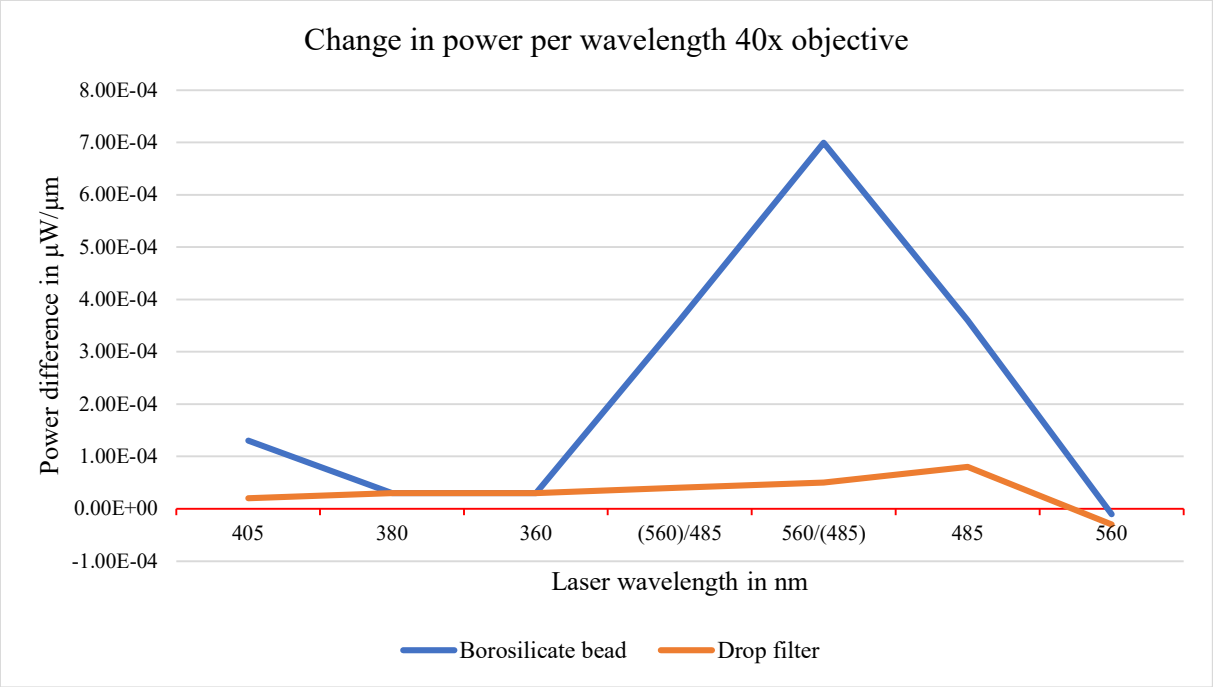

Figure 3.5: Chart showing the effect of water drops and beads on power of light sources 40x objective.

Figure 3.5 shows the changes in powers for the same filters utilizing the 40x objective. Both filters increased the power for all the wavelengths except the 560 nm laser. In this case, however, the 485 dichroic filter laser had the largest increase in power, followed by the 405 nm laser.

These changes in power can be attributed to the focal point light being changed to be located in the surface of the power meter, rather than past it. A similar effect was observed in the Egri (2010) study where the location and shape of water drop affects the magnification of the light. Shape and material impact the magnification and power of the light that passes through. Our wavelength of interest, 405 nm, was increased by all of the filters in all the trials which corresponds with our hypothesis that this wavelength is increased through droplets. The more defined shape of the borosilicate bead in comparison to the fluidity of the water droplet in the filter may have been of impact in the results.

3.4 Sunlight wavelength and power intensity

Data obtained from using the spectrometer and analyzing sunlight under clear weather conditions, 19°C temperature and 23% humidity showed the different wavelength counts obtained. The following graphic in Figure 3.6 was obtained from the spectrometer and details the counts of each wavelength perceived and the power of the light at that instance. The total power was 3.64×10^7 W/m^2/nm. The dominating wavelength was 590 nm and purity was 13.63.

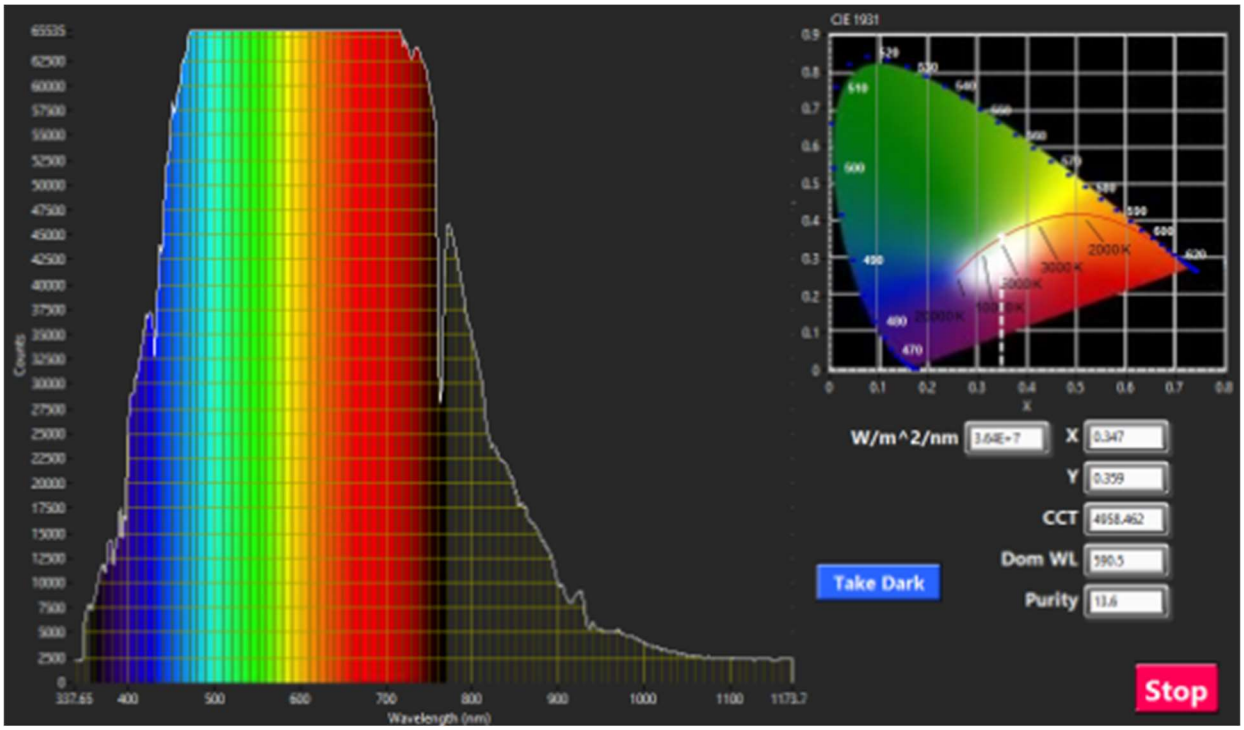

Figure 3.6: Spectrometer reading during a clear day.

Spectrometer readings of sunlight under cloudy weather conditions are shown in Figure 3.7. During that time, there was 23°C temperature and 69% humidity and total power of the light as 1.57×10^7 W/m^2/nm. The dominating wavelength was 587 nm and purity was 46.6.

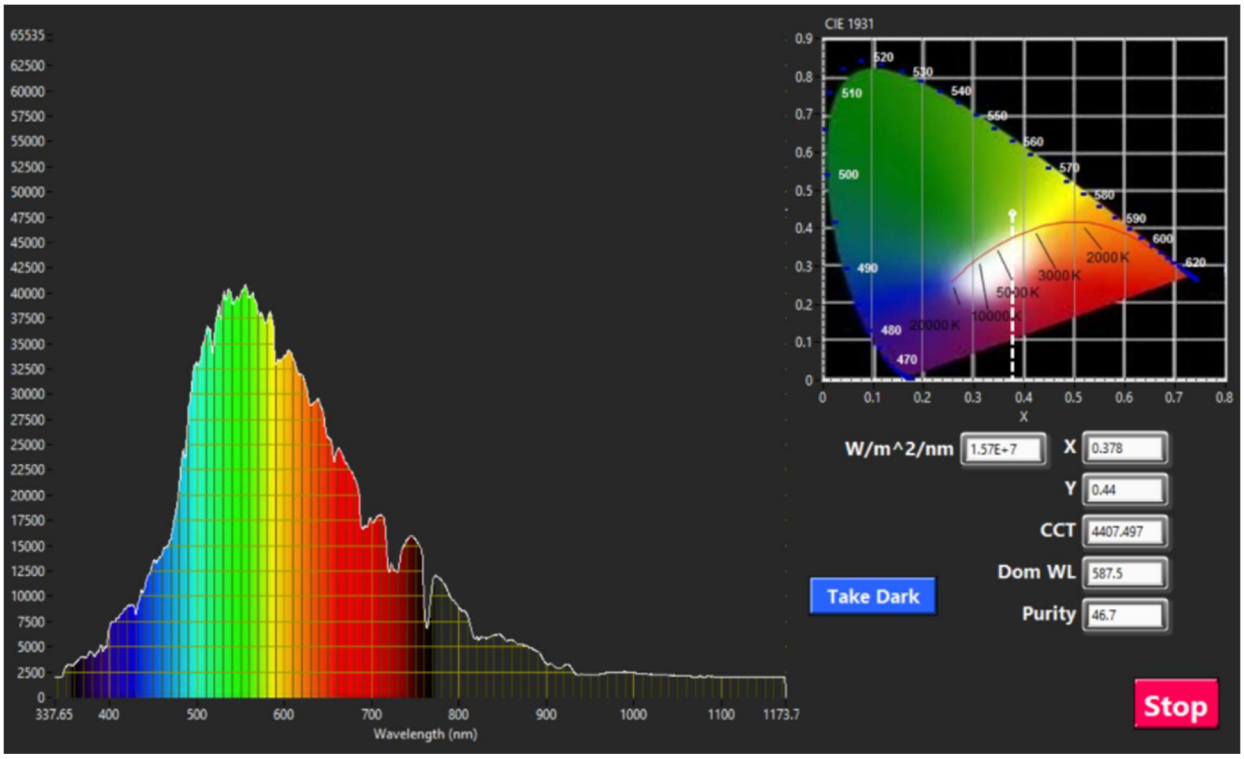

Figure 3.7: Spectrometer reading from a cloudy day.

Through obtaining various days of sunlight under various conditions, it was found that the 405nm counts were mostly present on days with sunny weather. All readings were taken at noon at the same place. The calculated power per area of the 80% laser was 4.66 $\mu W/\mu m^2$, compared to the 5.809 $\mu W/\mu m^2$ of the full power laser and the 2.837 $\mu W/\mu m^2$ of the 50% power laser.

3.5 Circadian rhythm

The experiments conducted with the plants grown under different conditions showed the following results in Figure 3.8. The calcium wave changed from the all-light growing conditions seedlings by showing more uniform results, with the delayed increase in calcium as the power of the laser decreased. There was a similar result with the 80% power laser calcium wave, where a fast increase in the calcium wave was observed, with an immediate recovery in contrast to all other powers.

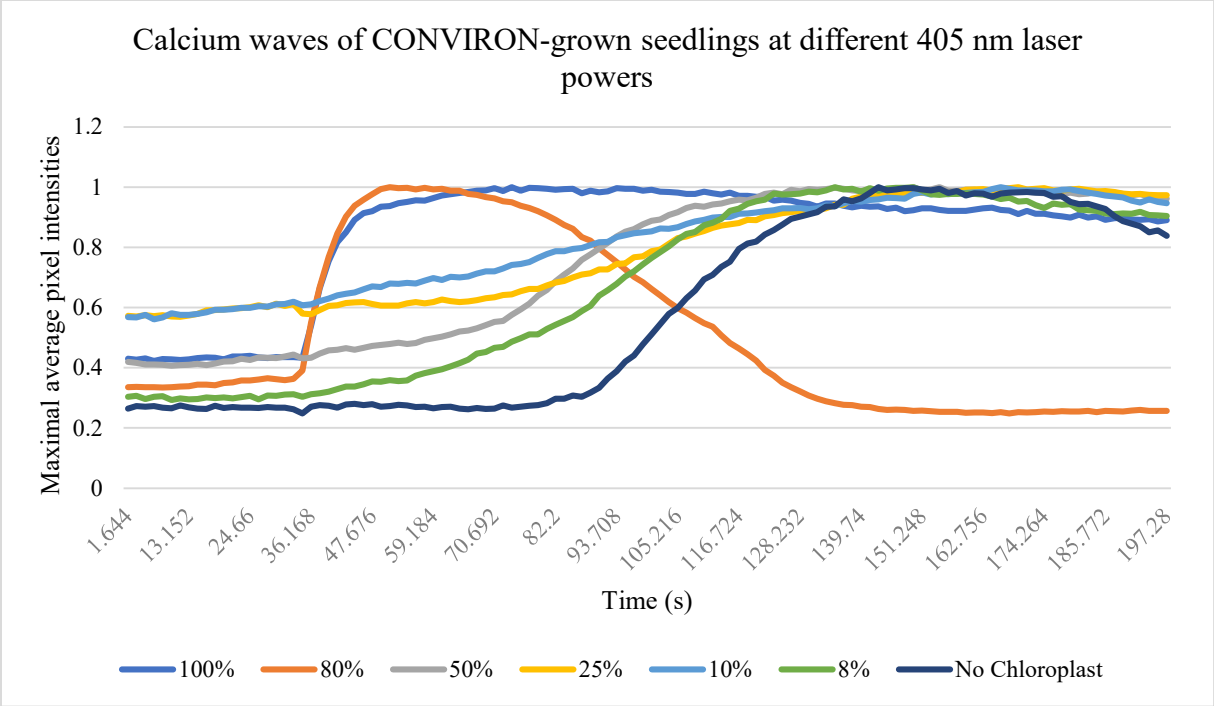

Figure 3.8: Calcium waves in CONVIRON-grown seedlings at different powers.

3.6 Summary

Typical photostimulation calcium wave observations show an immediate increase of brightness due to the localized calcium wave near the ER-chloroplast nexus. Upon adjusting the power dosage of the laser, it was found that a delayed wave manifested in the cells. Furthermore, both the borosilicate bead and water drop filters showed an increase in the power of the light utilizing the 405 nm laser. The sunlight conditions which show the highest power was under clear and sunny weather conditions. The effect of utilizing seedlings grown under circadian rhythm fostering conditions was similar to that of the all-light growth conditions seedlings, showing a delayed increase in the calcium.

4. CONCLUSION

4.1 Changes in calcium wave with changed power and magnification

The observations of our lab compared to the real-life conditions were analyzed. Our signature calcium wave was altered by the adjusted power parameters. This contrasts with our observed signature calcium wave through a delay in the appearance of the calcium wave. This is a previously observed effect, and the resulting calcium wave is not a result of our suggested glutamate receptor-like channels and therefore not ER originated. Rather, it is a stress response different from the ER circulatory system response and could be further studied and compared to the known blue light receptors.

The magnification of light on the surface of *A. thaliana* leaves similar to our observed power increase may be due to trichomes. These leaf hairs may be up to 0.5mm in height and their tips could shape droplets and have an impact on the focal point of the light magnified on the leaf surface. The flatter the drop is on the surface, the further in the focal point is, which also results in the disrupting of the light waves with the plant tissue. The distance of the drop, resulting from the trichomes, could lead to a focal point on the leaf surface and therefore increased focus of the light which could be correlated to our observed increased power dosage. Other physiological characteristics of leaves such as epicuticular waxes, could also affect the presence and shape of water droplets.

4.2 Significance of circadian rhythm

Regarding nature conditions in which this wave may manifest, the use of leaves grown under day and night conditions showed clearer results in the signature calcium wave. This could mean that the calcium wave is part of the intracellular communication of the circulatory network

of the cell. Furthering our understanding of the effect of circadian rhythms could provide more concrete evidence on the changes in calcium waves as a result of natural growth conditions. For instance, further experiments could obtain the calcium wave results at different times of the day and night

4.3 Dew drops formation correlation to calcium significance

As previously stated through the analysis of literature, dew drop formation depends on physiology and spatial parameters in a plant's environment. The known blue-light photoreceptors serve as signalers of growth and development through series of phosphorylation cascades and active site activations (Jones et al., 2012). In our case, the hypothesized receptors located on the ER-chloroplast nexus are responsible for then activating the glutamate receptor-like channels which release ER luminal calcium into the cytoplasm of the whole photostimulated plant cell.

Another important observation, the immediate recovery after the calcium wave of the 80% power laser, has been previously observed in our laboratory. This could be due to the light reaching past a threshold in the action spectrum, causing a much faster increase in calcium and recovery. The occurrence of this effect in both the all-light growing conditions seedlings and the CONVIRON-grown seedlings at the 4.66 $\mu W/\mu m^2$ power laser should be further investigated.

Tying the calcium wave back to its biological significance as a stress response, the hypothesized appearance of the light on days with sunny weather are likely to produce a biological response because it is the most likely to create optimal wavelengths and photon dosage through drops. The increased 405 nm power through the different filters has shown that physiological manifestation of dew drops in leaf surfaces can facilitate internal signaling responses to its environment.

4.4 Future research

Future research may include the use of seedlings at different developmental stages to observe the change in response since chloroplasts sink further into the older the seedling is in epithelial cells. The ratio of trichomes to leaf surface also decreases as the plant gets older, so exploring the locations and focusing of light in regards to the location of internal structures and organelles as they grow into the plant would be of interest. Our laboratory also focuses on the study of the calcium wave in the epithelial hypocotyl cells, so exploring how the different areas of the plant are affected by the presence of dew drops or rain drops could provide further evidence into the role drops play in the communication of the plant.

Furthering our understanding of the significance of these calcium waves can be translated to solving issues in healthcare and agriculture involving calcium stress signaling. Observing the changes in calcium waves by directly using drops of water to see the effect of magnifying in real time would also be helpful in providing evidence for our hypothesis model. Studying the shape of droplets in different *A. thaliana* structures through computational modeling can also help visualize the focal point of the light in the plant surface. This could be through the use of microstructures such as the trichomes or cuticular waxes can shape droplets and impact the photostimulation thresholds. Observing how microstructures change the power of light sources at different angles can also create more natural conditions and differing interactions of light with the droplets. These observations can even correlate how plant irrigation at different times of the day can change their signaling responses.

Limitations of utilizing laboratory settings to observe the calcium wave may also be due to confocal limitations (Tseng & Chu, 2017). Further imaging experiments that create conditions more closely related to those that can be observed in nature would provide better conclusion

results. Continuing to also explore the different calcium waves in other plant species can also provide insight to the overall communication and signaling of plants as a result of the different phenotypic surface structures and the respective dew drop formation.

4.5 Summary

Various aspects of interactions with light were analyzed and related back to the results obtained. The delay in appearance of the calcium wave as the power dosage decreased showed that there has to be a specific power to create the signature calcium wave. The increased power for the 405 nm laser in the water drop and borosilicate bead showed the effect that water and shape have on light that passes through them and supports our hypothesis that they facilitate light interactions needed for signaling. Connections between the observed wavelengths under different weather conditions and their effect on drops at different times of day could also enrich our conclusions. For future experiments, it is recommended to use differently aged seedlings and mimicking real life conditions more closely. An analysis of the photon dose and wavelength that manifests in different weather conditions can also help further the understanding of the biological significance of the signature calcium wave.

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APPENDIX: POWER MEASUREMENTS

Table A.1: Power changes per wavelength at different objectives.

Objective	Wavelength in nm	Borosilicate bead filter power (uW/um^2)	Water bead filter power (uW/um^2)	Empty filter power (uW/um^2)
10x	405	4.30E-04	4.48E-04	3.80E-04
	380	3.49E-04	3.21E-04	3.42E-04
	360	4.80E-04	4.83E-04	4.76E-04
	560/485	1.17E-03/1.28E-03	1.04E-03/1.52E-03	9.45E-04/1.38E-03
	560	2.29E-04	1.78E-03	1.53E-03
	485	1.82E-03	2.33E-04	2.36E-04
20x	405	1.12E-03	1.11E-03	1.03E-03
	380	1.17E-03	1.29E-03	1.22E-03
	360	1.86E-03	1.64E-03	1.54E-03
	560/485	1.41E-03/1.67E-03	1.43E-03/1.66E-03	9.42E-04/1.41E-03
	560	1.39E-03	1.01E-03	9.80E-04
	485	6.49E-04	7.89E-04	6.88E-04
40x	405	2.42E-03	2.31E-03	2.29E-03
	380	2.81E-03	2.81E-03	2.78E-03
	360	3.46E-03	3.46E-03	3.43E-03
	560/485 dichroic	1.84E-03/2.44E-03	1.52E-03/1.79E-03	1.48E-03/1.74E-03
	560	1.71E-03	1.69E-03	1.72E-03
	485	1.79E-03	1.51E-03	1.43E-03