JOURNAL OF UNDERGRADUATE RESEARCH

Effect of Sonication on Plant Stomatal Movement

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Abstract

The primary goal of this study was to determine the effect of sonication on stomatal movement. A minor goal was to determine the best time interval at which sonication is the most effective at removing mesophyll cells and enriching guard cells. For this study, abaxial leaf peels of *Arabidopsis thaliana* were sonicated for 1, 3, 5, and 7-minute intervals at a set amplitude to analyze the removal of mesophyll cells. To juxtapose the leaves and to determine guard cell enrichment, microscopic images were taken prior to and after sonication. Furthermore, to establish that the stomata are alive, neutral red staining was used in conjunct with 40x magnification. It was hypothesized that sonication is an effective method for the removal of mesophyll cells and enrichment of guard cells. The results of this study suggest that sonication is in fact an effective protocol for guard cell enrichment; however, it is not as effective for guard cell purification. This is due to the presence of mesophyll cells and epidermal layers present after sonication. Previous research dealing with sonication is very prevalent; however, research on sonication dealing with the removal of mesophyll cells in *A. thaliana* is not widely studied. Thus, previous information to support this study could not be attained. Results from the first part of the experiment were then extended to determine how sonication affects stomatal movement. It was determined that in the experimental group, the average stomatal aperture decreased over a two-hour period.

Keywords: Sonication, Stomata, Mesophyll cells, Arabidopsis, Guard cells

Introduction

Before delving deeper into sonication and its effect on stomatal movement, there are many concepts that should be understood. One is the anatomy of a leaf. The uppermost layer of the leaf is the epidermal layer. The epidermal layer retains water by secreting a waxy substance called the cuticle. Below follows the palisade mesophyll layer and the spongy mesophyll layer. The palisade layer contains chloroplasts, which converts light energy into sugars (chemical energy) for the plant. The spongy mesophyll layer contains nonuniform cells. Within the nonuniform cells are the xylem and phloem, which act as transport systems. The xylem is specifically responsible for transporting water and minerals throughout the plant. This allows leaves and roots to receive the vital nutrients for growth and establishment. The sugars made from photosynthesis are transported via the phloem. In the bottom-most layer are the guard cells. Guard cells surround the stomata and are responsible for controlling the size of the stomatal aperture. The changing aperture of the stomata allows exchange of gases, such as carbon dioxide

and oxygen with the environment and retain or release water. Lastly, guard cells can be examined to determine the functions of signaling molecules and enzymes they house in various studies.

Sonication is a widely used protocol to break down particles. For this study, sonication was used to remove mesophyll cells for guard cell enrichment. Sonication works by emitting ultrasound waves at about 20 kilohertz (kHz). The term, cavitation, is prevalent when dealing with sonication. Cavitation is a process in which "vacuum bubbles" form and interact, which leads to the breakdown of mesophyll cells. Furthermore, the friction that forms in the bubbles help with its breakdown. Unfortunately, there is one downside to the vigorous bubbles formed and the large influx of ultrasound waves— that is the heat produced. The heat produced can negatively impact the cells by damaging them. Thus, to curb this, the beaker containing the leaf peels were placed in an ice bath during sonication.

For the latter part of the study, magnesium chloride ($MgCl_2$) was used as mock treatment, and bacteria *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) was used in the experimental group. *Pst* DC3000 was utilized to help determine if the stomata are functioning properly after sonication. Plants utilize pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). This system works by recognizing bacteria on the surface of the leaf via receptors. When bacteria are detected, the stomata close as an innate immune response.

Methods

Leaf Peels

For this study, leaves of *Arabidopsis thaliana* were used. The method is adopted from Lawrence *et al.,* 2018. The leaves were extracted from five-week old plants, which grew in a short-day chamber (8 h light 16 h dark). The size of each leaf were two centimeters in length and one centimeter in width. The leaves were then taped on both sides and pressed upon firmly to release air bubbles and to make sure that the leaf is stuck evenly on the tape. After six leaf peels were prepared, tweezers were used to remove one piece of the tape from one side. The abaxial peels were then immersed in 100ml of distilled water.

Neutral Red Staining

0.03% neutral red was used to determine if the stomata were still alive after sonication. One leaf peel was immersed in neutral red solution for five minutes before sonication. The peel was then removed of excess stain and viewed using a Leica DM6000B light microscope. Another leaf peel was immersed in the neutral red solution for five minutes after the 7-minute interval of

sonication to be compared to the leaf peel that was stained with neutral red for five minutes prior to sonication.

Sonication

Probe sonication was used to remove mesophyll cells to enrich the guard cells. The probe was placed in the beaker at a fixed depth of one centimeter above the bottom of the beaker throughout the study. Furthermore, an amplitude of 100 (equivalent of 20 kHz) was used throughout the study.

To begin the study, an ice bath was prepared and the beaker containing the distilled water and leaf peels was placed in the ice bath. Afterwards, the sample was sonicated for 1, 3, 5, & 7-minute intervals. Leaf peel images were then taken with a microscope after each interval with 10x magnification setting. Before the 1-minute interval, an image was taken at 40x magnification of the leaf peel that contained the neutral red solution. The peel was discarded accordingly. After the 7-minute interval, another image was taken at 40x magnification containing the neutral red solution. 10x magnification was used to for an overall observation and to make visible more stomata. 40x magnification was used to focus on the stomata in greater detail and for ease in stomata aperture measurement.

$MgCl_2$ and *Pst* DC3000

First, using the tape-peel method, leaves of *A. thaliana* were taped and peeled. For each treatment and time interval, 3 peels of five-week-old *A. thaliana* leaves were used. The leaf peels were gathered and placed in two different sets of distilled water at 50ml each: mock and experimental group. The tape peels of the experimental group and mock group were sonicated for five minutes each. Magnified images of the stomata were then taken using the Leica light microscope (20x magnification).

Leaf peels of the mock group were then treated with 10 mM $MgCl_2$ and the leaf peels of the experimental group were treated with bacteria *Pst* DC3000 at optical density (OD) 0.2 ($OD_{0.2}$) in 10 mM $MgCl_2$ for a two-hour interval. The purpose of the bacteria was to stimulate stomatal movement to detect aperture change. Stomatal aperture was then measured using Image J to determine if the stomata were functional after sonication.

Results

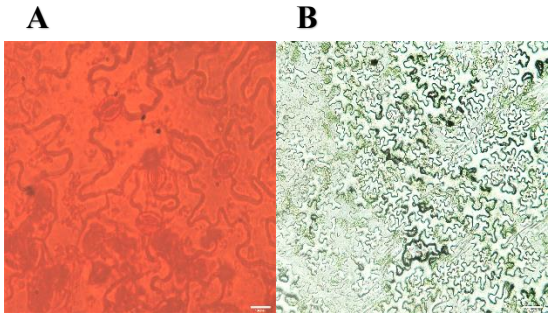

Figure 1: (A) Pre-sonication with neutral red stain (40x magnification). (B) Pre-sonication leaf peel (10x magnification)

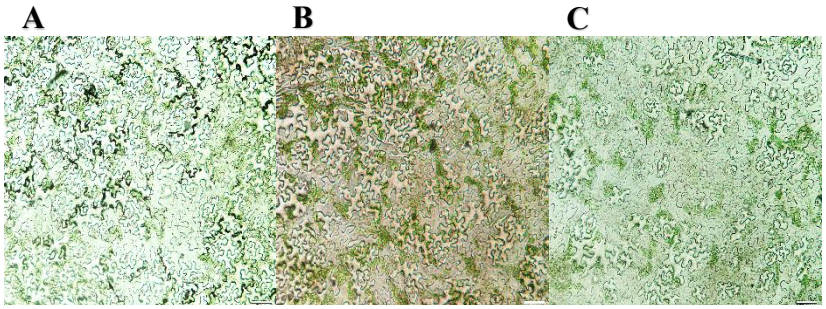

Figure 2: (A) Sonication 1-Minute (10x magnification). (B) Sonication 3-Minutes (10x magnification). (C) Sonication 5-Minutes (10x magnification).

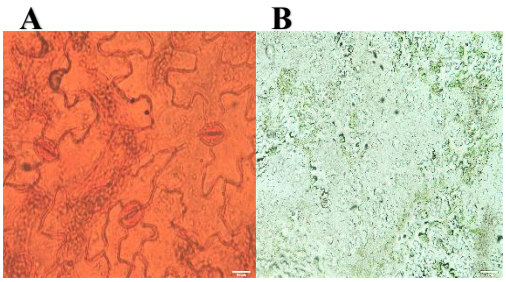

Figure 3: (A) Sonication 7-minutes with neutral red stain (40x magnification). (B) Sonication 7-minutes (10x magnification).

Prior to sonication, there were a lot of mesophyll cells and hefty epidermal layers present, in which the guard cells were hidden beneath (Figure 1). After one minute of sonication, there wasn't much of a difference (Figure 2A). As shown, mesophyll cells and the epidermal layer obscure the guard cell. After three minutes of sonication, some of the mesophyll cells and a very

little of the epidermal layer had been broken down (Figure 2B). However, after five minutes of sonication, there was a considerable difference in the amount of mesophyll cells removed and epidermal layers broken down (Figure 2C). After five minutes of sonication, more guard cells are present when viewed under the microscope. At the last interval of sonication, seven minutes, it was clear that there were still some mesophyll cells and epidermal layers present (Figure 3). There's also a substantial difference when comparing the images containing the neutral red stain. Prior to sonication, the epidermal layer was much more prominent. Moreover, the guard cells were hidden beneath them. In contrast, after seven minutes of sonication, it is evident that the guard cells were more enriched and were much more visible. The pavement cells were also red, which means that they were alive. This data helps arrive at the conclusion that sonication is a quality protocol for guard cell enrichment, but may not be a good method for guard cell purification. This is because even though the guard cells became more visible after sonication, the epidermal layer and some mesophyll cells were still present.

Regarding stomatal movement, mock group stomatal aperture averages present similar throughout the two-hour time interval (Figure 4). Experimental group stomatal aperture averages exhibited a decreasing pattern. After one hour of bacteria treatment, stomatal aperture average is about two micrometers. After two hours of treatment, stomatal aperture is 1.8 micrometers. Stomatal aperture averages show that after bacteria treatment, the stomata are functioning properly. Thus, it was concluded that sonication did not negatively affect stomatal function.

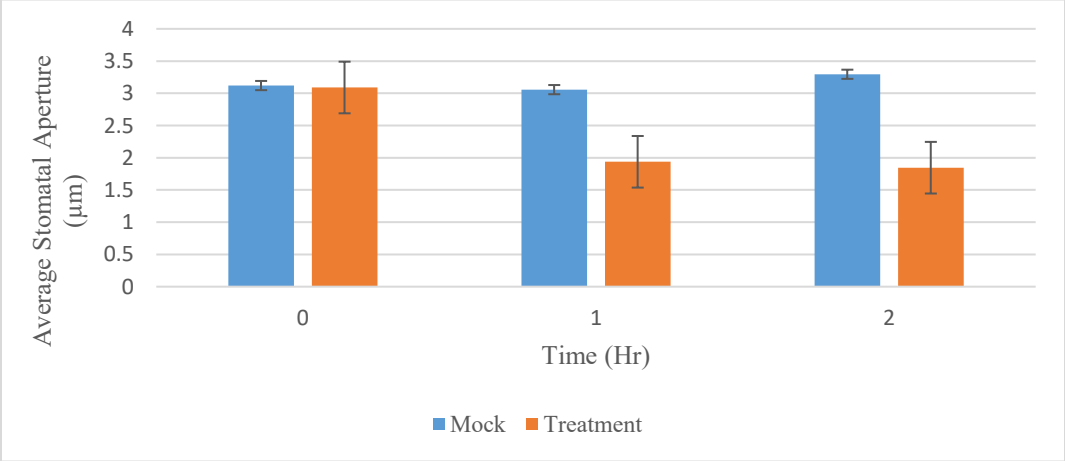

Figure 4: Effect of sonication on stomatal aperture under mock and bacterial treatment conditions. For each group, 30 stomata in three biological replicates were used. The data represent average ± standard deviation.

Discussion

After conducting the study and analyzing data, it was concluded that sonication is a viable method to remove mesophyll cells and to enrich guard cells. The microscopic images obtained after sonicating for five minutes showed that sonication is a promising method and that it is also a applicable method because of its low cost. The enrichment of guard cells allows for more single cell-type data to be obtained in future studies. For example, with the enriched cells, kinanes native to guard cells can be studied and the function of these kinases in stomatal guard cells can be determined without much interference from mesophyll cells and membrane layers. With the observation that five minutes is sufficient for the proper removal of mesophyll cells and guard cell enrichment, future researchers can save time and resources when guard cell enrichment is required for their studies.

To verify and obtain further results, there are many future experiments that can be conducted. Sonication contains many variables that can be controlled to determine changes in results. One of the variables that can be controlled is the time interval the leaf peels are sonicated for. In the future, the time interval can be either lengthened or shortened. Another variable that can be controlled is the amplitude. The amplitude is the power that is delivered to break down the peels. Amplitude and time interval can be controlled simultaneously. Amplitude can be increased while the time interval is decreased or vice versa. Another variable that can be controlled is the depth the sonication probe is placed in the solution. The probe can be placed at varying lengths into the solution. This may produce different results. Perhaps, the deeper the probe, the better the leaf peels are sonicated. If the probe is turned on when it is barely in the water, foam will form. Thus, to avoid this and to obtain accurate results, the probe should clearly pass the surface of the water and then vary depth length. Another future experiment that can be conducted is enzymatic digestion. Enzymatic digestion is a protocol that contains a mixture of cellulysin, cellulase RS, and Macerozyme R10. This mixture is specifically used to break down the plant's cell wall to enrich the guard cells. Every 100 leaf peels require about 20ml of enzyme solution. The mesophyll cells and the guard cells have different compositions which are washed away with water. This method is said to be effective, however, it is much more expensive. For example, a one-gram bottle of cellulose RS is priced at around $140. Thus, it is easy to see that if repeating an enzyme digestion study, the hefty price will play a big factor. However, by conducting a study

on enzymatic digestion, results can be compared with sonication to better determine which method is more effective for guard cell enrichment.

Acknowledgements

The research has been supported by an award from the National Science Foundation and United States Department of Agriculture (Project number 1920420) to S. Chen.

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