The Effects of Elevated Carbon Dioxide on the Density and Mutation rates of the Trichomes, as well as the Rosette Development of Arabidopsis thaliana in the Presence of Azacytidine

With the levels of atmospheric carbon dioxide rising, it is important to understand how flora will adapt to this new environment. To this end, the model plant Arabidopsis thaliana was utilized to examine phenotypic responses to changes in carbon dioxide levels in wildtype and epimutated plants. The primary goal of this research was to examine how the epimutagen Azacytidine alters patterns of trichome development in ambient and elevated carbon dioxide. This examination revealed that trichome and rosette development was not significantly affected by exposure to Azacytidine or elevated levels of Carbon Dioxide. However while bolting and flowering times did not respond to exposure to Azacytidine, both were altered by carbon dioxide levels decreased both flowering and bolting times of Arabidopsis thaliana.

Climate change

The previous century experienced the warmest temperatures of the last thousand years. The primary cause of this is an increase of atmospheric greenhouse gases. These gases, which prevent solar energy from being radiated away from the atmosphere, consist mainly of carbon dioxide, methane, nitrous oxide, and an entire artificial subgroup labeled fluorinated gases (containing the element fluorine). Of the gases mentioned, carbon dioxide is of most concern due to it being a byproduct of various industrial practices. The US Environmental Protection Agency estimates that carbon dioxide emissions in the United States increased by about 4 percent between 1990 and 2016. Overall these actions have increased atmospheric carbon dioxide from pre-industrial levels of 280 parts per million(ppm) to the current observed level of 400 ppm. If the current trend continues, by the year 2100 atmospheric carbon dioxide with reach 800ppm (Stott et al., 2006).

Utilizing historical data in conjunction with computerized climate models, potential outcomes of this increase in carbon dioxide can be predicted. Among these consequences: increased mean global temperatures resulting hotter summers and milder winters, acidification of oceans in the form of dissolved carbonic acid, loss of terrestrial glaciers and the resulting rise in sea levels, as well as the consequences of the strategies biological organisms employ to adapt to the new environments.

One such consequence, is a reduction in nutrition value of harvested crops. Increased carbon dioxide is associated with significant decreases in the concentrations of zinc and iron in all C3 (A plant in which the carbon dioxide is first fixed into a compound containing three carbon atoms before entering the of photosynthesis) grasses and legumes (Meyers et al., 2014).

C3 plants include beans, rice, wheat, potatoes, most temperate crops and all woody trees. These changes are the result of epigenetic plasticity on short time scales and evolution over long periods of time.

Evolution and Epigenetic Plasticity

Evolution

Evolution is the process in which different organisms adapt over generations to the environments they experience. The primary mechanisms of evolution are natural selection and genetic drift.

Genetic Drift

Genetic drift is a random change of frequency of certain genes and their subsequent traits from reproductive populations. This process requires populations to be relatively small and isolated. Without the introduction of new genetic information, genes can become common, known as fixed, or disappear without any influencing factor. This process accounts for a small percentage of a species evolution, with the primary factor being natural selection.

Natural Selection

There are four requirements for natural selection to occur; genetic variation, traits that are heritable, different reproduction rates and offspring mortality. Genetic variation, induced by errors in genetic replication known as mutation, is required to produce individuals that are and react to the environment uniquely. These variations must be heritable, allowing them to be passed along to future generations. This allows for a percentage of a population to possess

increased rates of reproduction, also known as fitness. Finally, not all the offspring produced can survive to reproductive age.

This selection process results in organisms that possess adaptions to a certain environment that increase their fitness allowing their offspring to out compete potential rivals. This process requires many generations to function; with the rate of adaption tied to generation length. This time component presents itself as the major drawback of natural selection and evolution in general. There are certain species of bacteria, such as *e. coli* that can produce new generations every half hour, allowing evolution to occur quickly. This is contrasted with other types of species, such as vertebrates which require millions of years to evolve. The slow nature of evolution, results in a scenario in which rapid changes in an environment can not be tolerated, leading to possible extinction. One strategy employed to combat rapid environmental changes is phenotypic plasticity.

Phenotypic Plasticity

Plasticity is the ability of an organism's individual genotypes (genes) to produce different phenotypes (observed traits) when exposed to different environmental conditions, allowing an individual organism to change its phenotypic state or activity; its metabolism for example (Fusco and Minelli, 2010). The main method of causing changes in plasticity is thru a process called DNA (deoxyribonucleic acid) methylation.

DNA Methylation

DNA methylation is an epigenetic process that modifies gene expression without altering any of the nucleotide's sequences. This process silences or activates genes with the addition or

removal of a methyl group. This methyl group, consisting of one carbon atom bonded to three hydrogen atoms, is bound covalently to the five-prime carbon of the cystine ring producing a compound known as 5-methylcytosine (Burcu 2018). Once attached the methyl group resides in the major groove region of DNA; interrupting the transcription process.

Due to their sessile nature, the role of DNA methylation in plants is more prominent when compared to other mobile organisms. When exposed to abiotic stresses such as drought, cold, changes in salinity, heat, UV radiation and biotic stresses such as pathogen infection or herbivores the only strategy plants have is to endure it (Burcu 2018). This ability to endure stress through DNA methylation and epigenetic alterations in general has resulted in a genome with increases survivability.

Phenotypic Changes

One area of study that has yet to be thoroughly explored is how elevated carbon dioxide affects trichomes. *Arabidopsis* leaf trichome comprises a stalk topped by three or four symmetrically arranged branches of equal length. Arabidopsis trichomes are an ideal model in part because their development is a dramatic example of directional cell expansion giving rise to a complex cell shape (Zang et al., 2005).

Another observable adaption of increased atmospheric carbon dioxide, that has been documented is alterations to stomatal density. Stomata, the Greek word for mouth, are specialized epidermal structures that act as biological valves for gas exchange, allowing carbon dioxide to enter a plant's leaf and oxygen to exit (Nadeau and Sac, 2002). The stomata are flanked by parenchyma (non-woody) cells called guard cells that regulate the size of the stomatal openings. Stomata size regulation is required to balance the uptake of atmospheric carbon

dioxide with the resulting loss of water caused by the opening (Daszkowska-Golec and Szarejko, 2013). This control of the size of the stomatal aperture optimizes a plants photosynthetic efficiency.

Photosynthesis is the manner in which plant life utilize light, either natural or artificial, along with carbon dioxide and water to create the energy required to survive. To transform the energy from a light source, chlorophyll is required. This pigment, in its various incarnations, absorbs most of the visual spectrum except for green light; this exception results in most photosynthetic organisms appearing green.

With increased atmospheric carbon dioxide, the number of stomata required to maximize efficiency is reduced, decreasing density. In conditions where carbon dioxide is doubled, a mean decrease of twenty-two percent in density was observed (Woodward et al., 2008). This observation required analyzing four separate regions of a leaf under a light microscope at a power of forty times. The number of stomata present in each region was catalogued and averaged to create an overall mean density.

Arabidopsis thaliana

A member of the mustard family, *Arabidopsis thaliana* (*Arabidopsis*) is native to Europe, Asia and North America. It is classified as an annual plant, with its life cycle lasting for a period of one year and dying. Arabidopsis became the favored plant model organism (a species used in a laboratory to study biological phenomena) in the 1980's with the release of a detailed map of its genetic makeup (Meinke,1998). When analyzed, it was discovered that *Arabidopsis*'s genome

(1.35 billion base pairs on five chromosomes) was relatively small in comparison to other plants. Adding to its appeal, mutations can be produced easily through irradiation or exposure to mutagenic compounds and the plants self-fertilizing natural allows for these mutations to be expressed with little effort. The main advantage of self-fertilization is that all the different specimens of *Arabidopsis* are genetically identical, dismissing the notion that any observed differences are the result of naturally occurring genetic variation.

Arabidopsis is a hearty plant that requires very little space. Standard in vitro conditions required for the successful growth of *Arabidopsis* include maintaining a constant temperature range of 20°C to 25°C, nitrogenic soil placed in plots 14x8.5x 6 centimeters, increased humidity to maintain soil moisture but hinder root immersion, as well as approximately 16 hours of indirect light (Li, 2011). Under these conditions, *Arabidopsis* rapidly progresses thru seven separate stages: Germination, Leaf and Rosette Production, Rosette Growth, Inflorescence Emergence, Flower Production, Silique Ripening, and Senescence.

The first stage, Germination which initiates the development process and is considered day zero, consists of plant matter immerging from the seed state and last approximately six days. The second stage, leaf production and rosette formation, begins at the six-day mark and continues for nineteen days. The Rosette Growth stage continues for five subsequent days. With full rosette growth *Arabidopsis*, ends its vegetative state and proceeds into its reproductive state with the Inflorescence stage. Signaled by the first growth of flowers, the Inflorescence state which may overlap with the previous growth stage, begins at the twenty-six day after germination and continues for five days. This stage begins with the plant bolting (the production of a flowering stem) and concludes with the first flower opening. Post the primary opening, the

Flower Production stage lasts for eighteen days and concludes with a complete opening of all flowers present on the plant. With flowering complete, the Silique Ripening (rupturing of mature seed pods), commences lasting approximately two days. With growth and reproduction complete, the Senescence stage is reached indicating the mature plant is no longer capable of reproduction (Boyes et al., 2001).

The main purpose of this research is to determine how the phenotypic plasticity of *Arabidopsis* is affected by an increased carbon dioxide environment when it is affected by 5-Azacytidine, a chemical that inhibits gene methylation. The hypothesis of this line of research being that trichome density will decrease, while the rate trichome mutation will increase when treated with Aza in a high Carbon Dioxide environment.

Methods

After being removed from their pods, the seeds of the two ecotypes Columbia (Col) and Landsberg (Lan) were placed in petri dishes with filter paper moistened with deionized (DI) water. The seeds were added to their assigned dishes, sealed with parafilm and placed in a refrigerator for a period of a week. Subjecting the seeds to cold temperatures mimics the "winter" period required for the seeds to germinate.

After the elapsed time, the seeds were removed from the refrigerator and equal amounts were transferred to three additional petri dishes with identical DI moistened filter paper, creating four dishes in total. Each of the petri dishes were labeled with one of the four the experimentally treatments: Low Carbon Dioxide (CO2) with only DI water (Control), High CO2 control, Low CO2 with seventy-five micromolar Azacytidine (AZA) treatment, and High CO2 AZA treatment.

The seeds were then allowed to germinate in their assigned environmental conditions for one week. Two samples of each ecotype (Col 8& 22, Lan 19 & 20) were chosen, based on overall seed germinating, for transfer to soil plots. Each sample was placed into a row of six plots, creating six replicants of each treatment. Two soil plot trays were created, low and high CO2, and returned to their respective environments. The plants that survived (Table 1 & 2) were allowed time to bolt and flower, then their leaves were sampled and placed under a light microscope and photographed to analyze trichome structure and density. The photographs of each leaf were then analyzed using the image processing software, *Image J,* and all statistical tests were conducted using the statistical analysis software *R.*

***The statistical test Kruskal-Wallis was used for data analysis. This test was chosen due to the non-parametric nature of the data. Also known as non-normal data, non-parametric data is asymmetrical regarding the mean and cannot be represented as a bell curve (normal distribution).*

*** A p value is the percentage that the hypothesis is false, but the data provided suggests it is true.*

***Alpha, or a predetermined p-value to accept the hypothesis, is set at 0.05*

*** Graphs 1-5 represent the mean values for each group*

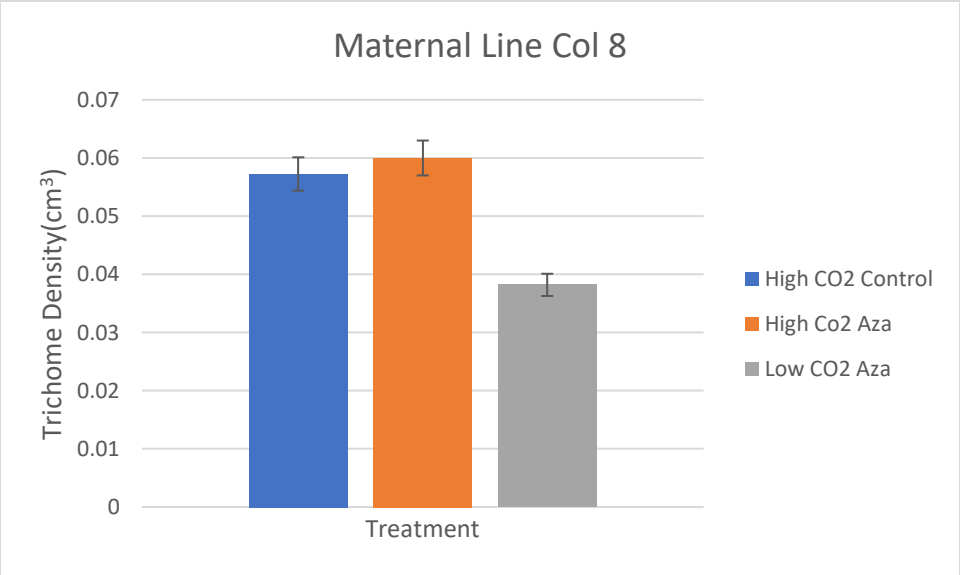
Table 2 Low CO2 **(X indicates plant presence)**

Col 8 Con						
Col 8 AZA		X		X	X	
Col 22 Con	X					X
Col 22 AZA		X	X	X	X	
Lan 19 Con	X		X	X	X	X
Lan 19 AZA		X	X		X	
Lan 20 Con		X	X		X	X
Lan 20 AZA	X	X	X	X	X	X

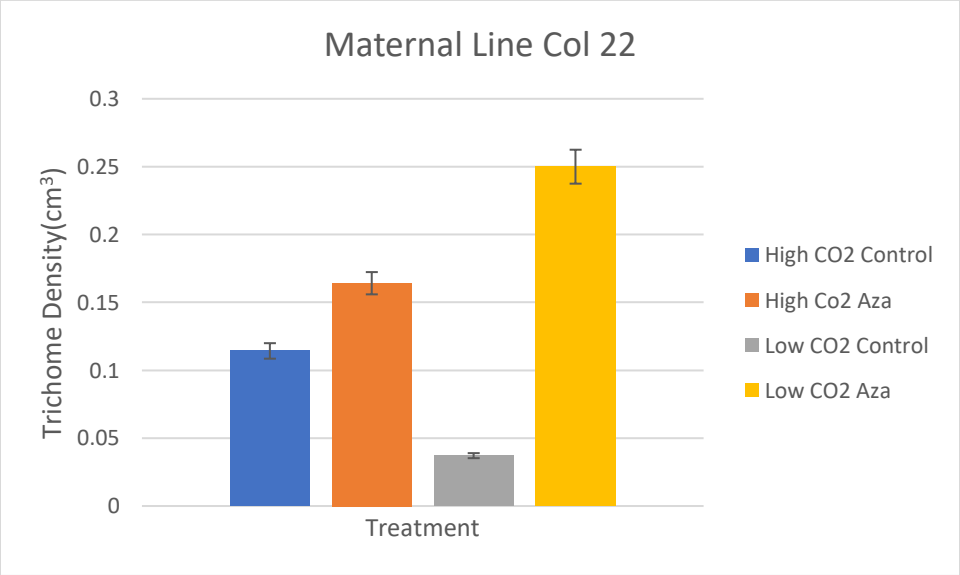
Table 3 High CO2 **(X indicates plant presence)**

Col 8 Con			X	X		
Col 8 AZA	X		X	X		
Col 22 Con	X	X	X		X	
Col 22 AZA	X	X	X	X	X	X
Lan 19 Con	X	X	X	X	X	X
Lan 19 AZA		X	X	X	X	X
Lan 20 Con	X	X	X		X	X
Lan AZA		X		X	X	

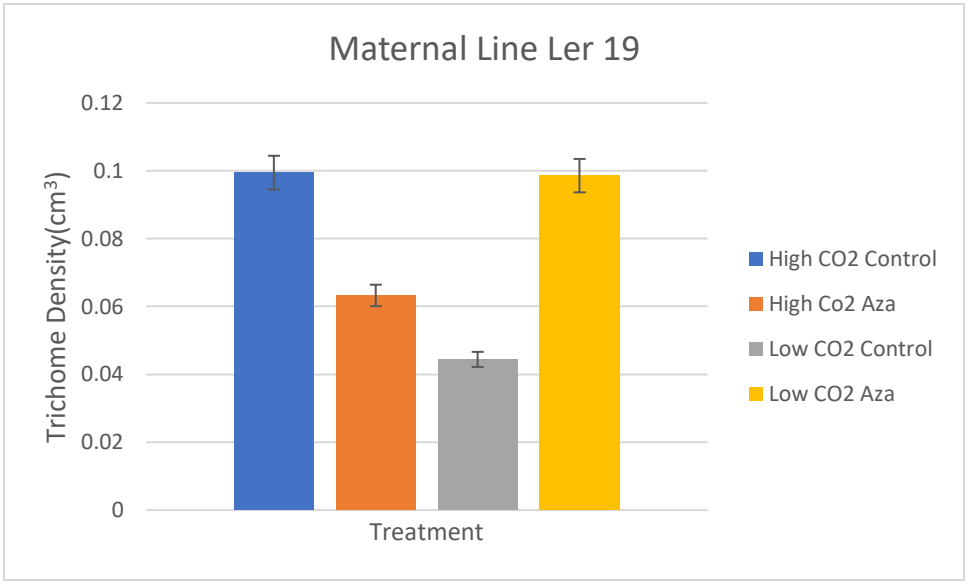
Results



Graph 1



Graph 2



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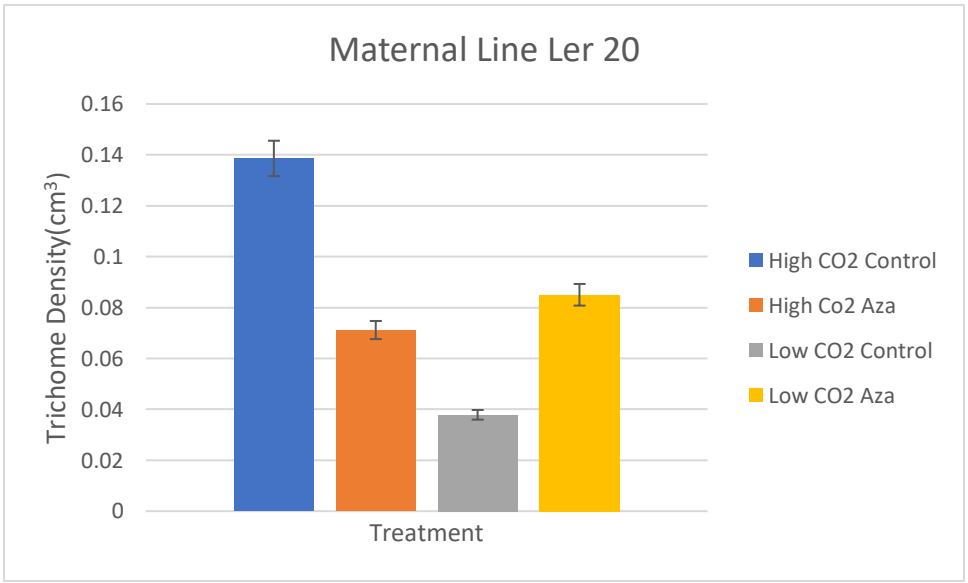

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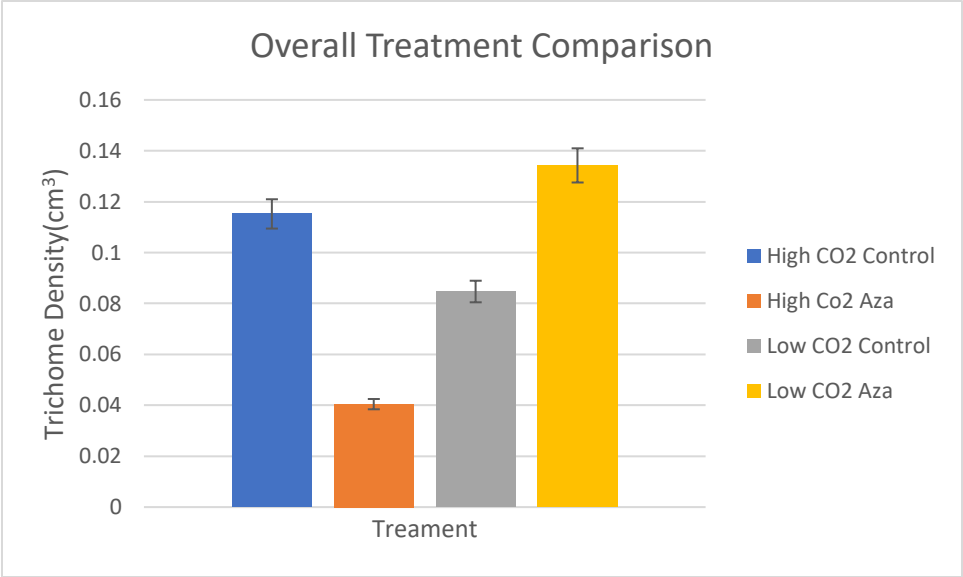

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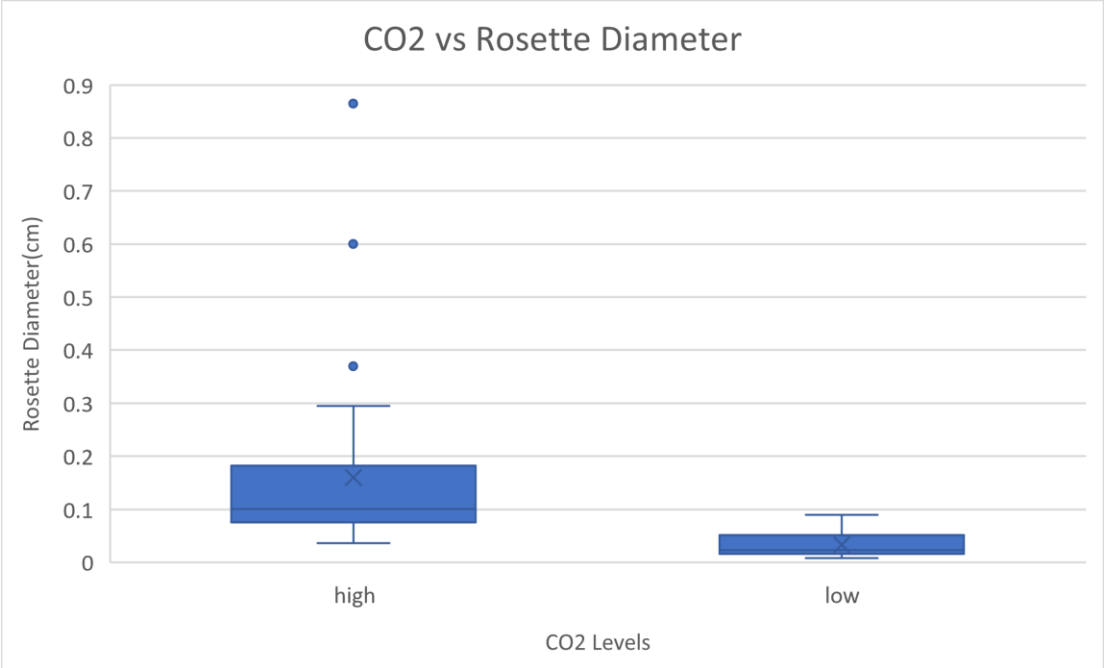

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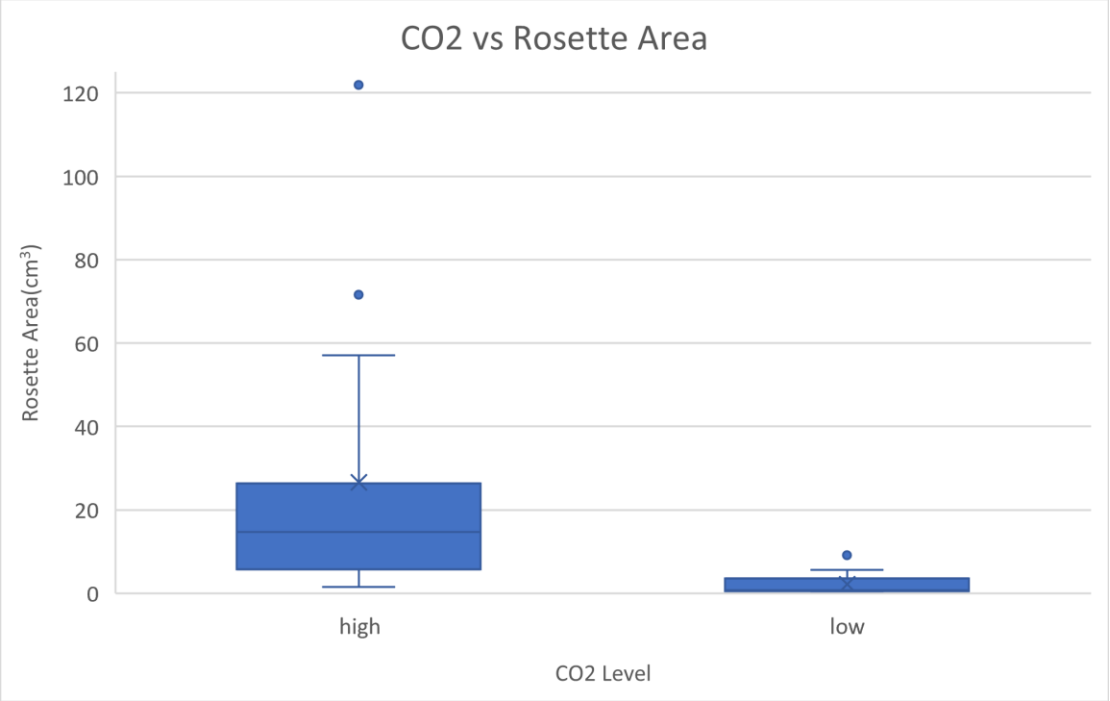

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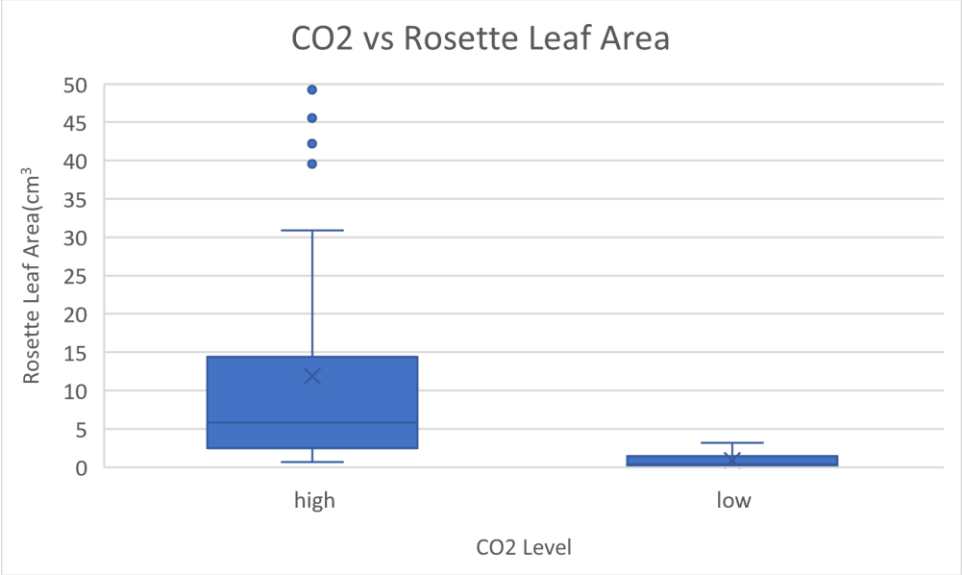

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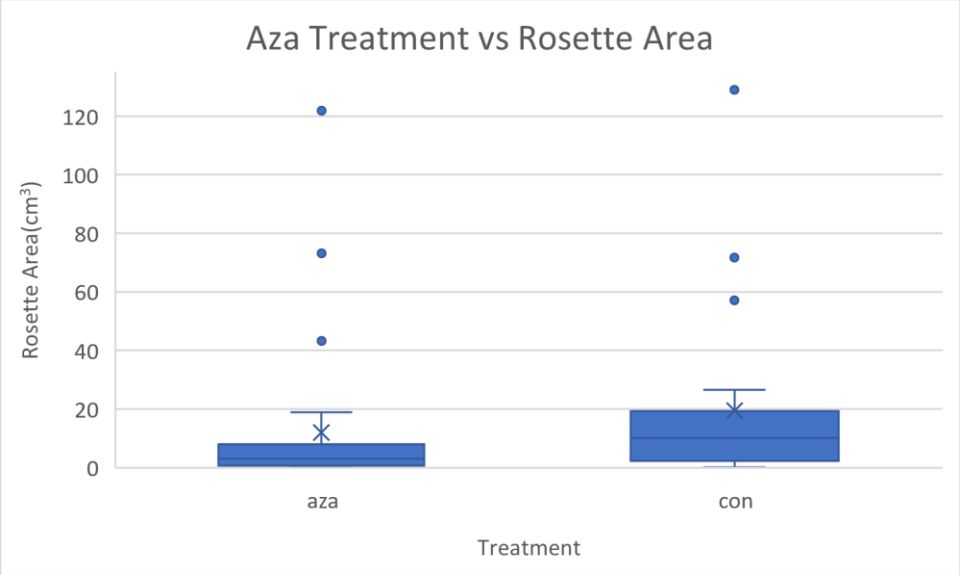

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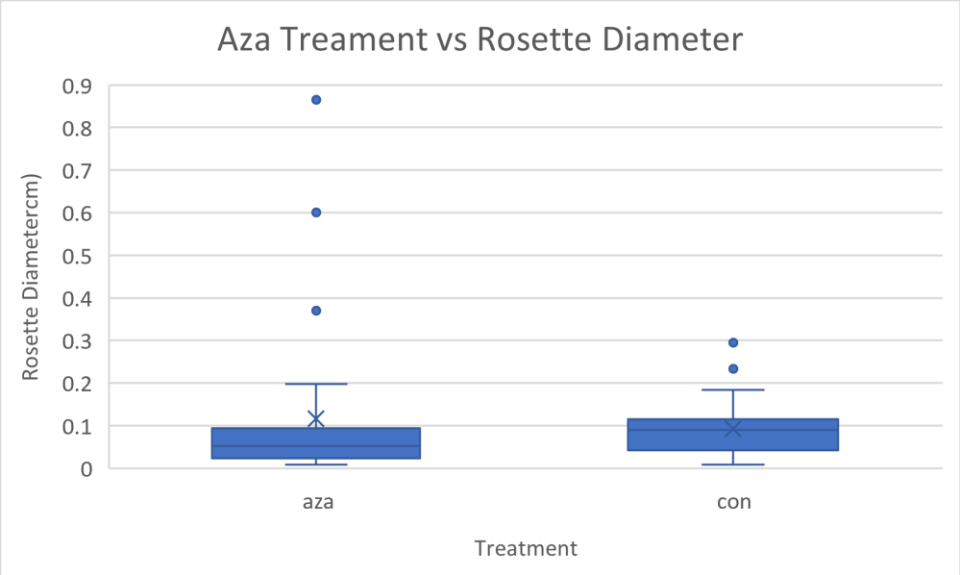

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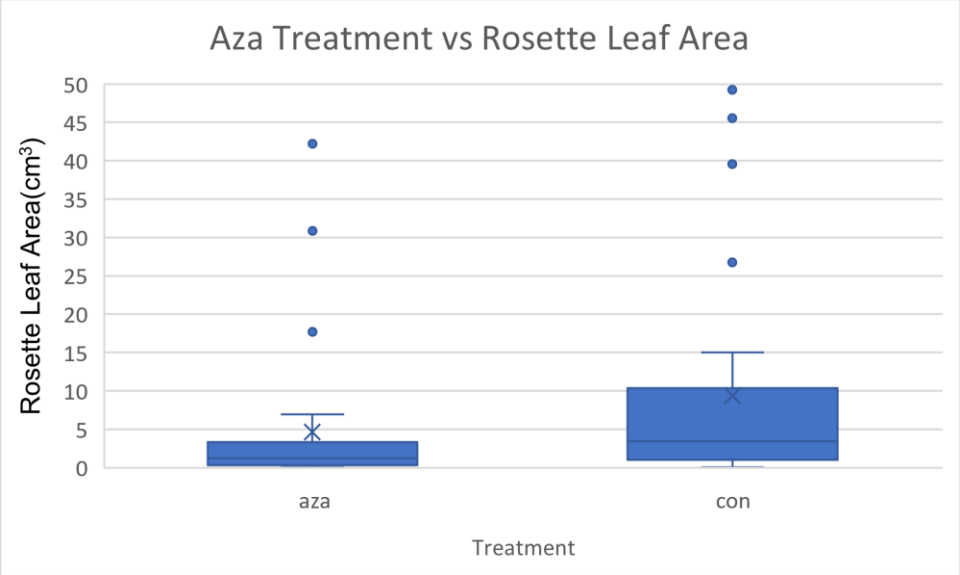

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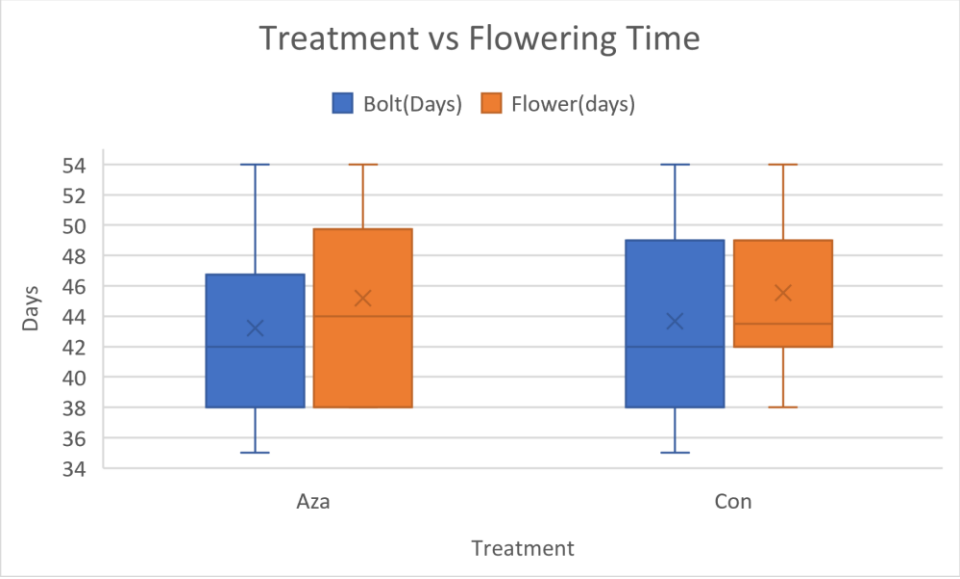
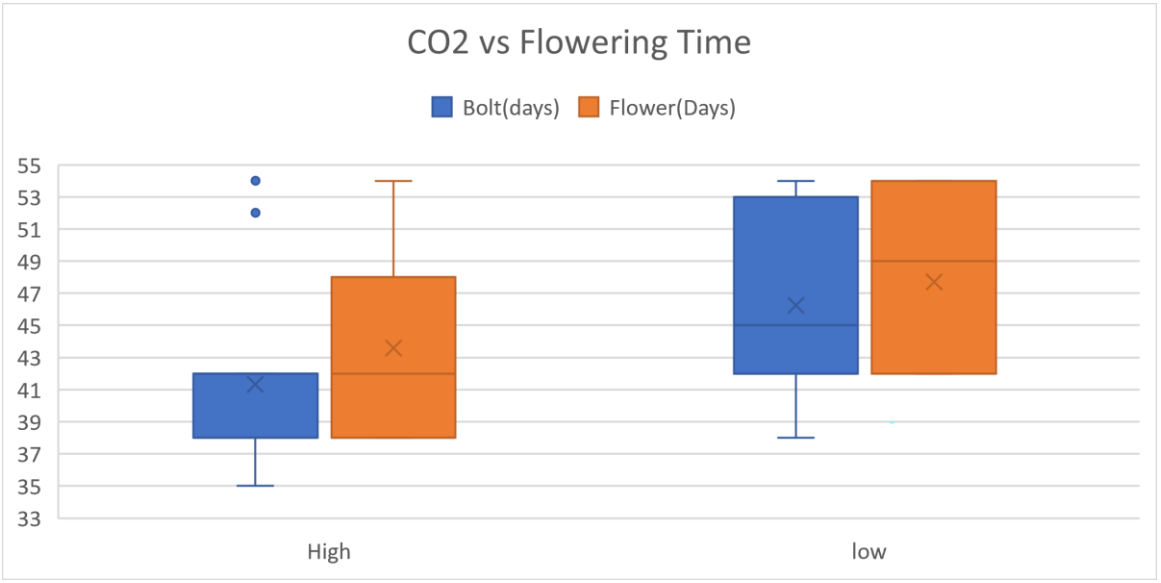


Graph 12



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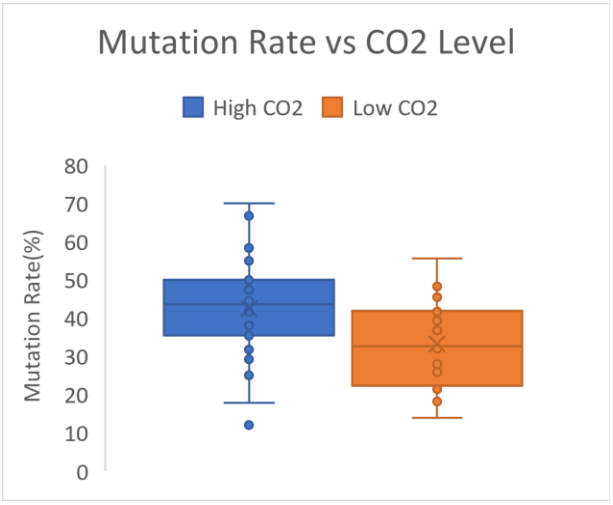
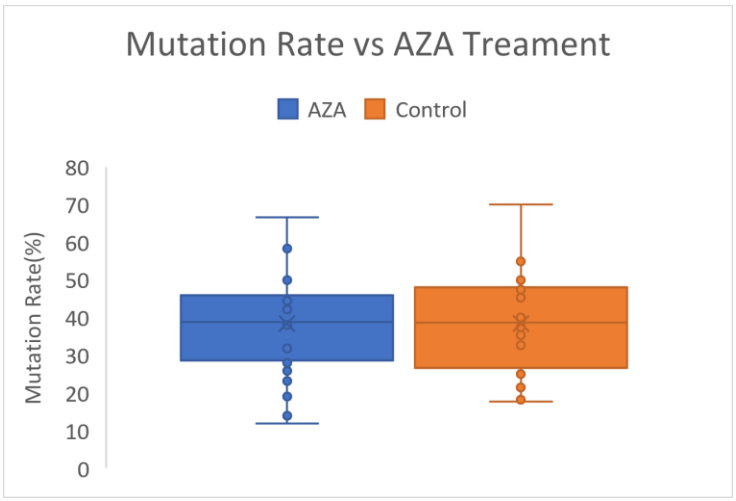
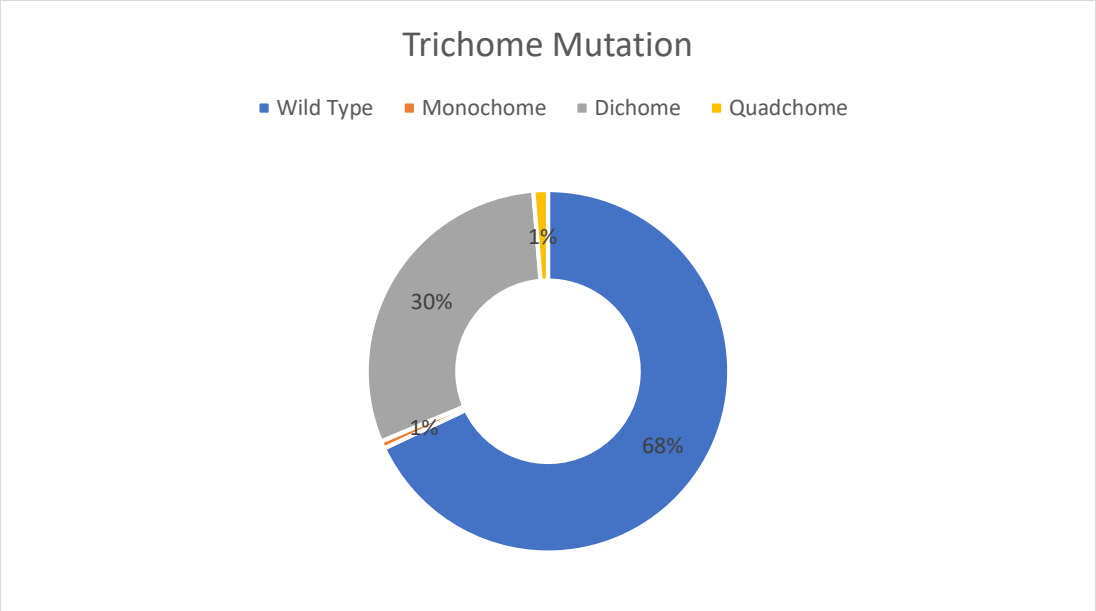




Graph 14



Graph 15

Discussion

The density of trichomes is not dependent on either CO2 levels or exposure to Aza, both producing data with a p value of 0.45 (graphs 1-5) well above the acceptable alpha. While there was a positive correlation between CO2 levels and leaf area; there was a corresponding increase in the number of trichomes, resulting similar densities.

Mutation rate of the trichomes was not significantly affected by either CO_2 levels or AZA treatment, producing a p-value of 0.7. 68% of the trichomes were wild type (unmutated trichomes, Picture 1) with 0.7% Monochomes (Picture 2), 30% being dichomes (Picture 3) and quadchomes (Picture 4) at 1.3% (graph 15).

The rosette stage of development also was not significantly affected by changes in CO_2 or exposure to AZA. With a p value of 0.4, the data for leaf Area, rosette diameter, and the area of influence of the plant (graphs 6-11) did not vary significantly between the controls and the experimental treatments.

Bolting and flowering time showed no significant difference between the different AZA treatments (graph 12), with p values of 0.863 and 0.591 respectively, however both are CO_2 dependent. With p values both well below the alpha at <0.001, the bolting and flowing times (graph 13) was faster in high CO_2, confirming the findings of Andalo et. al, 2001. When the plants were grown in a high CO_2 environment, the average bolting time was 5 days sooner (41 vs 46 days) and the average flowering time was 4 days quicker (44 vs 48 days), then the plants grown in a low CO_2 environment. This result is the only statistically significant finding of the experiment.

Conclusion

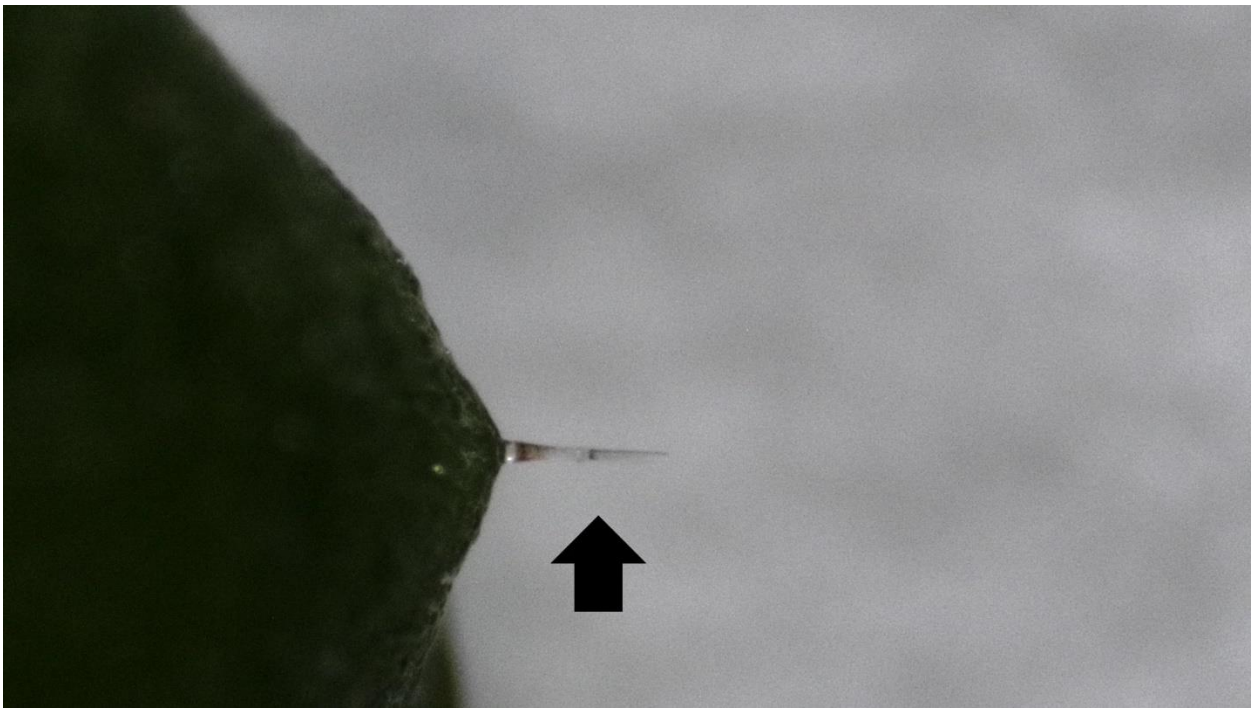
There was not enough evidence to support the original hypothesis, that trichome density will decrease, while the rate trichome mutation will increase when treated with Aza in a high Carbon Dioxide environment.

Due to the high mortality rate, both caused by human error, mainly damage to the root system in seed transfer from petri dish to soil, and exposure to experimental treatment; it is recommended that this experiment be replicated to provide a larger sample size. This increase should allow for more accurate and precise conclusions to be reached. However, based on the collected data trichome density and mutation rates are not affected by changes in CO2 levels or exposure to AZA.

Acknowledgements

The patience and guidance of Dr. Jonas was pivotal to the completion of this research and is apricated. Special thanks to Dr. Mcenroe for agreeing to be the secondary reader of this report.

Picture 1



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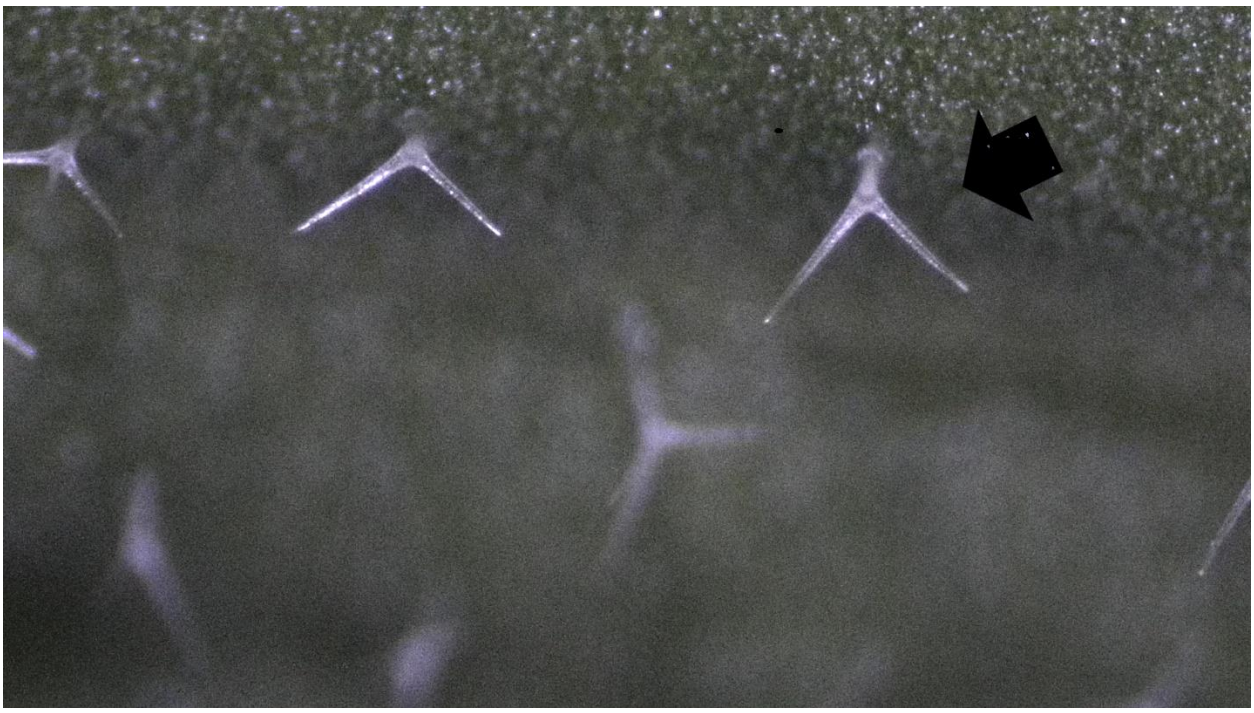

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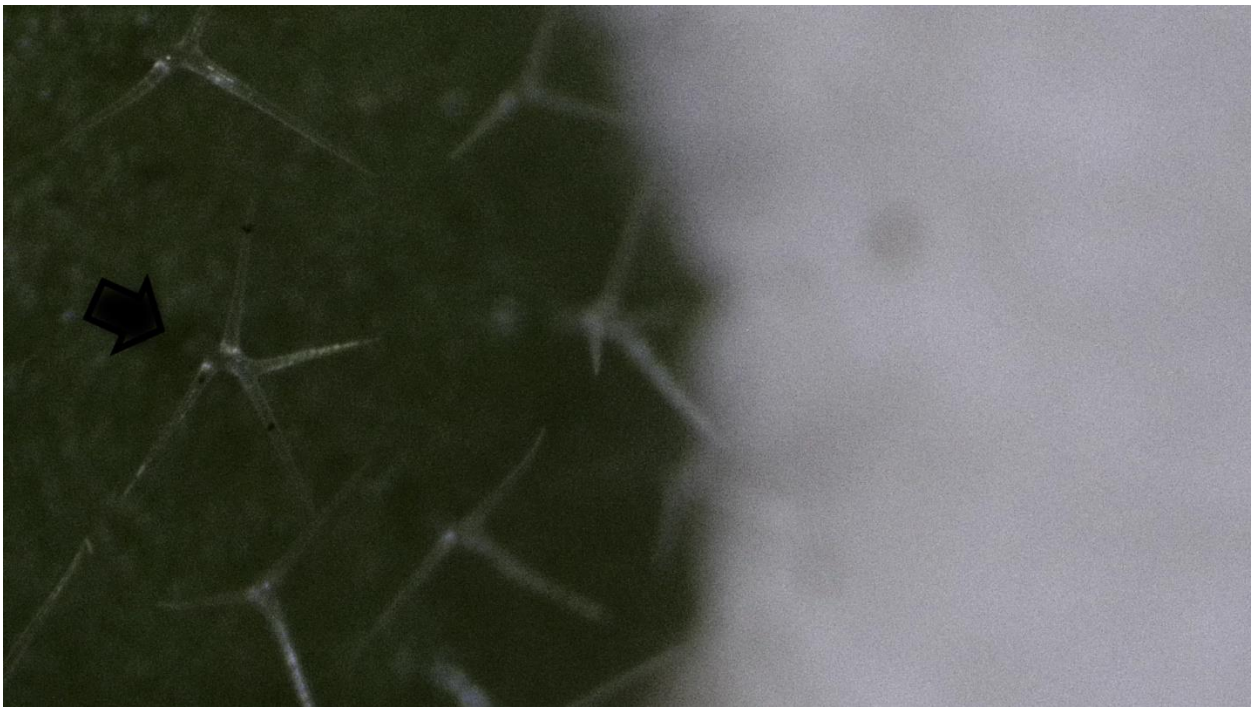

Picture 4

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