Ethylene Stimulated Growth and Plant Defence Effects due to Artificial Wind on *Glycine Max*

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Abstract

Ethylene is an important hormone that is produced in the process of thigmomorphogenesis, and thus is triggered by environmental stimuli. Ethylene is also involved in plant defence, therefore we created an experiment in which half of a set of *Glycine max* were inoculated with *Heterodera glycines* and then half of the inoculated and control groups were exposed to simulated wind on business days to test if ethylene production could be induced and increase plant defence. Data collection was taken every 2-5 days throughout the experiment, followed by statistical analyses using ANOVA and t-tests, which yielded no significant relationship between the growth of *Glycine max* and simulated wind exposure. Once plants were uprooted on the final day, it was found that the *Heterodera glycines* did not take to the inoculated groups, causing this portion of our research to remain unanswered. The vertical growth of plant height yielded significant differences on the two final days of data collection, giving reason to extend the length of this experiment in the future.

Keywords

Wind, wind simulation, ethylene, plant defence, *Glycine max*, *Heterodera glycines*, Alberta, thigmomorphogenesis

Running title

Simulated wind on growth in *G. max*

Introduction

Background

Despite agriculture in Canada only employing 4% of the labour force, it is still a vital part of the economy, producing for domestic and international markets and playing a large role in providing materials for retail industries (Britannica 2018). One of the most significant crops grown in Canada is *Glycine max,* the soybean, which is the third largest of Canada's field crops (SOY Canada 2018). Most of this production takes place in Southern Ontario, but has expanded to Quebec as well as the western provinces of Saskatchewan and Manitoba since the 1970s (SOY Canada 2018). Due to its stature in the agricultural industry, understanding how to maximize soybean crop yields while minimizing losses due to damage is important. The number one ranked disease agent for soybeans in North America is *Heterodera glycines,* the soybean cyst nematode (SCN), which is a parasitic roundworm that attacks the soybean plant by penetrating root tissue and establishing feeding sites (King 2019). When females reach maturity and die, they form hardened bodies, or cysts, which contain around 200 eggs which are protected and can remain dormant in the soil for up to 8 years (CABI 2019). SCN spread across crop fields through soil by many modes of transportation, including wind, farm machinery, and migration on human clothing (King 2019). This parasite can reduce crop yields by up to 25% depending on climatic conditions (dryness) and on the spread of the disease (Gabruch & Gietz 2014). The symptoms and severity of damage can vary, however, the most common is a yellowing of the plants, as well as stunted growth in the stem and roots (King 2019). These nematodes also compete for nutrients with the soybeans and can interfere with water uptake and nodulation. As the SCN does not outright kill these plants, they may spread unnoticed, making it a significant threat to crop yields (King 2019). Additionally, the spread of SCN has been known to facilitate the spread of other diseases which can kill the soybean, like the fungal disease “soybean sudden death syndrome” (King 2019). Due to the potency of the SCN pathogen, the goal of this study was to investigate if plant defences could be artificially induced, causing the soybean to be less susceptible to the effects of the SCN. This research could then be used to help soybean producers choose growing environments for the soybean that would naturally induce these plant defences, protecting the plant and ensuring a more consistent crop yield.

Due to the sessile nature of plants, they are much more limited in their ability to respond to stressors, as they cannot simply relocate to a new environment in the same way animals can (Jaffe & Forbes 1993). As a result, thigmomorphogenesis, which is defined as the physiological and morphological adaptations undergone by plants when responding to environmental perturbations, plays a vital role in how a plant grows (Jaffe & Forbes 1993). One of the major plant hormones believed to be involved in regulating these thirmomorphogenic responses is the phytohormone, ethylene (Pierik *et al.* 2007). Ethylene is also integrated into the complex network of plant defences and is generally accepted to be an antagonizer of salicylic acid (SA)-dependent resistance against biotrophic pathogens, along with being associated with growth inhibition (Adie *et al.* 2007). However, there has been much controversy in the role of ethylene as a growth and defence regulator in more recent years as conflicting results have been gathered from new studies (Adie *et al.* 2007). Cooperation between ethylene and SA pathways has been reported and ethylene has been implicated in the activation of defences against some biotrophic pathogens (Adie *et al.* 2007). Additionally, responses to mechanical stimuli, such as wind, which can elicit a change in plant morphology, are associated with increased ethylene production and have been documented to increase stem and root diameter and decrease stem elongation over time, suggesting ethylene can act as both a growth inhibitor and promoter (Pierik *et al.* 2007).

In this study, we used a simple organismal design to observe the effects the mechanical perturbation of wind had on plant growth and defence. We wanted to investigate whether artificial wind could noticeably affect the growth of a plant species, indicating that ethylene production was increased substantially. We then wanted to see whether this ethylene, that was originally induced to affect growth, would augment the defences of the plant, as ethylene is also involved in the SA defence network (Adie *et al.* 2007). For our study, the *G. max,* or soybean, was grown with the herbivorous nematode, *H. glycines* being introduced to the soil. We used an electric fan to generate artificial wind on an experimental group, which was able to elicit thigmomorphogenesis in these soybeans (Jaffe & Forbes 1993). We were looking to answer: '1) Does wind stimulation increase stem diameter and decrease stem height, indicating induction of ethylene production?', and '2) Do plants stimulated with wind exhibit better plant defences and decrease the effectiveness of the SCN?'. Our null hypothesis states wind will have no effect on the *G. max*'s growth or plant defence, whereas our alternative hypothesis states that wind will affect plant growth by increasing stem diameter and decreasing stem elongation, and will increase plant defence against *H. glycines*.

Study System

The plant-animal interaction occurring in this study is between *G. max* and *H. glycines*. While soybeans were introduced to Canada in the mid 1800s, the production of soybeans in Canada boomed post World War Two (Statistics Canada 2009). In 2014, Canada exported two-billion dollars worth of soybeans and 3.4 million metric tons with a gross domestic product of 5.64 million dollars, making this crop economically important to Canadian agriculture (Meyers Norris Penny LLP 2016).

Although soybeans are highly profitable, yield loss associated with *H. glycines* can be up to 25%, or about 4.3 million metric tons lost as a result of these pests which is even greater than the country's total soybean export (Gabruch & Gietz 2014). It is believed that SCN were introduced to North America from Asia in 1954 upon their first discovery in North Carolina, and reports in Canada were first filed in 1987 (Tylka & Marett 2014). If farmers can use parts of the natural environment to reduce the destruction caused by this invasive pest they will protect their crop yields.

Materials and Methods

Lab Setup

On Day One of the experiment, 34 newly seeded *G. max* plants were potted and divided into four groups of 8-9 plants, in a 2×2 factorial design (view Figure 1 for details). The four groups were divided into two shallow plastic trays, enclosing groups A and B together in one and groups C and D together in the other. Groups A and B were isolated from the wind disturbance using 3 sided cardboard dividers around groups C and D, which were situated 40 centimetres away from a fan. Groups C and D received wind for six hours total over a twelve hour period every business day, in which wind and no wind alternated hourly.

Insect and Plant Rearing

Prior to inoculation, *G. max* in groups C and D were exposed to artificial wind upon the seeding date, Day 1, with the intention of stimulating the production of ethylene. Prior to inoculation, each plant was watered once, and as the experiment progressed, the *G. max* were watered as needed in order to prevent a risk of death due to over or under watering. Furthermore, possible differences in soil environments which may have acted as confounding variables caused by potential overwatering of groups A and B due to the wind's drying effect on groups C&D (if the two sets of groups had been watered equally over the course of the experiment) were considered, in order to avoid them. The *H. glycines*, as well as the *G. max* came from Agriculture Canada's Harrow, Ontario Research Station.

Inoculation

On day five of the experiment, the *G. max* in groups B and D were inoculated with approximately two grams of sand inoculum mix containing an unknown quantity of *H. glycines.* The inoculum mix was added to the top layer of soil, leaving *H. glycines* to travel into the soil toward the roots.

Measurements

On day 3, data began to be collected from each plant, in regards to the number of leaves, diameter of plant base (using vernier calipers), height of plant (using a tape measure), and any plant deaths. Measurements were repeated every 2-5 days. On the final day of measurement, day 23, the plants were uprooted for SCN cysts to be counted.

Statistical Methods

In order to statistically analyze collected data from the experiment, two-way ANOVA, t-tests and graphical analysis were conducted. The two-way ANOVA analyzed the interaction between time and wind as independent variables, with individual measurements, such as plant height as the dependent variable. Following this, the mean of plant diameter and plant height in both wind and no wind environments on each day of the experiment were calculated and plotted as a scatter plot. T-tests were completed to find significant differences between means in both groups. In addition, for nonsignificant interactions found using ANOVA, a t-test was used to determine significance between the single independent variable, wind, and plant measurements. This is because wind is the one independent variable that relates directly to the hypotheses, and the purpose of the interaction term in the first place was only to account for the inherent link between time and plant growth.

Results

Nematodes

The final measurement yielded no nematode cysts to be counted, which is indicative that the introduced SCN failed to take to groups B and D where they were originally introduced. No plants in groups B and D (or in any group) appeared to have suffered any damage due to herbivory. Based on these results, the second research question, the relationship between wind and plant defence in the experiment's *G. max* was unable to be answered. Therefore, based on these inconclusive results, the null hypothesis cannot be accepted or rejected with respect to plant defence.

Plant Height

Using two-way ANOVA, we found that the p-values for the independent variables, time and wind independently, on the dependent variable, plant height, were significant ($p < 2 \times 10^{-16}$and $p = 0.01848$, respectively), whereas ANOVA on the interaction term between the two independent variables, did not reveal a significant relationship with plant height ($p = 0.60614$). Next, we chose to use a t-test to test the effect of wind independently on plant height. It was determined that there was no significant difference between plant height with or without wind (see Figure 2(a), *t Stat*$= - 1.6397795$, and $t\ critical\ two - tail = 1.96885517$). To view the differences in plant growth over the course of the experiment, we plotted the mean height from each day graphically, and ran t-tests between measures in both groups (see Figure 2(b)). There were only significant differences between means on the final two days of data collection, days 21 and 23, which is an interesting development that will be further addressed. In sum, a significant relationship was not found between wind and plant height.

Plant Diameter

Using two-way ANOVA, it was determined that only time individually had a significant relationship with plant diameter, and that wind and the interaction between wind and time did not ($p = 3.138 \times 10^{-16}$, $p = 0.3219$, and $p = 0.6913$, respectively). Despite the lack of significant results observed from ANOVA, t-tests were conducted on the mean measurements taken each day and it was found that there were no significant differences between mean diameters on any data collection day. The means of measurements taken each day of plants exposed to wind and no wind were plotted using a scatter plot, seen in Figure 3.

Leaf Count

An additional datum, leaf number, was collected for good measure within the dataset over the course of the experiment. The motivation behind counting leaves was multifaceted. Although leaf number is not as commented upon in the literature on ethylene production in plants, it has been looked at in some studies, and we thought it could still contribute to our findings. One such way could have been if the sprouting of leaves had abruptly slowed after inoculation, we could have gathered that such a phenomenon was related to reallocation of plant resources from respiration to defence. Due to the lack of successful inoculation, it is likely that the plants did not need to reallocate resources, which explains why leaf number between groups remained fairly uniform over the duration of the experiment, as seen in Figure 4. Two-way ANOVA was conducted again for the interaction between time and wind on leaf count, with similar results, significance-wise, to plant base diameter. Only a significant relationship was found between time and leaf number ($p = 2 \times 10^{-16}$), whereas wind and the interaction were nonsignificant ($p = 0.1406$, and $p = 0.9294$, respectively).

Discussion

Ethylene

The phytohormone ethylene has been well documented to be an important regulator of plant defences and growth, though controversy still surrounds its specific role in these processes (Adie *et al.* 2007). There has been conflicting research regarding whether ethylene is a growth inhibitor or promoter, as well as its function in activating SA pathways, although it is clear that ethylene is a key contributor to the intricate inducible defence signalling network. Typically, ethylene is associated with a characteristic "triple response": inhibition of hypocotyl and root elongation, induction of radial swelling (increasing stem diamater), and the formation of an exaggerated apical hook, which has been seen to be a general response across plant species (Pierik *et al.* 2007). Additionally, another classic effect of ethylene on plant growth is the inhibition of stem elongation (Pierik *et al.* 2007). Ethylene production can be induced by both biotic and abiotic factors, including mechanical perturbation which has been known to affect plant growth (Jaffe & Forbes 1993). We measured plant height and stem diameter in both a control and experimental group, and performed statistical and graphical analysis to determine whether or not ethylene production was increased in affected plants.

In our study we observed that soybeans grown with artificial wind had insignificant differences in their mean stem diameters or heights to those exposed to no wind. Interestingly, when the heights of the two groups over time were compared with a t-test, it was found that on days 21 and 23 there was a significant difference between plant heights, with the plants affected by wind having a lower mean height. These results suggest that the plants are responding to the wind with a corresponding thigmomorphogenic response, however it is unclear if ethylene is the primary driver of this change, as the other responses we were expecting were not exhibited by the affected soybeans (Pierik *et al.* 2007). Further research could be conducted that tests the plants in a similar way over a greater time span, to investigate whether this difference would become further exaggerated as the plants matured, as well as if differences could begin to be seen in diameter.

In addition to stem diameter and stem height, leaf count was also recorded each measurement day, as ethylene has also been known to affect leaf development in a concentration dependent fashion (Iqbal *et al.* 2017). However, similar to the other defence and growth responses, ethylene has been found to have both inhibiting and promoting effects, making it harder to discern what the specific effect would be to the soybean (Iqbal *et al.* 2017). Additionally, this effect is not as consistently discussed in the literature, and so we decided to not base our hypotheses on leaf count, but thought it was worth consistent observation, as it may have supported the conclusions we came to. In the end, when we compared mean leaf counts between the experimental and control groups, we found that there was no significant difference which supports our null hypothesis that the wind did not augment ethylene production, because neither of ethylene's current proposed effects on leaf number were observed to have taken place.

Growth indicators

With none of our growth indicators demonstrating the typical ethylene responses, we conclude that soybeans grown with wind stimulation do not seem to produce increased ethylene, or at least not enough to induce any consequential changes in the soybean's morphology. Our latter results with plant height suggest that the plants may be beginning to respond to the wind with a thigmomorphogenic response, however it is unclear if ethylene is the primary regulatory hormone behind this. Further research could be conducted to try to identify ethylene specifically. An example would be using gas chromatography to try to detect and quantify the ethylene content in the air around the plants, as ethylene is a gaseous hormone (Cristescu *et al.* 2013).

Nematodes and plant defence

The nematodes introduced to the soybean groups did not establish a population, therefore we were unable to conclude that plants stimulated with wind had a more effective defence in response to this biotrophic pathogen. As a precaution we still examined the effect of herbivory with our statistical models and found that there was no significant effect on the soybean heights or diameters, which supports the intuitive conclusion that the nematodes would not be able to affect any change in the soybean, as they would not be establishing feeding sites in the roots.

Soybean production in Canada

As a result of our inability to determine whether or not wind had an impact on plant defence against SCN, we cannot comment definitively upon whether moving soybean production to windier provinces would be a worthwhile investment. With that being said, there is already growing interest in producing soybeans in Southern Alberta, and it would be interesting to study the crop outputs there, compared to Ontario's (Gabruch & Gietz 2014). This growing interest is because soybeans usually bring greater returns when compared to wheat and barley, which are already grown in Alberta, and looking just south of the border, the input costs tend to be low since they have been growing soybeans there for many years (Gabruch & Gietz 2014). A large investment would be required in Alberta in order to introduce this production, as bacterial bacterial inoculation needs to occur for the soybeans to take to the soil, for the first few years of implementation. Furthermore, either processing plants for soybeans need to be made or the cost to export soybeans to other national and international soybean processors should be analyzed. Finally, soybean varieties should be considered, as there are varieties that are better suited to be produced in Alberta's climate (Gabruch & Gietz 2014).

Moving more soybean production to the Maritimes is also a suitable solution. There is already production occurring there, with an annual total output of 175 million dollars (Meyers Norris Penny LLP 2016). The start up costs we have to consider for Alberta could be avoided since there is already production in the Maritime provinces. Once future research is done that potentially supports the relationship between wind and plant growth and defence, more production could occur in the Maritimes.

Sources of error and future research

More research would need to be conducted in order to assess whether wind can cause an appreciable increase in plant defences that would protect crop yields. When considering where future research could go and how they could improve upon our study, one of the most obvious is the timeline. As previously stated, in the latter part of the experiment significant results were observed between the mean heights of the soybeans as a result of wind. Therefore, if a similar experiment is repeated, the duration should be prolonged in order to determine if this significant difference continues and/or exaggerates. One major source of error was that the inoculated SCN did not establish a viable population, so further research should be done to allow the SCN to take to the roots, allowing the effect of wind on plant defence to be observed. When considering ethylene in the context of this experiment, researchers could study a different producer and herbivore system in order to analyze how ethylene affects plant defence in different plants. Despite wind having no discernible effect on the soybean, the dual nature of ethylene as a key component in both growth and defence is still worth exploring. A final source of error was the fact that the soybeans were being rotated incorrectly at first, with wind blowing on 3 sides total when the intention was to simulate bi-directional wind, but that was fixed early on. The wind also was comparatively weak compared to the locations where we planned on having increased soybean production, so future experiments should have fans with higher wind speeds.

Conclusion

Our study was designed to determine if there is a correlation between wind and *G. max's* growth, as well as wind and *G. max's* defence against *H. glycines*. When looking at the first part of our hypothesis, our results supported the null with regards to wind and plant growth. Our results between wind and plant defence were inconclusive, as the SCN were not able to affect the plants. However, it cannot be assumed that plants affected with wind would fare better against the pathogen. As a result, other factors should be considered and further research conducted, prior to a large expansion of soybean production into Alberta or the Maritimes, since the soybeans would not necessarily be more resilient in windier provinces. This result simply implies that we have to do more research about the SCN to find out the best conditions for them to attach to the soybeans, and adjust our protocol if the experiment is repeated.

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Figure legends

Figure 1: A table describing groups A, B , C and D of the 2×2 factorial experimental design as they differ based on simulated wind and having been inoculated by *H. glycines*

Figure 2: A scatter plot plotting mean plant height of plants with and without simulated wind, which demonstrates that plants exposed to no wind conditions had larger mean growth than those exposed to wind, especially on the last two days, which are denoted with an asterisk to be significantly different, as determined by t-tests (a), and mean plant height over time of plants with and without simulated wind (b). Plants exposed to no wind had increased growth than those that were, when observed over time, especially near the end of the experiment.

Figure 3: A scatter plot plotting mean plant diameter over time of plants with and without simulated wind. This plot shows no significant differences between plant diameters of plants with and without wind on any data collection day of the experiment.

Figure 4: Scatter plot plotting mean leaf number over time for plants with and without simulated wind. It can be observed that plants exposed to no wind had a slightly higher mean leaf count over time, but significant differences were not found between the two groups on any days

Figure 1:

	Not Inoculated	Inoculated
No Wind	A	B
Wind	C	D

Figure 2(a)

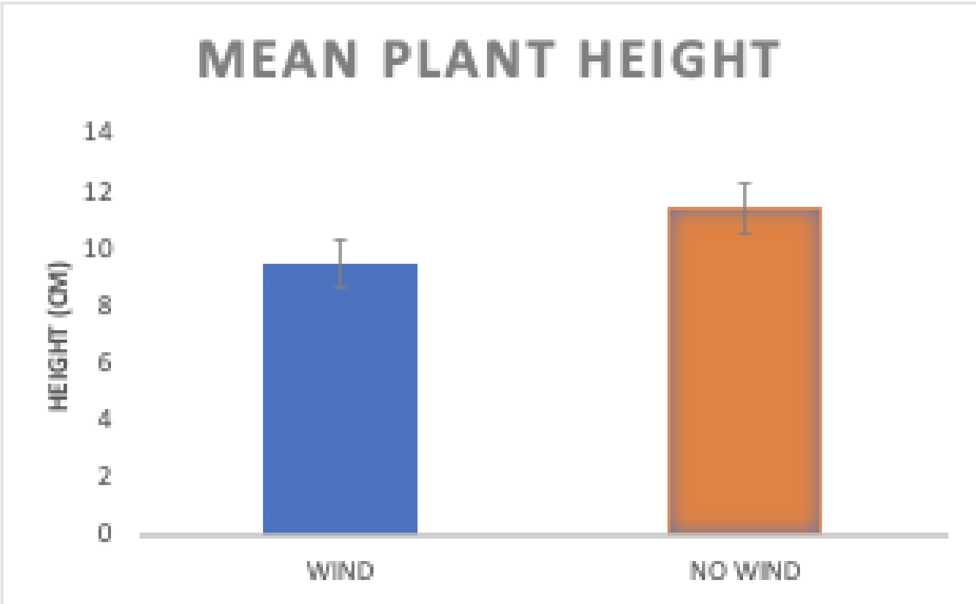


Figure 2(b)

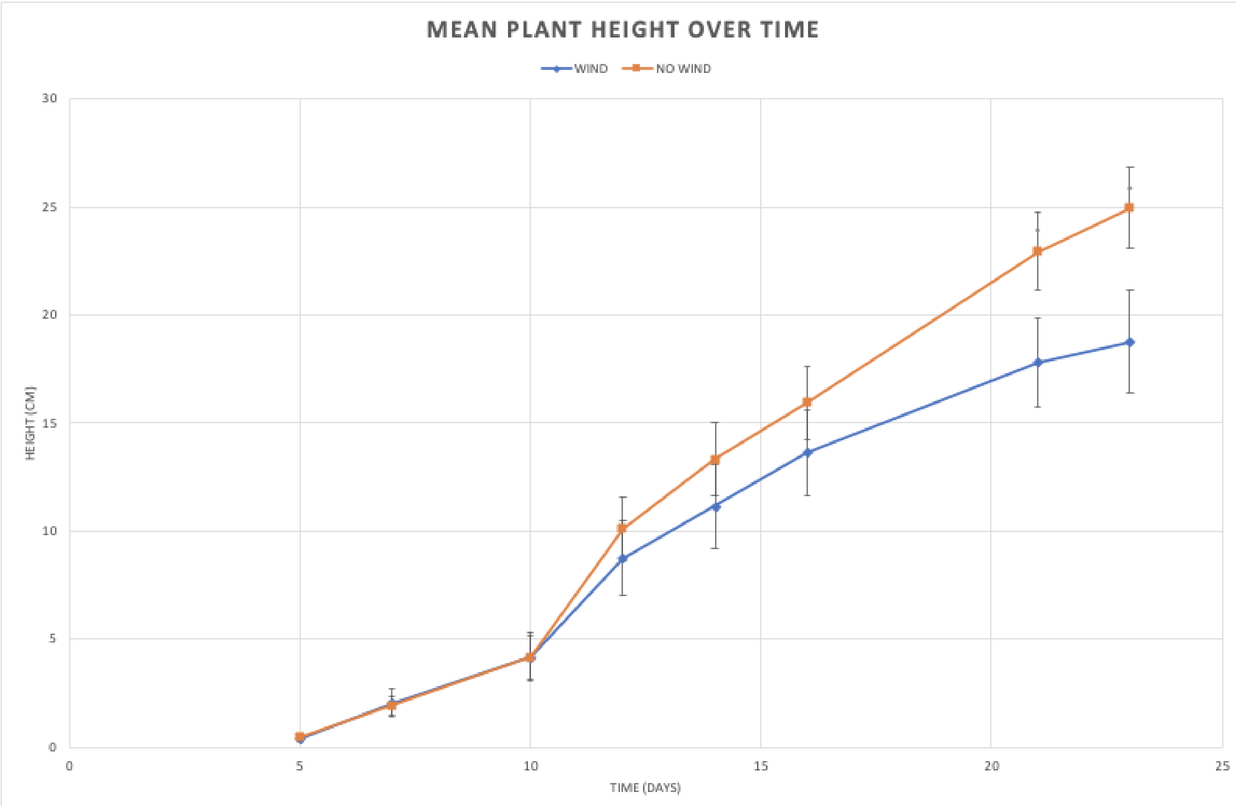

MEAN PLANT HEIGHT OVER TIME
WIND
NO WIND
HEIGHT (CM)
30
25
20
15
10
5
0
0
5
10
15
20
25
TIME (DAYS)

Figure 3

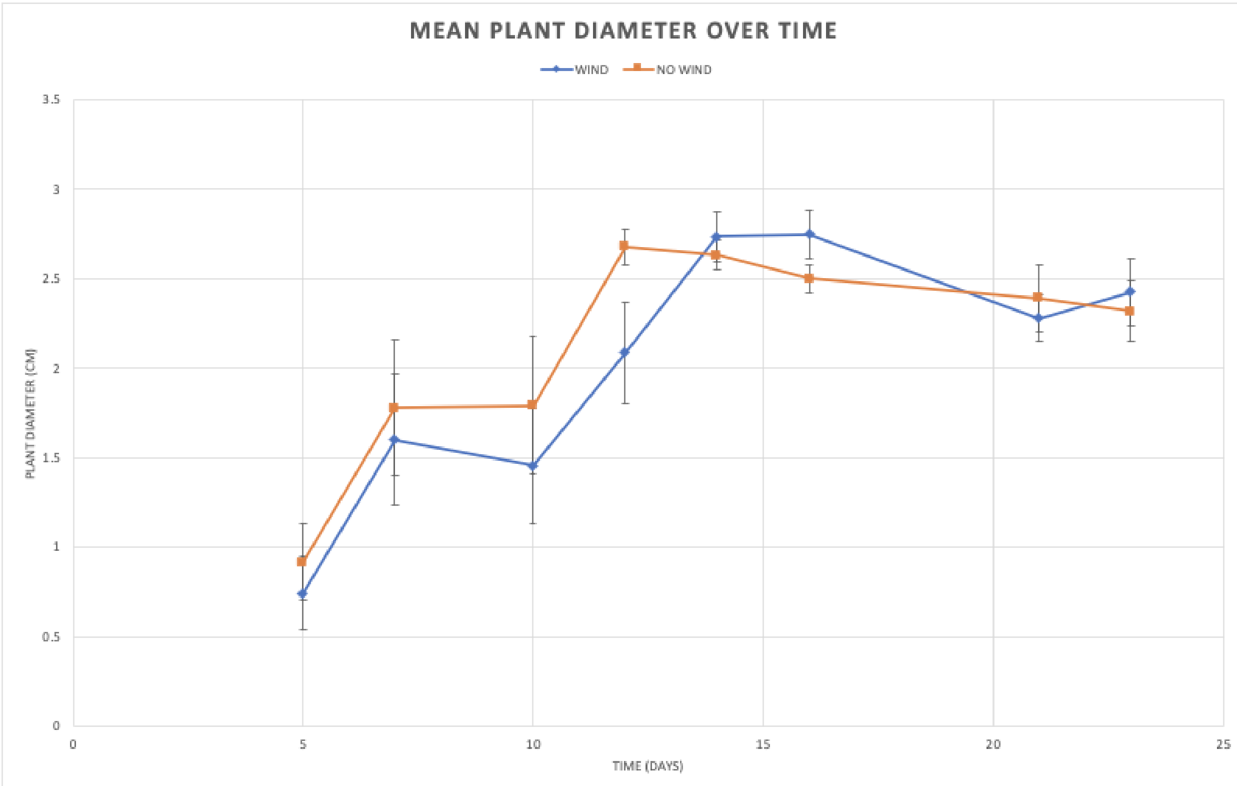
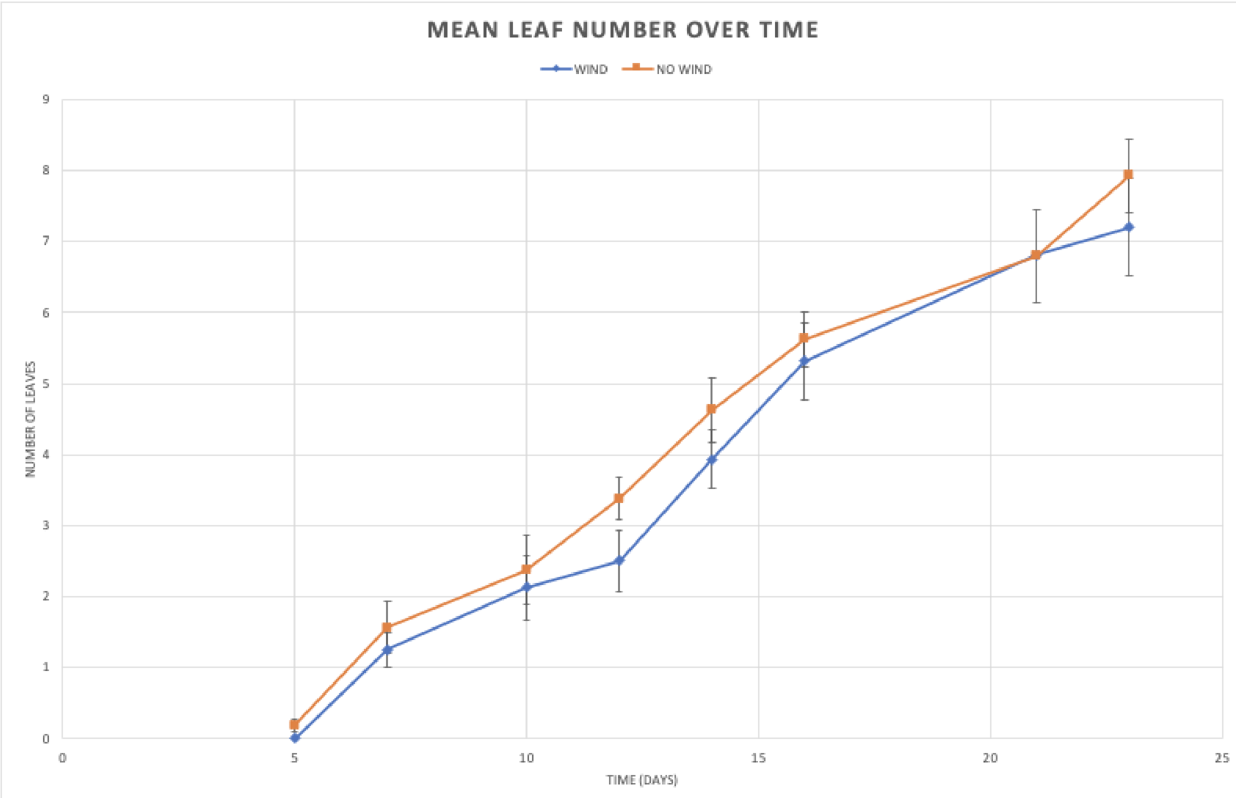

MEAN PLANT DIAMETER OVER TIME
WIND
NO WIND
PLANT DIAMETER (CM)
TIME (DAYS)
0
0.5
1
1.5
2
2.5
3
3.5
0
5
10
15
20
25

Figure 4


MEAN LEAF NUMBER OVER TIME
WIND
NO WIND
NUMBER OF LEAVES
TIME (DAYS)
0
1
2
3
4
5
6
7
8
9
0
5
10
15
20
25