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Article

Effects of Various Environmental Factors on Stomatal Density, Area, and Potential Conductance Index

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SUMMARY

Stomata, microscopic pores on a leaf flanked with flexible guard cells that open and close the stomatal opening, account for 95% of terrestrial movement of water vapor and carbon essential to the survival of plants. The climate crisis is challenging plants with elevated CO_2, drought, varying soil salinity, varying soil acidity, and increasing temperature. While research has been done on how stomata respond to elevated CO_2 alone, markedly less research has been done on the effect of elevated CO_2 in combination with other environmental factors. Thus, the purpose of this study was to determine the effects of elevated CO_2 in combination with other environmental factors on stomatal density, size, and conductance in radish, barley, tomato, and buckwheat. A controlled experiment with these plants and six conditions (Control, Elevated CO_2, Elevated CO_2 + Salinity, Elevated CO_2 + Acidity, Elevated CO_2 + Temperature, and Elevated CO_2 + Drought) was conducted, and data was collected. The results trend towards a decrease in stomatal density, stomatal area, and potential conductance index (PCI) in the elevated CO_2 conditions compared to the control conditions. Additionally, our results suggest that the other four conditions do not amplify the effect of elevated CO_2 levels alone. Overall, results showed variation in data among the tested plants, suggesting that making generalizations about the impact of CO_2in combination with other environmental factors is risky. Thus, further research on the effects of multiple environmental conditions on stomatal characteristics is warranted to determine the impact on agriculture adaptation and water management strategies.

INTRODUCTION

Climate change, resulting from the ongoing rise of heat-trapping gases such as CO_2 within Earth's atmosphere, is a pressing issue in the world today. The effects on agriculture include reduced crop yield from an increase in temperature, reduced soil moisture from a lack of precipitation, and higher net photosynthetic rates from an excess of CO_2 (1). Furthermore, the effect of climate change will vary throughout the world. Changes in rainfall patterns, temperature, and CO_2 levels may have significant effects on global agriculture, which are causing suitable land for key crops to undergo geographic shifts. Thus, in low-income populations that base their survival on isolated agricultural systems (systems in which the population creates and distributes their own food supply), climate change poses a major threat to their quality of life and economic stance (2).

Stomata, tiny pore-like structures on the leaf, play an integral role in gas exchange that drive photosynthesis (3). They are an essential part of important hydrological cycles and overall plant growth, because they regulate a majority of terrestrial movement of gases in plants (4). Stomata consist of kidney-shaped cells, also known as guard cells, that have an arrangement of microfibrils that allow the stomatal aperture to open and close. The opening and closing of these pores depend on external factors, such as concentrations of CO_2 and water supply. For instance, stomata tend to stay closed if water is scarce and open if water is available (3). Stomatal density is used to measure this response, which then reveals survival mechanisms that the plant develops based on its conditions. Oftentimes, higher stomatal density suggests more CO_2 uptake and water loss, whereas lower levels suggest the opposite (5). In fact, Arabidopsis plants that are genetically modified to have reductions in stomatal density have shown increased water-use efficiency, as fewer overall stomata are transporting water and gas in and out the leaf, leading to more water retention (6). In the same regard, stomatal area is another effective metric used to indicate how the individual stomata of a plant responds to its environment. For instance, larger stomatal sizes often indicate slower responses and guard cell movement, whereas smaller stomatal sizes indicate the opposite (7). If a plant were to have slower stomatal responses, this may negatively affect how quickly a plant may adapt, deterring plant growth and development.

However, the response of stomatal development to elevated CO_2 conditions is still unclear (8). Research has found both significant decreases in herbarium specimens and increases in stomatal densities of woody, herbaceous, and annual species of plants after exposure to elevated CO_2 concentrations (9, 10). Further, another study reported no stomatal response to CO_2 enrichment for non-vascular plants and some moss sporophytes. (11). As for stomatal area, elevated CO_2 may cause guard cells to induce stomatal closure, often resulting in a lower stomatal area. (12). Conversely, according to a study, elevated CO_2 may alleviate the impact of drought on barley by lowering stomatal conductance and area, which would improve water status (13). These stomatal trends may be used to create novel, more-efficient crop management appliances to regulate plants in the midst of an increasingly variable climate.

Thus, we investigated how stomatal density, stomatal area, and PCI are affected in common agricultural plants such as *Hordeum vulgare* (barley), *Raphanus sativus* (radish), *Solanum lycopersicum* (beefsteak tomato), and *Fagopyrum esculentum* (buckwheat) when exposed to elevated CO_2

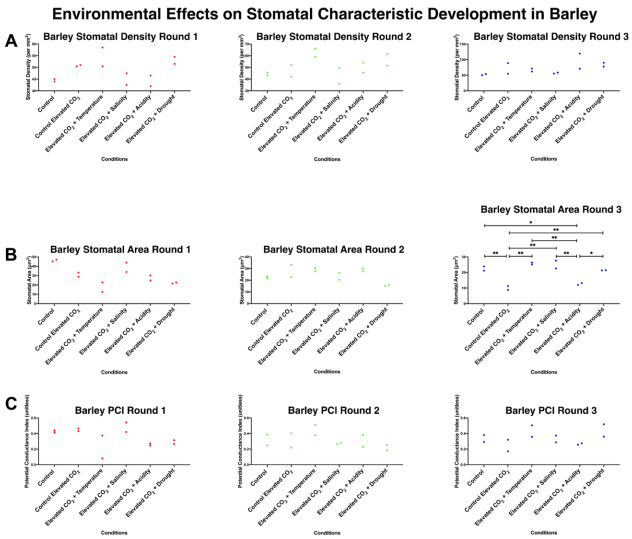

Figure 1: Stomatal density (A), stomatal area (B), and PCI (C) data collected over three rounds in barley plants. Each round of data was collected every two weeks over a total of six weeks, with two leaves of each plant being measured for every condition. The statistical analysis conducted on the round three data is presented with brackets connecting the groups with statistically significant differences. Statistically significant differences are marked with asterisks to denote *p*-values (* *p*-value < 0.05, ** *p*-value < 0.01).

levels alone and elevated CO_2 levels in combination with increased soil temperature, soil acidity, soil salinity, and drought stress. Stomatal density is a measurement describing the stomata present per square millimeter. Stomatal area is a calculation describing the general size of each stomata. PCI is a calculated value that describes the rate of gas exchange depending on stomatal density and guard cell length (14).

We hypothesized that elevated carbon dioxide conditions will decrease stomatal density, size, and PCI in radish, tomato, barley, and buckwheat plants as a result of increased gas exchange. Because a major function of the stomata is water regulation, environmental conditions that require the plant to retain water will limit the size, density, and PCI. Therefore, conditions such as increased salinity, increased temperature, increased acidity, and drought stress will further decrease stomatal characteristics, and thus amplify the impact of elevated CO_2 levels. Our results show varied data, which does not create any conspicuous trends across the plants, simply suggesting that different plants react differently to their conditions.

RESULTS

To study the effect of elevated CO_2 in addition to other environmental factors on stomata, six groups were created — a standard control in normal growing conditions (C group), a control with elevated CO_2 conditions (E group), and four groups with elevated CO_2 combined with one of the following factors: increased temperature (ET group), increased soil salinity (ES group), increased soil acidification (EA group), or drought stress (ED group). The testing conditions were determined based on projections for the developing natural environment, currently being impacted by climate change. For instance, elevated CO_2 conditions were kept at over 700 ppm compared to normal CO_2 conditions, which was kept at around 400 ppm (15). Increased temperature values were kept at the highest end of optimal growing temperatures relative to each plant (these specific temperatures can be found in the Methods section). Soil salinity was increased to a 5% saline concentration (slightly saline), which is compared to the control condition of a 0-3% concentration (non-saline) (16). Soil acidity was increased to a pH of 5-5.5 (strongly acidic), which is compared to the control conditions with a pH of 6-6.5 (slightly acidic) (17). Drought stress was induced by watering with 50% of the normal 46 mL per pot. Stomatal density and stomatal size were measured every two weeks for a total of three times throughout the six-week experiment. PCI was calculated from stomatal density and guard cell length. The data was observed in two ways. First, for each plant, we examined the development of stomatal characteristics over time in each of the six testing conditions by looking at the correlation between time and stomatal density, stomatal area, or PCI. Second, we examined the notable correlations among conditions by comparing two groups and looking at that difference across all four plants.

Barley

Stomatal Density: The ET, ES, and ED groups showed a positive correlation between time and stomatal density for barley plants. The C, E, and EA groups showed no trend in stomatal density for barley plants (**Figure 1A**).

Stomatal Area: The E group displayed a negative correlation between time and stomatal area for barley plants. The C, ET, ES, EA, and ED groups displayed no trend in stomatal area for barley plants (**Figure 1B**).

PCI: The E and ES groups both had a negative correlation between time and PCI for barley plants. The other conditions — the C, ET, EA, and ED groups — showed no trend in PCI for barley plants (**Figure 1C**).

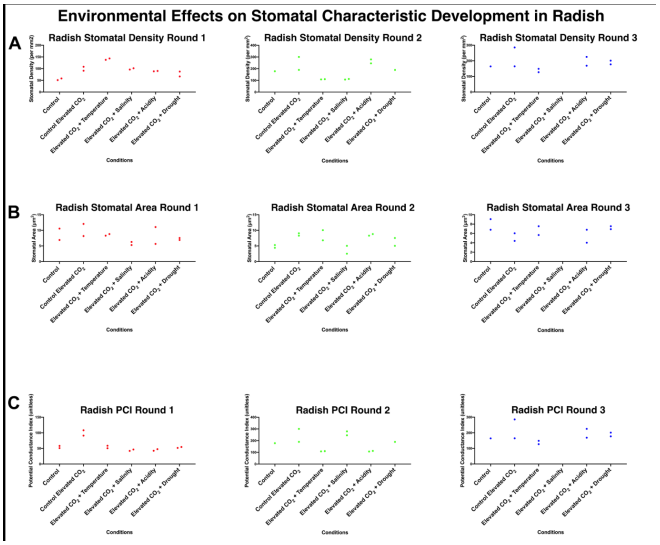

Figure 2: Stomatal density (A), stomatal area (B), and PCI (C) data collected over three rounds in radish plants. Each round of data was collected every two weeks over a total of six weeks, with two leaves of each plant being measured for every condition. The statistical analysis conducted on the round three data is presented with brackets connecting the groups with statistically significant differences. Statistically significant differences are marked with asterisks to denote *p*-values (* *p*-value < 0.05, ** *p*-value < 0.01).

Radish

Stomatal Density: The EA group showed a positive correlation between time and stomatal density for radish plants. The C, E, ET, and ED groups showed no trend in stomatal density for radish. Additionally, there was no data collected for the third round of the ES group for stomatal density in radish plants, so a meaningful trend cannot be determined (**Figure 2A**).

Stomatal Area: The E and ET groups displayed a negative correlation between time and stomatal area for radish plants. The C, EA, and ED groups displayed no trend in stomatal area for radish plants. Again, there was no data collected for the third round of the ES group for stomatal area in radish plants, so a meaningful trend cannot be determined (**Figure 2B**).

PCI: The C, EA, and ED groups all showed a positive correlation between time and PCI for radish plants. The E and ET groups showed no trend in PCI for radish plants. Like in stomatal density and stomatal area, a meaningful trend for PCI was not determined due to lack of data for the ES group (**Figure 2C**).

Tomato

Stomatal Density: The E group had a negative correlation between time and stomatal density for tomato plants, whereas the ED group had a positive correlation. The C, ET, ES, and EA groups had no trend in stomatal density for tomato plants (**Figure 3A**).

Stomatal Area: The EA and ED groups both had positive correlations between time and stomatal area for tomato plants. The C, E, ET, and EA groups had no trend in stomatal density for tomato plants (**Figure 3B**).

PCI: The C and ET groups had a negative correlation between time and PCI for tomato plants, whereas the EA and ES groups had a positive correlation. The C, E, and ED groups had no trend in PCI for tomato plants (**Figure 3C**).

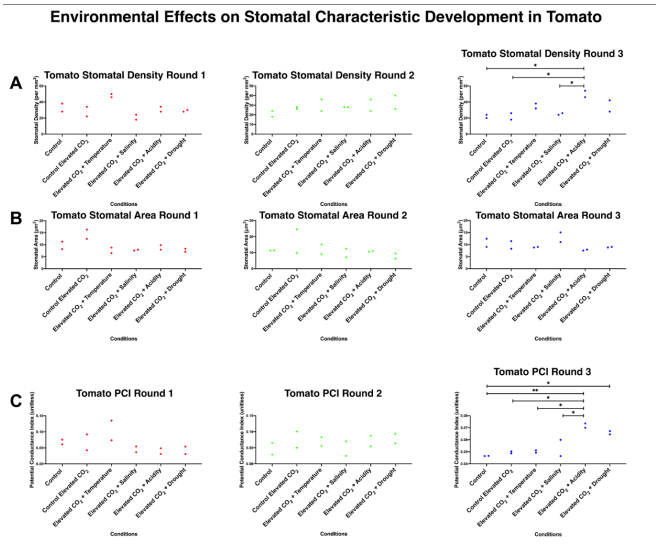

Figure 3: Stomatal density (A), stomatal area (B), and PCI (C) data collected over three rounds in tomato plants. Each round of data was collected every two weeks over a total of six weeks, with two leaves of each plant being measured for every condition. The statistical analysis conducted on the round three data is presented with brackets connecting the groups with statistically significant differences. Statistically significant differences are marked with asterisks to denote *p*-values (* *p*-value < 0.05, ** *p*-value < 0.01).

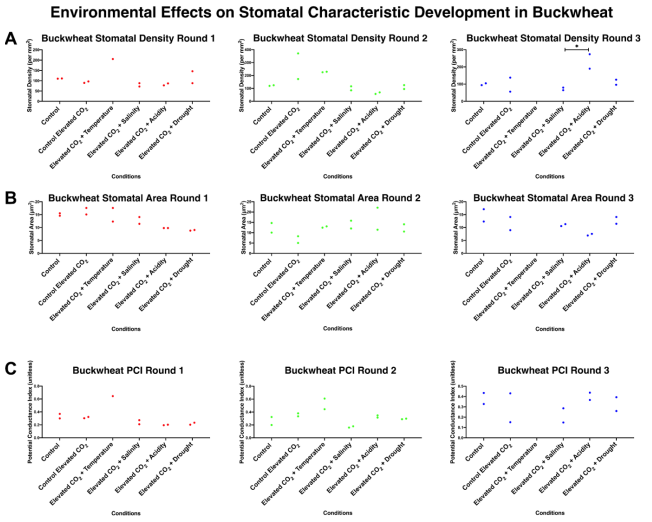

Figure 4: Stomatal density (A), stomatal area (B), and PCI (C) data collected over three rounds in buckwheat plants. Each round of data was collected every two weeks over a total of six weeks, with two leaves of each plant being measured for every condition. The statistical analysis conducted on the round three data is presented with brackets connecting the groups with statistically significant differences. Statistically significant differences are marked with asterisks to denote *p*-values (* *p*-value < 0.05, ** *p*-value < 0.01).

Buckwheat

Stomatal Density: The EA group had a positive correlation between time and stomatal density for buckwheat plants. The C, E, ED, and ES groups had no trend in stomatal density for buckwheat plants. The ET group did not have any data collected for round three, so a meaningful trend cannot be determined (**Figure 4A**).

Stomatal Area: The ED group had a positive correlation between time and stomatal area for buckwheat plants. The C, E, EA, and ES groups all no trend in stomatal area for buckwheat plants. The third round of data in the ET group was not collected, so a meaningful trend cannot be determined (**Figure 4B**).

PCI: The ED and EA groups both had positive correlations between time and PCI for buckwheat plants. The C, E, and ES groups had no trend in PCI for buckwheat plants. Similar to the stomatal density and stomatal area of buckwheat plants, the third round of data in the ET group was not collected, so a meaningful trend with PCI cannot be determined (**Figure 4C**).

Notable Correlations Among Conditions

Apart from the trends over time that occurred in individual conditions for each plant, trends based on comparisons between testing groups were also analyzed. Essentially, we analyzed and compared the differences between the round three values of stomatal density, stomatal area, and PCI between all six testing groups. Statistical analysis was performed exclusively on this interpretation of the data. There were some trends that were consistent throughout all plants in these comparisons, but after performing a One-way ANOVA with post-hoc Tukey HSD Test, only a few values

had a statistically significant difference. We found a decrease in stomatal area between the C group and the EA group in barley, buckwheat, radish, and tomato (**Figure 1B**, **Figure 2B**, **Figure 3B**, **Figure 4B**). Barley had a statistically significant decrease in stomatal area ($p < 0.05$, ANOVA test) (**Figure 1B**). We found an increase in stomatal density across all four plants in the EA group compared to the C and E groups (**Figure 1A**, **Figure 2A**, **Figure 3A**, **Figure 4A**) Tomato had a statistically significant increase in stomatal density ($p < 0.05$, ANOVA test) (**Figure 3A**). Finally, when comparing the E group to the EA group for PCI, there was also an increase among all four plants (**Figure 1C**, **Figure 2C**, **Figure 3C**, **Figure 4C**). Tomato had a statistically significant increase in stomatal density ($p < 0.01$, ANOVA test) (**Figure 3C**).

DISCUSSION

All three stomatal characteristics studied (stomatal density, stomatal area, and PCI) showed major variation in each of the four plants. These variations suggest that plants respond differently to their given environments based on their needs for survival. For example, barley tends to thrive in cool, dry, mild winters in droughty soil and can tolerate more alkaline and salty soils. (18). Radish tends to thrive in cool, moist weather with well-drained and slightly acidic soil, but it is not as tolerant to saline (19, 20). Tomato tends to thrive in very warm temperatures with well-drained, slightly acidic soils and is somewhat tolerant to saline (21). Buckwheat tends to thrive in warmer temperatures with airy, moist, slightly acidic soils and is less tolerant to drought stress but more tolerant to saline. (22, 23). Over the 6-week growth period, each plant responded to a certain condition in different ways. For instance, for the stomatal density measurement in the ES group, barley and tomato had no trend over time, whereas radish and buckwheat had a positive trend over time. Likewise, for the stomatal area measurement in the E group, barley and radish had a negative trend over time, whereas tomato and buckwheat had no trend over time. These are some of the many examples that indicate that there was never an instance where all four plants had the same type of trend in a certain condition for a specific measurement. Thus, the variation of results could emerge not only from the environmental condition, but the type of plant as well, which is a key theme throughout our data.

Additionally, to address our initial hypotheses, we observed how the testing groups compared between conditions in the last round of measurements for each plant. The majority (66.7%) of the data collected for the plants trended towards a decrease in stomatal characteristics for elevated carbon dioxide conditions as compared to the control conditions. However, much of this data was statistically insignificant. Nonetheless, it still suggests that, in some cases, elevated carbon dioxide decreases stomatal density, stomatal area, and PCI. The second hypothesis stated that environmental conditions requiring plants to retain water combined with the stress of elevated carbon dioxide will further decrease the stomatal density, stomatal area, and PCI in the four plants. However, a majority of the data suggests that the four conditions — the ES, EA, ET, ED groups — did not further amplify the effect of elevated carbon dioxide levels alone.

As mentioned before, there was a lack of statistically significant data, and this could be because we did not test a large enough sample size due to the limited time that we had to conduct the experiment. Only allowing six weeks for plant growth could also have led to a restricted, incomprehensive representation of stomatal development in these plants. Due to limited time and resources, we were unable to set up an additional control group of normal CO_2 concentrations and the environmental conditions, which, if conducted, would have provided more data that would help develop better analyses and conclusions about the effects of elevated CO_2 and the environmental factors. However, we were able to use previously published findings of these specific conditions to add to our analyses later on. If there were opposite effects between the elevated CO_2 control groups and environmental factor control groups, this could lead to more questions about individual and combined effects of CO_2 levels and changing environments in certain plants. Additionally, another possible error could be miscounting some of our data. We could not utilize the available computer program, ImageJ, to count the stomata, as the images taken from the light microscope were not compatible with the program. This led to manual counting of stomata and performing calculations, which lengthened the overall time for data collection and calculation and could have provided a possibility for human error. Lastly, carbon dioxide production was manually regulated with daily reactions of baking soda and vinegar. This reaction created carbon dioxide as well as a byproduct of water vapor. Water vapor, like the environmental factors, could have influenced the stomatal characteristics of the plants. It could have also impacted the results in the drought condition as it was adding back some water into the environment. The hand-crafted system we created may have presented inconsistencies with CO_2 levels in the plants' growing chambers. For future experimentation, prepared sources of CO_2 like carbon dioxide tanks could be used to eliminate problems with byproducts from CO_2 synthesis. There are also other inexpensive, homemade options that can create a pure source of CO_2. Additionally, sustained mechanisms can be used to automatically regulate CO_2 levels, which would alleviate issues of CO_2 level inconsistencies.

Aside from the main hypotheses, there were some other statistically supported findings that appeared in the data. These findings agree with prior research and warrant further study. For example, we found a statistically significant difference between the stomatal area of barley in the C group and the E group, 22.62 μm^2 and 10.02 μm^2 respectively ($p < 0.01$, ANOVA test). Similarly, Yamamoto *et al*. suggested that elevated CO_2 causes enhanced anion channel activity in guard cells which induce stomatal closure, often resulting in a lower stomatal area (12). Our data supports these findings. In the barley plants, the stomatal area in the E group was notably lower than the stomatal area in the C group.

Additionally, the stomatal density and area of barley increased from the E group to the ED group. Barley had a statistically significant difference between the stomatal area in the E group and the ED group ($p < 0.01$, ANOVA test). This evidence refutes previous studies which concluded that elevated CO_2 promotes plant water retention in drought conditions via stomatal closure and lower stomatal densities, leading to less water loss (13).

Based on the instances where our data refuted previous findings, further research can be conducted with more intensive focus on specific environmental conditions and stomatal characteristics in regard to the inconsistencies we saw within our own experiment. For example, the stomatal

area of barley decreased when comparing the E group to the ED group in rounds one and two. However, in round three, the stomatal area of barley increased. This was a contradiction to the established idea that the stomatal area would be generally smaller in conditions where retaining limited water is vital. Additionally, the stomatal density of barley showed a consistent increasing trend between the E group and the ED group condition. Considering the previous conclusion that stomatal density would decrease in order to limit water loss in a drought environment, the trend observed in our data is contradictory. This brings about the question of whether these trends in barley are real or just anomalies in our data. More studies specifically focusing on the individual and combined effect of drought and elevated CO_2 on stomata would confirm or deny the trends visualized in our data as well as previous studies.

Similar inconsistencies arose when looking at the effects of other environmental factors on plants. For instance, previous findings have generally shown that stomatal conductance decreases with elevated CO_2 and increases with rising temperature (24, 25). With a few exceptional cases, stomatal conductance generally decreases in the presence of both increased temperature and elevated CO_2 concentrations (8). However, some of our data refutes this statement. When comparing the E group to the ET group in round three data, the tomato plant had shown hardly any increase or decrease in PCI in the third round. But barley had shown an increase in PCI in the third round, following the previously established trend in past research. This could be due to the fact that the two plants have very different thresholds for optimal temperature ranges. Tomato, which has a higher temperature range of 21-32°C, is more heat tolerant than barley, which has a lower temperature range of 10-20°C (26, 27). Thus, their distinct ideal growing conditions could be the reason why the trends between tomato and barley differed.

Furthermore, increased salinity has also shown to have the same effect. According to previous findings, stomatal conductance generally decreases in elevated CO_2 concentrations and decreases in increased soil salinity (24, 28). In combination, increased soil salinity and elevated CO_2 concentrations have shown to decrease stomatal conductance (8). When comparing the E group to the ES group in round three data, although barley and tomato increased in PCI, buckwheat had decreased and radish had no data available. These variances could be related to each plant's individual salt tolerances and/or reactions to elevated CO_2.

Markedly less research has been done on the effect of soil acidification on stomatal characteristics. Nonetheless, similar to the other environmental factors, all four plants did not unanimously exhibit the same trend in round three data. For the few studies that have been done, stomatal conductance has been found to decrease when soil acidity increases (29). When comparing the E group to the EA group, barley, tomato, and buckwheat showed an increase in PCI, whereas radish showed a decrease in PCI. This variation in the results reverts back to the specific ideal growing conditions of each plant. The four plants have their own soil and environmental needs for optimal growth, meaning that they develop their own tolerances to acid levels in soil.

With these variances observed, more experimentation with environmental factors and elevated CO_2 could further contribute to this project and add clarifications regarding stomatal response to changing environmental conditions. Overall, this experiment emphasizes the importance of studying the impact of changing environmental conditions on crops, which is essential to agricultural productivity. Thus, the conclusions from this type of data can be used to develop novel technology, crop management, and adaptation strategies in order to continue to grow the plants optimally and efficiently.

METHODS

The plants used in this experiment were H*ordeum vulgare* (barley), *Raphanus sativus* (radish), *Solanum lycopersicum* (beefsteak tomato), and *Fagopyrum esculentum* (buckwheat). Beefsteak tomato seeds were purchased from Seed of Change on Amazon, and buckwheat seeds were purchased from Old Cobblers Farm on Amazon. Both barley and buckwheat seeds were purchased from Carolina Biological. Seeds were first potted in 3.5-inch diameter pots in potting soil from Carolina Biological as per the sowing instructions for each type of seed. These pots were transferred to fish tanks which would serve as the growing chambers for each plant. A total of 14 covered, 2.5-gallon betta fish tanks were used to simulate the six growing conditions. Two tanks containing pots of all four plant types were the baseline control. Since there were two types of plants per tank, plants were divided by cardboard dividers in the tanks. Each section of the tank had two pots of the same plant. These plants were grown according to their normal instructions. They were grown under natural light and LED/fluorescent plant lights. Plants in the temperature conditions received the LED plant lamps along with natural light because they helped raise the temperature, and all other plants had fluorescent light along with natural light. All plants were watered with 46 mL of tap water and kept in a room with a baseline temperature between 20–22°C. Reusable ice packs were placed under the pots of radish and barley plants to cool the soil because their average growing temperature was under the room's average temperature. Barley plants were grown at 15°C (25); radish plants were grown at 18°C (28); tomato plants were grown at 29°C (26); buckwheat plants were grown at 27°C (29). The soil temperatures for all the plants were monitored by thermometers in the soil.

The remaining 12 tanks all required elevated levels of carbon dioxide. To achieve this, individual apparatuses were created to synthesize carbon dioxide. A system of gallon milk jugs, water bottles, and tubing was fixed together, and daily reactions of approximately 1/6 gallon of vinegar and 2 tablespoons baking soda yielded the amount of carbon dioxide gas needed to keep the carbon dioxide level above the predetermined amount of 700 parts per million in the 12 tanks (**Figure 5**). Carbon dioxide levels were measured with a Pasco sensor. This method to create carbon dioxide gas for the chambers could be done with other alternatives such as carbon dioxide gas tanks or CO_2 synthesis through yeast. Of these 12 carbon dioxide tanks, 2 were part of the second control group: the E group. This group was grown the same way as the control group, but with the addition of an increased amount of carbon dioxide in the growing chambers.

The remaining 10 tanks would represent the 4 specific environmental factors chosen to be studied along with an elevated carbon dioxide setting. Four of these 10 tanks would be used to represent the ET group. Each of the four tanks would have one of the four plants, and heating pads and growing lights were used to bring the tank temperatures up to

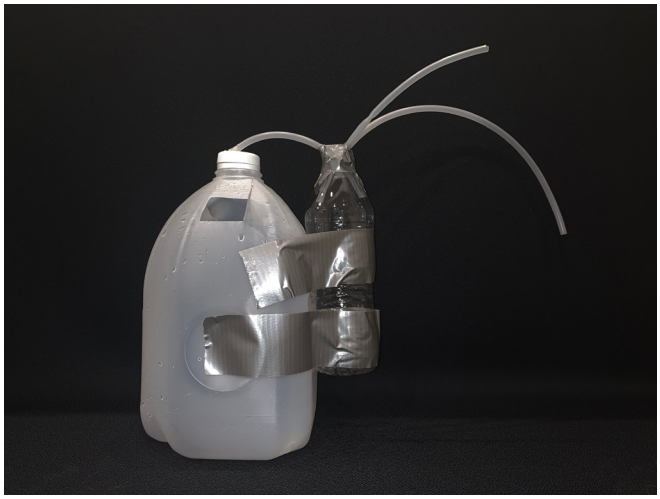

Figure 5: Experimental apparatus used to elevate CO_2 conditions. A milk gallon jug is fastened to a water bottle. A sealable opening in the gallon jug is where vinegar and baking soda would be added in to cause a reaction which produces CO_2 gas. The gas and any excess liquid that overflowed from the strong reaction would pass through the tube connecting the milk jug and the water bottle. Excess liquid would collect in the water bottle, and gas would flow through the remaining two tubes into the growing chambers. Each of the two tubes would be attached to each of the two sections of a growing chamber. The baking soda and vinegar would be replenished regularly to maintain CO_2 levels of 700 ppm or greater.

the highest temperature in the specific plant's growing range. Radish and barley were grown without ice because the room temperature represented the highest temperature in their growing range. Radish was grown at 21°C and barley was grown at 20°C (30, 26). Buckwheat and tomato were heated to their highest temperatures with heating pads. Buckwheat was grown at 37°C and tomato was grown at 32°C (31, 27). Two more tanks were the EA group, in which an acidic mixture of 1 cup vinegar and 1 gallon of water was tested and determined to drop the soil pH from the normal soil pH, 6-6.5, to about 5, which was checked with pH paper to make sure the acidity levels were maintained (17). Two tanks simulated the salinity and elevated carbon dioxide condition, in which a 100 mM solution of 5.844 grams Kosher salt and 1 Liter of water was used to water the plants. The final two tanks were the ED group, in which the amount of water used to water the plants was halved from 46 mL to 32 mL to put a stress on the ability of water retention of the plants. All plants were grown with natural light and LED/fluorescent plant lights, and they were watered twice a week with the water that each condition called for. Plants were subjected to their conditions at the point where the cotyledon emerged during germination.

Data collection started when plants started growing. The plants grew over the course of six weeks, and approximately at every two-week interval, two leaves from each of the four plants in each of the six conditions were collected for stomatal analysis. Two of the most developed, matured leaves were selected for a leaf imprint. Leaf size is a factor in stomatal density and area, which was kept in mind during the experiment. Leaves of similar sizes from each type of plant were used for leaf impressions. Even though the values of stomatal density and area were different by plant, they were recorded nonetheless. This is because, in our analysis, we were focusing on the differences and trends over time among testing groups, not necessarily the numerical values themselves. Thus, these differences and trends over time revealed whether or not our plants adapted to their environments throughout the experiment.

The underside of the leaf was painted in Sally Hansen brand clear top coat nail polish so that light could pass through for analysis under a compound light microscope. Once dry, the layer of nail varnish was peeled off, put on a microscope slide, and labeled for analysis under a Swift brand light microscope with an ocular micrometer as well as any compound light microscope with a one square millimeter viewing range. Stomatal density is a measurement describing the stomata present per square millimeter. Stomatal density was measured once on each leaf by creating a one millimeter by one millimeter viewing window out of a piece of tape and sticking it on the slide being analyzed. Stomata were counted in that square millimeter field and the number was recorded. Alternatively, this process could be done using a program designed for stomatal analysis like ImageJ and a microscope that could directly view leaf tissue, eliminating the need for a clear leaf impression. Stomatal area is a calculation describing the general size of each stomata. Stomatal area was calculated by measuring the length and width of the stomata in micrometers with the Swift microscope and then using an ellipse area formula to calculate the stomatal area ($A = \pi ab$). One stoma was measured on each of the two leaf samples. PCI is a calculated value that describes the rate of gas exchange depending on stomatal density and guard cell length. PCI was calculated by squaring the stomatal length, multiplying it by the stomatal density for that leaf, and then multiplying that by 10^{-4} ($PCI = (\text{guard cell length})^2 \times \text{stomatal density} \times 10^{-4}$). This leaf impression and data collection process was done three times throughout the 6-week time period. Once the third round of measurements and calculations were collected, data analysis along with statistical analyses were performed. One-way Analysis of Variance (ANOVA) tests with a post-hoc Tukey Honestly Significant Difference (HSD) tests were conducted to see if there was significant difference between any of the test groups across all plants and conditions (32). Raw data for each plant and condition were inputted into the website's calculator, and the results of the test were analyzed.

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