DNA Methylation and Phenotypic Plasticity in Tomatoes and Peppers

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Abstract

This study examines the relationship between the epigenetic mechanism of DNA methylation and phenotypic plasticity, a measure of adaptability, in tomatoes and sweet peppers. Groups of plants were treated with zebularine in order to cause experimental demethylation of their genomes and were grown in two different environments in order to determine how phenotypic variability between the two environments differed between normal plants and demethylated plants. Phenotypic plasticity was measured in terms of differences in size, color, development, and mortality. High rates of mortality and stunted growth in plants treated with zebularine during germination suggests that the treatment concentration used was too high for plants to be able to reach maturity, making some measurements of phenotypic plasticity difficult or impossible to compare between control and experimental groups. However, statistically significant differences in phenotypic plasticity were observed in the sizes and colors of tomato plants. Differences in phenotypic plasticity were not observed in sweet pepper plants by any of the measures used. It was also determined that treating plants with zebularine after the plants have already reached full-size does not affect phenotypic plasticity.

Introduction

DNA methylation is one of the mechanisms of epigenetics, which can be defined as inheritance or gene expression based on mechanisms other than DNA sequence (Bossdorf et al., 2010). The mechanism of DNA methylation describes the presence of a methyl group at the 5' position of a cytosine base (Xiao et al., 2020). The addition of a methyl group changes the physical structure of the DNA, affecting its ability to coil and produce proteins by causing DNA compaction (Bossdorf et al., 2010). This results in transcriptional silencing, which is useful for the regulation of gene expression, especially in the context of developmental changes (Bossdorf et al., 2010; Xiao et al., 2020). This process is largely catalyzed by a type of gene called DNA methyltransferase genes (Xiao et al., 2020). Just as methyl groups can be added to cytosine bases, they can also be lost. Loss of methylation can happen passively during DNA replication or actively by demethylase genes, which remove a methylated cytosine and replace it with an unmethylated cytosine (Xiao et al., 2020).

The presence of both methyltransferase genes and demethylase genes in plants aids in their ability to regulate gene expression. Addition of methyl groups can slow or stop the production of encoded proteins, while removal of the methyl groups allows the proteins to be produced again, and both of these can occur over the course of a plant's life to control different stages of development (Xiao et al., 2020). This phenomenon has been widely observed in the process of fruit ripening, and it is especially important for plants considering their lack of mobility, which causes them to be sensitive to environmental change (Xiao et al., 2020; Verhoeven et al., 2016). Studies examining the role of DNA methylation often experimentally demethylate plant genomes by inhibiting methyltransferase genes so that methyl groups cannot be added to cytosine bases (Baubec et al., 2008; Bossdorf et al., 2010; Xiao et al., 2020). Popular methyltransferase inhibitors include 5-azacytidine and zebularine, both of which are cytidine analogues (Baubec et al., 2008; Bossdorf et al., 2010).

Phenotypic plasticity is the extent to which a given genotype can produce different phenotypes in response to the environment (Herrera & Bazaga, 2012). High phenotypic plasticity makes an organism more resilient, as it is more prepared to adapt to change, making this evolutionarily advantageous. The mechanisms behind phenotypic plasticity still require more research, but some studies show a

correlation between epigenetic differences and phenotypic differences (Bossdorf et al., 2010; Herrera & Bazaga, 2012).

This experiment studies the relationship between DNA methylation and phenotypic plasticity in two agriculturally important plant species: tomato and sweet pepper. Specifically, it asks if experimental demethylation of the genome results in decreased phenotypic plasticity in the plants. This would look like less significant differences in size, color, development, and mortality between different environments for the treated plants. A relationship between epigenetic differences and phenotypic plasticity would suggest not only that epigenetic factors influence phenotype but also that regulation of gene expression by epigenetic factors is influenced by the environment. Studying this relationship can help us better understand plant evolutionary development and how plants are affected by and adapt to environmental changes.

Materials and Methods

Plant Material and Treatment:

Plant material used in this experiment was commercially available cherry tomato (*Solanum lycopersicum* var. *cerasiforme*) and sweet pepper (*Capsicum annuum*) seeds. Seeds were germinated in plastic six-pack pots using commercially available potting mix. The treatment used was a methyltransferase gene inhibitor called zebularine. Environmental groups consisted of warmer conditions (greenhouse/indoors) and cooler conditions (indoors/outdoors). The environmental conditions are described further under the experimentation section. By the end of the experiment, there were four control groups and six experimental groups, each with a unique combination of plant species, environment, and treatment. These groups will be referred to by a combination of letters: 'T' refers to tomatoes and 'P' refers to peppers; 'G' refers to plants that started in the greenhouse and moved indoors and 'I' refers to plants that started indoors and moved outdoors; 'C' refers to control/untreated plants, 'Z' refers to peppers treated with zebularine during germination, 'Z1' refers to tomatoes treated with zebularine during germination, and 'Z2' refers to tomatoes treated with zebularine once full-grown. Only tomato plants had groups treated at different times, as only tomato plants reached full size. Also note that for analyses in which all of the data is from before tomato plants reached full size, there are only 'C' and 'Z' groups. The abbreviated labels for each group are listed below in **Table 1** and **Table 2**.

Tomatoes	Control	Treated at Germination	Treated at Full Size
Warmer Environment	TGC	TGZ1	TGZ2
Cooler Environment	TIC	TIZ1	TIZ2

Table 1: Abbreviated Labels for Control and Experimental Groups of Tomatoes

Peppers	Control	Treated
Warmer Environment	PGC	PGZ
Cooler Environment	PIC	PIZ

Table 2: Abbreviated Labels for Control and Experimental Groups of Peppers

Experimentation:

The first step of the experiment was to create the treatment solution by dissolving 10 mg of zebularine (228 g/mol) in water to yield 11 mL of 400 µM zebularine solution, as modeled after a study that used this concentration of solution to germinate demethylated wheat (Finnegan et al., 2018). This solution was used to moisten filter paper in two petri dishes, and water was used to moisten filter paper in two more petri dishes. 20 or more tomato seeds were placed in one petri dish with treatment solution and 20 or more were placed in one petri dish with only water. The same was done with pepper seeds. The petri dishes were placed in a cool, dark environment where the seeds were left to germinate for one week.

After germination, 12 successfully germinated seeds were planted in soil. Half of the planted seeds of each species were left to grow in the greenhouse, where temperature ranged from 68 to 114 degrees Fahrenheit (20 to 46 degrees Celsius), and the other half were left to grow indoors, where temperature ranged from 60 to 64 degrees Fahrenheit (16 to 18 degrees Celsius). The plants were watered and transplanted to larger containers as needed.

As they grew, the plants were checked daily and data regarding developmental progress was collected, including time to sprouting in days, time to flowering in days, and time to the first instance of fruit maturity in days. The height, measured in centimeters from the base of the stem to the apical meristem, and the diameter, measured in centimeters across the longest distance from one side to the other, were measured weekly. Photos of each plant were taken every other week at 11 a.m. in the same spot in the greenhouse for color analysis. When the untreated tomato plants reached full size, half from each location were watered with the zebularine solution to determine effects of later treatment compared to treatment during germination.

Twelve weeks into the experiment, the plants needed to be relocated from San Diego, CA to Phoenix, AZ. The plants all survived the trip with few problems. The plants previously in the greenhouse were then grown indoors and the plants previously indoors were then grown outdoors so as to maintain a warmer environment for the former and a cooler environment for the latter. The plants continued to be cared for in these environments until all fully grown tomato plants reached fruit maturity. Data continued to be collected in the new environments with the exception of photos for color analysis, as changes in lighting could skew this data. The new environments had more variable temperatures as well as differences in lighting, but they were kept as similar as possible to the original environments, as the experiment is designed to measure phenotypic plasticity by comparing plants in the warmer environment and the cooler environment rather than comparing plants before and after a change in environment.

When the fully grown tomato plants reached fruit maturity, all the plants were cut at the base and left to dry for five days. This included sweet pepper plants and treated tomato plants, as they had ceased to show any significant growth or new developmental changes. The total number of flowers produced, including ones that had been pollinated and produced fruit, as well as the total number of fruit produced were recorded for each plant. After the drying period, the final biomass was recorded for each plant.

Data Analysis:

Data cleaning took place in Excel. There was no redundant data, but there were some fields without entries where the category was not applicable to the individual. For example, a plant that did not produce fruit would not have an entry for number of days until first production of fruit. These fields were left blank and the individuals were not included in calculations for those categories. After the death of a plant, heights and diameters were entered as 0 and were still included in calculations.

Analysis was conducted using Spyder, Excel with Visual Basic for Applications (VBA), and RStudio. First Spyder was used as the environment for writing a Python program. The program was written for the purpose of averaging RGB arrays in images. When run, it asks for an image to open, calculates the RGB array of each pixel in the image, averages the RGB arrays of all the pixels, and returns the average. These averages were used for colors analysis of the plants and are located in **Table 4** and **Table 5**. Images taken of the plants were organized by group and cropped to show only leaves with no background pixels. There were 6 replicates for each group – two replicates from each of three dates, each date being two weeks apart. The cropped images were run through the Python program and the results were recorded in Excel. The code for this program can be found in the appendix.

All statistical tests were done in Excel. For the color analysis, three ANOVA tests were run: a two-factor ANOVA with replication for red content, a two-factor ANOVA with replication for green content, and a two-factor ANOVA with replication for blue content. Heights and diameters of plants were also analyzed using two-factor ANOVA tests with replication. There were also two-factor t-tests for heights and diameters of each treatment group between the two environments as well as a two-factor t-test between the differences in environment for peppers and a one-factor ANOVA for the differences in environment for tomatoes. Final biomass for tomatoes was analyzed using a two-factor ANOVA with replication. The other characteristics (time to sprouting, time to flowering, and time to fruit maturity) were analyzed using two-factor ANOVA tests without replication. These tests were done on the means of each group due to inconsistent numbers of data points that prevented the use of ANOVA tests with replication.

All of the tables were also created in Excel, and graphs were created using both VBA and RStudio. VBA is a programming language used to automate Microsoft Office applications. Line graphs of height and diameter were created automatically using VBA code that caused graphs to be generated at the click of a button. VBA code for one of the graphs can be found in the appendix. Code for the other graphs is almost exactly the same. Boxplots were created in RStudio. Data on time to sprouting and data on final biomass were each put in separate Excel workbooks, arranged into stacked format, and saved as CSV files. These CSV files were then loaded into RStudio for the creation of the boxplots.

Results

Plants demethylated at germination had significantly higher mortality than control plants and plants treated with zebularine after reaching full size. Only 11% of tomato plants treated at germination survived the full length of experimentation compared to 94% for control and late-treated tomato plants. For sweet pepper plants, 50% of demethylated plants survived the full length of experimentation, compared to 100% for control plants. For tomatoes, 50% of early-treated plants that survived the full length were in each environment. For peppers, 80% of treated plants that survived the full length were

in the warmer environment, compared to only 20% in the cooler environment. Only control and late-treated tomatoes produced flowers and fruit.

Categories with the greatest statistical significance included height, diameter, and color. These also happen to be the most important categories for analysis because they apply to every group of plants. Final biomass showed significant differences between the warmer and cooler environments but not between control tomatoes and late-treated tomatoes. Tomatoes treated at germination were not included in the analysis of final biomass because there were not enough data points for a reliable conclusion to be drawn due to the low levels of survival. Statistical tests for biomass also were not performed on pepper data due to low sample sizes. Time to sprouting, time to flowering, and time to fruit maturity did not yield statistically significant results, but there were still some differences, which are shown in the figures and tables below.

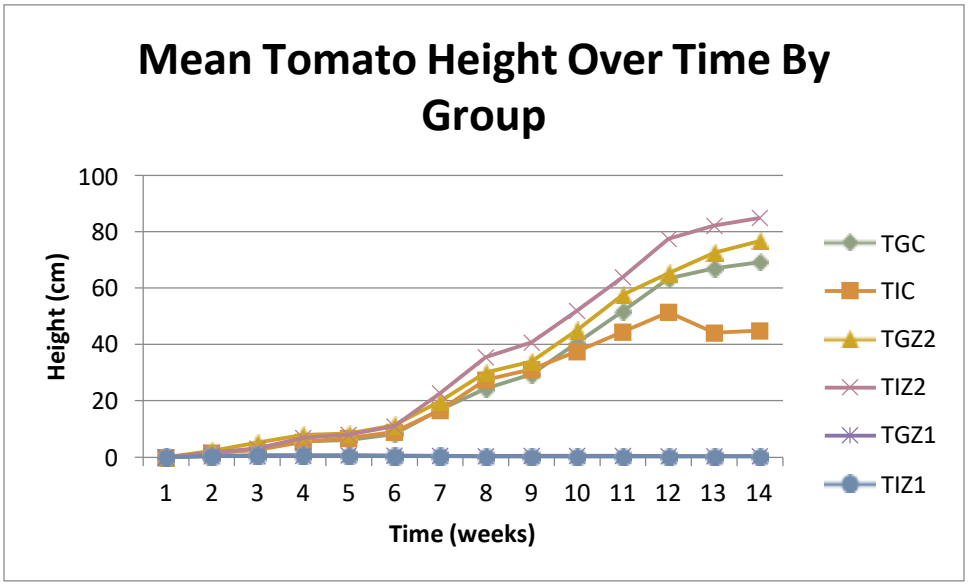

Figure 1: Greater Height in Control and Late-Treated Tomato Plants Than Tomatoes Treated During Germination. There is a statistically significant difference in tomato height based on treatment group (p-value = 6.0605E-07), but not based on environment (p-value = 0.955887). While the height differences were not significantly different between environments, the mean differences were calculated by subtracting heights from one environment from heights in the other environment, and there was a significant difference between the differences for each treatment group (p-value = 0.0287808). Control plants had the greatest height difference based on environment and plants treated at germination had the least height difference based on environment. This suggests a difference in phenotypic plasticity based on methylation levels.

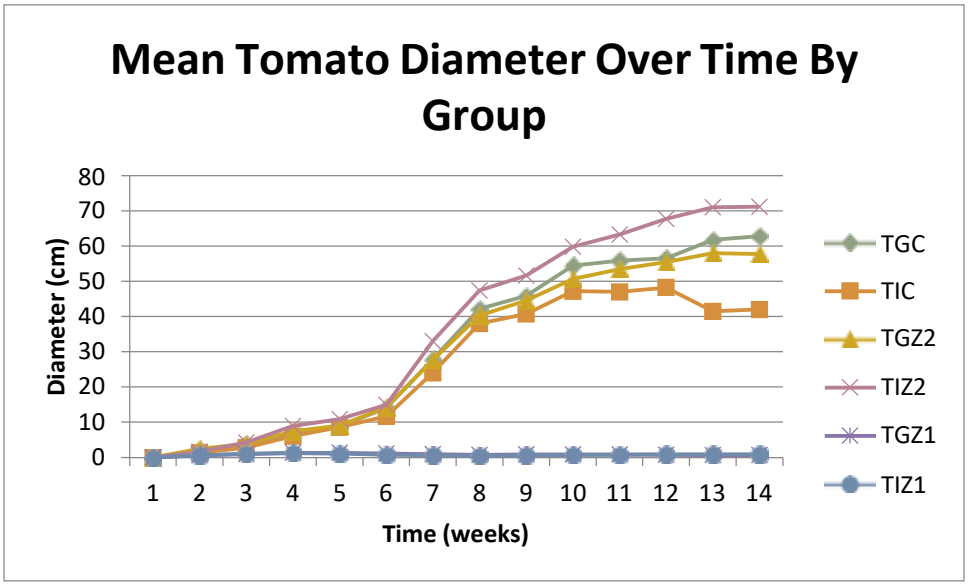

Figure 2: Greater Diameter in Control and Late-Treated Tomato Plants Than Tomatoes Treated During Germination. There is a statistically significant difference in tomato diameter based on treatment group (p-value = 2.52512E-08), but not based on environment (p-value = 0.980074). While the diameter differences were not significantly different between environments, these differences were calculated, and there was a significant difference between the differences for each treatment group (p-value = 0.00384628), with control plants having the greatest diameter difference based on environment and plants treated at germination having the least diameter difference based on environment. This suggests a difference in phenotypic plasticity based on methylation levels.

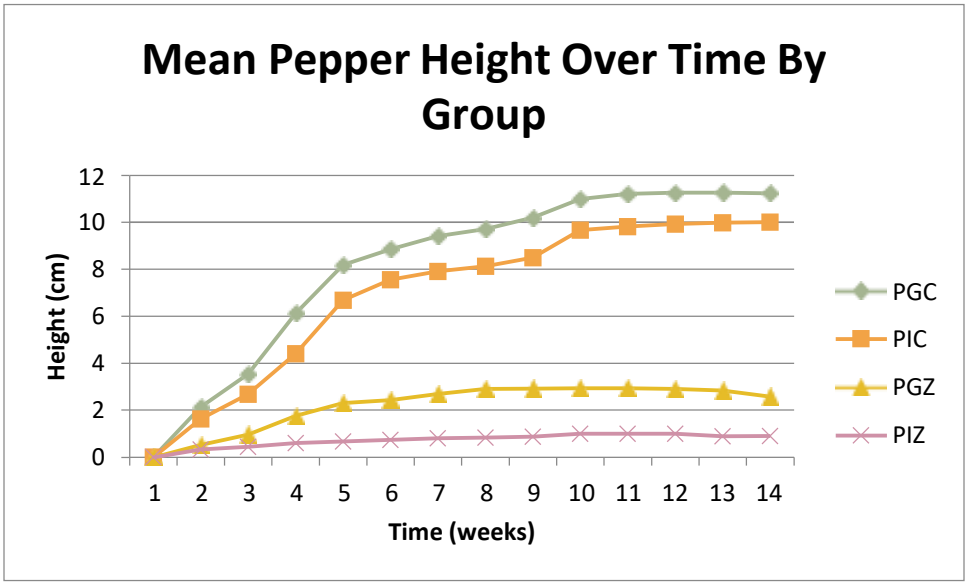

Figure 3: Greater Height in Control Peppers Than Treated Peppers. There is a statistically significant difference in pepper height based on treatment group (p-value = 6.88802E-12). T-tests between the heights in different environments did not result in a statistically significant difference for control plants

(p-value = 0.371637) but did show statistical significance for demethylated plants (p-value = 1.49408E-05). This would suggest higher phenotypic plasticity in the demethylated plants. However, the height differences between the environments were calculated, and there was not a significant difference between the height differences for the two environments (p-value = 0.301421), suggesting that there actually is not a significant difference in phenotypic plasticity.

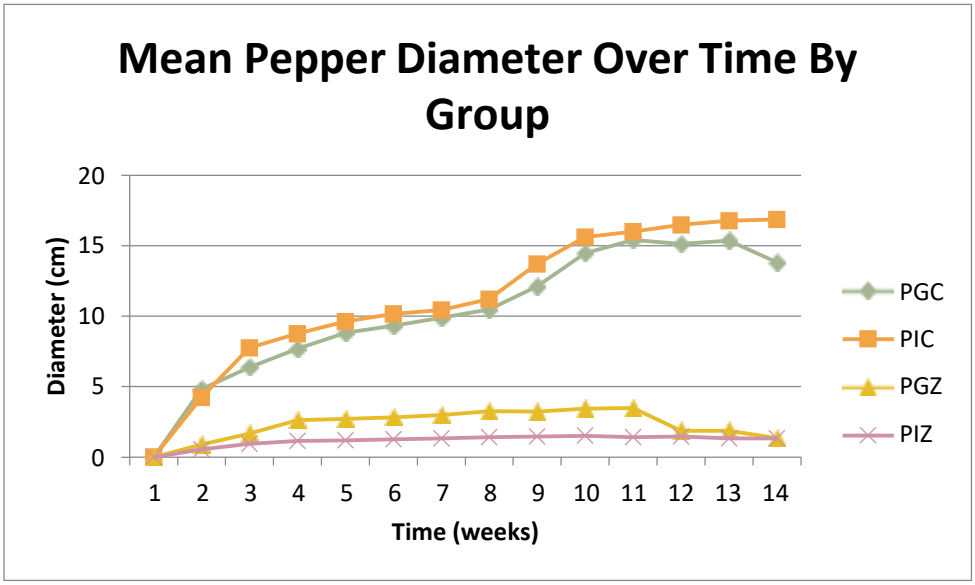

Figure 4: Greater Diameter in Control Peppers Than Treated Peppers. There is a statistically significant difference in pepper diameter based on treatment group (p-value = 2.35402E-13). T-tests between the diameters in different environments did not result in a statistically significant difference for control plants (p-value = 0.591413) but did show statistical significance for demethylated plants (p-value = 0.000930876). This would suggest higher phenotypic plasticity in the demethylated plants. However, the diameter differences between the environments were calculated, and there was not a significant difference between the diameter differences for the two environments (p-value = 0.836436), suggesting that there actually is not a significant difference in phenotypic plasticity.

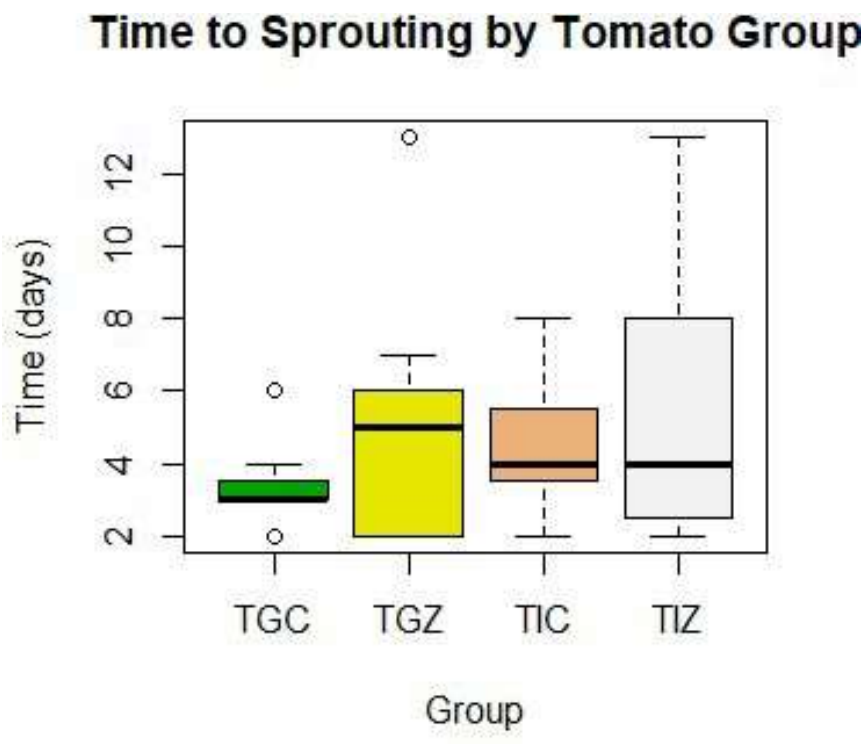

Figure 5: Time to Sprouting for Tomatoes. A two-factor ANOVA without replication did not show a statistically significant difference in sprouting time based on treatment (p-value = 0.165541) or environment (p-value = 0.282521). Despite the differences not being statistically significant, the boxplot shows demethylated plants taking slightly longer to sprout and shows more variation in time for demethylated plants.

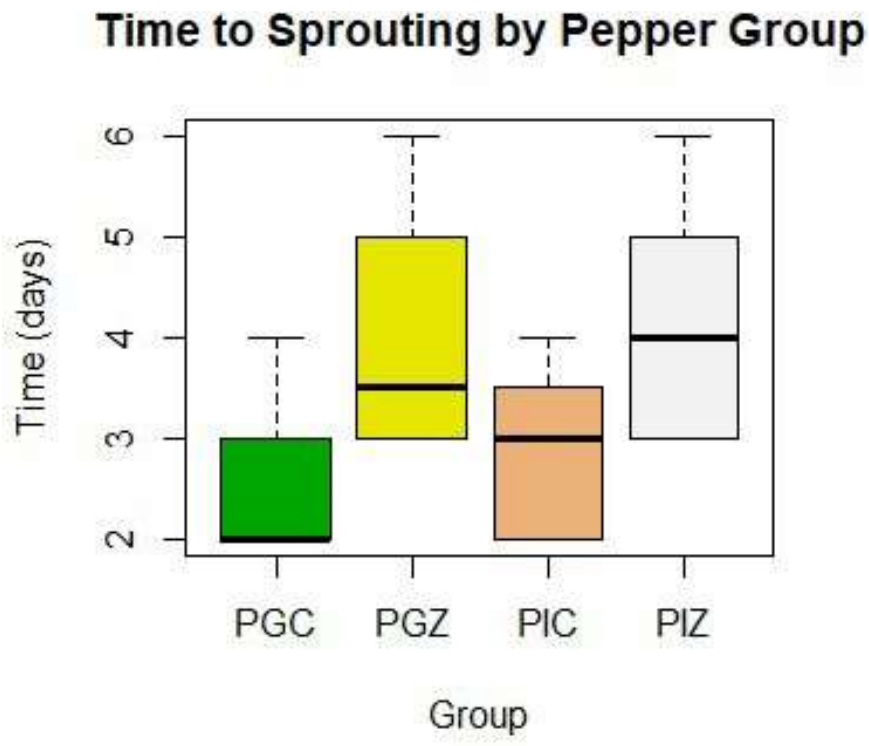

Figure 6: Time to Sprouting for Peppers. A two-factor ANOVA without replication did not show a statistically significant difference in sprouting time based on treatment (p-value = 0.0588117) or environment (p-value = 0.239913), although the difference based on treatment was close to being significant. Despite the differences not being statistically significant, the boxplot shows demethylated plants clearly taking longer to sprout and shows slightly more variation in time for demethylated plants.

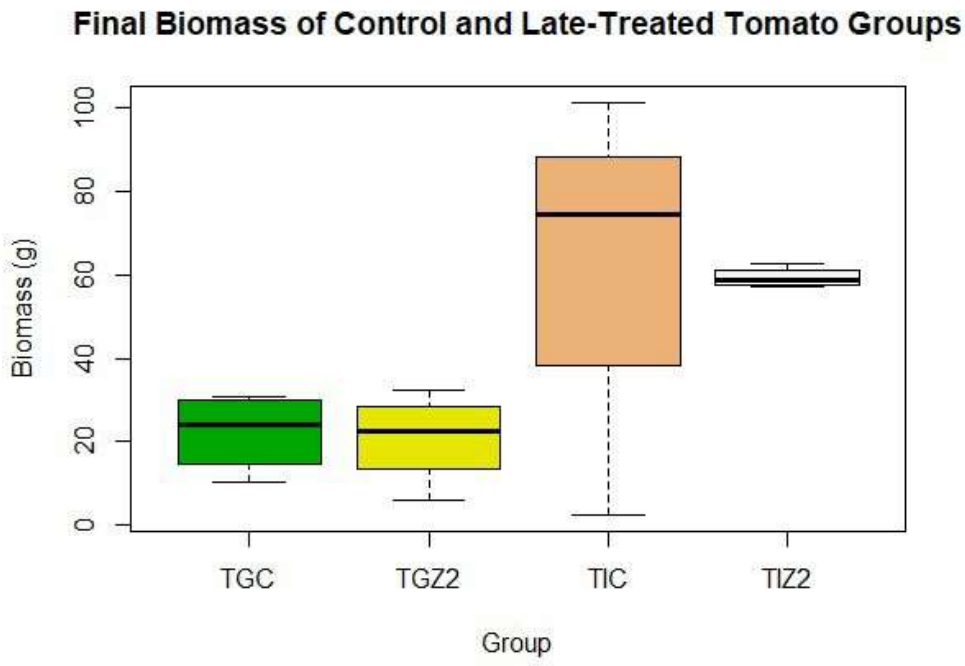

Figure 7: Final Biomass of Control and Late-Treated Tomatoes, With Greater Biomass in Cooler Environment. A two-factor ANOVA with replication showed a statistically significant difference in final biomass based on environment (p-value = 0.00403467) but not based on treatment (p-value = 0.818320). This shows phenotypic plasticity being present in both control tomatoes and late-treated tomatoes. This suggests that phenotypic plasticity was not affected by treatment once plants were full-sized.

	Group	Mean Time (days)
Flowering:	TGC	60.50
	TIC	64.25
	TGZ2	59.75
	TIZ2	60.75
Fruit Maturity:	TGC	121.0
	TIC	148.3
	TGZ2	115.5
	TIZ2	154.3

Table 3: Time to Reach Developmental Stages for Control and Late-Treated Tomatoes. Two-factor ANOVA tests without replication showed no statistically significant difference in time to first instance of flowering based on treatment group (p-value = 0.365614) or environment (p-value = 0.334095) as well as no statistically significant difference in time to first instance of fruit maturity based on treatment group (p-value = 0.976597) or environment (p-value = 0.108945). Despite there not being statistical significance, the values in the table show plants taking slightly longer to flower and reach fruit maturity

in a colder environment. This is seen in both control and late-treated tomatoes, suggesting no difference in phenotypic plasticity when plants are treated after reaching full-size.

TGC			TGZ			TIC			TIZ		
R	G	B	R	G	B	R	G	B	R	G	B
59.28616	88.82967	34.98385	100.323	120.1757	65.07108	75.64266	107.967	41.09242	110.4879	128.0091	117.5148
116.0922	135.6327	52.37614	150.3727	180.4599	102.7605	101.6028	131.7562	50.49082	93.6102	88.42133	71.22844
59.85354	88.42033	39.48783	153.965	187.5964	156.5664	53.8855	83.50495	27.00009	115.2089	130.0494	66.64254
58.83807	86.38892	34.33455	159.6221	200.2384	119.7981	64.48344	96.45718	12.04312	103.7382	118.6069	28.1926
66.81592	90.7201	33.09211	174.834	194.4231	108.6325	58.58134	87.33688	39.61585	79.48009	95.72075	34.00892
54.73202	81.0975	31.60419	118.564	130.008	85.02527	86.07236	115.3867	58.34891	60.03607	73.83188	21.53658
69.26965	**95.18153**	**37.64644**	**142.9468**	**168.8159**	**106.309**	**73.37802**	**103.7348**	**38.09853**	**93.76022**	**105.7732**	**56.52065**

Table 4: Average RGB Arrays for Tomato Groups Show Darker Greens in Control Plants. Each number in an RGB array can be anywhere from 0 to 255, with 0 meaning the color is not present at all and 255 meaning the color is fully present. All 0s result in black and all 255s result in white. A healthy plant would be expected to have a dark green color, represented by low numbers overall but a high proportion of green. The mean RGB array for all replicates of each group is shown in the bottom row. The colors shown at the top are from these mean RGB arrays, showing the average color of plants in each group. Two-factor ANOVA tests with replication were performed to compare red content, green content, and blue content between groups. For red, there were statistically significant differences based on treatment (p-value = 6.14037E-05) and environment (p-value = 0.0251764). There was also interaction evident between treatment and environment (p-value = 0.00966092). For green, there were statistically significant differences based on treatment (p-value = 0.00132819) and environment (p-value = 0.0142591). There was also interaction evident between treatment and environment (p-value = 0.00211832). For blue, there were statistically significant differences based on treatment (p-value = 0.000479896) and environment (p-value = 0.0286195). There was also interaction evident between treatment and environment (p-value = 0.0261370).

PGC			PGZ			PIC			PIZ		
R	G	B	R	G	B	R	G	B	R	G	B
78.633 07	109.83 83	27.383 28	122.63 57	157.20 4	67.670 02	76.959 06	107.00 03	29.825 41	93.726 18	119.96 48	27.748 86
85.393 73	113.10 21	56.259 39	100.82 76	130.06 07	73.231 02	75.239 41	99.525 41	28.512 05	105.99 47	129.51 15	37.573 07
82.439 34	113.62 58	48.869 59	119.15 36	145.62 53	101.13 85	90.393 28	115.96 06	54.737 36	100.51 08	120.08 9	33.600 49
90.037 45	114.62 3	45.100 98	142.43 15	179.53 81	71.067 26	100.64 14	119.04 48	41.860 24	107.52 66	141.64 57	53.290 84
119.17 22	149.33 12	55.462 62	136.80 82	174.56 07	73.847 25	98.067 24	125.57 75	25.928 88	148.00 9	193.11 15	85.751 68
145.93 58	171.22 36	113.17 39	108.29 14	125.90 96	54.166 1	96.414 43	132.17 21	54.721 26	116.22 8	151.25 36	27.022 51
100.26 86	**128.62 4**	**57.708 29**	**121.69 13**	**152.14 97**	**73.520 02**	**89.619 13**	**116.54 68**	**39.264 2**	**111.99 92**	**142.59 6**	**44.164 57**

Table 5: Average RGB Arrays for Pepper Groups Show Darker Greens in Control Plants. Each number in an RGB array can be anywhere from 0 to 255, with 0 meaning the color is not present at all and 255 meaning the color is fully present. All 0s result in black and all 255s result in white. A healthy plant would be expected to have a dark green color, represented by low numbers overall but a high proportion of green. The mean RGB array for all replicates of each group is shown in the bottom row. The colors shown at the top are from these mean RGB arrays, showing the average color of plants in each group. Two-factor ANOVA tests with replication were performed to compare red content, green content, and blue content between groups. For red, there was a statistically significant difference based on treatment (p-value = 0.0107648) but not based on environment (p-value = 0.206404). For green, there was a statistically significant difference based on treatment (p-value = 0.0144783) but not based on environment (p-value = 0.256469). For blue, there was not a statistically significant difference based on treatment (p-value = 0.241072) but there was a significant difference based on environment (p-value = 0.0113484).

Discussion

There is growing support for the importance of methylation in plants, especially due to its role in regulating plant development (Bossdorf et al., 2010; Herrera & Bazaga, 2012; Xiao et al., 2020). Demethylation of the DNA of tomatoes and peppers has been studied for the investigation of fruit ripening regulation, and demethylation of Arabidopsis DNA has been studied for the investigation of phenotypic plasticity (Bossdorf et al., 2010; Xiao et al., 2020). This study combines these two concepts by investigating how experimental demethylation affects phenotypic plasticity in tomatoes and peppers, with the hypothesis that plants treated with zebularine would exhibit less phenotypic plasticity due to less control over what genes are transcribed at what times. This study also differs from other studies by treating groups of plants both during germination and after plants have reached full-size to see whether demethylation affects them differently at different stages of development. This research is important for gaining a greater understanding of the role of genomic methylation in plant development. Looking at phenotypic plasticity based on environmental temperature in particular is important due to our changing climate. Understanding how methylation affects phenotypic plasticity can help us to better

understand, among other things, how climate change could impact plant life and how plants can respond to this.

The results of this study suggest a decrease in phenotypic plasticity by demethylation during germination in some characteristics but not others, and only for tomato plants. Most notably, there were significantly greater differences in tomato height (p-value = 0.0287808) and diameter (p-value = 0.00384628) between the warmer and cooler environments for control and late-treated tomato plants than for tomato plants treated at germination. The control tomatoes tended to have smaller height and diameter in the cooler environments than in the warmer environments. Not seeing this difference in the tomatoes demethylated during germination suggests that these tomatoes might not have had as much control over their growth in response to the environment. These trends are seen in **Figure 1** and **Figure 2**. There were also statistically significant differences in color based on both environment and treatment. Red, green, and blue contents were significantly different based on the interaction between environment and treatment (R p-value = 0.00966092; G p-value = 0.00211832; B p-value = 0.0261370), supporting the idea that demethylation affects phenotypic plasticity in tomatoes. The control plants had darker green colors than the demethylated plants, and the control plants in the warmer environment had a darker green color than the control plants in the cooler environment, as control plants in the warmer environment had the lowest numbers for every color. However, for demethylated plants, the plants in the cooler environment were darker than the ones in the warmer environment, as demethylated plants in the warmer environment had the highest numbers for every color. This suggests that DNA methylation regulates leaf pigmentation in tomato plants based on environment, with demethylation having an opposite effect. This phenomenon could be used to regulate photosynthesis or might simply be an indicator of plant health. These values and the colors they represent are shown in **Table 4** and **Table 5**. The pepper plants did not have any significant differences in phenotypic plasticity in relation to treatment. It can also be noted that the differences in the various measures of phenotypic plasticity between the two environments could have been due, at least in part, to other environmental factors in addition to temperature. These include differences between more direct sunlight and more indirect sunlight and artificial light, as differences in lighting were difficult to isolate from differences in temperature. The most important thing was to keep the two environments distinct from one another so that the plants would be more likely to respond differently to each of them.

There was not any significant difference in phenotypic plasticity when plants were treated with zebularine after reaching full-size. Some characteristics did not show phenotypic plasticity in either control or treated plants, so differences could not be measured for these characteristics. However, there were characteristics that showed phenotypic plasticity in both control and late-treated plants. Final biomass was significantly different between the warmer and cooler environments (p-value = 0.00403467) but not between control and late-treated plants (p-value = 0.818320), as seen in **Figure 7**. Time to flowering and time to fruit maturity were both greater in cooler environments for both control and late-treated plants. The differences were not statistically significant (p-value = 0.3340954; p-value = 0.108945), but it is also worth noting that it is more difficult to get significance with an ANOVA test without replication as it cannot account for sample size. These results are shown in **Table 3**. While zebularine is a known inhibitor of methyltransferase genes, all other plant studies included treatment during germination only. This begs the question of whether the plants were not affected by demethylation at the fully-grown stage or whether the zebularine was not effective in demethylating

the plants in the first place in the fully-grown stage. This would be an interesting point of further study by using bisulfite sequencing to measure methylation levels across the genome.

The most notable limitation of this study is that the high death rates and stunted growth for plants demethylated at germination prevented several characteristics being studied between the control groups and the early treated groups. The plants were not able to grow to full-size and produce flowers and fruit. The high death rates and stunted growth suggest that the concentration of zebularine used was too high for the plants to grow to maturity. While the concentration used was similar to that of another study, most studies use lower concentrations, ranging from 40 µM to 100 µM (Baubec et al., 2009; Finnegan et al., 2018). Repetition of the study with lower concentrations of zebularine could yield more significant results. Including bisulfite sequencing or another kind of technology to measure methylation levels would be another way to improve the experiment in future trials by providing more information that could be used to draw more precise conclusions.

References

Baubec, T., Pecinka, A., Rozhon, W., Scheid, O.M. (2009). Effective, homogeneous and transient interface with cytosine methylation in plant genomic DNA by zebularine. *The Plant Journal, 57*, 542-554. http://doi.org/10.1111/j.1365-313X.2008.03699.x

Bossdorf, O., Arcuri, D., Richards, C.L., Pigliucci, M. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in *Arabidopsis thaliana*. *Evolutionary Ecology, 24*, 541–553. https://doi.org/10.1007/s10682-010-9372-7

Finnegan, E.J., Ford, B., Wallace, X., Pettolino, F., Griffin, P., Schmitz, R., Zhang, P., Barrero, J.M., Hayden, M.J., Boden, S.A., Cavanagh, C.A., Swain, S.M., Trevaskis, B. (2018). Zebularine treatment is associated with deletion of FT-B1 leading to an increase in spikelet number in bread wheat. *Plant Cell Environment, 41*, 1346– 1360. https://doi.org/10.1111/pce.13164

Herrera, C. M. & Bazaga, P. (2012). Epigenetic correlates of plant phenotypic plasticity: DNA methylation differs between prickly and nonprickly leaves in heterophyllous *Ilex aquifolium* (Aquifoliaceae) trees. *Botanical Journal of the Linnean Society, 171*(3), 441–452. https://doi.org/10.1111/boj.12007

Verhoeven, K.J.F., vonHoldt, B.M. & Sork, V.L. (2016), Epigenetics in ecology and evolution: what we know and what we need to know. *Molecular Ecology, 25*, 1631-1638. https://doi.org/10.1111/mec.13617

Xiao, K., Chen, J., He, Q., Wang, Y., Shen, H., & Sun, L. (2020). DNA methylation is involved in the regulation of pepper fruit ripening and interacts with phytohormones. *Journal of Experimental Botany, 71*(6), 1928–1942. https://doi.org/10.1093/jxb/eraa003

Appendix

Python Code for Color Analysis:

```
from PIL import Image
'''loop through each pixel and average rgb'''
```

```
def averagePixels(pic_image):
    r, g, b = 0, 0, 0
    count = 0
    imgData = pic_image.load()
    for x in range(pic_image.size[0]):
        for y in range(pic_image.size[1]):
            clrs = imgData[x,y]
            r += clrs[0]
            g += clrs[1]
            b += clrs[2]
            count += 1
    #calculate averages
    return (r/count), (g/count), (b/count), count
#ask for picture file
openfile = input ("please enter the name of the file: ")
pic_image = Image.open(openfile)
pix = averagePixels(pic_image)
print ("Below is the average red content, average green content, average blue content, and total
number of pixels in the image")
print (pix)
```

Visual Basic for Applications Code for Line Graphs:

```
Private Sub CommandButton1_Click()
ActiveSheet.Shapes.AddChart.Select
ActiveSheet.Shapes(1).Top = 10
ActiveSheet.Shapes(1).Left = 10
ActiveChart.ChartType = xlLineMarkers
ActiveChart.PlotArea.Select
ActiveChart.SetSourceData Source:=Range("='Tomato Heights'!$G$1:$G$15,'Tomato
Heights'!$L$1:$L$15,'Tomato Heights'!$R$1:$R$15,'Tomato Heights'!$W$1:$W$15,'Tomato
Heights'!$AJ$1:$AJ$15,'Tomato Heights'!$AV$1:$AV$15")
ActiveChart.HasTitle = True
ActiveChart.ChartTitle.Text = "Mean Height Over Time By Group"
ActiveChart.Axes(xlCategory, xlPrimary).HasTitle = True
ActiveChart.Axes(xlValue, xlPrimary).HasTitle = True
ActiveChart.Axes(xlCategory, xlPrimary).AxisTitle.Characters.Text = ("Time (weeks)")
ActiveChart.Axes(xlValue, xlPrimary).AxisTitle.Characters.Text = ("Height (cm)")
End Sub
```