jbk284

Internal Assessment

Biology HL

What is the effect of different caffeine concentrations (0.025%, 0.05%, 0.1%, 0.2%, 0.4%) on the dry mass (in g) of *Glycine max* in a 15-day experiment?

INTRODUCTION

Glycine max, commonly known as soybean, originates from the East Asia region but has spread to the entire world as one of the main species of modern agriculture. In the 2016/2017 production season, Brazil produced around 114 million tons of *G. max*. It also represents a significant market for Brazil, representing 1.74% of the country's GDP in 2019 (Embrapa, n.d.). Investing in new technologies to be used on crops has been a key factor in Brazil's rise as the second-biggest soybean producer in the world (Apex-Brasil 2020).

Moreover, caffeine is an alkaloid that causes the also famous sense of alertness in humans. It is considered the most used psychoactive substance in the world, with 80% of the Western world adult population making use of it frequently (Daly, Holmén, and Fredholm 1998). In Brazil, the per capita consumption of coffee reaches 5.8 kg/year (ReviewCafe 2020). The consumption of caffeinated beverages in my family is daily and cultural. The effects of caffeine interested me and when researching for this experiment I started questioning whether caffeine had the same stimulant effect in plants as it has in humans.

Several studies were already conducted on the field of human physiology and the effects of caffeine in humans is mostly understood. On the other hand, the effects of caffeine on plant growth is a topic that has not gotten much attention overall. Even so, a study developed analyzing the effect of caffeine on plant growth came to the conclusion that caffeine in smaller concentrations helped positively plant growth on *Helianthus annuus L.* after 30 days (Khursheed, Shahab, and Ansari 2009, 58). On the other hand, higher concentrations had a decrease in their height (Khursheed, Shahab, and Ansari 2009, 58). A more researched area is the weed control technology. A study in this field came to the conclusion that high caffeine concentrations could be used to kill weeds (Shettel and Balke 1983, 297).

The study aims to study the effect of caffeine concentrations on the soybean dry mass. A benefit from a positive outcome in this experiment is that an increase in productivity would be achieved, while using one of the most common molecules in people's daily diet. Increasing productivity also means being able to meet the market demand without an aggressive expansion of the agricultural frontier.

Research Question

What is the effect of different caffeine concentrations (0.025%, 0.05%, 0.1%, 0.2%, 0.4%) on the dry mass (in g) of *Glycine max* in a 15-day experiment?

VARIABLES AND MATERIALS

Independent Variable

Caffeine ($C_8H_{10}N_4O_2$) on six treatments:

- Treatment 0.00% (Control)
- Treatment 0.025%
- Treatment 0.05%
- Treatment 0.1%
- Treatment 0.2%
- Treatment 0.4%

Dependent Variable

Dry mass (g) of *G. max* after 15 days from germination.

Controlled Variables

- Light intensity and source: 176 lux ± 0.1 lux from 24W LED Strip
- Photoperiod: 10 hours a day ± 0.5 min
- Frequency of watering: daily ± 0.5 min at 9 AM
- Quantity of water when watering: 4 ml ± 0.5 ml
- Soil type: black soil
- Quantity of soil: 100 g ± 0.001 g
- Water source for all purposes during the experiment: tap water
- Integrity of the seed coat previous to the soaking: a visual check of the presence of the seed coat without any damage and apparent colour of seed
- Duration of the experiment after seed germination: 15 days ± 12 hours from the day the sprout reaches soil surface level)
- 54 *G. max* seeds
- Distance from the soil surface the seed is planted: 1 cm ± 0.5 cm
- Temperature: 22℃ ± 8℃
- Distance of the plants from the light source: 15 cm ± 0.5 cm
- How and how long the caffeine solution was homogenized when prepared: glass rod for 5 min ± 0.5 min

Materials

1. 72 150 ml disposable plastic cups (Brand: Copobras)

2. One 90 x 43 x 20 cm styrofoam box
3. 20000 ml of tap water
4. Six 10 ml syringes with ml marks ± 0.5 ml (Brand: Descarpack)
5. One 24W 5 m LED strip (Brand: Gaya)
6. 1 kg of *G. max* seeds (Brand: Pop House)
7. Ruler with centimetres marks ± 0.5 cm (Brand: Waleu)
8. 7.2 kg Black soil (Brand: Matsubashi)
9. One digital thermometer ± 0.1℃ (Brand: Eos)
10. Six 1000 ml beakers (Brand: Global Glass)
11. One lab oven (Brand: De Leo)
12. One 10 m power cord extender
13. One precision scale ± 0.001 g (Brand: Marte)
14. 10 500 ml plastic bottles (Brand: GoodPack)
15. One 38 x 28 x 3 cm drying pan
16. One black permanent marker (Brand: Maxprint)
17. One pack of plastic seals (Brand: Hellermann Tyton)
18. One luxmeter (Brand: ICEL)
19. 0.015 g of caffeine (Brand: Química Anastacio)
20. Ten Petri dishes (Brand: Global Plast)
21. One 5 L bucket bought locally
22. One kitchen knife
23. One office stapler (Brand: Eagle)
24. Disposable gloves (Brand: Descarpack)

METHODS

This experiment aimed at answering the research question “What is the effect of different caffeine concentrations (0.00%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4%) on the dry mass of *G. max* in a 15-day experiment?”. To assure a better accuracy in the results of the experiment, the number of replicates was defined to be nine, for each of the six treatments. This number was considered ideal because even considering the possibility of some replicates not germinating, the author would still have sufficient data to apply the statistical procedures. To detail more the methodology developed for this experiment, the methods section was divided into “Preparation of the styrofoam box”, “preparation of the caffeine solutions”, “preparation of the soil”, “preparation of the seeds”, “during the experiment” and “after the experiment”.

In order to assess the ideal water volume provided for plants daily (with or without caffeine), a preliminary experiment composed of 3 replicates was done using all the methodological procedures proposed for the experiment and described below. The volumes tested were 2 ml, 3 ml and 4 ml given daily to the plants for 10 days after germination. The result achieved in the preliminary experiment was that two out of the three replicates from the 4 ml sample presented the biggest dry mass, thus 4 ml was considered the ideal water volume for the experiment.

Preparation of the styrofoam box

The LED strip was installed following five lines which were designed to coincide with the rows present in the plant nursery pot (Figure 1). To fixate the LED strip in the box's lid, a stapler was used. A hole in the space between the lid and the box itself was done carefully using a kitchen knife, to allow the passage of the LED strip power cable and the thermometer ± 0.1℃. The thermometer ± 0.1℃ was attached to the top of the lid using two plastic seals. Whenever connecting or disconnecting the LED strip from the power grid, electrical insulated shoes, dry hands and a safety check on the system were done, if identified any possible risk, the lab's power grid was turned off and the issue was solved. The LED strip power extension cord was then connected to the power, which when the photoperiod started was put into the power outlet. Before the beginning of the experiment, a luxmeter was used to measure the amount of light throughout the surface of the styrofoam box. The measurement assured that all the surface received the same light intensity, 176 lux ± 0.1 lux.

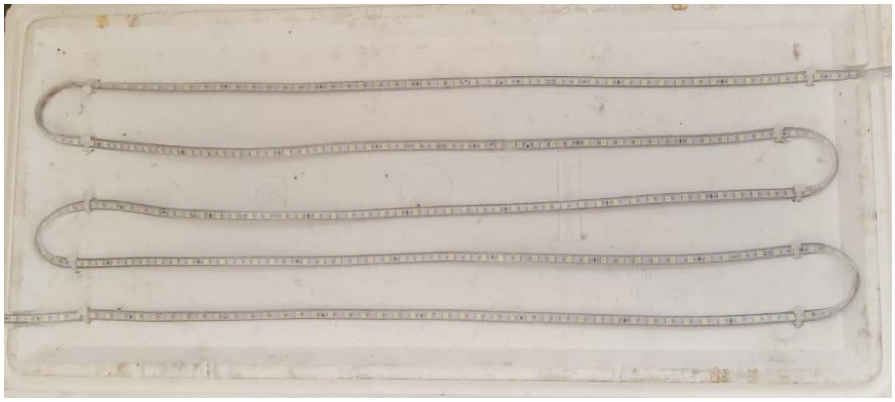

Figure 1 - Installed LED Strip on the lid of the styrofoam box

The cups were placed inside the styrofoam box 0.5 cm apart from each other and 15 cm from the light source.

Preparation of the caffeine solutions

Five treatments received caffeine in the experiment, they are 0.025%, 0.05%, 0.1%, 0.2%, 0.4%. To have the solutions with different concentrations, an initial stock solution was prepared by diluting 0,1g of caffeine into 1 L of tap water. Precaution measures were taken when manipulating the concentrated caffeine by using disposable gloves and appropriate lab clothing. The use of gloves is justified because there is a risk of absorbing caffeine through the skin (van de Sandt et al. 2004, p. 271). After the preparation of the solutions was finished the disposable gloves were disposed of in the appropriate garbage bin. From that stock solution, all the other concentrations were prepared through dilution using tap water. The calculations to obtain the other concentrations from the stock solution were performed by the use of the https://www.physiologyweb.com/calculator. The treatments were then bottled and stored in the refrigerator at a temperature of around 8℃ to be better conserved throughout the experiment.

Preparation of the soil

Using the Petri dishes and the precision scale ± 0.001 g, 100g of black soil (Brand: Matsubashi) was measured for each of the 54 replicates, corresponding to the 9 replicates of the 6 treatments. After the quantity of soil was weighted, it was placed in the 150 ml disposable plastic cups always having attention to keep the soil soft. After the 54 pots were filled with the soil, labels were applied to the lateral of the plant nursery pot, and, with the aid of a permanent black marker, the different treatments were labelled, diving into 0.00%, 0.025%, 0.05%, 0.1%, 0.2%, 0.4%. After the labelling of the different treatments, every individual pot was labelled using a sequential order from 1 to 9, considering replicates.

Preparation of the seeds

The integrity of the seed coat before the pre-planting soaking was assured through a visual check by observing the the presence of the seed coat without any damage and whether the seed presented its expected colour or not. If the seed didn’t present its expected colour, it was rejected. Then, using the drying tin, the 54 *G. max* seeds were placed on the bottom of the tin and sufficient tap water to cover all seeds was applied. A cell phone was used to set a 12-hour timer and the seeds were let submerged for this period. This was done to break the seed dormancy. After the time elapsed, the seeds were taken out of submersion and submitted to a process of seed scarification to accelerate and ensure a better germinator to seed planted ratio. The method used was to do two parallel cuts on the seed, as shown in the picture below (Figure 2).

Figure 2 - *G. max* seed showing the scarification process with two parallel cuts. The image was digitally modified to allow a better comprehension of the process.

With the aid of a ruler ± 0.5 cm, 1 cm was measured in every pot, considering the soil surface, and the seeds were planted at this soil depth. The image below (Figure 3) shows the experiment set.

Figure 3 - Styrofoam box on day 1 of the experiment

During the experiment

Six syringes ± 0.5 ml, one for each treatment, were used in the process of watering the plants every day at 9 AM with 4 ml of either water (control treatment) or of the caffeine solution according to the treatment.. Once all plants were watered the light source was turned on. A cell phone was used to set a 10-hour timer that represented the daily photoperiod. After 10 hours the light source was turned off. This procedure was repeated every day until the end of the experiment, which lasted for 18 days. Once the seed sprout reached the soil surface the information was put in a spreadsheet and the period of plant growth would start to be counted lasting for 15 days. The germination day of each replicate was recorded.

After the experiment

Once each replica reached the 15-day mark, the plant was carefully unearthed and then submerged in a 5 L bucket with tap water in it, to take all the earth out of the roots. Once this was done, the replica was placed on the drying pan, labelled using the same logic presented before of Treatment X, Replicate Number X, and put in the lab oven at a temperature of 60℃ overnight. On the morning of the next day, with the help of the precision scale ± 0.001 g, the mass of each replica was measured and recorded in the spreadsheet. This procedure was applied for each replica.

All of the disposable materials used in this experiment (72 150 ml disposable plastic cups; Six syringes with ml marks ± 0.5 ml; Plastic film; One pack of plastic seals) were cleaned with water and then sent to the local recycling center. The solutions containing caffeine were disposed of in the sink since their caffeine concentrations were very low and could be considered unharmful for the environment.

Statistical procedure

Once all data were collected, mean and standard deviation were calculated. The mean of each treatment (Figure 4) was compared against the mean of the control by using the t-test. The results were displayed in bar graphs with standard deviation (Figure 5) and error bars.

$$\bar{x}\,(mean) = \frac{sum\ of\ the\ terms}{numbers\ of\ terms}$$

Example:

$$\bar{x} = \frac{(4+3+11)}{3}$$

$$\bar{x} = \frac{18}{3}$$

$$\bar{x} = 6$$

Figure 4 - Explanation, with an example, of mean calculation.

$$\sigma(standard\ deviation) = \sqrt{\frac{\sum(x-\bar{x})^2}{n-1}}$$

x = each value of the population that is being analyzed

n = size of the population

Example:

$$\sigma = \sqrt{\frac{(4-6)^2 + (3-6)^2 + (11-6)^2}{2}}$$

$$\sigma = \sqrt{\frac{(-2)^2 + (-3)^2 + 5^2}{2}}$$

$$\sigma = \sqrt{\frac{4 + 9 + 25}{2}}$$

$$\sigma = \sqrt{\frac{38}{2}}$$

$$\sigma = \sqrt{19}$$

$$\sigma = 4.36$$

Figure 5 - Explanation, with an example, of standard deviation calculation.

The p-value from t-test was calculated using an excel spreadsheet. All *p-values* were calculated between the mean of the Control Treatment and the Treatment at question. *p-values* smaller than 0.05 reject the null hypothesis, which states "There is not a significant difference between the two groups; any observed differences may be due to chance and sampling error" (Biology for Life, n.d.). If the null hypothesis is rejected, the alternative hypothesis is accepted, which states "There is a significant difference between the two groups; the observed differences are most likely not due to chance or sampling error. On the other hand, *p*-values of $p > 0.05$ accepts the null hypothesis.

The experiment was conducted considering nine replicates for each treatment, but only three out of the nine replicates in each treatment germinated. Considering this fact, the Student's statistical test isn't the most adequate for small sample sizes, therefore the Mann–Whitney U test was also run using the calculator in the following website "https://www.statskingdom.com/170median_mann_whitney.html".

RESULTS

A trend observed in the replicates from Treatment 0.10% in relation to Control Treatment 0.00% is the tendency of having longer and more developed root systems. This probably had an effect on the measurements presented on Table 1, since bigger roots means more mass. Besides the root observation, all the other replicates that germinated presented the same qualitative characteristics. The leaves were a bright green with a healthy appearance. They are curved to the

sides, as if they had grown upwards but hadn't enough place to continue growing, it has connection with the height of the styrofoam box.

Table 1 shows the dry mass of *G. max* at the end of the experiment in all treatments. It also shows the mean, standard deviation, the *p-values* from t-test and the *p-values* from the Mann–Whitney U test. The MW U test was considered to be necessary in the data analysis due to the small number of replicates that germinated.

Table 1 - Dry mass (g) of *G. Max* after 15 days of growth from the time of germination alongside the mean for each treatment, the standard deviation for each treatment and the p-values acquired through a t-test between Treatment 0.00% (Control) and the treatment in question. **p-values* acquired through t-test ***p-values* acquired through the Mann–Whitney U test.

Trials	**Caffeine Solutions (± 0.001 g)**					
	0.00%	0.025%	0.05%	0.10%	0.20%	0.40%
1	0.092	0.046	0.098	0.128	0.163	0.122
2	0.108	0.067	0.103	0.137	0.137	0.153
3	0.115	0.099	0.124	0.126	0.193	0.136
Mean	0.105	0.071	0.108	0.130	0.164	0.137
S. Dev.	0.012	0.027	0.014	0.058	0.028	0.015
p-values*		0.111	0.770	0.029	0.028	0.047
p-values**		0.608	1.000	0.494	0.494	0.494

Figure 6 shows the mean dry mass of *G. max* growing under different caffeine concentrations. It is possible to observe that the only treatment that the caffeine produced a negative impact on growth was Treatment 0.025%. Replicates under Treatment 0.05% had a mean almost equal to the Control Treatment, with both statistical tests also showing a high *p-value.* (Table 1) Treatment 0.10%, Treatment 0.20% and Treatment 0.4% had a positive growth in relation to the Control Treatment. The three treatments had statistically relevant *p-values* in the t-test, but didn't have in the MW U test. Treatment 0.025%, Treatment 0.1%, Treatment 0.2% have a high and unusual standard deviation.

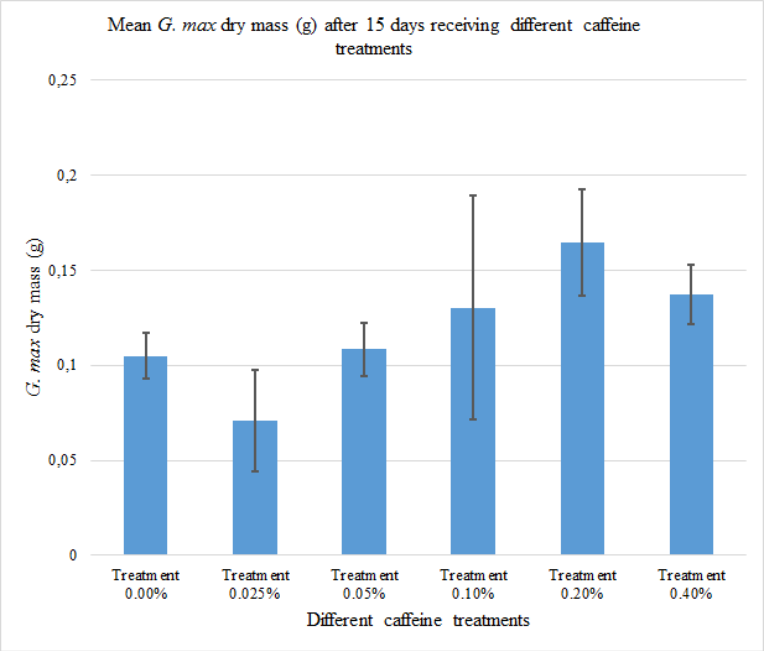

Figure 6 - Mean dry mass (in g) of *G. max* after 15 days growing under different caffeine concentrations. Error bars represent standard deviation (± SD).

DISCUSSION

The research question for this experiment was "What is the effect of different caffeine concentrations (0.025%, 0.05%, 0.1%, 0.2%, 0.4%) on the dry mass (in g) of *Glycine max* in a 15-day experiment?" Table 1 shows that all treatments were statistically similar to the control treatment but treatment 0.025% and Treatment 0.05%. However, according to the MW U test, all treatments had the null hypothesis accepted. The p-values from Mann Whitney U test were also calculated because the sample size at the end of the experiment was considerably small. For this experiment, nine replicates were planned for each treatment, mostly because of difficulties to germinate and cultivate plants, which is a difficulty in every biological experiment that uses plants. However, only three replicates were able to germinate in each treatment. This caused the t-test to become inappropriate as the only statistical test of the experiment, The small sample size with large standard deviation is probably also the reason why the MW U test presented such high *p-values* (Table 1).

The results seen in Figure 6, of intermediate caffeine concentrations having the greatest impact on plant growth, whereas higher concentrations had less or no effect on plant growth, were also found by a study in *Helianthus annuus L.* (Khursheed, Shahab, and Ansari 2009). The authors argue that a possible reason for this decrease in plant growth once a certain concentration is reached is that some mutagenic properties of caffeine may interfere with the growth

(Khursheed, Shahab, and Ansari 2009). Therefore, it can be concluded that higher caffeine concentrations would not be beneficial for plant growth.

The failure in germination may be explained by the interaction of caffeine molecules with the cell wall and phospholipids of the embryo, making both structures more rigid (Tanti et al. 2016). According to the same study, this prevents water uptake, which causes some seeds to fail germinating. This information is interesting to analyze because it may mean that even though the addition of caffeine had a positive effect in plant growth, the fact that germination is affected by caffeine also influences the possibility of it being used on real-life crops. Another possible cause of the failure in germination is the low luminous intensity used throughout the experiment, of 176 lux. Researches have found that for *Copaifera oblongifolia*, seeds that were planted under low luminosity had a smaller percentage of germination than the ones planted under high light and dark conditions (Veloso et al. 2017, 736).

Putnam (1988) also brings the fact that various studies have found great concentrations of caffeine in fields near *Coffee arabica* L. plantations. The author suggests that, if caffeine lasts for a long time on the soil, it might also present an issue of long-time contamination of the soil. Putnam (1988) adds that even though caffeine could be used not only to increase *G. max* growth it could also be used to control the control of other species of plants, commonly referred to as weeds. An issue raised by the author, however, is that weed control using caffeine would need high concentrations in the soil and that would not compensate both economically because it would be too expensive and biologically, since a caffeine-contaminated soil would inhibit germination and some plant growth, as seen in the present experiment (Table 1). Adding to that, when looking at the experimental data, it is possible to observe a decrease in dry mass on concentrations above 0.2% (Table 1, Figure 7), suggesting that the positive effects of caffeine on plant growth may decrease as caffeine concentrations increase above 0.2%.

In conclusion, the addition of caffeine presented a positive impact on the dry mass of *G. max* in Treatments 0.1%, 0.2% and 0.4%, when taken into consideration the t-test, in relation to the control treatment (Table 1 and Figure 6). The lack of a bigger replicate number, alongside its most impactful effect of a high standard deviation, causes the data to be unreliable and so the conclusion based on that data also becomes untrustworthy. Furthermore, the conclusion takes into consideration a laboratory approach to the use of caffeine in *G. max*, therefore the application of caffeine in real-life crops needs to be further investigated. Those investigations should be focused on improving the number of replicates and questions such as caffeine preventing water uptake, caffeine being used both as a weed control agent and as an agent to help

the biomass growth and whether these two actions would be compatible regarding caffeine concentration.

EVALUATION

A measurement with a lux meter was conducted while the experiment was being executed to make sure that the entirety of the styrofoam's interior received an equal amount of luminosity. The amount of luminosity overall, however, might have been too low, at only around 176 lux. In a real-life situation, the *G. max* crops would receive direct sunlight, which is around 100 000 lux. This doesn't compromise the reliability of the experiment because if every replicate had an issue with low luminous intensity, that would be considered as a controlled variable and, thus doesn't directly affect the results. The issue that this raises is that it detaches the experiment from reality and that affects indirectly the results. Since the goal of the experiment was to bring some of the real-life characteristics of *G. max* crops, it is recommended that future experiments try to achieve the 100 000 lux luminosity.

Another possible issue that could have affected the results was the number of replicates. Conducting the experiment with nine replicates per treatment was considered ideal when designing the experiment but in the light of the difficulty cited by Tanti et al. (2016) regarding the diminished water uptake caused by caffeine on the seed's embryo, more replicates would have caused an increase in the reliability of the experiment. Considering the necessity of having a minimum of 30 replicates for the Student's test to be reliable and considering that, because of the difficulty in water uptake, caused only 33% of the replicates in this experiment to germinate. The suggestion is for future experiments to have at least 90 replicates. The reason for that is that the standard deviation would not be as big, which is a characteristic from experiments with a low number of replicates. Another measure that could have helped mitigating the diminished water uptake would be an increase in the volume of water given, which was 4 ml every 24 hours for this experiment, to to 6 ml for the first 5 days of experiment. Then, 4 ml again for the rest of the 15 days.

Another aspect of the experiment that could be improved is the uncertainty of the measurements using the precision scale. The scale used in the experiment had an uncertainty of ± 0.001 g. For the measurement of the dry masses the uncertainty represented around 1% of the final values, which is not a worrying information. However, when considering the measurement of caffeine for the caffeine solutions, the uncertainty goes to 2% of the final value. Therefore, a more precise scale, with an uncertainty of ± 0.0001 g, for example, would increase even more the reliability of the data acquired.

REFERENCES

Apex-Brasil. 2020. “Brazil leverages breakthroughs in technology, productivity and safety to lead global agriculture and livestock market amid COVID-19.” *PR Newswire*, August 03, 2020. https://www.prnewswire.com/news-releases/brazil-leverages-breakthroughs-in-technology-productivity-and-safety-to-lead-global-agriculture-and-livestock-market-amid-covid-19-301102477.html.

Biology for Life. n.d. “Independent T-test.” Biology for Life. Accessed March 8, 2021. https://www.biologyforlife.com/t-test.html.

Daly, J W, J. Holmén, and B B Fredholm. 1998. “[Is caffeine addictive? The most widely used psychoactive substance in the world affects same parts of the brain as cocaine].” *Lakartidningen* 95 (December): 51-52. https://pubmed.ncbi.nlm.nih.gov/9889511/.

Embrapa. n.d. “Embrapa Soja.” Embrapa. Accessed January 19, 2021. https://www.embrapa.br/soja/cultivos/soja1#:~:text=O%20Brasil%20%C3%A9%20o%20segundo,de%203.362%20kg%20por%20hectare.

Khursheed, Tara, Danish Shahab, and Mohd. Y. Ansari. 2009. “Studies on the effect of caffeine on growth and yield parameters in Helianthus annuus L. variety Modern Article.” *Biology and Medicine* 1, no. 2 (February): 56-60. 10.4172/0974-8369.1000014.

Putnam, Alan R. 1988. “Allelochemicals from Plants as Herbicides.” *Weed Science Society of America* 2, no. 4 (October): 510-518. http://www.jstor.com/stable/3987390.

ReviewCafe. 2020. “Dados sobre Café no Brasil: Consumo, Produção e Exportação.” ReviewCafe. https://reviewcafe.com.br/dicas-e-receitas/dados-sobre-cafe-no-brasil/.

Shettel, Nancy L., and Nelson E. Balke. 1983. “Plant Growth Response to Several Allelopathic Chemicals.” *Weed Science* 31, no. 3 (May): 293-298. https://www.jstor.org/stable/4043708.

Tanti, Amar J., Pranaba N. Bhattacharyya, Sosanka P. Sandilya, and Prasanta Dutta. 2016. “Allelopathic potential of caffeine as growth and germination inhibitor to popular tea weed, Borreria hispida L.” *Current Life Sciences* 2, no. 4 (October): 114-117. 10.5281/zenodo.163671.

van de Sandt, J J M, J A van Burgsteden, S. Cage, P L Carmichael, I. Dick, S. Kenyon, G. Korinth, et al. 2004. “In vitro predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-centre comparison study.” *Regulatory Toxicology and Pharmacology* 39, no. 3 (June): 271-281. 10.1016/j.yrtph.2004.02.004.

Veloso, Aniele C., Priscila S. Silva, Wallison K. Siqueira, Karen L. Duarte, Ingrid L. Gomes, Henrique T. Santos, and Marcilio Fagundes. 2017. “Intraspecific variation in seed size and light intensity affect seed germination and initial seedling growth of a tropical shrub.” *Acta Botanica Brasilica* 31, no. 4 (October-December): 736-741. 10.1590/0102-33062017abb0032.