Investigating the Effect of Increasing Acetylsalicylic Acid Amounts on the Stomatal Density of *Nasturtium officinale*

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HIGHER LEVEL IB BIOLOGY

INTERNAL ASSESSMENT

INTRODUCTION

Acetylsalicylic acid (ASA), also known as aspirin, is a drug commonly used to relieve pain, fever and inflammation— symptoms associated with a variety of sicknesses, not limited to: common colds, toothaches, burns and back pain (National Centre for Biotechnology Information, 2020). Along with these uses, ASA has its role in agriculture, as it helps increase germination capacity, prolongs the longevity of plant cuttings, and accelerates plant growth in many aspects, such as growth in leaf diameter (Pallag et al. 2014). Rivas & Plasencia (2011) notes that ASA is derived from salicylic acid (SA), a hormone produced in many plants that plays an active role in regulating both physiological and biochemical responses. Larqué & Martin (2007) explains that SA can influence the number of leaves, stem diameter, shoot fresh, and dry mass in ornamental plants, while also influencing biochemical responses such as photosynthesis. SA solute concentrations can influence photosynthetic rate, by influencing net carbon dioxide assimilation, however higher SA concentrations have been found to reduce photosynthetic rate, and decrease RuBisCO activity in barely plants (Pancheva et al. 1998). In the IB biology higher level curriculum, photosynthesis and plant biology are studied in depth. During plant transpiration, it is known that the plant stomata regulates the movement of CO_2 into the leaves, and the CO_2 is then used to synthesise sugars through photosynthesis. With this in mind, ASA concentration may affect stomatal density and size, as previous research has shown its influences on CO_2 movement, leaf growth, and photosynthesis rate.

According to CABI (2019), *Nasturtium officinale* or commonly known as "Watercress" is a semi-aquatic herb commonly grown in fresh water, it is characterised by its cauline leaves, and creeping or floating stems. This species is native to many European, and east/southeast Asian countries. *Nasturtium officinale* is optimally grown in soils with a pH of 7.2, and in very humid conditions. The reason for choosing *Nasturtium officinale* is because of its popularity as a vegetable, and having the highest nutrient density score of any other vegetable. According to Di Noia (2014), nutrient densities are calculated with regards to the quantity of various nutrients including but not limited to: Vitamin A, Vitamin C, Iron, Zinc and Potassium, per calorie serving. Watercress is able to provide high amounts of all 17 nutrients of public health importance considered in the nutrient density scale. With this being said, diets particularly in western continents are high in calorie, and low in nutrition (Drewnowski, A. 2005), the addition of high nutrient density foods in diets will overall lead to an improvement in the daily nutrient intake of many individuals, subsequently leading to better health outcomes. Therefore, this investigation is able to aid in determining the effects of a common nonsteroidal anti-inflammatory drug, that may influence the propagation of watercress, in light of real-life implications of greatly improving the nutritional intake of many people when consumed. Additionally, *Nasturtium officinale* was chosen due to its prevalence in the Philippines thus easily accessible for the researcher, and its ability to grow very quickly given the time constraints of this investigation (CABI, 2019). This study's findings are able to investigate the effect of increasing ASA aqueous concentration solutions on stomatal density and size, which can hopefully be used by agricultural farmers, and gardeners to influence stomatal density of *Nasturtium officinale* for optimal CO_2 uptake used in photosynthesis, while minimising water loss, potentially leading to increase in growth. Having said that, this report offers an investigative study on the stomatal density of *Nasturtium officinale* under varying concentrations of acetylsalicylic acid.

Research Question

What effect will increasing concentrations of acetylsalicylic acid aqueous solution (0 mol/L, 0.00005 mol/L, 0.0001 mol/L, 0.0005 mol/L, 0.001 mol/L) have on stomatal density (s/mm^2) of Watercress (*Nasturtium officinale)* after a period of twenty-one days?

Background Information

Stated by Clarke et al. (2001), ASA in small doses can stimulate cell proliferation, while in higher doses can become detrimental to the plant as it will trigger cell death. Songul & Omer (2007) investigated the concentration of ASA on plant growth parameters that included leaf length, chlorophyll amount, root growth and hypocotyl growth. The study indicated that (high) 0.005M ASA aqueous solution inhibited leaf length, hypocotyl growth, root growth, shoot length and reduced chlorophyll amounts, 0.001M ASA prevented root growth and chlorophyll amount, while concentrations as little as 10^{-5}M ASA promoted plant growth during a one week time period. These values served the basis for the concentrations chosen in this study, the range of $5x10^{-5}$M to 10^{-3}M ASA concentration is well within concentrations tested in the study that brought significant change to plant growth parameters, while limiting the detrimental effects of higher ASA concentrations that may lead to plant death.

Acetylsalicylic acid is derived from SA. Mitchell and Broadhead (1967) states that ASA hydrolyzes into its derivative, SA in aqueous solutions. For this reason, the effect of SA must be considered. SA plays a major role in the physiological development to a plant, including growth in leaves **(**Rivas-San Vicente, M., & Plasencia, J., 2011), while also influencing biochemical responses such as photosynthesis through the net assimilation of CO_2 being diffused into the stomata (Pancheva et al. 1998). Noting that varying concentrations of SA influence growth in leaves, net assimilation of CO_2, and photosynthetic rate of a plant, this could subsequently lead to the development of more stomata, thus increasing stomatal density, as previous research has already shown positive correlations between stomata density and size with CO_2 uptake (Bertolino et al. 2019).

Hypotheses

H_0: With increasing concentrations of acetylsalicylic acid, there will be no significant decrease in stomatal density in lower leaf epidermis even as ASA becomes inhibitory for plant growth.

H_1: With increasing concentrations of acetylsalicylic acid, there will be a decrease of leaf size, shoot length, and CO_2 uptake as ASA become inhibitory for plant growth. Hence, there will be a significant decrease of stomatal density in lower leaf epidermis in response to the decreasing growth factors, and decreasing net assimilation of CO_2 regulated by the stomata.

METHODOLOGY

The field of view was calculated to be 0.154 mm^2 ($\pm 0.022 mm^2$) at 400x magnification.
A Worked example of how to calculate for field of view is shown below:

$$\text{Field of view at 400x magnification} = \pi \times \left(\frac{Measured\ diameter\ of\ microscope}{2}\right)^2$$
$$= \pi \times \left(\frac{14\mu m (\pm 1\mu m)}{2}\right)^2$$
$$= \pi \times (7 \pm 0.5\ \mu m)^2$$
$$= 153.938 \mu m^2\ (\pm 22 \mu m^2)$$
$$\approx 0.154 mm^2\ (\pm 0.022 mm^2)$$

An uncertainty of ±1μm was used as the smallest increment of the stage micrometer. After solving, the field of view had an uncertainty of (±0.022mm^2)

A worked example of how stomatal density is calculated is shown below:

Stomatal Density (s/mm^2) = # of stomata in field of view (s) / field of view at 400x magnification (mm^2)
= 44s / 0.154 mm^2 (±0.022mm^2)
= 285.714s/mm^2 (±41.7s/mm^2)
≈ 286 s/mm^2(±42s/mm^2)

The uncertainty is calculated from the uncertainty of (±0.022mm^2) at 400x microscope field of view. The uncertainties and stomatal densities are rounded up to whole numbers as the nature of the data must be discrete (no fractions or decimals of the number of stoma).

All stomata measured was from the lower epidermis of the leaf given that stomata is more concentrated in the lower epidermis in *Nasturtium officinale*.

Selection and Monitoring of Variables

Independent Variable

The level of ASA concentration (mol/L) is manipulated so that a series of experiments can be performed over a range of increasing amounts of ASA. The mass of ASA is carefully measured using an electronic balance capable of measuring up to three decimal places of mass in grams. As the balance is only capable of measuring up to three decimal places, an uncertainty margin of 1mg is used. The volume of water is measured using a 500ml beaker with the smallest increment being 5ml, hence the margin of error for a 2L volume. The selection of ASA concentrations were based on previous research that had influence on the growth of different plant parameters. The experiment will use five different conditions and repeats, treated with varying ASA concentrations (0 mol/L, 0.00005 mol/L, 0.0001 mol/L, 0.0005 mol/L, 0.001 mol/L) to provide more data collection, thereby increasing the reliability of results. A graduated cylinder measured 20ml of ASA aqueous solution to give the seeds everyday, hence another uncertainty margin of 1ml was used.

Dependent Variable

The dependent variable for this investigation is stomatal density and size. Stomatal density and size was chosen due to their role in CO_2 assimilation, and as a growth parameter of plants. Prior research has shown the effects of ASA and SA on leaf growth and CO_2 assimilation, hence may affect the the stomatal density and size as the stomata is located in plant leaves, and plays an important role for net CO_2 assimilation. The ability to manipulate stomatal density and size is valuable in agriculture as it heavily influences CO_2 uptake and the rate of water loss, which influences plant growth, and withering due to water loss (Bertolino, et al. 2019). A Stomata at the edge of the field of view will be counted if approximately half of the stomata is seen. The changes in these variables from the control condition (0 mol/L ASA), are able to show the effects of an increase of ASA concentration on stomatal density (s/mm^2).

Table 1. Selection and monitoring of controlled variables

Controlled variables	Significance/Justification	Control method
Temperature	Temperature greatly affects plant growth. Higher temperatures lead to an increase in enzymatic activity in the plant, hence allow for metabolism needed for growth. However, when temperature increases beyond optimal, proteins in plant cells may denature severely harming the plants. Temperature plays a role in plant transpiration which may have an effect on stomatal density.	Plants will be placed in the same area, hence in an environment where all conditions are subject to the same temperature. The room temperature was approximately 25ºC across 21 days.
Sunlight	Sunlight is an important factor in photosynthesis. Chlorophyll in plants absorb sunlight triggering the release of high energy electrons (photoactivation) needed to produce ATP through phosphorylation. Greater levels of photosynthesis will affect plant growth, due to the increase in available energy in plants for cell division, ergo may affect stomata growth.	Plants will be assumed to have received the same amount of sunlight as the experiment was conducted entirely in the same location (enclosed room with exposure to sunlight through a window)
Volume of ASA aqueous solution given to each condition per day	Water is an essential component for photosynthesis and plant growth. Deficiencies slow plant growth thus may influence stomatal density and size. Furthermore, solutions must be given in the same volume to investigate the effects of the these varying ASA concentrations in plant growth.	20ml (±1ml) of ASA aqueous solution was is added to each container at the same time each day (7:45am) throughout the 21 days. There is a ±1ml error allowance as a graduated cylinder was used to measure the solution.
Plant (*Nasturtium officinale)*	Different plants may have different rates of growth and stomata size, hence will be a confounding variable unless controlled.	*Nasturtium officinale* will be used for all conditions of this investigation.
Cotton	Cotton was used for the experiment as it does not contain nutrients that other solids may have. This could have potentially affected the rate at which the watercress samples grew. Furthermore, other nutrients that could potentially affect the growth of *Nasturtium officinale* are in very small concentrations.	Cotton balls were used as the medium of of planting for all conditions.
Time of growth	A period of 21days was chosen in order to ensure that leaves were able to grow larger in diameter. The time of growth was also sufficient for multiple plant parameters to be compared for qualitative observation (e.g. shoot length).	The *Nasturtium officinale* seeds were planted at the same time, and were grown for the same amount of time (21 days).

Materials

- 500mg of acetylsalicylic acid
- 200 *Nasturtium officinale* seeds
- 10L Distilled Water
- 40g Cotton
- 5x 2L plastic bottles
- 1x Electronic balance (uncertainty: ±0.001g)
- 1x microscope with magnification up to 400x
- 1x 500ml Beaker (uncertainty: ±5ml)
- 1x graduated cylinder (uncertainty: ±1ml)
- Stage micrometer (uncertainty: ±1μm)
- 5x Plastic Tupperware (25x15cm)
- 20ml Clear Nail Varnish
- Transparent sticky tape
- 5x Microscope slides
- 1x Scalpel
- 1x Marker
- 1x Stage Micrometer

Procedural Design

1. Prepare the 5 Plastic Tupperwares 25cm in length and 15cm in width
2. Using a marker, label the containers with the respective aqueous acetylsalicylic acid concentrations.
3. Label 5 plastic bottles for each acetylsalicylic acid concentration condition (0 mol/L, 0.000025 mol/L, 0.00005mol/L, 0.000075 mol/L, 0.0001 mol/L).
4. To prepare the aqueous acetylsalicylic acid concentrations, thoroughly mix the volume of distilled water (L) with the respective masses of the pulverised acetylsalicylic acid (mg). Weigh the respective masses of acetylsalicylic acid using an electronic balance (±1mg), and measure the volume of distilled H_2O with the 500ml beaker (±1ml). Quantities of both materials are indicated in **Table 2**.

A worked example of how to calculate for acetylsalicylic acid concentration from mass of ASA (g) and volume of H_2O(L) is shown below:

$$\begin{aligned}\text{Acetylsalicylic acid concentration} &= \text{moles of acetylsalicylic acid / volume of } H_2O \text{ (L)}\\ &= 0.0002 \text{ mol / 2L } H_2O\\ &= 0.0001 \text{ mol/L}\end{aligned}$$

$$\begin{aligned}\text{Mass of acetylsalicylic acid (g)} &= \text{moles of ASA} \times \text{molar mass of ASA (g/mol)}\\ &= 0.0002 \text{ mol} \times 180.158 \text{ g/mol}\\ &\approx 36 \text{ mg}\end{aligned}$$

The derived values for volume of H_2O in litres needed, and mass of ASA in milligrams to prepare varying levels of ASA concentration used in this experiment is tabulated in **Table 2.**

Table 2. Mass of ASA and Volume of H_2O with respective ASA concentration levels.

Approximate mass of ASA (±1mg)	Volume of H_2O (±20ml)	ASA concentration (±0.0000055)
0 mg	2 L	0 mol/L
18 mg	2 L	0.00005 mol/L
36 mg	2 L	0.0001 mol/L
180 mg	2 L	0.0005 mol/L
360 mg	2 L	0.001 mol/L

5. Fill the labelled plastic bottles with ASA aqueous solutions respective to their labels.
6. Fill all 5 plastic containers with cotton
7. Pour 40ml of ASA aqueous solution in each labelled container
8. Scatter 40 *Nasturtium officinale* seeds in each condition approximately 1cm apart.
9. Pour 20ml (±1ml) of ASA aqueous solution to the respective concentrations of each condition to each labelled container at 7:45am everyday for 21 days.
10. After 21 days, measure stomatal density:
11. Using the scalpel, cut off one leaf from the plant
12. Identify the upper and lower surfaces of the leaf, and spread a thin layer of clear nail polish on the lower surface. Leave it to dry for approximately 15 minutes.
13. Place a strip of clear stick tape over the clear nail polish. Press the tape down to make a good connection with the nail polish.
14. Peel the tape from the surface of the leaf, there should be an impression made by the dried nail polish.
15. Place the tape with leaf impression on a microscope slide. Use the scalpel to trim the excess sticky tape from the edge of the slide.
16. View under the microscope at 400x magnification. Count the number of stomata to solve for stomatal density (s/mm^2).
17. Repeat steps 11 to 16 to take the stomatal density of 5 plants in each condition.

Risk Assessment

Table 3. Safety, environmental, and ethical considerations

Concerns	**Hazards**	**Precautions**
Safety	Nail polish may cause respiratory irritation, headache and nausea when inhaled. Acetylsalicylic acid may cause serious eye irritation and respiratory irritation when inhaled.	As a personal safety preclude, provide adequate ventilation for indoor use. Wear a mask when mixing the pulverised ASA with water. Wear eye protection so as to avoid eye irritation. Additionally, when either are inhaled, allow the victim to move to fresh air, and let them rest in a position comfortable for breathing. If nausea, headache or respiratory irritation persists, consult a licensed physician.
Environmental	Although ecotoxicity of acetylsalicylic acid is very low, disposal must abide according to local environmental control regulations (Global Safety Management, 2015)	Given the low concentration, it is possible to dispose of ASA solute concentrations down the drain (University of Wisconsin, 2021).
Ethical	Given this investigation complies with the IB ethical guidelines, no ethical issues need to be taken into consideration.	

ANALYSIS

Raw Data

Table 4: Number of stomata (s) in 400x field of view, and stomatal density (s/mm2) of Watercress (Nasturtium officinale) exposed to increasing increasing concentrations of acetylsalicylic acid aqueous solution (0 mol/L, 0.00005 mol/L, 0.0001 mol/L, 0.0005 mol/L, 0.001 mol/L) after a period of twenty-one days.

	ASA aqueous solution concentrations									
	0 mol/L		0.00005 mol/L		0.0001 mol/L,		0.0005 mol/L,		0.001 mol/L	
Sample	s	(s/mm^2)	s	(s/mm^2)	s	(s/mm^2)	s	(s/mm^2)	s	(s/mm^2)
Plant 1	44	286 (±42)	45	292 (±43)	46	299 (±44)	43	279 (±41)	38	247 (±36)
Plant 2	43	279 (±41)	48	312 (±45)	45	292 (±43)	44	286 (±42)	45	292 (±43)
Plant 3	50	325 (±47)	49	318 (±46)	44	286 (±42)	43	279 (±41)	43	279 (±41)
Plant 4	47	305 (±43)	52	338 (±49)	43	279 (±41)	45	292 (±43)	43	279 (±41)
Plant 5	45	292 (±43)	50	325 (±47)	45	292 (±43)	41	266 (±39)	43	279 (±41)

Processed Data

Calculation of average stomatal density: $\frac{\sum stomatal\ density\ of\ samples\ in\ the\ same\ condition}{number\ of\ samples}$

Worked Example:

Average stomatal density of 0mol/L condition: $\frac{286 + 279 + 325 + 305 + 292}{5} = 297.4 \approx 297\ (\pm 43)\ \text{s/mm}^2$

The average of the uncertainty is also calculated.
The resultant is rounded up to whole numbers as data should be discrete.

Descriptive Statistics

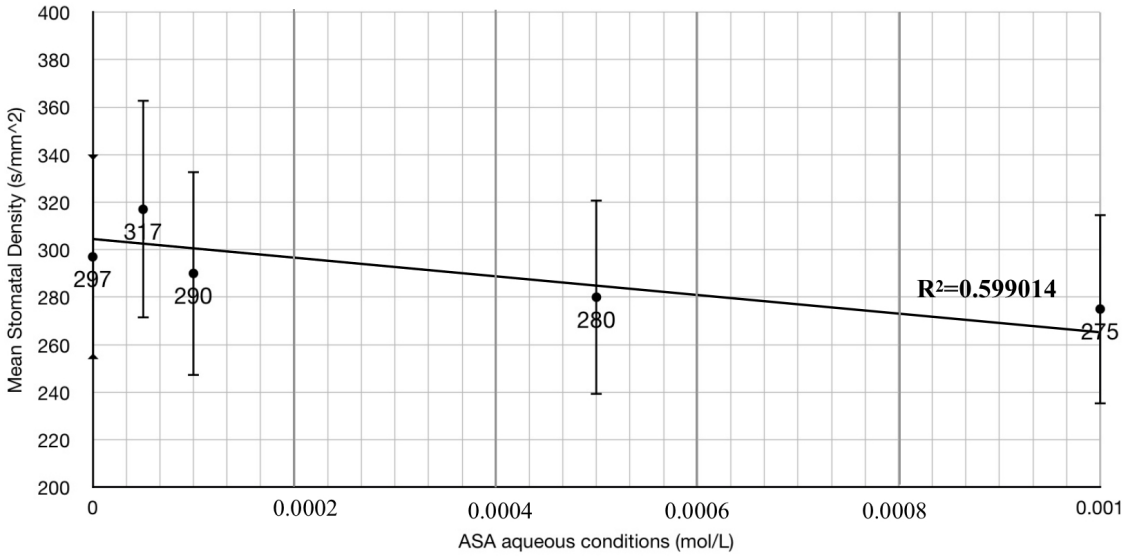
Table 5: The average stomatal density of Nasturtium officinale exposed to increasing increasing concentrations of acetylsalicylic acid aqueous solution over a period of twenty-one days.

	ASA aqueous solution concentrations				
	0 mol/L	0.00005 mol/L	0.0001 mol/L	0.0005 mol/L	0.001 mol/L
Mean Stomatal Density	297 (±43) s/mm^2	317 (±46) s/mm^2	290 (±43) s/mm^2	280 (±41) s/mm^2	275 (±40) s/mm^2

The mean was used as the central tendency. From the mean, it is noticed that a trend of decreasing stomatal density is seen from conditions treated with 0.0001mol/L, 0.0005 mol/L to 0.001 mol/L ASA aqueous solution. The stomatal densities were also less in comparison to the control condition (0mol/L). However, it can be seen that the mean stomatal density of the 0.00005 mol/L condition that was greater in comparison to the control condition.

From the mean alone, it appears as though that increasing ASA may have some influence over stomatal density (s/

Graph 1: Graph showing how increasing ASA concentration affects the stomatal density of *Nasturtium officinale* after a period of twenty-one days.



mm^2) of *Nasturtium officinale* over a period of twenty-one days.

The moderate R^2 value of the line of regression, and downwards slope indicates a moderate negative correlation between increasing ASA aqueous concentration, and the average stomatal density. The error bars of the graph represent standard error on each averaged data point, and the R^2 value determines the suitability of the line of best fit. The data points are relatively close to the line of best fit, thus may indicate higher certainty of results, however, due to the large error bars, there are large chances of errors, thus decreasing the certainty and accuracy of the data.

Inferential Statistical Test

To establish a statistical difference between the 5 different groups, a one-way ANOVA (analysis of variance) test was conducted on the raw data Table 4. This test allowed me to test for a statistical relationship across conditions with regards to stomatal density.

Table 6: Table of how ANOVA test was carried out

Summary of Data

	Treatments					
	0 mol/L	0.00005 mol/L	0.0001 mol/L	0.0005 mol/L	0.001 mol/L	Total
N	5	5	5	5	5	25
∑X	1487	1585	1448	1402	1376	7298
Mean	297.4	317	289.6	280.4	275.2	291.92
$\sum X^2$	443551	503601	419566	393498	379796	2140012
Std.Dev.	18.1466	17	7.5033	9.7108	16.7392	19.979

Result Details

Source	*SS*	*df*	*MS*	
Between-treatments	5383.44	4	1345.86	$F = 6.41436$
Within-treatments	4196.4	20	209.82	
Total	9579.84	24		

The *f*-ratio value is 6.41436. The *p*-value is .001722. The result is significant at $p < .05$.

*screenshot taken from online calculator: https://www.socscistatistics.com/tests/anova/default2.aspx

Results Format: F [degrees of freedom (d.f) between treatments, d.f total]= F value p (probability level)
Results: F (4, 24)= 6.41436 p= 0.001722

Based on the low probability value of 0.001722 ($p \leq 0.05$), the null hypothesis was rejected, hence, the alternative hypothesis is accepted: With increasing concentrations of acetylsalicylic acid, there will be a significant decrease of stomatal density in lower leaf surface of Watercress (Nasturtium officinale) after a period of twenty-one days. However, the test does not show whether the mean stomatal density of the 0.00005 mol/L condition was significantly greater in comparison to the control, and other conditions given that it was noticed that the 0.00005 mol/L condition had a much larger mean in comparison to all other conditions. This can indicate that the 0.00005 mol/L condition could have an increase on stomatal density. Hence a Post Hoc Turkey HSD test was conducted for comparison between the means of the treatments.

Table 7: Table of how the Post Hoc Turkey HSD test was carried out

Pairwise Comparisons		$HSD_{.05}$ = 27.4141 $HSD_{.01}$ = 34.2898	$Q_{.05}$ = 4.2319 $Q_{.01}$ = 5.2933
T_1:T_2	M_1 = 297.40 M_2 = 317.00	19.60	Q = 3.03 (*p* = .24279)
T_1:T_3	M_1 = 297.40 M_3 = 289.60	7.80	Q = 1.20 (*p* = .91098)
T_1:T_4	M_1 = 297.40 M_4 = 280.40	17.00	Q = 2.62 (*p* = .37158)
T_1:T_5	M_1 = 297.40 M_5 = 275.20	22.20	Q = 3.43 (*p* = .14982)
T_2:T_3	M_2 = 317.00 M_3 = 289.60	27.40	Q = 4.23 (*p* = .05015)
T_2:T_4	M_2 = 317.00 M_4 = 280.40	36.60	Q = 5.65 (*p* = .00570)
T_2:T_5	M_2 = 317.00 M_5 = 275.20	41.80	Q = 6.45 (*p* = .00158)
T_3:T_4	M_3 = 289.60 M_4 = 280.40	9.20	Q = 1.42 (*p* = .85036)
T_3:T_5	M_3 = 289.60 M_5 = 275.20	14.40	Q = 2.22 (*p* = .53099)
T_4:T_5	M_4 = 280.40 M_5 = 275.20	5.20	Q = 0.80 (*p* = .97831)

*T_1=0mol/L condition, T_2=0.00005mol/L condition, T_3=0.0001mol/L condition, T_4=0.0005mol/L condition, T_5=0.001mol/L condition
*screenshot taken from online calculator: https://www.socscistatistics.com/tests/anova/default2.aspx

Given the high probability value of 0.24279 ($p>0.05$), the mean of the 0.00005 mol/L condition was not significantly higher in comparison to the control condition. Thus it can be inferred that the higher stomatal density mean of the 0.00005 condition was most likely due to chance. However, the mean of 0.00005mol/L condition was significantly greater in comparison to both the 0.0005 and 0.001 mol/L conditions, indicating p-values of 0.0057, 0.00158 respectively.

Table 8: Table with qualitative observations throughout the experiment.

Timeline	Notes and qualitative observations
First Week	1. Approximately 80% of seeds in the 0.001 mol/L condition germinated, in comparison to the control condition that had 95% of the seeds germinate.
Second Week	1. Stems in the 0.001mol/L condition had a brown tinge in the internode space. 2. The leaves in the 0.0005 and 0.001 mol/L condition were more 'yellowish' in comparison to the conditions with lower ASA concentrations.
After 21 days	1. The shoot length of the 0.0005 and 0.001 mol/L condition were much shorter, while the 0.00005 mol/L condition were longer in comparison to the control condition. 2. Stems in the 0.001 mol/L condition had stems that were purple, other conditions had white stems.

EVALUATION

Conclusion

From the results of the experiment, I can conclude that as ASA mol/L concentration increases, the stomatal density of *Nasturtium officinale* after a period of twenty-one days decreases. Therefore, the null hypothesis is rejected, and my alternative hypothesis is accepted. This relationship is shown in graph 1, using the line of best fit, and R^2 value. The R^2 value of 0.599014 and the downwards slope indicates a moderate negative correlation between increasing ASA aqueous concentration, and stomatal density of *Nasturtium officinale*, this correlation is then tested for significance using the one way ANOVA test. A 5% chance was accepted, hence, results were only accepted at the $p \leq 0.05$ level. The low probability value of 0.001722 states that results of a moderate negative correlation were significant, and thus not due to chance.

A possible explanation is that ASA hydrolysed into its derivative, SA in aqueous solutions, therefore with increasing concentrations of ASA, plants will also be subject to higher levels of SA. Noted by Gust & Nürnberg (2012), SA plays a vital role in Effector-triggered immunity (ETI) in plants. ETI is an immune response mechanism that allows plants to respond to an infection. During ETI, SA is secreted in response to a microbial attack, and binds to the non-expresser pathogenesis related genes 3 (NPR3) which causes the degradation of the cell-death suppressor NPR1, hence triggering programmed cell death and ETI. However, in lower concentrations, SA binds to the receptor NPR4 inhibiting the degradation of NPR1, therefore favouring cell survival. For this reason, there was a decrease in plant growth factors as ASA concentrations increased noted by Songul and Omer (2007). The increase of programmed cell death may have also played a role in triggering the decreased development of stomata, given that other growth factors were also largely influenced by programmed cell death. This is evident from the data collected as the low (0.00005mol/L) ASA condition showed significantly greater mean stomatal density in comparison to the 0.0005 and 0.001 mol/L conditions. Noting that SA in smaller concentrations would not bind to bind to the NPR4 receptor, this will not induce cell death, hence the mean stomatal density for the 0.00005 mol/L concentration was significantly greater in comparison to higher ASA concentration treatments (0.0005 mol/L, 0.001 mol/L) indicated by the HSD test.

Additionally, the ETI response in plants also explains why the shoot length of the 0.0005 and 0.001 mol/L condition were considerably smaller in comparison to to both the 0.00005mol/L and control condition, and why only 80% of seeds in the 0.0001mol/L condition germinated, in comparison to the 95% germination rate of the controlled condition. My results are consistent with that of Songul and Omer (2007). Their investigation found that ASA concentrations as little as 0.00005 mol/L induced plant growth (e.g. greater shoot length found in this experiment). However, the high uncertainties of the apparatus used decreased the certainty and accuracy or results. The small sample size per condition (N=5) may have also further confounded results.

Strengths

One strength of the experiment was the use of 5 different ASA concentration conditions. This provided many opportunities for comparison, and allowed more accurate conclusions gained from the inferential statistic test. Furthermore, the mean stomatal densities were also very close to the line of best fit, indicating greater certainty and accuracy of results. The experiment is also highly reproducible and replicable given that the uncertainties, computational steps, materials, and procedure are stated.

Weaknesses

Many environmental factors were not maintained nor measured, the humidity of the room was not measured with a hygrometer. This lack of measure may have resulted in different degrees of condensation occurring in the leaves, stems, or the container. This could have diluted the ASA aqueous solution, albeit only slightly. However, given the already small concentrations of the solution, any additional increases of water could greatly confound results. This could mean that the results gained could only correspond to lower ASA concentrations instead of the ASA concentrations being investigated. This can be rectified by monitoring the changes in humidity and wiping off any droplets on the inner sides of the containers.

Additionally, the samples of *Nasturtium officinale,* cotton, and container were not sterilised, thus may have triggered an ETI response, further causing the increased secretion of SA in samples. This may have caused further cell death, hence decreasing stomatal density among samples. Furthermore, given that microbes can enter through the stomata, this may have also caused samples to develop less stomata to prevent microbial entry. This means that there could have been a greater decrease in the stomata, in response to greater concentrations of SA than provided by the different ASA concentrations. To rectify this, containers can be wiped throughly with disinfectants to kill any microbes prior to planting.

The use of cotton did not provide the nutrients and minerals for *Nasturtium officinale* growth, hence potentially decreasing the development of stomata. This was concerning as *Nasturtium officinale* requires more nutrients for proper development in comparison to other plants. A modification would be to use clay soil as it retains moisture, while providing essential nutrients such as nitrogen, phosphorus or potassium for better growth outcomes.

The treatments and concentrations that this study chose were not spaced with even increments (particularly the inclusion of the 0.00005mol/L condition), hence there may have been type II errors in the inferential statistics test. This can be mitigated by choosing concentrations that have progressing solution concentrations with even increments (e.g. redacting the 0.00005 treatment, replacing it with a 0.0015mol/L treatment). The concentrations of the ASA utilised in the different conditions are in a very narrow range (0.00005mol/L to 0.001mol/L), this limits the extent to which this study examines how much larger, or lower concentrations will influence stomatal density. This can be rectified by conducting the experiment across more ASA concentrations. Additionally, the experiment had a small sample size as it only examined 5 plants out of the 40 plants in each treatment, hence decreasing reliability for the experiment. Naturally, if more samples have been tested, this could arise to a more reliable conclusion.

This experiment used apparatus that had large uncertainties, causing decreases in certainty and accuracy of measures. The mass of ASA was measured with an electronic balance with an uncertainty of ±1mg, and water volume with an uncertainty of ±4ml, thus leading to an uncertainty of ±0.0000055mol/L, this was a relatively large uncertainty considering the small concentration conditions, particularly on the 0.00005 and 0.0001 condition. The measure of stomatal density also had a similar problem. Given the ±1μm uncertainty of the stage micrometer, this caused a larger uncertainty of 0.022mm^2 in the 400x microscope field of view, this was further amplified as stomata had to be divided by the field of view, hence causing larger error bars in stomatal density. In addition, Plants were watered using a graduated cylinder with an uncertainty of 1ml, thus may have also largely confounded results given the small volume of solution the plants are given each day (20ml). A possible modification is to utilise more precise apparatus, with smaller uncertainties.

Extensions

A potential extension of this investigation is to investigate the influence of microbial attacks on plant stomatal density and other growth factors when treated with varying levels of ASA concentrations. Given that ASA hydrolyses to SA, and SA plays a large role on the plant immune system—would high levels of SA be beneficial to ensure plant survival in plant disease spread (e.g. Crown Gall Disease) although compromising plant growth? It would also be interesting to uncover if concentrations below 0.00005mol/L will significantly increase stomatal density given that this investigation found that only concentrations above 0.00005 mol/L had decreasing stomatal density. Furthermore, this study did not find a possible explanation for the purple colour of the stems in the 0.001 mol/L condition, it would be an interesting extension to know how SA may also influence the colour of *Nasturtium officinale* stems and how might this be linked to the decrease in plant growth parameters.

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