A study regarding the effect of the microalgae Chlorella zofingiensis as a potential biostimulant on the height and fresh weight of leaf celery (Apium graveolens var. secalinum)

Research question:
how will the use of microalgae extract (*Chlorella zofingiensis*) affect the height (cm) and fresh weight (gr) of leaf celery (Apium graveolens var. secalinum) when given (via Fertigation) at 3 different concentrations (0.5%, 0.1%, 0.01%) in comparison to positive and negative control?

Aim:
The aim of this study is to explore the effects of the microalgae *Chlorella zofingiensis* extracts as a potential biostimulant. This was done by evaluating the effects of the microalgae's extract in diffrent concentrations (0.5%, 0.1%, 0.01%) on the fresh weight (gr) and height (cm) of leaf celery (Apium graveolens var. secalinum) comparing it to a negative and a positive control group.

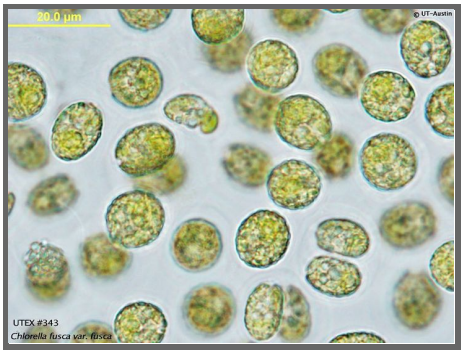
Background:
Biostimulants are considered to be any substance or microorganism applied to a plant with the purpose of stimulating\natural processes, enhancing or benefiting nutrient uptake, nutrient use efficiency, abiotic stress tolerance, crop quality and yield, regardless of its nutrients content (du Jardin, 2015). In recent years those have been developed as a possible alternative and a natural solution to the environmental impact and harm of fertilizers used in agriculture(Rajabi Hamedani, Rouphael, Colla, Colantoni & Cardarelli, 2020). Many of the biostimulants available on the market consist of algae and seaweeds extracts. This is due to the fact that many seaweeds and algae product exhibit growth-stimulating activities, containing compounds such as macro- and micro-element, nutrients, amino acids, vitamins, cytokinins, auxins, abscisic acid (ABA), and other growth-stimulating substances which affect cellular metabolism in treated plants leading to enhanced growth of crops yield. (Khan et al., 2009). One example for an algae based biostimulant which is widely utilized today is the brown algae Undaria pinnatifida (fig 1.).
U. pinnatifida is a brown algae native to Northeast Asia and Russia but can also be found in Europe, North America, South America and on the coast of Australia and New Zealand as an invasive species (Ohno & Mizuta, 2005). Extracts of the of the algae have found to contain vicarious bioactive compounds and metabolites such as phenolic compounds, terpenoid, nitrogen compounds and phlorotannins which are attributed with antioxidant, anti-inflammatory, antidiabetic, anti-proliferative, and antibacterial activities as well as the intrinsic ability to precipitate proteins (Dong et al., 2019). The extracts of the algae are commercially distributed as biostimulants by the company "Waikaitu"; an agriculture and biotech company based in Nelson, New Zealand develop and distributes organic fertiliser and biostimulant (Waikaitu, 2019).
Their product claim to "accompaniment both conventional and organic operations, to increase crop quality and production, providing over 70 key nutrients as well as a range of naturally

occurring carbon compounds (amino acids, plant hormones, alginates, sugars, etc.) that work with soil and plant biology to facilitate nutrient uptake, enhance immune response and help reduce leaching losses."(Waikaitu, 2019).

Fig. 1. The brown algae Undaria pinnatifida, retrieved from ("Southland Pest Hub", 2021); no credit was provided

In recent years there has been research into the potential use of microalgae as possible biostimulants. This is due to the fact many micro-algaes such as the soil surface green microalgae Chlorella zofingiensis (fig.2) which contains various proteins, polysaccharides, oils, vitamins, carotenoids, Astaxanthin and other biologically active compound, and is currently being researched as a potential biostimulant (Liu et al., 2014; private information, Barak & Levi, 2020).

Fig. 2 green microalgae Chlorella zofingiensis image by the university of texas culture collection of algae (Algae, 2021)



Hypothesis :

There will be an improvement in the height and fresh weight of the celery treated with the microalgae when compared to the negative control group due to the presence of bio active compounds and metabolites in the treated plants, the similar result should be observed in the positive control group (those treated with the brown algae extracts) when compared to the negative control group.

The experiment was conducted in the ecological greenhouse near the school.

Variables:

Independent variables: concentrations of microalgae (*Chlorella zofingiensis*) extract (0.5%, 0.1%, 0.01%).

Dependent variable: the height (cm) and the fresh weight (gr) of the treated celery plants.

Controlled Variables:

- Irrigation; amount of water applied to plants via drip irrigation was constant and controlled by an irrigation system.
- Soil: The soil used was provided from the greenhouse.
- Amount of treatment applied to the plants: 10 ml per week over the course of 3 weeks.
- Time: the course of the experiment was identical to all treated groups.
- Sample size: a constant number of 10 repetitions per treatment; overall a population of 50 plants.

Apparatus:

- An irrigation table equipped with an irrigation system.
- 55 leaf celery seedlings.
- Tap water.
- 50 ml of concentrated brown algae extract (U. pinnatifida); provided by Dr. Eran Barak which specializes in Innovative Plant Nutrition.
- 50 ml of concentrated microalgae extract (C. zofingiensis); provided by Dr. Eran Barak.
- Two 250 ml glass beakers.
- Five 200 ml erlenmeyer flasks.
- 100 ml graduated cylinder ± 0.5 ml.
- Two 1 ml glass pipette ±0.05 ml
- 10 ml syringe ± 0.25ml.
- A notebook and a pan.
- Soil provided by the greenhouse.
- 55 pots of about 500 ml in volume.
- Plastic cap.
- scissors.
- 30 cm ruler ± 0.05 cm
- The Electric scale used at the green house ± 0.1 gr (no label was provided).
- A large basket.
- Gardening shovel.
- 50 ml graduated cylinder 0.5 ± ml.

Pictures of the procedure equipment and apparatus can be found in appendix

Description of treatments

Table 1.treatments table

Treatment's number	Treatment 1 (negative control)	Treatment 2 (positive control)	Treatment 3	Treatment 4	Treatment 5
contains	water	U. pinnatifida	C. zofingiensis	C. zofingiensis	C. zofingiensis
Concentration of algal extract	0%	0.1%	0.5%	0.1%	0.01%
Amount of solution applied to plant (ml)	5 (ml)	5 (ml)	5 (ml)	5(ml)	5 (ml)

Justification for concentrations: the concentration used for the positive control (treatment 1)was decided upon based on the recommended amount described in the manufacture website (Waikaitu, 2019). The concentration of the microalgae extract (treatments 3, 4 & 5) were decided upon concerns over acidic properties of the microalgae extract in high concentrations (those of which might be damaging to the plant) and values found in literature regarding the use of other similar microalgaes (private information, Barak & Levi, 2020; Mutale-joan et al., 2020).

Overview of experiment

The experiment was conducted over the course of 5 weeks starting at 09/07/2020 until 06/08/2020.The table below outlines the course of the experiment and average temperature over the five weeks.

Table 2. Overview of experiment and average temperatures

	Week 1 (09/07/2020)	week 2 (16/07/2020)	Week 3 (23/07/2020)	Week 4 30/07/2020	Week 5 06/08/2020
Description	Planting and preparation	First course of treatments	Second course of treatment	Third course of treatment	Harvest and measuring
Average temperature Per week (℃)	28.6	28.7	29.6	30	29.8

Methodology

Initial preparation and planting

The first week of the investigation consisted of preparation of equipment, materials, irrigation system and the planting and preparation of the celery seedlings.

Beginning by transferring and planting the 50 celery seedlings into same sized pots using the soil provided by the greenhouse. This was done by filling up about two thirds of the pot with soil by either using a small gardening shovel or hands, then using a finger to create a small hole in the soil. The celery seedling was then put in the pot, before filling up the rest of the pot with soil.

After all celery plants were prepared, an irrigation table consisting of four rows was cleared and connected to the greenhouse automatic irrigation system. A thorough examination of the taps and pipes used for the irrigation inorder to avoid leaking and ect. After preparation of the irrigation table was made, each celery plant was marked by a serial number (1-50), put on the irrigation table and was connected to the drop irrigation system. The newly planted celery seedlings were left untouched for a week before beginning the course of treatments. This Was done to let the newly planted seedlings to accumulate to their new environments and its abiotic conditions.

Preparation of treatments

Treatment 1 (negative control)- no preparations were made; untreated plants.

Treatment 2 (positive control) - using a pipette 0.1 ml of the concentrated brown algae extract (U. pinnatifida) was dissolved in an erlenmeyer flask containing 100 ml of water then stirred for 30 seconds by hand.

Treatment 3: using a pipette 0.5 ml of the microalgae concentrated extract C. zofingiensis was dissolved in an erlenmeyer flask containing 100 ml of water then stirred for 30 seconds by hand.

Treatment 4: using a 50 ml graduated cylinder 20 ml of the 0.5% solution (treatment 3) were taken and poured into an erlenmeyer flask containing 80 ml of water, the solution was then stored for 30 seconds by hand resulting in a concentration 0.1%.
Treatment 5:: using a 10 ml syringe, 10 ml of the 0.1% solution were taken and poured into an erlenmeyer flask containing 90 ml of water, the solution was then stored for 30 seconds by hand resulting in a concentration 0.01%.An excess amount of the treatments were prepared in order to avoid errors; Preparation of treatments was repeated for each course of treatments.

Application of treatments

Treatments were applied once a week over the course of 3 weeks. In order to avoid errors derived from nuisance factors, those of which might influence the spread of the data in the population, the experimental method of randomized complete block design (RCBD) was utilised to ensure an equal distribution of conditions throughout all treatments (Rzewnicki,1992). following the RCBD method the population of plants were numbered and assigned into blocks of five plants (10 blocks in total).Treatments were distributed similar to the following order "A-B-C-D, B-C-D-A, C-D-…" as can be observed in the raw data table. treatments were applied to plants via the method of fertigation, allowing absorption of the treatments from the roots. Using a syringe 5 ml of the desired treatments was applied to the plant. The treatments were applied following the order of treatment.

Description of harvest and measuring

Height: Using a ruler the height of each plant was measured from the beginning of the stem to the end of the system's highest leaf. the measured height was then written next to the plant's serial number.
Fresh weight: the measuring of the fresh weight was done by cutting the planet using scissors and measuring its weight on an electric scale. the measured height was then written next to the plant's serial number. Plants were measured one by one according to their serial number. With the measured plants collected in a basket and recycled as animal food (donkeys).
The data collected was then transferred to the raw data table (table 3) in excel.

Risks and ethical consideration

Risks: The experiment performed in this investigation involved prolonged exposure (2 hours at a time) to sunlight and hot temperatures. The risk associated with prolonged exposure to sunlight and UV radiation are skin damage, sunburns and the increased risk of developing skin cancer (Jakuboski, 2015) as well as heatstroke and dehydration due to exposure to high temperature (OSH Answers, 2021). In order to avoid the risks mentioned the use of protection measures such as hats and sunscreen as well as the repeated drinking of water were utilized.
Ethical and environmental: no intentional or unintentional harm was imposed on humans, animals or the environment during the course of this investigation. The investigation was planned according to IB ethical and safety guidelines.

Analysis

Table 3. Raw data table with outliers marked in yellow

Plant number	treatment	height (cm ±0.05 cm)	weight (gr ±0.1 gr)
1	1	21	13
2	2	20.1	10
3	3	20.2	15
4	4	21.7	12
5	5	25	12
6	2	21.2	9
7	3	20.3	9
8	4	19.9	8
9	5	22.2	12
10	1	18.1	10
11	3	21.7	13
12	5	22.1	10
13	2	21.3	11
14	3	20.1	10
15	4	22.3	10
16	5	19.2	8
17	1	21.5	10
18	2	20.5	12
19	4	19.4	12
20	5	20.9	10
21	1	23	9
22	2	19.6	7
23	3	18.6	12
24	5	21.6	7
25	1	24	12
26	2	18	10
27	3	24.6	16
28	4	21.9	12
29	1	21	7
30	2	20.1	10
31	3	21.8	11
32	4	20.4	10
33	5	22.2	15
34	2	20.2	8
35	1	18.8	8
36	3	23.8	10
37	5	19.5	7
38	1	21.4	10
39	3	22.7	12
40	4	19.6	9
41	5	21	7
42	1	23	13
43	2	23	13
44	1	20.5	8
45	4	20	7
46	3	24.1	14
47	4	22.8	13
48	5	23	15
49	2	20.2	10
50	4	21.1	8

Processed data

Table 4. Showcasing the mean, standard deviation, sample variance of the height (cm) measured

	water	brown algae 0.1%	microalgae 0.5%	microalgae 0.1%	microalgae 0.01%	general population
mean (cm ± 0.05 cm)	21.23	20.42	21.79	20.91	21.67	21.20
Standard Deviation	1.837	1.286	1.997	1.213	1.682	1.644
Sample Variance	3.376	1.653	3.988	1.472	2.829	2.704
count	10	10	10	10	10	50

Table 5. Showcasing the mean, standard deviation, sample variance of fresh weight (gr.) measured

	water	brown algae 0.1%	microalgae 0.5%	microalgae 0.1%	microalgae 0.01%	general population
mean (gr ± 0.1 gr)	10	10	12.2	10.1	10.3	10.52
Standard Deviation	2.108	1.764	2.299	2.079	3.129	2.384
Sample Variance	4.445	3.111	5.289	4.322	9.789	5.683
count	10	10	10	10	10	50

Fig.3 mean height of treatments

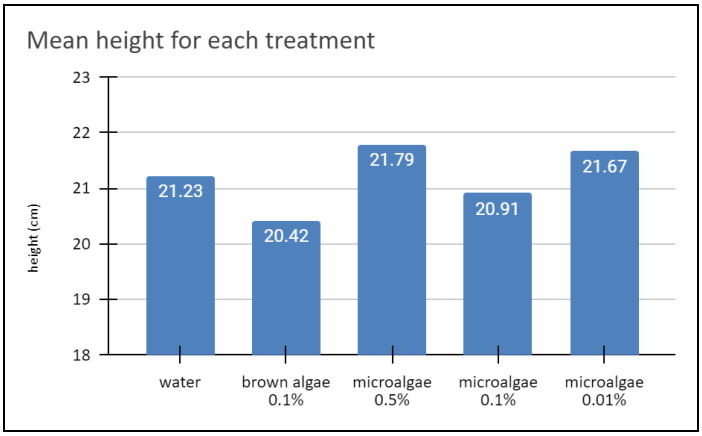
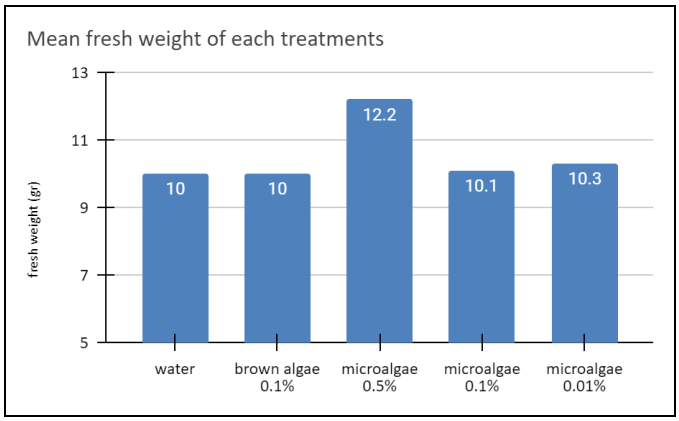


Fig.4 mean fresh weight of treatments



analysis of variability (one way ANOVA)

Null Hypothesis (H_0):Means are equal "*H0: $\mu 1 = \mu 2 = \mu 3 \ldots = \mu k$*"

Alternative hypothesis (H_1): Means are not all equal.

Level of significance is α=0.05

Fig.5 the result of the one way anova test performed in excel for height

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
water	10	212.3	21.23	3.3757
brown algae 0.1%	10	204.2	20.42	1.6523
microalgae 0.5%	10	217.9	21.79	3.988
microalgae 0.1%	10	209.1	20.91	1.472
microalgae 0.01%	10	216.7	21.67	2.829

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	12.6232	4	3.1558	1.1848	0.33036	2.57874
Within Groups	119.856	45	2.6635			
Total	132.4792	49				

Fig.6 the result of the one way anova test performed in excel for fresh weight

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
water	10	100	10	4.4445
brown algae 0.1%	10	100	10	3.1111
microalgae 0.5%	10	122	12.2	5.2889
microalgae 0.1%	10	101	10.1	4.3222
microalgae 0.01%	10	103	10.3	9.7889

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	35.88	4	8.97	1.663849959	0.17504	2.57874
Within Groups	242.6	45	5.3911			
Total	278.48	49				

Table.6 results and analysis of ANOVA for height and fresh weight

Perimeter	**Height**	**Fresh weight**
Results of ANOVA	P-VALUE > α (0.33036>α)	P-VALUE > α (0.175046>α)
Conclusion	P-value is larger than level of significance. Therefore the alternative hypothesis (H1is) is rejected and the null hypothesis (H0) is accepted. There is no significant difference between the group's means.	While the p-value is relatively small, it doesn't coordinate with the required level of significance there for the null hypothesis (H0) is accepted. There is no significant difference between the group's means.

Qualitative observations

The observed height of the celery seedlings was around 5-7 cm in the first week of the experiment. By the second week of the experiment most plants have already doubled in size. By the end of the experiment all plants have grown significantly, developing new leafs and stems. Around the third week of experiment (second course of treatments) a suspected infestation of a pest known as broad mites (Polyphagotarsonemus latus) was observed. Those appeared to have caused deformities to plant such as mild distortions to older leaves, severe deformities to newer leaves as well as distortion and bumps on the stemms (as can be seen in fig. 7).

Fig.7 broad mites (left image) and their effect on leaf celery (right image); both images were taken by the author using a portable microscope at a magnification of 10x and a smartphone.

The distribution of affected plants appeared throughout all groups with no specific relation to blocks or treatments. While hard to determine due to the effects of the mites, some of plants treated with treatment 3 and 5 appeared to be slightly higher than those treated with the other treatments (highlighted in raw data), it appeared that the plants treated with the positive control were relatively smaller compared to both the other groups. Block placement did not appear to affect the growth of the plants.

Calculations

In this investigation calculation measures the distribution and speared of the data such as the calculation of means, standard deviation and sample variance. Calculations such as mean were used to summarize the data gathered for each treatment, while calculations such as standard deviation and sample variance were used to showcase and measure the spread and dispersion of the data and were used as tools to assess the effect of the mites. Additional statistical tests such as an anova test ant were conducted to measure and assess the significance of the data in regards to a null hypothesis.

Table 3. Description of calculations.

name	formula	Description
mean	$\underline{x} = \frac{\sum_{i=1}^{n} x_i}{n}$	average value of a given population; used in analysis.
Standard deviation	$\sigma = \sqrt{\frac{\Sigma(x_i - \mu)^2}{n}}$	Measures variation in a set of value; used in analysis.
Sample variance	$S^2 = \frac{\sum(x_i - \bar{x})^2}{n-1}$	Used to show how varied a sample is. Used to calculate the depression and variation of the data.
analysis of variability (one way ANOVA)	$F = \frac{MST}{MSE}$ $MST = \frac{\sum_{i=1}^{k}(T_i^2/n_i) - G^2/n}{k-1}$ $MSE = \frac{\sum_{i=1}^{k}\sum_{j=1}^{n_i} Y_{ij}^2 - \sum_{i=1}^{k}(T_i^2/n_i)}{n-k}$	The anova function compares the sample means of n groups, exploring the distribution of the groups and was used in data analysis.

Results of data analysis and discussion

Height: according to table 4. The standard deviation and sample variance of the group treated with the treatment 1 (water) and treatment 3 (0.5% microalgae) appears the largest out of the rest of the groups with treatment 2 (0.1% brown algae) having the lowest standard deviation and sample variance.with fig. 3 not showing a significant difference between the heights. Based on the ANOVA test results there is no significant difference between the group's means.

Fresh weight: according to table 5. The standard deviation and sample variance of the group treated with the treatment 5 (microalgae 0.01%) appeared to be the highest out of the groups, with the rest having similar standard deviation and sample variance. According to fig.5 the mean fresh weight of treatment 3 (0.5% microalgae) appears to be larger than the rest of the groups all of which have similar means. While the anova test did not reveal any significance of the data, the relatively small P-value does hint on a difference between the means and corresponds with those observed in figure 5. According to the data presented there is no observed difference between the control groups, with the negative control mean height value being larger than that of treatment 3.

The range as results described in table 4, fig. 5 as well the relative high p-value when compared to those of the fresh can be associated with the deformities caused by the mites affecting the height of the plants more severely than those of its fresh weight.

Conclusion

Based on the result of this study there is lack of sufficient evidence to either support or reject the original hypothesis of this study. While the mean fresh weight of treatment 3 (0.5% microalgae) does appear to be higher than the rest of the groups there is no sufficient evidence that the results are attributed to the treatment. With the results of anova showcasing a small statistical significance to the measured data, but still above the level of significance. while there is no sufficient evidence to support the improvement in the height and fresh weight of the treated celery. The effects of the broad mites as shown in fig.7 undermine the reliability of the measured data. I would argue that therefore no sufficient conclusion can be drawn.

Based on the results of this investigation and reliability of the data a repetition of the experiment is recommended according to the suggestions made in the evaluation.

Evaluation

While several strategies were used in order to avoid errors and uncertainties (such as the sample size and the use of a random complete blocks design) there are many limitations and errors that should be acknowledged.

Broad mites: broad mites (Polyphagotarsonemus latus) are polyphagous pests which are the most active throughout the summer months and are commonly found in the tropical and subtropical areas as well as greenhouses of countries with warm climate. The mites are easily spreadable and persistent due to their small size (0.2 to 0.3 mm) short life span (13 days producing 30- 70 offspring). The mites consume soft tissue motley found at the lower parts of the plant and leaves causing the deformities shown in fig. 7. The mites probably originated from another plants

greenhouse. The effects of the mites have been completely devastating, affecting the reliability of the data measured. An attempt to combat the mites with the use of nim oil was thought of, but was abandoned due to a low availability of the extract.

Uncertainties: uncertainties in the data as well as flaws in the method used to measure the data should be addressed, as well as the uncertainties of using a ruler, specifically due to the effects of the mites on the plants.

The effect of the pandemic on the procedure: regulations and limitations of the pandemic have influenced and affected this investigation, such as limited equipment and repetitions of the experiment due to the lockdown.

Applications and suggestions

The following are suggestions and applications regarding the limitations previously described.

The use of pest resistant plants such as basil: while conducting the experiment in the greenhouse I have noticed that while plants such as papers and leaf celery have been severely affected by the mites, basil (Ocimum basilicum) did not appear to be affected by the mites. Basil contains certain essential oils which repel pests such as the mites, repeating the experiment with basil might prevent the possibility of broad mites infestation.

Diffrent measurement of growth and biomass: biostimulants have diverse range effects in regards to plants enzymatic activity, nutrients and more(du Jardin, 2015). Assessing the effects of the biostimulant on the plants measuring chlorophyll level, dry weight and protein content will allow for a better understanding of the effects the microalgae extract has on the growth of plants, and it's possible use as a biostimulant.

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