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EXPLORING THE BIOFERTILIZATION EFFICIENCY OF *CHLORELLA VULGARIS* ON TOMATO (*LYCOPERSICON ESCULENTUM* L.) GROWTH AND PRODUCTIVITY

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Abstract

Rapid global population growth has increased food demand, leading to the extensive use of synthetic fertilizers that degrade soil health and contribute to greenhouse gas emissions. As a sustainable alternative, algal biofertilizers—particularly those derived from Chlorella vulgaris—offer a promising approach to enhance crop productivity while maintaining ecological balance.

This study evaluated the effects of a Chlorella vulgaris-based biofertilizer on the growth and biochemical attributes of tomato (Lycopersicon esculentum L.). The nutrient-rich microalga C. vulgaris was cultivated in Bold's Basal Medium, identified through microscopy, PCR amplification, and Sanger sequencing, and formulated into a biofertilizer (OD 0.27). Tomato seedlings were divided into three groups: control, Chlorella-treated, and urea-treated.

Application of the Chlorella biofertilizer significantly improved plant growth, as evidenced by increased root length, plant height, and leaf number. Biochemical analyses demonstrated elevated levels of chlorophyll a, chlorophyll b, and carotenoids, indicating enhanced photosynthetic efficiency and overall plant vigor. Higher proline accumulation suggested the induction of a mild stress response.

These findings highlight the potential of Chlorella vulgaris as an eco-friendly biofertilizer for promoting plant growth and productivity. However, further investigation is required to better understand the associated stress responses and to optimize its application before large-scale agricultural implementation.

INTRODUCTION

Food demand has increased drastically over the past few years due to increasing population and is tremendously pressuring the agriculture systems around the world. According to conventional methods, farmers have excessively been using synthetic or destroys the structure as well as the microflora present in the soil deteriorating its long-term viability and capacity to sustain robust crop production. Due to the use of these synthetic fertilizers, nitrogen also runs off into the water bodies and causes contamination leading to eutrophication and biodiversity loss. Even when these fertilizers are manufactured on a commercial scale, they emit many greenhouse gases causing the greenhouse effect, global warming, and climate change (Chaudhary *et al.*, 2022).

As chemical fertilizers have more harmful effects than benefits, a new concept of biofertilizers has been introduced to the world by the scientific community. chemical fertilizers to increase the yield of the crops and fulfill the food demand. However, like every other thing, chemical fertilizers also have some disadvantages such as they deteriorate the soil and deplete useful nutrients and minerals.

Biofertilizers are composed of organic beneficial microbes as well that help to protect soil integrity and Regular use of these fertilizers along with pesticides structure, increase soil fertility, and promote healthy plant development. According to studies, biofertilizers can prove to be potential alternatives for chemical fertilizers as they maintain and improve soil health, increase the diversity of microbes, improve and enhance the nutrient cycle, and reduce the environmental impacts of chemical fertilizers. Biofertilizers also play an important role in carbon sequestration as they take carbon dioxide from the environment sequestration, which involves removing

carbon dioxide from the environment and holding it in the soil, is another important function of biofertilizers. Thus, biofertilizers are becoming more and more recognized as a vital instrument in sustainable farming (Chaudhary *et al.*, 2022).

After the development of biofertilizers, algae-based biofertilizers have now become quite famous in the scientific world because of their many advantages. Although algae is known to contaminate the water bodies as it depletes the water of nutrients and causes problems for aquatic life it also contains so many potential minerals, nutrients, and beneficial microbes that can help maintain soil health and promote healthy plant development. According to studies, many types of algae have the potential to be used as biofertilizers for plants such *Echinops dimorphus, Spirulina platensis, Scenedesmus dimorphus, Azolla chinensis, and Nostoc* as they can improve the quality of soil, enhance plant growth and reverse the environmental damage (Tiwari *et al.*, 2017).

Recently, studies have been done to further explore the potential of different species of algae to synthesize a potent biofertilizer that can be used in the agricultural sector to meet the food demand by increasing the crop yield will less damage to soil and the environment (Mahapatra *et al.*, 2018).

Chlorella vulgaris is a species of green algae that is naturally found in freshwater and marine water as well. It is unicellular and is also found in some soil conditions. It is abundant in nutrients, antioxidants, and other important pigments for plant growth such as phycocyanin, carotenoids, and chlorophyll. Other important factors necessary for plant growth and development such as proteins, vitamins, carbohydrates, trace elements like zinc, manganese, and copper and minerals such as calcium, phosphorous, potassium, magnesium, and iron are also present in it (Ammar *et al.*, 2022).

Chlorella vulgaris also contains several bioactive substances such as enzymes, antioxidants, and phytohormones including auxins, gibberellins, and cytokinins which help in plant growth and development regulate nutrient uptake by the plants strengthen the overall defense system, and help it overcome disease attacks and environmental stressors. So, chlorella vulgaris as a biofertilizer can not only enhance healthy plant growth but can also help build the resilience of plants (Ammar *et al.*, 2022).

Another important advantage that *Chlorella vulgaris* has over chemical fertilizers is that it exhibits several important chemicals due to which nutrients are naturally added to the soil and the microbes present in it make it easier for plants to uptake nutrients while chemical fertilizers add nutrients in an unbalanced way in the soil due to which soil structure and health deteriorate over time. So, chlorella vulgaris can be a potent alternative to chemical fertilizer as it can restore soil health and fertility and is an eco-friendly practice for sustainable agriculture (Ammar *et al.*, 2022).

MATERIALS AND METHODS

Source of planting material and algal culture

The seeds of the tomato plant were purchased from NARC (National Agricultural Research Centre), Islamabad. The selected pure algae *Chlorella vulgaris* was obtained from the Biobank of the University of Haripur and cultured in sterile environmental conditions required for its growth using Bold's basal medium. This nutrient-rich solution was added to the algae suspension and observed after 16 hours. The algae cells were cultured at a constant room temperature (Paterson *et al.*, 2024).

Establishment of Model Plant (*Lycopersicon esculentum)*

Hybrid tomato seeds, F1 variety were sown in composite soil containing pots placed in the greenhouse of CUST at an optimum temperature of 30±2°C and relative humidity of approximately 45-50%. After 15 days, the seeds germinated, and young saplings were noted. The plant's growth was monitored for another 30 days before being moved to 4-by-4-inch pots and watched for an additional 5–10 days for the experiment.

Experimental Plan

After the plants reached a height of approximately 13cm or more they were stable enough to start the experimental treatment. A total of nine pots of tomato plants were divided into three groups i.e., control, standard, and treated groups each having three pots of tomato plants (Fig. 1). The control group was given water only with maximum controlled temperature whereas the standard group which was based on the application of synthetic fertilizer Urea available in the market and the last group was the treated group or the *Chlorella vulgaris* based biofertilizer group. The standard group was given 10ml diluted urea in soil to each plant by diluting 2.5g urea in 1L water and the treated or Biofertilizer group was given 10ml purified *Chlorella vulgaris* dilutions in soil daily having an O.D value of 0.27.

Figure 1. Progeny development of *L. esculentum*

Preparation of Media and Protocol
Bold's Basal Medium (BBM), which was made using Stein's (1973) approach, is used to cultivate a variety of algal cultures. One liter of deionized water, 1.0 mL of a 0.1% sulfuric acid solution, and 0.705 g of B1675 were combined to create the standard medium. KOH was used to bring the final solution's pH down to 6.6 +/- 0.1 (Stein, 1973).

Confirmation of Strain
Microscopy and analytical techniques were then applied to the algal suspension (Alberto *et al.*, 2020). DNA extracted from the confirmed algal strains was subjected to PCR amplification against primers designed for the 18SrRNA region (Alberto *et al.*, 2020). To determine the concentration of cells, present in the culture, spectrophotometric analysis was performed to check the optical density (O.D.) of the algae counts (Arora and Philippidis, 2021).

DNA extraction and PCR amplification
Total DNA extraction from the purified culture of Chlorella was done with the help of a DNA extraction kit by Sigma Aldrich. After that DNA extracted was quantified with the help of Nanodrop. 221ng/µL of DNA was obtained by subjection a 1 ul sample on a nanodrop sensor. Gradient PCR was performed at 55°C annealing temperature. Primers used for amplification are ITS region 18S rRNA.

Progeny Development and Treatment Tomato plants were developed by sowing seeds in composite soil (sand, soil, and peat, 1:1:1) provided the necessary conditions. The plant after growth of 30 to 40 days was divided into three groups; control, standard, and treated groups. The control group was given no treatment, the standard group was given market-based NPK fertilizer in the form of urea and the treated group was given the biofertilizer developed using *Chlorella vulgaris* daily for 20 days (Liu *et al.*, 2023).

Biofertilizer Formulation and Spectrophotometry
To prepare the *Chlorella*-based biofertilizer, the culture was used in its suspension form without any prior modifications. To achieve the desired concentration, two sequential dilutions were made. In the first step, 1 ml of the original culture was taken and mixed with 9 ml of distilled water in a culture tube, making a total of 10 ml. This preserved the microalgae's key characteristics while lowering its density. To further lower the concentration for the second dilution, 3 ml of the first diluted sample was moved into a different tube and 7 ml of distilled water was added (Park et al., 2022).

Morphological Assessment of Tomato Plants
The morphological analysis of plants was done to assess the effects of various treatments on plant development by primarily focusing on three important growth parameters: the number of leaves, root length, as well as shoot length. Plant height and total leaf number were recorded with an interval of 5 days for all groups. Shoot length was also measured for all groups using a measuring scale every five days to monitor and assess vertical length for every treatment type. After the experiment was completed, plants were harvested, and root length was also measured with the help of measuring tape as root development also plays an important role in the overall health of the plant (Liu *et al.*, 2023).

Biochemical Assessment of Tomato Plants

To check the impact of synthesized *Chlorella vulgaris* biofertilizer on plants, the treatment group was compared to the control and standard groups through biochemical testing. The concentration of photosynthetic pigments and proline was determined in the plants using different biochemical tests. Total chlorophyll and carotenoids were measured using acetone extraction along with spectrophotometric analysis as stated by Zarafshar *et al.*, 2016. Proline concentration was measured according to the methods of Bates *et al.*, 1973.

Statistical Evaluation

After the morphological and biochemical analysis was done, the data was subjected to statistical analysis using ANOVA as stated by Bumandalai *et.al,* 2019.

RESULTS AND DISCUSSION

Confirmation of *Chlorella vulgaris*

Chlorella vulgaris was provided by the Biobank of the University of Haripur in suspension form under the accession number SIDB00000000 as shown in Fig. 2. To confirm the identity of the strain and culture, it smear was subjected to morphological analysis through microscopic examination shown in Fig. 3. Light microscope was used to make and analyze wet mount slide at 40X magnification. Under the microscope, the culture appeared to be green-colored, with green-colored unicellular and spherical cells as mentioned in the literature (Das and Deka, 2019). No microbial or other contamination was observed in the culture under the microscope.

Figure 2. Chlorella culture acquired from the University of Haripur, KPK

According to the literature, Chlorella vulgaris is unicellular and eukaryotic. It is a species of green algae and is known to contain a high concentration of chlorophyll due to which the green color is evident in the culture. This pigment plays an important role in increasing oxygen and regulating the nutrient cycle for plants to perform photosynthesis, especially in aquatic environments. Under the microscope, the cells of C. vulgaris appear to be unicellular, rounded, and small with a diameter from 2 to 5 µm. Its unique identity is more evident by the presence of distinct cell walls, chloroplasts, and consistent green color (Pawlita, M. et al., 2018).

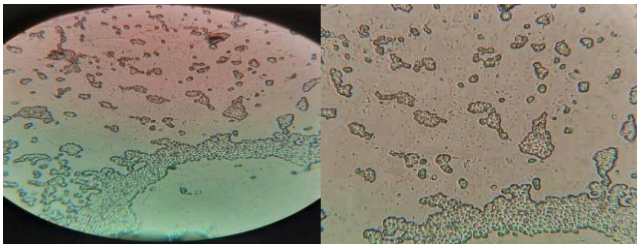

Figure 3. Chlorella strain microscopic evaluation utilizing a 40x lens and a light microscope.

To use Chlorella vulgaris in soil for plant growth and development, a diluted solution of the suspension is used so that excess algae is not accumulated as excess accumulation can disrupt the soil health and nutrient

uptake of the plants which can ultimately result in unhealthy plants. As present in literature, C. vulgaris when applied to the soil as a biofertilizer supplies important elements such as vitamins, proteins, and minerals to the soil in abundance due to which soil fertility, and root and shoot development of plants is enhanced with less harmful impact on the environment and ultimately promotes sustainable agriculture and farming practices.

3.2 PCR Amplification

DNA extraction was done first from the pure strain of obtained *Chlorella vulgaris*. After DNA extraction, the strain was subjected to PCR amplification and a DNA fragment of size 470 bp was obtained which matched the anticipated amplicon size as reported by S. *et al.*, 2021. The product of amplification was then run on 1.5% agarose gel for confirmation with a 100 bp DNA ladder as shown in Fig. 4 as confirming the results of PCR using gel electrophoresis is a vital step in molecular biology.

Gel electrophoresis further guarantees that the amplified product is correct and can be used in subsequent processes including gene expression analysis, cloning, and sequencing. No extra or unexpected bands in gel electrophoresis confirm that the amplification process was done with precision and makes the DNA fragment authentic to be used for further research S. *et al.*, 2021.

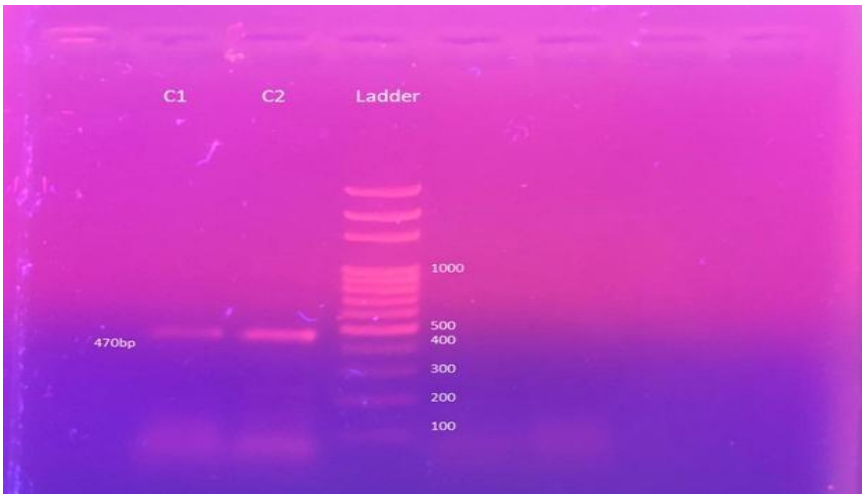

Figure 4. Gel Electrophoresis of Amplified PCR product

Biofertilizer Formulation and Spectrophotometry

Two dilutions were formulated using the suspension culture. The first dilution contained 1 ml culture and 9 ml distilled water and the second dilution was formulated using 1 ml diluted culture and 9 ml distilled water.

These dilutions were then analyzed using a spectrophotometer at 680 nm wavelength with distilled water as blank to find the ideal concentration that can be applied to the soil as biofertilizer. Using the spectrophotometer, the optical density (O.D) value was measured to help determine the amount of Chlorella vulgaris in the suspension. An O.D. of 0.88 for the first dilution indicated a rather high microalgae concentration. As anticipated, the distilled water had an O.D. of 0.06, whereas the second dilution had a much lower O.D. of 0.27 (Fig 5). After spectrophotometric analysis, the second dilution was selected for the biofertilizer group application as per the findings of Jui et al. (2024).

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Figure 5. Spectrophotometric analysis of *C. vulgaris* dilutions

Morphological Assessment of Tomato Plants

To evaluate the effect of each treatment group on leaf growth, differences between them were recorded. For morphological assessment and analysis, data from plants was first taken before the treatment to know the difference in average height and number of leaves between the three groups as shown in Table 1.

Table 1. Average no. of leaves and height of tomato plants recorded before the start of the treatment

	No. of leaves	**Height (cm)**
Control	20.6 ±3.0	13±0.36
Standard	21.3±4.1	13.4±0.5
Treatment	22.0±3.5	13.3±0.6

During the 20 days of the experiment, the number of leaves and height were recorded at the interval of 5 days using a measuring scale. Using this data, graphs were generated to determine which treatment showed the best results as shown in Figure 6. After 20 days of experiment, the control group had a short height compared to other groups and lost many leaves due to excessive heat present in the environment. The treated group had good height and number of leaves. The standard group was slightly smaller in height and had an almost equal number of leaves to that of treated group. Similar results of *Chlorella vulgaris* biofertilizer on height and number of leaves were seen in Swiss Chard as reported by Hajnal-Jafari *et al.*, 2020.

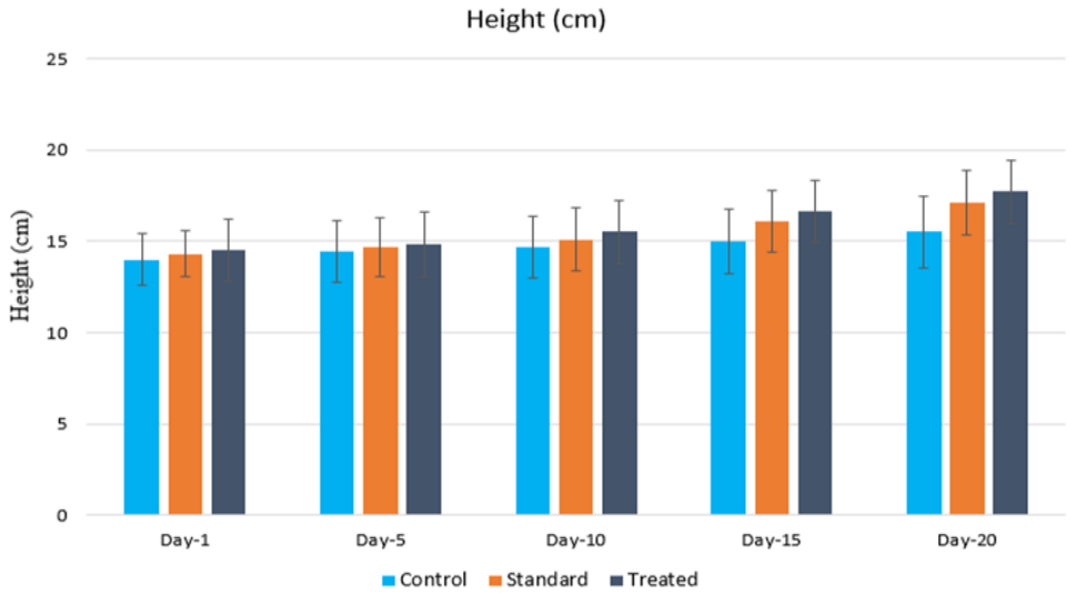

Figure 6. Average height comparison of three

treatment groups with 5 days interval After the treatment, the plants were also harvested tomeasure and compare the average root length of the three plant groups as demonstrated in Figure 7. The root length of plants in each group was measured using measuring tape and the average was taken to generate a mean graph. The control group had the shortest root length, the standard group had a root length greater than the control and the treatment group
had the greatest root length as shown in Figure 8. An increase in root length as a result of the application of Chlorella biofertilizer was also observed in different agricultural plants such as maize, wheat and lettuce already referred to by Hajnal-Jafari *et al.*, 2016.

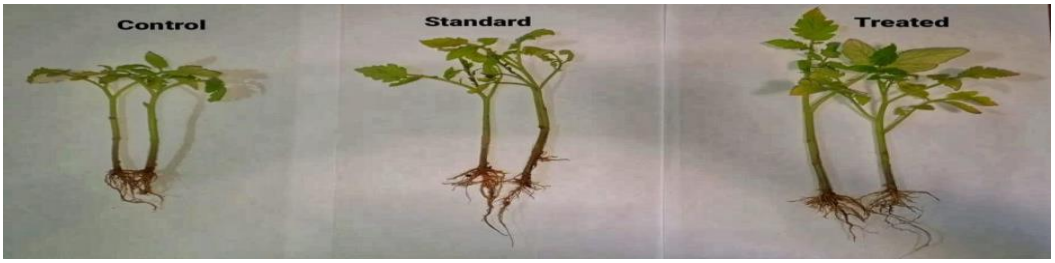

Figure 7. Root length of control, Standard (Urea)and treated (Biofertilizer) group

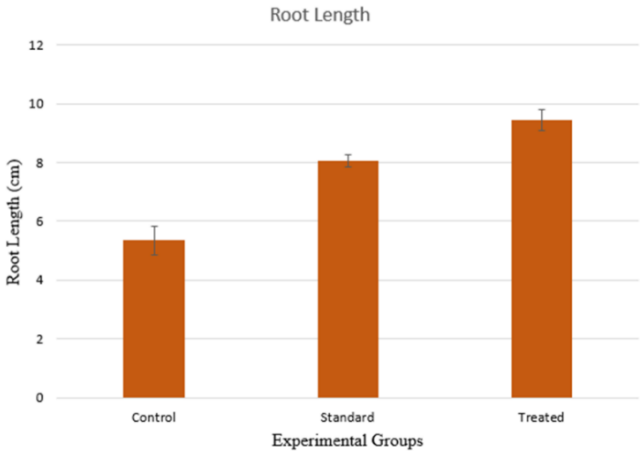

Figure 8. Mean graph showing average root length of the three group

Biochemical Assessment
Photosynthetic Pigments
Photosynthetic pigments play an important role in plant photosynthesis, growth and development. Acetone-based extraction was used to isolate chlorophyll a, chlorophyll b, and carotenoids. The extraction was followed by spectrophotometry to measure the concentration of pigments in leaves of *L. esculentum* using acetone as blank.
The photosynthetic pigments were highest in the treated group as compared to the control and standard groups as shown in Figure 9 and Table 2. The application of synthesized biofertilizer from *Chlorella vulgaris* in the soil had a positive impact on these pigments. Similar results were reported in Swiss chard when *Chlorella vulgaris* spray was applied to the soil (Hajnal-Jafari *et al.*, 2020) and in Red Russian Kale (Park *et al.*, 2022). Increased photosynthetic pigments demonstrated an increased photosynthetic rate.

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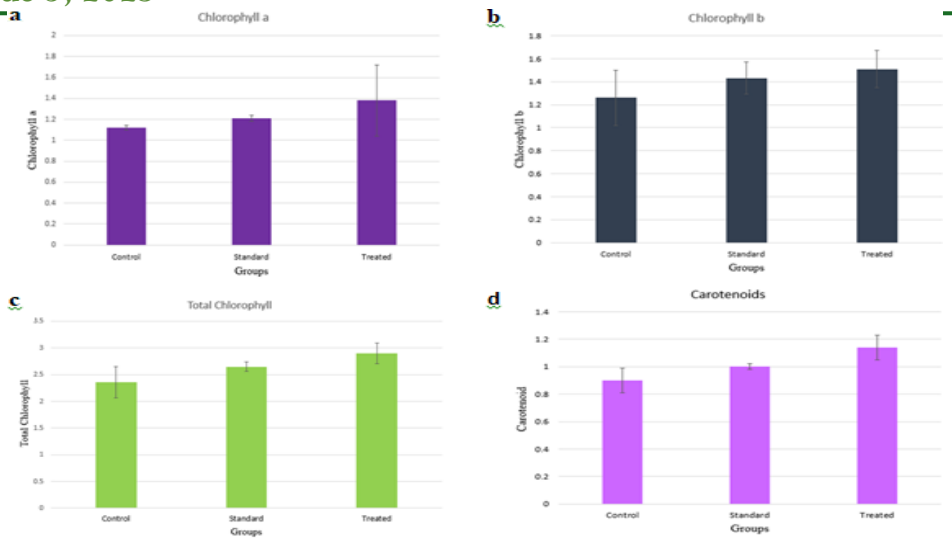

Figure 9. Mean graph off chlorophyll a (a), chlorophyll b (b), total chlorophyll (c) and carotenoids (d) present in leaves of three treatment groups; control, standard, and treatment of *L. esculentum*.

Proline Content

A valuable role is played by proline when plants are under stress. When plants are under stress, they produce proline which allows them to better cope with environmental stress. Proline was extracted from the leaves of the plants from each group. The absorbance was noted using a spectrophotometer and calculations were done.

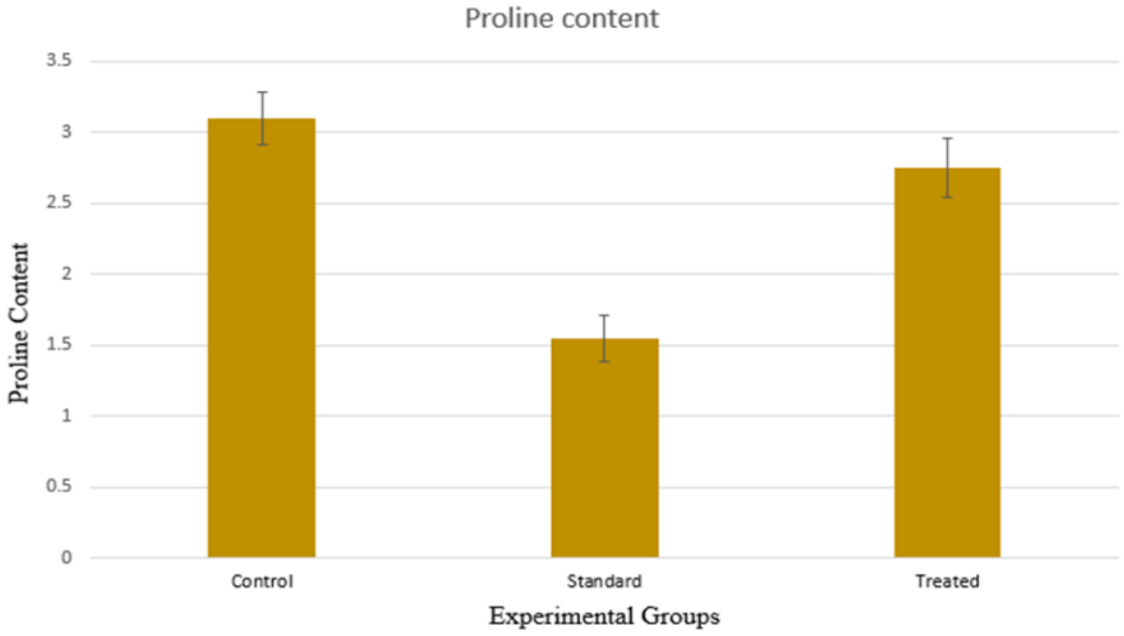

Figure 10. Proline content in leaves isolated from *L. esculentum*. Data shown is the average of plants from each group; control, standard and treated

The treated group's proline content was higher than the standard group but the control group had the highest proline content as shown in Table 2 and Figure 10.

High proline content indicates that plants were under stress. The plants were not provided with any kind of stress in this research so the highest proline content in the control group could be due to the excessive heat present in its environment (Kumar et al., 2013) (Sarkar et al., 2018). High proline content in treated also indicates that the Chlorella biofertilizer had a stressful impact on the plants which caused an increase in

proline content. More research needs to be done at this particular point as it is unknown whether the heat in the environment or the treatment was responsible for the stress.

Table 2. Biochemical parameters of control, standard, and treated groups of *L. esculentum* plants

Sr. No.	Parameter (mg/ml)	Control	Standard	Treated
1	Proline	3.1±0.19	1.55±0.16	2.75±0.21
2	Chlorophyll a	1.119±0.02	1.219±0.03	1.387±0.34
3	Chlorophyll b	1.26±0.24	1.43±0.14	1.51±0.16
4	Total Chlorophyll	2.36±0.29	2.65±0.09	2.90±0.19
5	Carotenoids	0.90±0.09	1.0±0.02	1.14±0.09

CONCLUSION

Overall, the morphological parameters showed that *Chlorella vulgaris* as a biofertilizer had a positive impact on the height, number of leaves, and root length of the plant as the most plant height, number of leaves, and root length were also observed in Swiss Chard, Maize, Wheat and Lettuce plant (Hajnal-Jafari *et al.*, 2016; Hajnal-Jafari *et al.*, 2020). Biochemical analysis of photosynthetic pigments also showed a positive impact on plants as the concentration of chlorophyll a, chlorophyll b, and carotenoids were higher in the treated group than in the other two groups also reported in Red Russian Kale (Park *et al.*, 2022). More photosynthetic pigments indicate a higher photosynthetic rate which means a positive impact on the overall growth and development of plants. In addition to the above results, Proline concentrations showed that the plants treated with synthesized biofertilizer were stressed. As there was no stress provided to the plant, excessive heat in the environment could be the reason for stress. The control group also showed high proline concentrations, so it is unclear if something in the environment caused the stress or if the biofertilizer was the cause which requires further research and field trials.

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