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ORIGINAL RESEARCH ARTICLE

Bioactivation effects of spirulina microalga and humic acid on safflower (*Carthamus tinctorius*)

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ABSTRACT

The *Carthamus tinctorius*, commonly known as safflower, is an annual plant with numerous branches and thorns from the Asteraceae family. For this experiment, three treatments were applied to the pots: humic acid, spirulina microalgae, and a mixture of both to analyze their bioactivation effects. These treatments were applied three times per week over the course of two weeks, with irrigation taking place every other day. The wet weight of the aerial parts of the harvested plants was measured and placed in liquid nitrogen, then stored in a freezer. Chlorophyll, carotenoids, proline, protein, phenol, antioxidants, and malondialdehyde were measured. The results show that several bioactivators significantly increased the growth, chlorophyll, carotenoids, protein, and proline of safflower plants when compared to the control. The three treatments reduced the antioxidant and malondialdehyde content significantly. In contrast to the control condition, the mixture of humic acid and spirulina microalgae, as well as humic acid alone, decreased the phenolic content. The findings demonstrated that humic acid and spirulina microalgae can serve as positive plant bioactivators for safflower by boosting its growth and reducing stress.

Keywords: Bioactivation; *Carthamus Tinctorius*; Humic Acid; Spirulina Microalgae; Safflower

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1. Introduction

Bioactivators have varied effects, both positive and negative. Positive plant bioactivators, also known as biostimulants, alter plant growth and development patterns by influencing gene expression and protein synthesis, ultimately affecting seed germination, seedling growth, and mature plant growth. The application of bioactivators also enhances macro and micronutrient uptake by acting as a natural chelating agent, leading to improved vegetative growth, as well as flower and fruit development[1].

Humic substances are natural, organic, polymeric compounds that include humic acid (HA) and flavonoid acid (FA). They originate from coal, soil, and organic substances like lignin, tannin, cellulose, and cutin. HA, which contains approximately 60% organic carbon (C), plays a vital role in soil microorganism growth. Besides carbon, humic acid (HA) also contains nitrogen (N), oxygen (O), hydrogen (H), and sulfur (S). Humic acid plays important roles in soil, such as enhancing the physical and biochemical activities by improving soil structure, texture, and water-holding capacity. Moreover, humic acid increases the availability of soil nutrients, including micronutrients, and facilitates the transportation of micronutrients to plants, thus reducing the

transfer of toxic heavy metals by their precipitation[2]. Humic acid also enhances crop growth by stimulating plant hormones such as auxin and cytokinin and increasing flower production. The primary benefit of HA is an improved rooting system that provides greater access to nutrients in the soil[3,4]. Additionally, HA darkens the soil to absorb more solar energy, improves soil health by strengthening beneficial microorganisms, and eliminating harmful ones[5,6]. The interaction of HA with plant leaves or the plant root may generate a mild stress signal, activating the hormonal and molecular pathways of the root to regulate responses to biotic and abiotic stresses[7].

Microalga consist of microscopic, eukaryotic algae and prokaryotic cyanobacteria, also known as photosynthetic bacteria[8]. Blue-green microalgae, specifically spirulina-based fertilizers, have proven effective in enhancing plant growth[9]. Spirulina is a filamentous, multicellular blue-green microalga that grows in water and can be easily harvested and processed. It contains significant amounts of macronutrients, micronutrients, amino acids, protein, vitamins, and other metabolites. This microalga is abundant in protein, 60%–70% essential fatty acids, betacarotene, and mineral elements. Spirulina grows in shallow waters with a salinity of about 7%–8% and high alkalinity, and its optimal survival pH is 9.23. In regard to micronutrients, spirulina includes vitamins B_{12}, A, and E, as well as minerals like iron, calcium, magnesium, manganese, potassium, and zinc[10,11].

Safflower (*Carthamus tinctorius*), which belongs to the Asteraceae family, grows in temperate climates, and is cultivated as an oilseed crop. Most commercially-grown safflower species can be planted in late winter or early spring[12]. The extracts from safflower plants are used in the pharmaceutical industry. The seed oil content ranges from 30% to 50%, depending on the variety, growth conditions, and environment[13]. This safflower plant is a common source of essential oil, food, and medicine in many regions worldwide[14]. The effectiveness of spirulina microalgae and HA as a fertilizer and their impact on the physiology of safflower plants (*C. tinctorius*) were examined to evaluate their bioactivation effects.

2. Materials and methods

Twenty pots were cultivated for six months in the biological greenhouse at Shahid Bahonar University of Kerman (30°283937' N, and 57°083363' E), Iran in 2022 year. Five pots were designated as the control group, while five pots were treated with HA under normal conditions, another five pots were treated with spirulina under normal conditions, and the final five pots received a mixture of humic acid and spirulina microalga treatment under normal conditions. The seeds in each pot were irrigated every other day until the soil was sufficiently moist for germination to occur. After sprouting, each pot was watered every other day with 150 mL of water. All pots were placed in the greenhouse under the same conditions. The soil in the pots consisted of sand (1/3 of total volume), clay (1/3 of total volume), and perlite and coco peat (1/3 of total volume). **Table 1** displays the soil analysis.

Table 1. Analysis of soil used for the experiments

Analysis	Value
EC ms/cm	2.50
pH of paste	8.13
TNV	57.20
OC (%)	0.86
OM (%)	1.48
TN (%)	0.09
P ava (ppm)	13.20
K ava (ppm)	325.00
Sand (%)	57.00
Silt (%)	31.80
Clay (%)	11.20
Cu ava (ppm)	1.14
Mn ava (ppm)	38.70
Fe ava (ppm)	6.40
Zn ava (ppm)	3.78

EC: electrical conductivity; TNV: total neutralising value; OC: organic carbon; OM: organic matter; TN: total nitrogen; P ava: available phosphorus; K ava: available potassium; Cu ava: available copper; Mn ava: available manganese; Fe ava: available iron; Zn ava: available zinc.

The solutions were initiated two weeks post-seed planting and applied thrice weekly for two weeks. For preparing the HA solution, 0.9 grams of HA powder was dissolved in 1.5 liters of water, and 150 milliliters of the solution was added to the humic acid treated pots thrice weekly. The spirulina algae solution was prepared by dissolving 20 grams of spirulina alga powder in 1.5 liters of water and applying the same treatment as described. To

provide a solution of HA and spirulina alga, dissolve 20 g of spirulina alga powder with 0.9 g of humic acid in 150 mL of solution and apply to the treatment pots three times weekly.

The plant in each pot was harvested by excising it from the soil level with a cutter and weighed using a scale with an accuracy of one-thousandth of a gram before submerging it in liquid nitrogen. Arnon's technique[15] was authorized to generate a solution for measuring chlorophyll and carotenoid. Chlorophyll a was measured at a wavelength of 663 nm, chlorophyll b at 645 nm, and carotenoid at 470 nm using the Spekol 1300 spectrophotometer for reading absorptions. The Equations (1), (2), and (3) were utilized to compute the quantities of chlorophyll a, b, and carotenoids, respectively. The data obtained was in µg/mL, which was then converted through unit conversion to present the amounts of chlorophyll and carotenoid in µg/g.

$$\text{Chlorophyll a} = [12/7\ (OD_{663}) - 2.69\ (OD_{645})]\ (\mu g/mL) \quad (1)$$

$$\text{Chlorophyll b} = [22/9\ (OD_{645}) - 4/68\ (OD_{663})]\ (\mu g/mL) \quad (2)$$

$$\text{Carotenoid} = [1{,}000\ (OD_{470}) - 1/82\ (\text{Chl a}) - 85/02\ (\text{Chl b})]/198\ (\mu g/mL) \quad (3)$$

Proline content was measured through the ninhydrin acid method, based on Bates *et al.*[16]. The plant's anti-oxidant potential was determined using the DPPH stable radical, following the method by Shimada *et al.*[17]. The Folin-Ciocalteu reagent was employed to determine the plant extract's phenolic compounds[18,19]. Lipid peroxidation was quantified by measuring the malondialdehyde content[20], while total protein content was measured via the Bradford method[21]. To generate the standard curve, initially, bovine albumin was prepared at concentrations of 50, 100, 200, and 400 mg/L. Thereafter, all test steps, as described earlier in unknown samples, were repeated on them using the reagent. A spectrophotometer was used to read the absorbance at a wavelength of 595 nm, and subsequently, the standard curve was plotted, and the equation of the line was calculated. The Biurea reagent was prepared by dissolving 0.1 g of Coomassie Brilliant Blue G250 in 50 mL of 95% ethanol for 1 h. Subsequently, 100 mL of 85% phosphoric acid was added drop by drop, and the total solution volume was adjusted to one liter with distilled water. Finally, the resulting solution was filtered using filter paper. Filter the resulting solution with filter paper. The equipment employed included a vortex (S0100 model, Labnet, USA), shaker (CR100 model, Finepcr, South Korea), stirrer (MS300HS model, MTops, South Korea), centrifuge (EBA20 model, Hettich, Germany), and spectrophotometer (UV-120-02 model, Shimadzu, Japan). Data analysis was conducted using a randomized complete block design with five replications for each treatment. The 20 pots were organized into four experimental blocks. Statistical analysis was performed through a one-way ANOVA using SPSS version 20 software, followed by Duncan's multiple range tests. The results are expressed as mean ± standard error.

3. Results and discussion

3.1 Fresh weight of safflower shoot

As demonstrated in **Figure 1**, the employment of spirulina microalga and HA treatment alongside the combination of HA and spirulina microalga resulted in a significant increase in the fresh weight of the safflower shoot. As the graph shows the treatments shoot fresh weight have increased significantly ($P = 0.027$) from 0.3 to 0.37 g (18% increase) when compared to the control.

3.2 Chlorophyll content of safflower leaves

As demonstrated in **Figure 2**, the use of treatments containing spirulina microalga, HA, and a combination of humic acid and spirulina microalga resulted in a significant increase ($P = 0.02$) in the chlorophyll a content of safflower leaves when compared to the control group. Among the treatments, spirulina microalga yielded the highest increase from 2.8 to 3.8 mg/g FW in chlorophyll content. But HA, and a combination of humic acid and spirulina microalga have increased from 2.8 to about 3.4 mg/g FW in chlorophyll content.

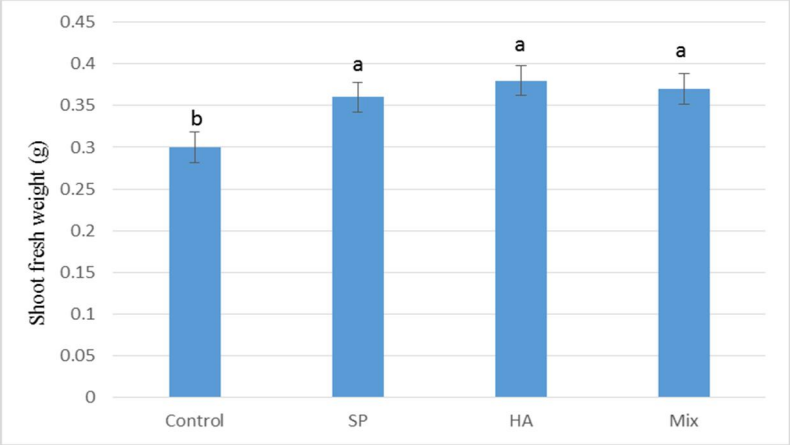

Figure 1. Effects of spirulina microalga and humic acid on the shoot fresh weight (g) of safflower shoot.

Note: Control, spirulina alga (SP), humic acid (HA) and mixture of spirulina alga and humic acid (Mix) from left to right. Different letters indicate significant differences at the $P < 0.05$ level. Error bars are based on standard error (± SE).

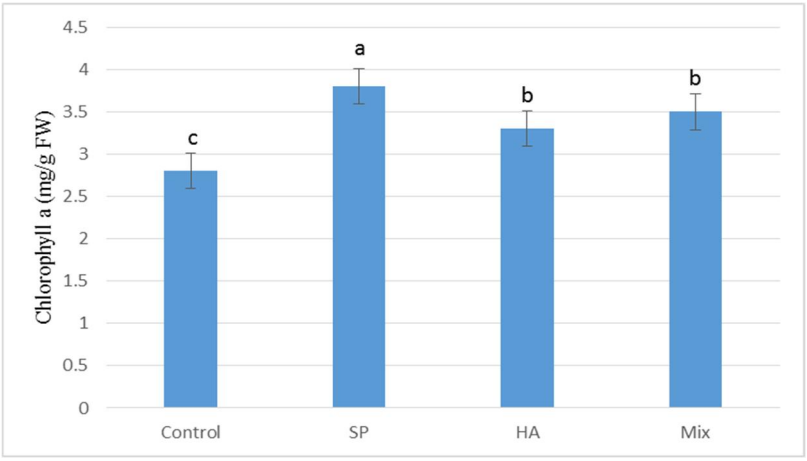

Figure 2. Effects of spirulina microalga and humic acid on the chlorophyll a content (mg/g FW) in safflower leaves.

Note: Control, spirulina alga (SP), humic acid (HA) and mixture of spirulina alga and humic acid (Mix) from left to right. Different letters indicate significant differences at the P < 0.05 level. Error bars are based on standard error (± SE).

3.3 Carotenoid content of safflower leaves

The treatments using spirulina microalga, HA, and a mixture of humic acid and spirulina microalgae led to a significant increase in carotenoid content in safflower leaves when compared to the control. Of these treatments, spirulina microalgae was significantly better than HA ($P = 0.03$). The carotenoid content increased from 3.5 to 4.8 mg/g FW. But HA and a combination of humic acid and spirulina microalga have increased from 3.5 to about 4.2 mg/g FW in carotenoid content (**Figure 3**).

3.4 Antioxidant content of safflower leaves

The impact of spirulina microalga and HA on the total antioxidant content of safflower leaves is depicted in **Figure 4**. Treatment with spirulina microalga, HA, and a combination of HA and spirulina microalga significantly ($P = 0.03$) decrease the relative antioxidant content of safflower leaves when compared to the control. The total antioxidant content of control reduces from 8.2 to about 7.2 µmole TE/g FW.

3.5 Proline content of safflower leaves

As presented in **Figure 5**, only the treatment of spirulina microalgae, significantly ($P = 0.03$) increased the proline content of the safflower leaves from 1.5 to 1.8 µmole/g FW when compared to the control. It is related to high protein (about 70%) and amino acid of spirulina microalgae.

3.6 Phenolic content of safflower leaves

The impact of spirulina microalga and HA on

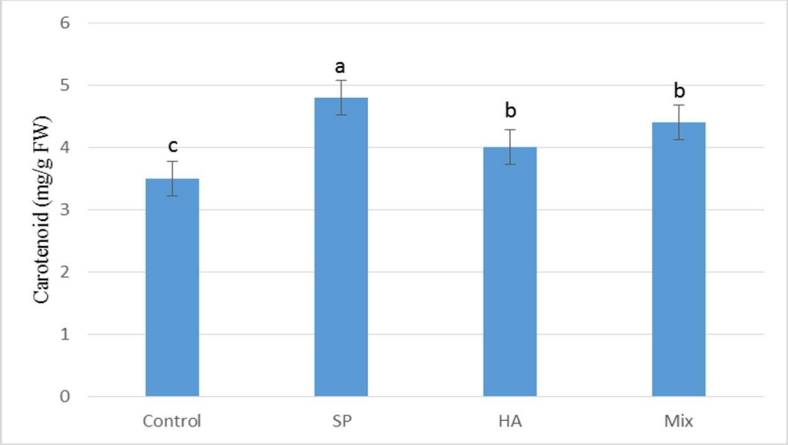

Figure 3. Effects of spirulina microalga and humic acid on the carotenoid content (mg/g FW) in safflower leaves.

Note: Control, spirulina algae (SP), humic acid (HA) and mixture of spirulina alga and humic acid (Mix) from left to right. Different letters indicate significant differences at the $P < 0.05$ level. Error bars are based on standard error (± SE).

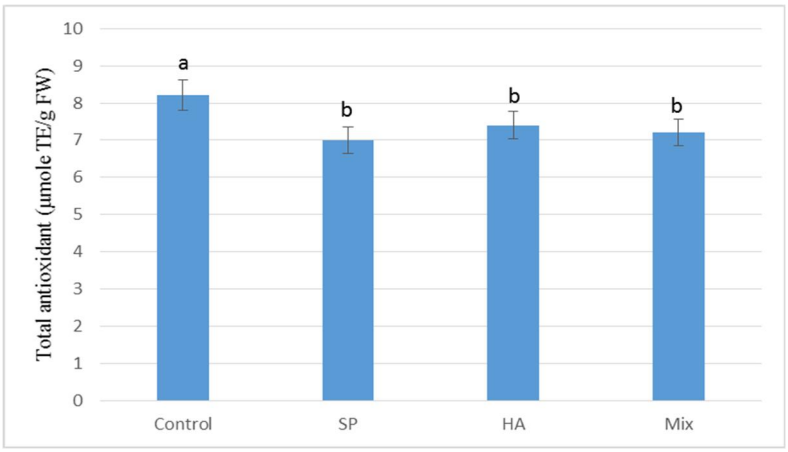

Figure 4. Effects of spirulina microalga and humic acid on total antioxidant (µmole TE/g FW) in safflower leaves.

Note: Control, spirulina alga (SP), humic acid (HA) and mixture of spirulina alga and humic acid (Mix) from left to right. Different letters indicate significant differences at the $P < 0.05$ level. Error bars are based on standard error (± SE).

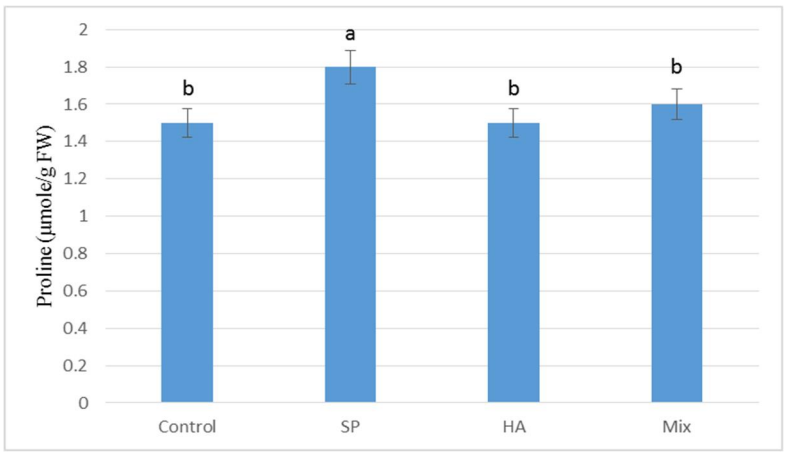

Figure 5. Effects of spirulina microalga and humic acid on proline (µmole/g FW) in safflower leaves.

Note: Control, spirulina alga (SP), humic acid (HA) and mixture of spirulina alga and humic acid (Mix) from left to right. Different letters indicate significant differences at the $P < 0.05$ level. Error bars are based on standard error (± SE).

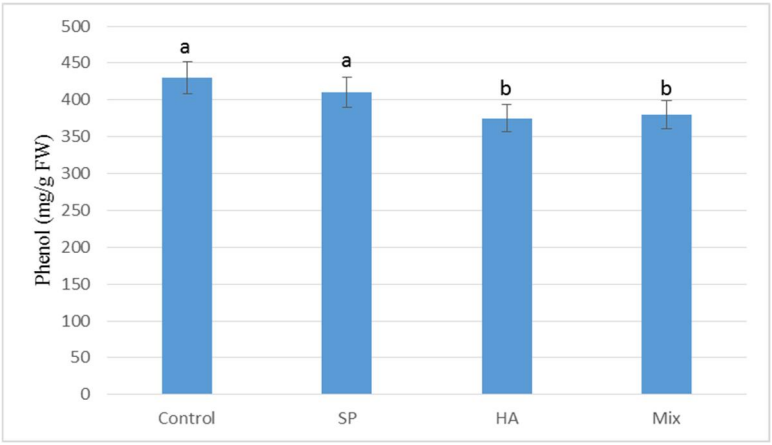

Figure 6. Effects of spirulina microalga and humic acid on phenol (mg/g FW) in safflower leaves.

Note: Control, spirulina alga (SP), humic acid (HA) and mixture of spirulina alga and humic acid (Mix) from left to right. Different letters indicate significant differences at the $P < 0.05$ level. Error bars are based on standard error (± SE).

the phenolic content of safflower plant leaves is displayed in **Figure 6**. The utilization of spirulina microalga did not result in a significant ($P = 0.1$) difference in the phenolic content of safflower leaves when compared to the control. However, HA and a combination of HA and spirulina microalga decreased the phenolic content of safflower leaves from 430 to about 377 mg/g FW when compared to the control. Phenolic compounds regulate crucial physiological functions in plants to provide resistance against various biotic and abiotic stress conditions.

3.7 Protein content of safflower leaves

As shown in **Figure 7**, the incorporation of spirulina microalga, humic acid, and a humic acid-spirulina microalga mixture led to a significant increase ($P < 0.02$) in safflower leaf protein content from 44 to about 57 mg/g FW when compared to the control. The findings demonstrate that spirulina microalga and HA enhance amino acid synthesis via increased nitrogen absorption, ultimately promoting protein production.

3.8 Malondialdehyde content in safflower leaves

As illustrated in **Figure 8**, the utilization of spirulina microalga, HA, and a combination of humic acid and spirulina microalga significantly reduced ($P = 0.03$) the malondialdehyde content in safflower leaves from 8.8 to about 7.7 µmole/g FW when compared to the control. Malondialdehyde level is a well-known indicator of oxidative stress, therefore suggesting that the treatments have lessened the stress on the safflower. Spirulina microalga and HA may have a crucial function in safe-

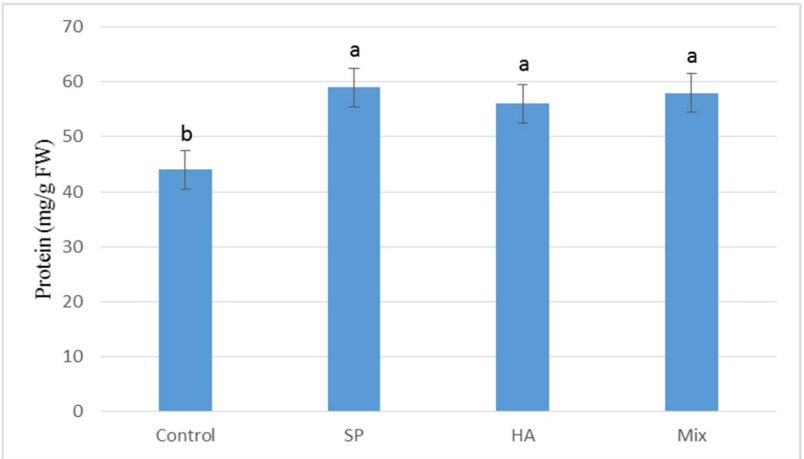

Figure 7. Effects of spirulina microalga and humic acid on protein (mg/g FW) in safflower leaves.

Note: Control, spirulina alga (SP), humic acid (HA) and mixture of spirulina alga and humic acid (Mix) from left to right. Different letters indicate significant differences at the $P < 0.05$ level. Error bars are based on standard error (± SE).

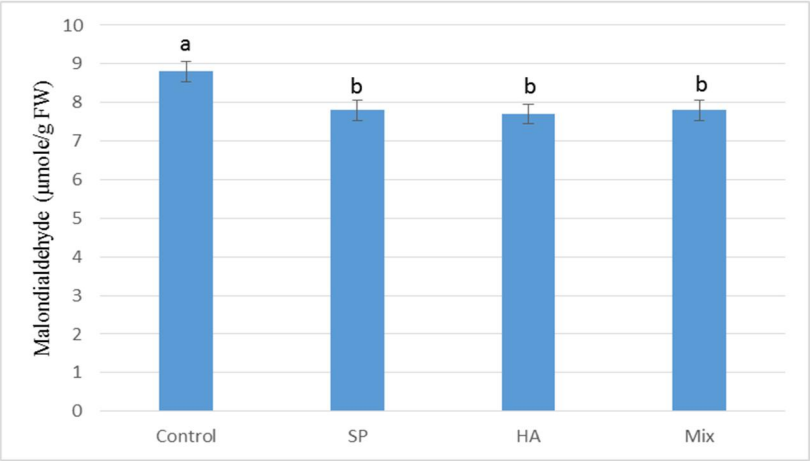

Figure 8. Effects of spirulina microalga and humic acid on the malondialdehyde (µmole/g FW) in safflower leaves.
Note: Control, spirulina alga (SP), humic acid (HA) and mixture of spirulina alga and humic acid (Mix) from left to right. Different letters indicate significant differences at the $P < 0.05$ level. Error bars are based on standard error (± SE).

guarding the photosynthetic system and cellular membranes against both regular and drought-induced stress in safflower, as they have the potential to reduce malondialdehyde levels[22].

Safflower is an important oilseed plant with commercial value and is known by different names around the world, including American saffron, dyer saffron, wild saffron, and Zaferan[23]. According to the results, several experimented bioactivators significantly increased safflower's growth, chlorophyll a, carotenoids, protein, and proline levels when compared to the control. However, the antioxidant content remained unchanged, but the malondialdehyde content was significantly reduced for all three treatments. The phenolic content was reduced with the application of HA or a mixture of humic acid when compared to the control.

Stimulation of the activity of the H-ATPase in the cell membrane suggests that the effects induced by HA are not limited to the root structure but may also impact critical biochemical pathways. This is because the electrochemical gradient across the plasma membrane is the primary facilitator of nutrient uptake. The effects of humic acid on root secretion, primary, and secondary metabolism vary depending on the environmental conditions and plant type[24]. However, it generally promotes plant rooting by increasing nutrient accessibility in the soil[4]. The use of spirulina-based fertilizers, a type of blue-green microalgae, has been shown to effectively enhance plant growth[9]. Spirulina biofertilization enhances growth, photosynthetic capacity, and yield according to research by Shedeed *et al.*[25]. *S. platensis* contains bioactive compounds such as plant hormones, including auxin and cytokinin, as well as macro and micronutrients which promote plant growth[26]. Generally, plant growth stimulators have been found in this article and other studies to significantly affect the remobilization of nutrients from soil to the plant and subsequent metabolic and synthesis changes[27,28]. Environmental growth stimulators, such as inorganic, organic, and biological factors, serve as positive plant bioactivators that aid plant survival under both normal and stressful environmental conditions[29]. Proper environmental management is essential in promoting their effectiveness.

Spirulina alga, which contain nitrogen, exhibited the highest growth rates on seedlings[30]. Chlorophyll is a crucial pigment in photosynthesis and reflects the state of plant growth. Changes in the ratio of chlorophyll a and carotenoids lead to variations in the performance of photosynthesis. Changes in chlorophyll content in leaves could potentially result from the biosynthesis of chlorophyll by stimulators. Based on the data obtained, the use of spirulina treatment in canola seedlings resulted in a significant increase in the content of chlorophyll when compared to the control. Additionally, the content of chlorophyll a did not significantly differ between the stressed and non-stressed conditions treated with spirulina, indicating that spirulina enhances photosynthetic performance[31,32].

In many plant species, proline levels increase under various conditions[33]. Proline plays a crucial role in plant stress tolerance[34]. Furthermore,

elevating proline levels assists the rapeseed plant in maintaining its water balance and enhancing its stress tolerance[35].

The function of antioxidants and antioxidant enzymes is affected by environmental conditions. Each treatment in low and high stress conditions results in an increased antioxidant content compared to the same treatment in no-stress conditions. Treatment with spirulina alga, under non-stress conditions, has significantly reduced antioxidant content compared to the control, indicating that spirulina treatment acts as an antioxidant regulator in the plant[22,36].

When plants are exposed to environmental changes, such as stress or fertilizer, their protein levels can increase[37]. This study's findings show that spirulina microalgae and HA, two bioactivators, enhance amino acid synthesis by increasing nitrogen absorption, ultimately promoting protein production.

Malondialdehyde content is commonly used to measure lipid peroxidation in plant tissue, which increases during oxidative stress. The safflower's malondialdehyde activity ranged from 5.85 to 8.90 µmole/g of fresh weight[38], consistent with the present study. The decrease in malondialdehyde content from treatments with spirulina microalga, HA, and a combination of humic acid and spirulina microalga indicates that these three bioactivators help to induce non-stress conditions.

4. Conclusion

Environmental growth stimulators include inorganic, organic and biological factors can help plant to survive in normal conditions by environmental management. The present study observed significant growth increases, as well as elevated levels of chlorophyll a, carotenoids, protein, and proline in safflower due to the application of bioactivators when compared to the control. The treatments reduced the antioxidant and malondialdehyde content, significantly. The use of HA and a mixture of humic acid and spirulina microalga reduced the phenolic content in comparison to the control condition. So, the use of these treatments can improve the efficiency of photosynthesis in safflower. Safflower requires growth activators for optimal growth, such as the spirulina alga activator which is high in nitrogen and HA. The research demonstrates that spirulina alga promotes the rapid growth of seedlings in comparison to the control group. Furthermore, the spirulina treatment and HA act as antioxidant regulators in the plants. The results indicate that HA and spirulina microalgae can act as positive plant bioactivators for safflower by promoting growth. Effective management of different fertilizers and microbiomes is necessary for the protection of the Earth's natural resources, and both scientists and engineers must take a key role in this management in soil environments.

Conflict of interest

The authors declare that they have no conflict of interest.

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