

Original Research Article

Unraveling the Biostimulant Impacts on Growth Traits and Biochemical Composition of *Amaranthus dubius*

ABSTRACT

The study aimed to evaluate the effects of different biostimulants on the growth, yield, biochemical properties, and economic viability of *Amaranthus dubius* and identify the most effective biostimulant or combinations for improving crop performance and profitability while providing an eco-friendly alternative to traditional practices. The experiment was conducted at Chozhapandi, Mannargudi district, Tamil Nadu (latitude 10°36'22.7" N, longitude 79°29'02.0" E & 29 MSL) from October to November 2021 and March to April 2022 and was arranged in a randomized block design over two seasons with 11 treatments involving single or combined applications of Moringa leaf extract (MLE), seaweed extract, salicylic acid (SA), and humic acid (HA). Growth parameters (plant height, leaf area index etc.), biochemical properties (chlorophyll a, b, total chlorophyll, carotenoids etc.), fresh leaf yield, and economic analysis (net returns, benefit-cost ratio) were assessed. Statistical analysis was performed using one-factor analysis with OPSTAT software at a 5% significance level against F-test values. Among the treatments, T11 (MLE 3% + seaweed extract 8% + SA 200 ppm + HA 0.4%) showed the highest plant height (56.8 cm), leaf area index (2.48), chlorophyll a (3.173 mg/g), chlorophyll b (0.752 mg/g), total chlorophyll (3.387 mg/g), carotenoids (1.391 mg/g), and fresh leaf yield (2.67 kg/plot), with net returns of ₹10,200. T10 (MLE 2% + seaweed extract 6% + SA 100 ppm + HA 0.2%) was the second-best treatment. The control (T9) exhibited the lowest total chlorophyll (1.423 mg/g) and carotenoids (0.550 mg/g). T2 (MLE 3%) and T8 (HA 0.4%) were the most cost-effective treatments, with benefit-cost ratios of 2.44 and 2.30, respectively. Logistic regression confirmed significant associations between biostimulant combinations and improved crop performance. Biostimulants, particularly in combination, are promising tools for enhancing the yield, nutritional quality, and profitability of *Amaranthus dubius*, offering an environmentally sustainable alternative to conventional practices.

Keywords: *Biostimulant. Amaranthus dubius. Moringa leaf extract. Seaweed extract. Ascophyllum nodosum. Salicylic acid. Humic acid*

1. INTRODUCTION

In Tamil Nadu and Kerala, *Amaranthus dubius* is the principal green leafy vegetable. It is cultivated as a grain crop, for leafy green (Hoidal et al., 2019), and for ornamentation (Artemyeva, 2021; Ruth 2021). Since the growth cycle is short, responsive to fertilizers, the yield is high (Bang et al., 2021), easy to grow (Ruth et al., 2021), and adaptable to different

conditions (Hoang et al., 2019), farmers want to cultivate Amaranthus; however, the crop is short-lived and gets damage and wilts quickly because of short shelf life (Nighita & Mathew, 2019) and vulnerability to pests (Seni, 2018). Vegetable farmers are increasingly applied plant biostimulants to increase crop productivity, nutritional efficiency, quality, stress resistance, and environmental safety.

Biostimulants are of biological origin and are large groups of biochemical compounds or microorganisms that can stimulate the biological processes to improve nutrient uptake, tolerance to stress factors, yield quality and environmental health. Some of these might reduce the deleterious effects of chemical fertilizers (Calvo et al., 2014; Posmyk and Szafranska, 2016; Van Oosten et al., 2017). This mainly includes increasing applications in agriculture and more controlled agricultural settings to promote yield and quality of crops for vegetables, thereby contributing to agriculture that is based on sustainability.

Moringa Leaf Extract (MLE) is one of the most promising biostimulants, which is found in nutrient-rich, phytohormone-dense, and bioactive *Moringa oleifera* leaves, enhancing growth and increasing tolerance to stress and improving crop yield and quality (Yuniati et al., 2023). MLE has been shown to enhance seed germination, increase root growth, and stimulate plant vigor under normal and stress conditions (Khan et al., 2022; Yuniati et al., 2023). An application of MLE could be an eco-friendly alternative to synthetic fertilizers (Mashamaite et al., 2022). Foliar application of MLE with a 2% concentration has been shown to improve most of the growth parameters such as plant height, leaf number, and leaf size compared to the control treatments in cabbage (Hoque, 2020). Merwad (2017) showed that moringa leaf extract improves pea plant characters such growth, yield and quality. Foliar application of 3% aqueous MLE has been shown to enhance growth, biological and grain yields, and biochemical parameters in wheat. This improvement is attributed to the presence of ascorbic acid and phenolics in MLE (Mashamaite et al., 2022). In pea (*Pisum sativum*), studies indicate that foliar spraying with MLE concentrations ranging from 1% to 4% enhances protein content and nutrient uptake, with 3% MLE being particularly effective (Merwad, 2017). Application of 3% MLE on rocket (*Eruca sativa*) has been found to increase chlorophyll, carotenoid, protein, sugar, phenol, and ascorbic acid content in rocket leaves, indicating improved nutritional quality (Abdallah, 2013).

Seaweed extract of *Ascophyllum nodosum*, a brown alga, enhances plant growth, root development, and tolerance against drought, salinity, and pathogen stresses. Rich in cytokinins and auxins that stimulate plant growth regulators, this enhances nutrient uptake and improves yield (Ali et al., 2019). The bio-stimulant being a natural alternative to synthetic fertilizers ensures healthier crops (Kumari et al., 2023). *Ascophyllum nodosum* was found to increase tomato and sweet pepper production in tropical environments (Ali et al., 2019). Studies have demonstrated that *A. nodosum* extract at a 0.5% concentration can induce defense mechanisms and promote growth in tomato and sweet pepper plants in tropical environments (Ali et al., 2019). Foliar application of *Ascophyllum nodosum* extract has been shown to enhance strawberry (*Fragaria × ananassa*) plant health and yield. In a study evaluating its effects on powdery mildew progression, concentrations of 0.1%, 0.2%, and 0.3% were tested. The 0.2% concentration was most effective, reducing spore germination by 75% and decreasing disease incidence by 37.2% under field conditions. This treatment also increased total phenolic and flavonoid content, indicating an induced defense response in the plants (Bajpai et al., 2019).

Salicylic Acid (SA) is an essential signaling molecule in plants which confers increased tolerance towards several biotic and abiotic stresses, thereby enabling a plant to thrive and grow more in challenging environmental conditions (Jayakannan et al., 2015; Emamverdian et al., 2020). It increases salt tolerance in barley (Fayez & Bazaid, 2014) and drought tolerance

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in faba beans (Abdelaal, 2015). It increases the growth, yield, and quality of vegetables. It increases the available nutrients and their uptake for garden thyme as studied by Haghighi et al. (2014). Salicylic acid at 100 ppm improved growth parameters, yield, and fruit quality. Combined applications (e.g., SA + Vitamin E) further enhanced early and total yields in tomato (Mady, 2009). In soybeans, foliar application of SA at 100 and 200 ppm increased chlorophyll content, reduced lipid peroxidation, and enhanced seed yields significantly (Kuchlan & Kuchlan, 2021).

Humic acid (HA) is an organic substance resulting from the degradation of plant and animal residues, which has been acknowledged as a powerful biostimulant in agriculture. It has been naturally beneficial in promoting plant growth, nutrient absorption, and yield enhancement, as well as in pollution tolerance of various crop species. This treatment significantly increased root biomass and improved tomato and melon seedling growth, resulting in a 13.1 cm increase in root growth at 36 days (Dunoyer et al., 2022) with 0.2% humic acid. Shuqin (2008) reported a decrease in nitrate accumulation and an increase in the activity of nitrate reductase that improves nitrogen metabolism in the plants when the plants were treated with 0.4% humic acid in cabbage, lettuce and spinach.

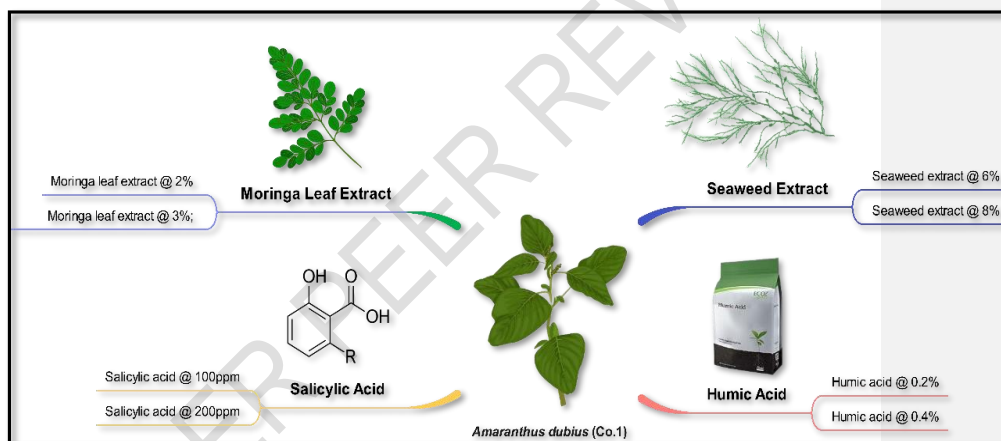


Fig.1 Schematic representation of treatments applied to *Amaranthus dubius* (Co-1). The study involved the application of Moringa leaf extract (at 2% and 3%), Seaweed extract (at 6% and 8%), Salicylic acid (at 100 ppm and 200 ppm), and Humic acid (at 0.2% and 0.4%).

The impact of Moringa leaf extract, SA, humic acid, and seaweed extract and also their interactivity (Fig.1) on *Amaranthus* plant growth, yield, biochemical contents, and shelf life is discussed in the present study.

2. METHODOLOGY

Two seasons of field experiments were conducted in a shade net house at Chozhapandi, Mannargudi district, Tamil Nadu (latitude 10°36'22.7" N, longitude 79°29'02.0" E & 29 MSL) from October to November 2021 and March to April 2022 using randomized block design (RBD) with three replications. The treatment details used in field experiments are: T1-Moringa leaf extract @ 2%; T2-Moringa leaf extract @ 3%; T3-Seaweed extract @ 6%; T4-Seaweed

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extract @ 8%; T5-Salicylic acid @ 100ppm; T6-Salicylic acid @ 200ppm; T7-Humic acid @ 0.2%; T8-Humic acid @ 0.4%; T9-Control; T10- Combination (T1+T3+T5+T7); T11-Combination (T2+T4+T6+T8).

Biostimulant concentrations selected were based on potential efficacy shown in previous studies in enhancing plant growth, yield and biochemical properties. The phytohormone content present in Moringa leaf extract (MLE) at 2% and 3% has been proved effective in improving growth parameters in crops like cabbage and peas (Hoque, 2020; Merwad, 2017). Auxins and cytokinins in seaweed extract at 6% and 8% enhance stress tolerance and yield in tomatoes and strawberries (Ali et al., 2019; Bajpai et al., 2019). Chlorophyll content and stress tolerance improvement in faba beans and soybeans is found from SA at 100 ppm and 200 ppm (Abdelaal, 2015; Kuchlan & Kuchlan, 2021). At concentrations of 0.2 and 0.4%, humic acid significantly increases root growth and nutrient uptake by leafy vegetables such as spinach (Shuqin, 2008; Dunoyer et al., 2022). At the same time, combination treatments that exploit synergistic effects of these biostimulants for maximum plant performance and minimal risks of phytotoxicity (Toscano et al., 2021).

2.1 Seed material and biostimulants

Amaranthus seed was of the CO1 (TNAU) variety (D. Thilokchand Seeds, Chennai), Seaweed extract BIO VITA (PI Industries, India), and Salicylic acid (Sisco Research Laboratories Pvt. Ltd., India), and Humicil (Corteva Agrisciences, India) were used in the study. Additionally, Moringa Leaf Extract (MLE) was prepared in laboratory (Fig.2) by dehydrating and grinding Moringa leaves and soft parts and mixing with distilled water. This mixture was autoclaved at 121 °C, 15 psi for 20 min, filtered and cooled to 4 °C, was centrifuged and the supernatant was collected as 100% MLE, as per Rama Rao (1990) and Yasmeen (2011) method. The extract was diluted in distilled water to 2% and 3% concentrations. The application of field foliar sprays including all the biostimulants was performed on the 7th and 14th days after seeding.

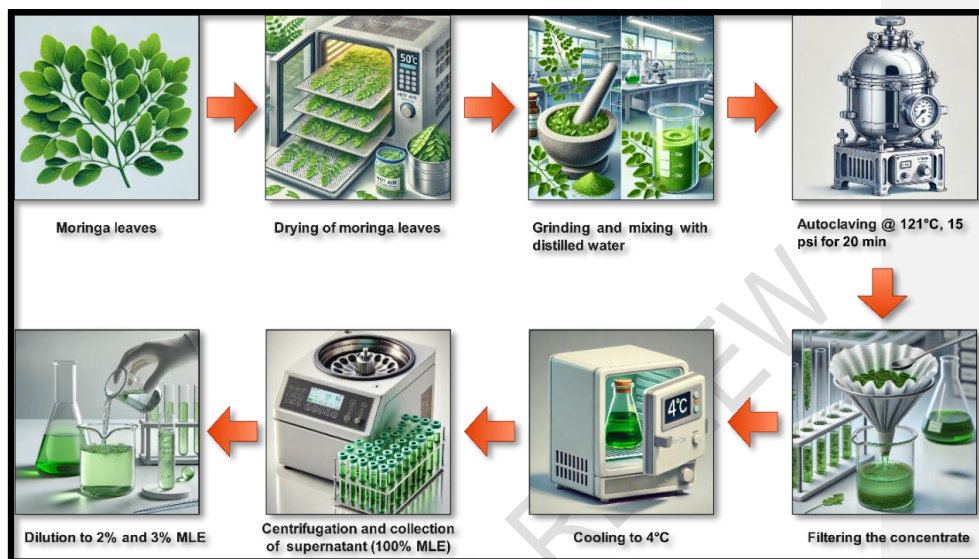


Fig. 2 Illustration of the preparation of Moringa Leaf Extract (MLE) and dilution to desired concentration

2.2 Observations

2.2.1 Growth and yield parameters: Plant height (cm), number of leaves per plant, leaf area (cm²), number of branches per plant, root length (cm), stem girth (cm), leaf/stem ratio, fresh weight of leaves per plant (g/plant), and yield per plot (g) were measured.

2.2.2 Physiological parameters

2.2.2.1 Relative water content (%): The leaves, shoots, and roots were washed and weighed immediately after harvest (Fresh Weight- FW). The plants were dried in a hot air oven at 80°C for 24 h to find out dry weight (Dry Weight- DW). Relative water content was calculated from weights obtained as described by Barr (1962).

$$\text{Water Content (WC)} = \left(\frac{\text{FW} - \text{DW}}{\text{FW}} \right) \times 100$$

2.2.2.2 Physiological loss in weight (PLW): The weight of the plants lost cumulatively was recorded and expressed as percentage physiological loss of weight (Srivastava and Tandon, 1968). PLW was measured on days 2, 4, and 8 of storage for all treatments.

$$\text{Physiological Loss in Weight (PLW)} = \left(\frac{P_0 - P_1}{P_0} \right) \times 100$$

Where P_0 - Initial Weight; P_1 - Final Weight

2.2.2.3 Membrane stability Index (MSI): The Membrane Stability Index (MSI) was determined by modifying the method of Khongwir et al. (2015). Leaf samples (5g) were placed in 100 ml

of double-distilled water for each treatment. One set was refrigerated at 4°C for 30 minutes and their electrical conductivity (C1) was measured using a Hanna Instruments E.C. & pH meter (model: HI5222). The other set, after a 15-minute 100°C water bath, had their electrical conductivity (C2) measured. The MSI of the samples was then calculated using the below formula,

$$\text{MSI \%} = \left(1 - \frac{C_1}{C_2}\right) \times 100$$

2.2.2.4 pH: After MSI analysis, the samples (C1) of all treatments are macerated and filtered in a beaker to measure the pH using the E.C & pH meter (Hanna instruments model: HI5222).

2.2.3 Biochemical parameters

2.2.3.1 Total Phenolic Content: A modified Singleton and Rossi (1965) method was used, using Folin-Ciocalteu reagent to determine the total phenolic content of *Amaranthus* leaf extracts. Leaf samples (1 g) were dried and powdered, and dried and powdered leaf samples (1 g) were extracted with 10 ml methanol for 30 minutes in an ultrasonic bath, filtered through Whatman filter paper No. 1. A reaction mixture was prepared by mixing 0.5 mL of the extract with 2.5 mL of Folin-Ciocalteu reagent (diluted 1: 10) and incubating at room temperature for 5 minutes. Then after 30 minutes, the mixture was kept in the dark with 2 mL of 7.5% sodium carbonate solution. UV-Vis spectrophotometer was used to measure absorbance at 765 nm, methanol as the blank. A calibration curve was prepared with gallic acid, and expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW) to quantify total phenolic content.

2.2.3.2 Oxalic Acid Content: The amount of oxalic acid in *Amaranthus dubius* leaves was determined by the permanganometric titration method. Fresh leaf samples (40 g) were washed with distilled water and boiled in 400 mL of distilled water for 3.5 min and cooled. Thereafter, the boiled leaf extract was transferred into a 100 mL volumetric flask (2.5 mL) and made up to 100 milliliters (mL) with distilled water and mixed properly. A volume of 10 mL diluted extract was placed into the conical flask, where 5 mL 1 M sulphuric acid (H_2SO_4) was subsequently added. Then, 0.3 ml of sample solution (0.1 ml twice) was added to the mixture; the mixture was titrated against standard 0.01N KMnO_4 till the production of faint pink color developed, which indicates the end-point. The calculation of oxalic acid content was done by using the formulae:

Normality of oxalic acid

$$V_{\text{KMnO}_4} \times N_{\text{KMnO}_4} = V_{\text{H}_2\text{C}_2\text{O}_4} \times N_{\text{H}_2\text{C}_2\text{O}_4}$$

Mass of oxalic acid

$$\text{Mass of oxalic acid (mg)} = \frac{V_{\text{H}_2\text{C}_2\text{O}_4} \times N_{\text{H}_2\text{C}_2\text{O}_4}}{\text{Equivalent weight of oxalic acid}}$$

Oxalic acid content

$$\text{Oxalic acid content (mg/g)} = \frac{\text{Mass of oxalic acid (mg)}}{\text{Weight of sample (g)}}$$

Equivalent weight of oxalic acid is 63 (Lestari & Dewi, 2020)

2.2.3.3 *Chlorophylls*: Chlorophyll a, chlorophyll b, and total chlorophyll content were determined using a UV-Vis spectrophotometer. A leaf sample (0.1 g) was ground with the help of a mortar and pestle and dissolved in 10ml of 80% acetone. The extract was filtered with a Whatman Filter Paper No. 3, then absorbance of the extract was measured using the UV-Vis spectrophotometer at 663 nm and 646 nm. Chlorophyll a, chlorophyll b and total chlorophyll content were calculated using the below formula:

$$\text{Chlorophyll a (mg/g)} = (12.3 \times A_{663} - 0.86 \times A_{646}) \times \frac{V}{1000 \times W}$$

$$\text{Chlorophyll b (mg/g)} = (19.3 \times A_{646} - 3.6 \times A_{663}) \times \frac{V}{1000 \times W}$$

$$\text{Total Chlorophyll} = (17.3 \times A_{646} + 7.18 \times A_{663}) \times \frac{V}{1000 \times W}$$

Where,

V= volume of chlorophyll extract in 80% acetone

W= fresh weight of leaf sample (Harbone *et al.*, 1987)

Where A_{663} , and A_{646} represent the absorbance values at their respective wavelengths, V is the extract volume (i.e., 10 mL), W is the sample weight (i.e., 0.1 g), and 1000 is a factor for unit conversion to mg/g. The coefficients constituting (12.3, 0.86, 19.3, 3.6, 17.3 and 7.18) are empirical constants obtained from standard calibration studies to estimate chlorophyll a, chlorophyll b and total chlorophyll with great accuracy.

Indeed, the method takes into consideration the possible interaction between the chlorophyll pigments by applying specific absorbance coefficients. All steps are performed under controlled conditions (e.g., under dim light) to minimize photooxidation of the pigments. This approach provides a consistent measurement of chlorophyll a, chlorophyll b, and total chlorophyll in the leaf samples.

2.2.3.4 *Total Carotenoids*: Carotenoids were estimated as per the extraction procedure for estimating chlorophyll. Fresh leaf tissue (0.1 g) was macerated by mortar and pestle, and 10 mL of 80% acetone was added to extract the pigments. The extract was passed through Whatman filter paper No. 3 in order to record the absorbance of the chlorophyll's filtrate at 470 nm (carotenoids), 663 nm (chlorophyll a) and 646 nm (chlorophyll b) using a UV-Vis spectrophotometer. The blank for calibration was the solvent (80% acetone). Total carotenoids were calculated based on the following formula:

$$\text{Total Carotenoids (mg/g)} = \frac{(1000 \times A_{470}) - (2.05 \times A_{663}) - (114.8 \times A_{646}) \times V}{1000 \times W} \quad (\text{Lichtenthaler \& Wellburn, 1983}).$$

where A_{470} , A_{663} , and A_{646} represent the absorbance values at their respective wavelengths, V is the extract volume (e.g., 10 mL), W is the sample weight (e.g., 0.1 g), and 1000 is a factor for unit conversion to mg/g. The coefficients (2.05 and 114.8) are empirically determined constants that correct for interference from chlorophyll a and chlorophyll b at 470 nm. The denominator value 198 is another empirical constant derived from calibration studies to ensure accurate estimation.

Since chlorophyll pigments could interfere with measurements in the assay, the procedure is utilized to minimize any potential interference from these pigments and all steps are

performed in dim light to avoid photooxidation of carotenoids. This approach allows for an accurate quantification of total carotenoids visible in the leaf extracts.

2.2.4 Benefit-Cost Ratio (B:C ratio)

The Benefit-Cost Ratio (B:C ratio) was calculated to evaluate the economic feasibility of biostimulant treatments. The cost of cultivation (Rs.) for each treatment was recorded, and the yield (kg) was multiplied by the market price (Rs. 30/kg) to calculate the gross income:

$$\text{Gross Income (Rs.)} = \text{Yield (kg)} \times \text{Market Price (Rs./kg)}$$

The B:C ratio was then determined as:

$$\text{B:C Ratio} = \frac{\text{Gross Income (Rs.)}}{\text{Cost of Cultivation (Rs.)}}$$

This ratio was used to compare the profitability of the treatments, where values >1 indicate profitability.

2.3 STATISTICAL ANALYSIS

The statistical evaluation of the datasets used one-factor analysis utilizing OPSTAT software obtaining relevant results and at a 5% significance level against values of F-test.

3. RESULTS AND DISCUSSION

3.1 Influence of biostimulants and their impact on growth parameters

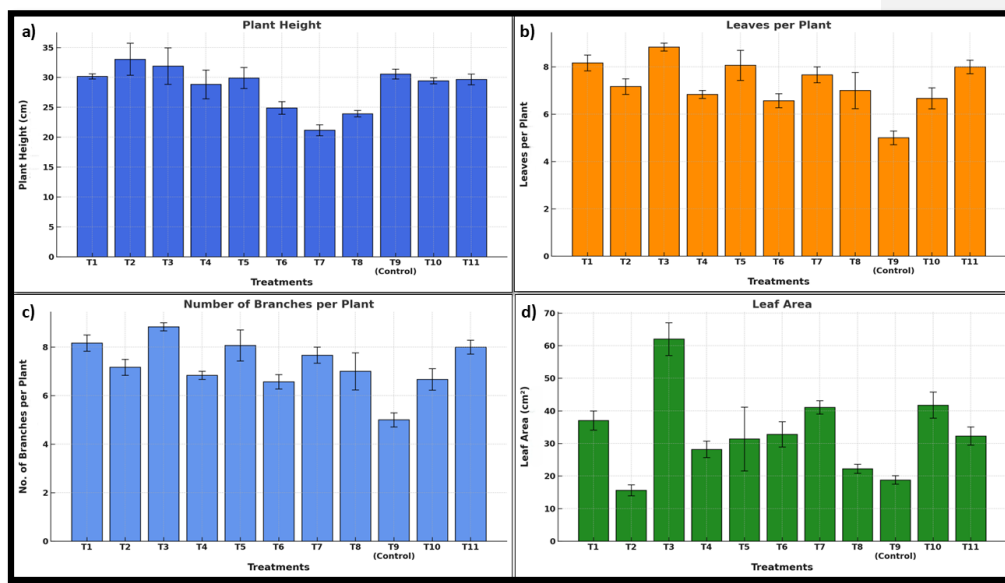


Fig. 3 Effect of various treatments (T1 to T11, including control) on growth parameters of *Amaranthus* plants. (a) Plant height (cm), (b) Number of leaves per plant, (c) Number of branches per plant, and (d) Leaf area (cm²). Data are presented as mean ± standard error (SE) for each treatment.

All growth parameters were significantly different in this experiment. Table 1 illustrates the effect of biostimulants on growth parameters. Foliar spray of moringa leaf extract enhanced the plant height. The data indicates that the maximum plant height was recorded in moringa leaf extract @ 3% (T2) with 33.03 cm (Fig. 3a) Foliar spray of seaweed extract at 6% (T3) showed the highest number of leaves (8.83) (Fig.3b), branches (8.83) (Fig.3c), and leaf area (62.03 cm²) (Fig.3d). Humic acid @ 0.2% (T7) increased stem girth (5.33 mm) (Fig.4a) and root length (9.23 cm) (Fig.4b).

Table 1 Effect of biostimulants on growth parameters of *Amaranthus*

Treatment	Plant Height (cm)	Leaves per Plant	Branches per Plant	Leaf Area (cm ²)	Stem Girth (mm)	Root Length (cm)
T1	30.16 ± 0.44 ^b	8.17 ± 0.33 ^a	8.16 ± 0.33 ^a	37.05 ± 2.91 ^b	4.13 ± 0.48 ^b	8.60 ± 0.36 ^a
T2	33.03 ± 2.68 ^{a*}	7.17 ± 0.33 ^b	7.17 ± 0.33 ^b	15.62 ± 1.69 ^f	3.37 ± 0.62 ^d	7.47 ± 0.58 ^c
T3	31.90 ± 3.06 ^a	8.83 ± 0.17 ^{a*}	8.83 ± 0.17 ^{a*}	62.03 ± 5.00 ^{a*}	4.23 ± 0.12 ^b	8.47 ± 0.32 ^b

T4	28.83 ± 2.40 ^c	6.83 ± 0.17 ^b	6.83 ± 0.17 ^b	28.18 ± 2.53 ^c	4.00 ± 0.50 ^b	7.93 ± 0.35 ^c
T5	29.90 ± 1.76 ^b	8.07 ± 0.64 ^a	8.07 ± 0.64 ^a	31.37 ± 9.78 ^c	3.03 ± 0.69 ^d	5.77 ± 0.14 ^d
T6	24.90 ± 1.07 ^d	6.57 ± 0.30 ^b	6.57 ± 0.30 ^b	32.80 ± 3.88 ^c	2.83 ± 0.24 ^e	9.17 ± 0.93 ^a
T7	21.16 ± 0.93 ^e	7.67 ± 0.33 ^b	7.67 ± 0.33 ^b	41.10 ± 2.04 ^b	5.33 ± 0.88 ^a	9.23 ± 0.20 ^a
T8	23.93 ± 0.52 ^d	7.00 ± 0.76 ^b	7.00 ± 0.76 ^b	22.20 ± 1.40 ^e	2.80 ± 0.15 ^e	5.87 ± 0.19 ^d
T9	30.56 ± 0.81 ^b	5.00 ± 0.29 ^c	5.00 ± 0.29 ^c	18.80 ± 1.30 ^f	2.03 ± 0.26 ^f	5.33 ± 0.17 ^d
T10	29.43 ± 0.52 ^b	6.67 ± 0.44 ^b	6.67 ± 0.44 ^b	41.78 ± 4.03 ^b	4.80 ± 0.17 ^a	5.57 ± 0.14 ^d
T11	29.66 ± 0.88 ^b	8.00 ± 0.29 ^a	8.00 ± 0.29 ^a	32.30 ± 2.80 ^c	3.63 ± 0.19 ^c	8.47 ± 0.09 ^b

Values in the table are averages of two seasons. Values are presented as mean ± standard error (S.E.).

Values followed by the same letter within a column are not significantly different at $p < 0.05$. Asterisks

(*) denote treatments with the best result for a given parameter.

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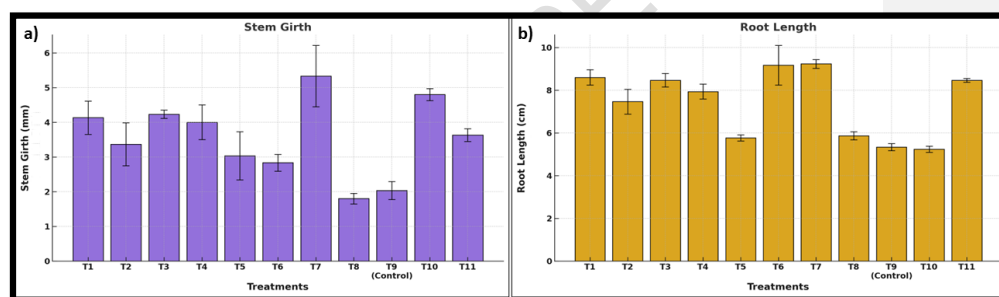


Fig. 4 Influence of various treatments (T1 to T11, including control) on stem girth and root length of *Amaranthus* plants. (a) Stem girth (cm) and (b) Root length (cm) under different treatments. Data are shown as mean ± standard error (SE).

It has been discovered that 4% MLE foliar spray on California Capsicum, the phytochemicals like zeatin, carotenoids, ascorbates, phenols, potassium, and calcium in the extract helped wonder pepper seedlings grow better (Hala et al. 2017; Chattha et al. 2018). Seaweed extract spray on saline-irrigated amaranth plants reduced salt stress and increased leaf number, possibly due to cytokinins such as trans-zeatin riboside (Saravanan et al. 2003). Humic acid, which lacks rooting hormones, prevents their oxidation and prolongs IAA activity, affecting the acid growth mechanism and the H^+ pump, according to Canellas et al. (2002) and Zandonadi et al. (2007), making root growth faster after application.

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3.2 Influence of biostimulants and their impact on yield parameters

Table 2 and Fig.5 depicts the impact of biostimulants on yield parameters. The leaf/stem ratio was highest with 0.2% Humic acid at 1.07 (T7) (Fig.5a), while 0.90 with 2% moringa leaf extract at T1 was the second. Karakurt et al. (2009) reported that foliar spray of humic compounds enhances growth, yield, and quality across species. Ugur et al. (2013) also reported that an increase in cress, rocket, and sorrel yields was brought about by 0.8% humic

acid. Humic acid's functional groups attach to K⁺, Ca⁺, and Mg²⁺ to aid *Gerbera jamesonii* L. in nutrient uptake (Nikbakht et al. 2008). It also activates bacteria in the soil responsible for producing auxin, cytokinin, and gibberellins that enhance plant growth (Rahni, 2012).

Table 2 Influence of biostimulants and their impact on yield parameters of Amaranthus

Treatment	Leaf/Stem Ratio	Fresh Weight of Leaves per Plant (g)	Yield per Plot (kg)
T1	0.90 ± 0.09 ^b	2.30 ± 0.22 ^a	4.97 ± 0.77 ^d
T2	0.72 ± 0.01 ^c	0.95 ± 0.06 ^c	8.95 ± 1.80 ^{a*}
T3	0.57 ± 0.01 ^d	2.70 ± 0.07 ^{a*}	6.05 ± 0.98 ^b
T4	0.64 ± 0.01 ^c	1.33 ± 0.11 ^b	4.31 ± 1.48 ^d
T5	0.61 ± 0.01 ^c	1.38 ± 0.14 ^b	4.46 ± 0.70 ^d
T6	0.70 ± 0.01 ^c	1.02 ± 0.10 ^c	4.91 ± 1.08 ^d
T7	1.07 ± 0.12 ^{a*}	1.67 ± 0.13 ^b	5.03 ± 0.35 ^c
T8	0.58 ± 0.01 ^d	0.69 ± 0.02 ^d	6.91 ± 1.81 ^b
T9	0.30 ± 0.00 ^e	1.21 ± 0.17 ^c	4.06 ± 0.72 ^d
T10	0.45 ± 0.01 ^d	1.98 ± 0.17 ^b	6.57 ± 0.64 ^b
T11	0.53 ± 0.01 ^d	2.02 ± 0.04 ^a	5.42 ± 1.22 ^c

Values in the table are averages of two seasons. Values are presented as mean ± standard error (S.E.).

Values followed by the same letter within a column are not significantly different at $p < 0.05$. Asterisks

(*) denote treatments with the best result for a given parameter.

The maximum fresh weight of leaves per plant was 2.70 g (Fig.5b) with 6% seaweed extract (T3), followed by 2.30g with 2% moringa leaf extract (T1) and 2.02g with a combination treatment (T11). Seaweed and moringa leaf extracts were more beneficial than other treatments, supporting the findings of Chrysargyris et al. (2018) on lettuce. Seaweed extracts boost plant nutrition and growth hormone uptake (Gupta et al. 2021). Best yield per plot (8.95 kg) was 3% moringa leaf extract (T2) (Fig.5c). Zeatin, a cytokinin hormone, in moringa leaf extract boosts cabbage growth, yield, and nutrient content, according to Hoque et al. (2020). Moringa leaves are rich in nutrients that boost agricultural output (Busani et al. 2011).

Commented [MHM6]: Why T2, provided highest yield? Treatment 2 (T2) provided highest plant height only, in case of other traits this treatment showed low values. If I compare T2 and T3, T2 revealed height plant height 33.03 ± 2.68^a , where T3 gave close values 31.90 ± 3.06^a . At the same time, (T3) showed the highest number of leaves (8.83) (Fig.3b), branches (8.83) (Fig.3c), and leaf area (62.03 cm^2) (Fig.3d), apart from these height traits values Stem Girth (mm), Root Length (cm), Leaf/Stem Ratio, Fresh Weight of Leaves per Plant (g) exhibited higher value compare to T2. But in case of yield T2 approximately 2 kg more yield than T3, weakening the creditability of the research. I think you may have made a mistake at the time of calculation. If not, please explain the reason behind it, otherwise this article would fail to prove its authenticity.

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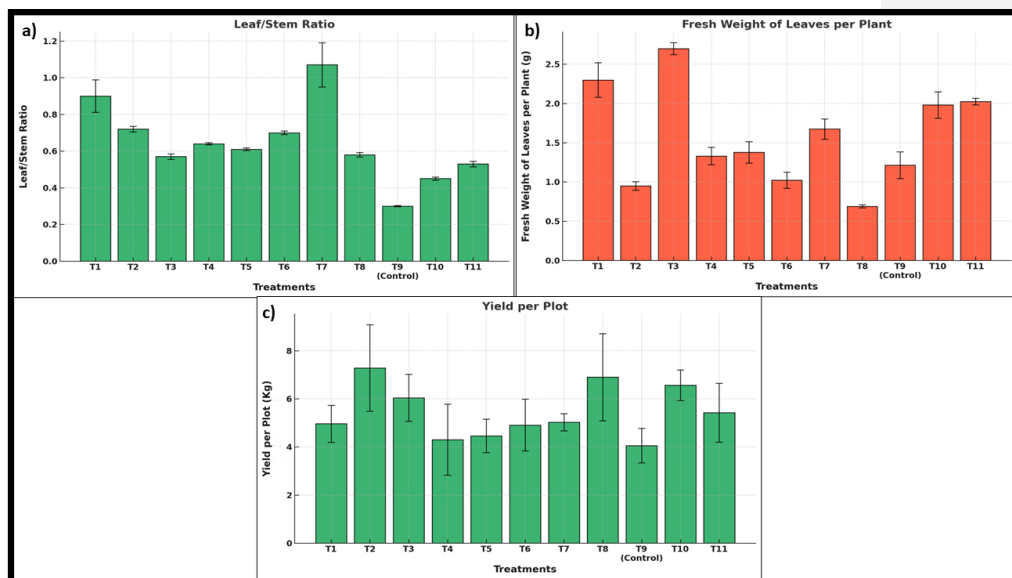


Fig. 5 Impact of various treatments (T1 to T11, including control) on yield-related parameters of *Amaranthus* plants. (a) Leaf-to-stem ratio, (b) Fresh weight of leaves per plant (g), and (c) Yield per plot (kg). Data are expressed as mean $\pm$ standard error (SE).

3.3 Influence of biostimulants and their impact on physiological and biochemical parameters

Biostimulants considerably affect physiological and biochemical characteristics (Table 3 & Table 4). MLE, Seaweed extract, Salicylic acid, and Humic acid (T10) had a greater relative water content (93.33%) (Fig.6a), whereas T9-92.83% and other treatments were equal. Merwad (2015) discovered that spinach (*Spinacia oleracea* L.) plants treated with aqueous and ethanolic Moringa leaf extracts (1, 2, 3, and 4%) had more moisture. Higher dry matter in these plants. Seed soaking in SA and foliar spraying with MLE raised plant dry weight of *Phaseolus vulgaris* L. to the highest (Rady et al. 2015). Haghighi and Najafi (2020) observed that 500 mg of humic acid increases, chlorophyll, fresh weight, and dry weight at 25% field capacity. *A. nodosum* biostimulants may increase leaf antioxidant activity (Pacheco et al. 2019). Hamza et al. (2001) demonstrated that high antioxidant levels increase shoot and root growth, preserve leaf moisture, and reduce disease incidence under optimal growing circumstances and environmental stress. Humic acid retains 80–90% water, which may have resulted in higher relative water content (Rahni et al. 2012).

Table 3. Influence of biostimulants and their impact on Relative Water Content, Physiological Loss in Weight, Membrane Stability Index & Total Phenol Content

Treatment	Relative Water Content (%)	Physiological Loss in Weight (%)	Membrane Stability Index (%)	Total Phenol Content (mg/100g)
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T1	90.59 ± 0.07 ^b	16.23 ± 1.12 ^c	16.28 ± 0.09 ^d	165.78 ± 1.06 ^{a*}
T2	89.98 ± 0.21 ^c	12.12 ± 0.72 ^e	23.05 ± 0.09 ^c	101.55 ± 0.61 ^d
T3	92.38 ± 0.04 ^a	31.94 ± 1.08 ^a	38.11 ± 0.15 ^b	109.41 ± 0.60 ^c
T4	90.38 ± 0.12 ^b	15.06 ± 0.99 ^d	44.92 ± 0.15 ^a	119.68 ± 0.47 ^b
T5	91.17 ± 0.10 ^b	20.95 ± 0.69 ^b	38.90 ± 0.15 ^b	134.97 ± 0.57 ^b
T6	90.18 ± 0.17 ^c	14.09 ± 0.99 ^d	26.49 ± 0.15 ^c	72.72 ± 0.92 ^e
T7	92.04 ± 0.02 ^a	28.91 ± 1.09 ^a	12.09 ± 0.09 ^d	140.95 ± 0.78 ^b
T8	92.06 ± 0.07 ^a	28.02 ± 1.00 ^a	16.58 ± 0.09 ^d	70.64 ± 0.60 ^e
T9	92.83 ± 0.16 ^a	34.65 ± 0.93 ^{a*}	19.51 ± 0.09 ^c	135.58 ± 0.27 ^b
T10	93.33 ± 0.21 ^{a*}	30.95 ± 1.14 ^a	42.63 ± 0.10 ^a	140.25 ± 0.45 ^b
T11	92.27 ± 0.01 ^a	31.49 ± 1.31 ^a	47.60 ± 0.15 ^{a*}	130.74 ± 0.15 ^c

Values in the table are averages of two seasons. Values are presented as mean ± standard error (S.E.). Values followed by the same letter within a column are not significantly different at $p < 0.05$. Asterisks (*) denote treatments with the best result for a given parameter.

Moringa leaf extract @ 3% (T2) caused the lowest physiological weight reduction (12.12%) (Fig.6b). Moringa leaf extract prevents *Amaranthus dubius* leaf respiration, minimizing physiological weight loss and preserving produce freshness.

MLE, Seaweed extract, Salicylic acid, and Humic acid (T11) exhibited the highest membrane stability index (47.60%) (Fig. 6c). Batool et al. (2020) observed that foliar moringa leaf extracts at 3% increased seedling membrane stability index by 45% above the control group. Due to their mineral nutrient and phytohormone concentration, MLE components may have improved chlorophyll fluorescence and osmoprotectant production by stimulating leaf photosynthetic pigment biosynthesis. Translocating moringa leaf extract osmoprotectants such soluble sugars and free proline to plant vitals could have also raised their levels. Higher photosynthetic activity due to MLE might have also enhanced membrane integrity, increasing cell health and turgidity (Abd El-Mageed et al. 2017).

Moringa leaf extract @ 2% (T1) had the highest total phenolic content (165.78 mg /100g) (Fig.6d). This is consistent with Toscano et al. (2021), who found that MLE increased *Brassica* phenolic content. MLE's vitamins, minerals, amino acids, and antioxidants possibly enhanced phenolic content (Hanafy, 2017).

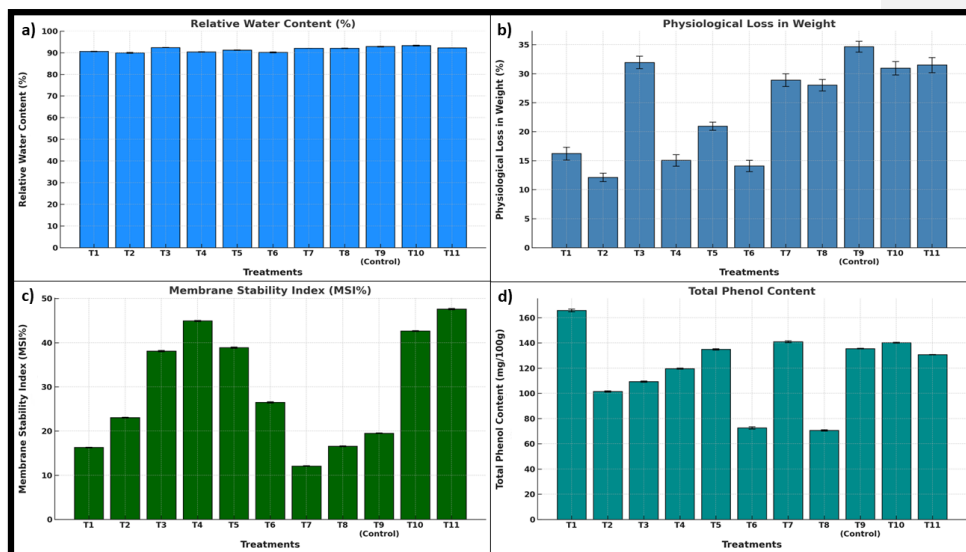


Fig. 6 Effect of various treatments (T1 to T11, including control) on physiological and biochemical parameters of *Amaranthus* plants. (a) Relative water content (%), (b) Physiological loss in weight (%), (c) Membrane stability index (MSI %), and (d) Total phenol content (mg/100g). Data are presented as mean $\pm$ standard error (SE).

The treatments had a significant impact on the chlorophyll content in *Amaranthus*, enhancing both chlorophyll 'a', chlorophyll 'b', and total chlorophyll levels compared to the control (T9).

Highest chlorophyll a was in the treatment T11 (3.173 mg/g) (Fig.7a) in experience with moringa leaf extract + seaweed extract + salicylic acid + humic acid. This combination is effective for the biosynthesis of photosynthetic pigments, possibly due to the combined effects of phytohormones, antioxidants, and mobilization of nutrients. Treatment T10 (2.857 mg/g) at lower concentration of them bio-stimulants was the second-best treatment in enhancing chlorophyll a content. The control (T9) reported significantly lower chlorophyll a content (1.427 mg/g) compared to the treatments, highlights the role of biostimulants in improving photosynthetic efficiency. Supporting evidence from research demonstrates the efficacy of biostimulants such as seaweed and moringa extracts to increase chlorophyll a levels through nutrient uptake and photosynthetic efficiency (Soliman et al., 2020).

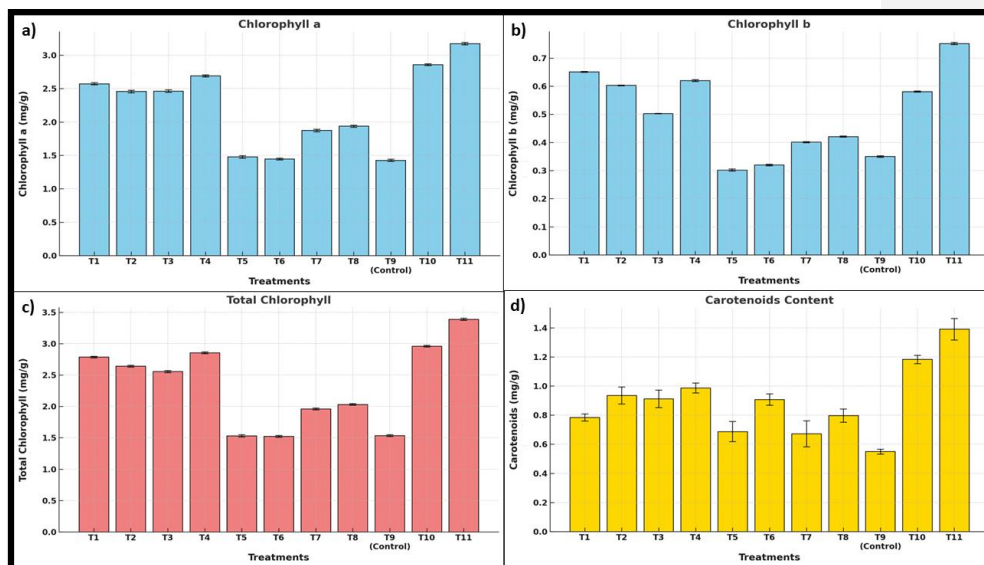


Fig. 7 Effect of various treatments (T1 to T11, including control) on photosynthetic pigment content in *Amaranthus* plants. (a) Chlorophyll a (mg/g), (b) Chlorophyll b (mg/g), (c) Total chlorophyll (mg/g), and (d) Carotenoids content (mg/g). Data are represented as mean $\pm$ standard error (SE)

For chlorophyll b, the highest record was being treated under T11 (0.752 mg/g) (Fig.7b) while the second was T10 (0.581 mg/g). Chlorophyll b is important for broadening the light absorption spectrum and increasing the energy transfer efficiency. The lowest was obtained by the control treatment (T9) (0.350 mg/g), which highlights the influence of treatments involving biostimulants on maintaining pigments. Moringa leaf extract (MLE) is established to improve chlorophyll levels, owing to the high levels of phytohormones and antioxidants in it (Abdalla, 2014).

A similar trend was observed in the total chlorophyll content where T11 with the value of 3.387 mg/g and T10 with the value of 2.960 mg/g enhanced other treatments (Fig.7c). These outcomes highlight the possibility of interaction of various biostimulants regulating combined pigment synthesis in plants. The control treatment, T9 had the least total chlorophyll content (1.534 mg/g) which corresponded to least activity of photosynthesis in plants which were not treated. As reported in earlier literature, sea weed and humic acid used as plant growth factor has a positive impact on chlorophyll biosynthesis specially when the stress conditions are prevailed (Kavipriya & Boominathan, 2018). Total chlorophyll content in the T11 and T10 can also be explained on the basis of multiple biostimulants working synergistically for biosynthesis and non-degradability of pigments as discussed by Orlov et al. (2005). It is postulated that MLE, seaweed extract, salicylic acid and humic acid may influence membrane stability and oxidation thereby stabilizing chlorophyll molecules. This accounts for the variation in total chlorophyll values across the treatments in relation to the control treatment.

Table 4. Influence of biostimulants and their impact on Chlorophyll a, Chlorophyll b, Total Chlorophyll, Carotenoids, pH & Oxalic Acid Content

Treatment	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total Chlorophyll (mg/g)	Carotenoids (mg/g)	pH	Oxalic Acid Content (mg/g)
T1	2.573 ± 0.015 ^a	0.651 ± 0.002 ^a	2.785 ± 0.013 ^e	0.784 ± 0.024 ^c	4.49 ± 0.007 ^e	0.0303 ± 0.0000 ^c
T2	2.457 ± 0.020 ^b	0.603 ± 0.001 ^b	2.642 ± 0.016 ^e	0.935 ± 0.059 ^b	4.97 ± 0.017 ^b	0.0254 ± 0.0020 ^e
T3	2.463 ± 0.020 ^b	0.503 ± 0.001 ^c	2.554 ± 0.016 ^d	0.913 ± 0.060 ^b	5.37 ± 0.067 ^{a*}	0.0275 ± 0.0010 ^d
T4	2.690 ± 0.015 ^a	0.620 ± 0.003 ^a	2.855 ± 0.015 ^d	0.987 ± 0.034 ^b	4.38 ± 0.009 ^e	0.0314 ± 0.0000 ^b
T5	1.477 ± 0.019 ^e	0.302 ± 0.004 ^e	1.531 ± 0.019 ^c	0.687 ± 0.069 ^c	4.90 ± 0.010 ^c	0.0329 ± 0.0020 ^b
T6	1.447 ± 0.015 ^e	0.320 ± 0.003 ^e	1.523 ± 0.014 ^b	0.908 ± 0.039 ^b	4.62 ± 0.010 ^d	0.0342 ± 0.0010 ^a
T7	1.873 ± 0.018 ^d	0.401 ± 0.002 ^d	1.960 ± 0.016 ^b	0.672 ± 0.089 ^c	5.32 ± 0.040 ^a	0.0237 ± 0.0000 ^e
T8	1.937 ± 0.015 ^d	0.421 ± 0.002 ^d	2.032 ± 0.013 ^b	0.797 ± 0.046 ^c	5.16 ± 0.020 ^b	0.0268 ± 0.0010 ^d
T9	1.427 ± 0.017 ^e	0.350 ± 0.003 ^e	1.534 ± 0.014 ^a	0.550 ± 0.017 ^d	4.54 ± 0.010 ^e	0.0368 ± 0.0010 ^a
T10	2.857 ± 0.015 ^b	0.581 ± 0.002 ^b	2.960 ± 0.013 ^a	1.183 ± 0.029 ^a	4.80 ± 0.010 ^c	0.0218 ± 0.0000 ^{e*}
T11	3.173 ± 0.018 ^{a*}	0.752 ± 0.004 ^{a*}	3.387 ± 0.017 ^{a*}	1.391 ± 0.074 ^{a*}	4.75 ± 0.007 ^c	0.0283 ± 0.0020 ^c

Values in the table are averages of two seasons. Values are presented as mean ± standard error (S.E.). Values followed by the same letter within a column are not significantly different at $p < 0.05$. Asterisks (*) denote treatments with the best result for a given parameter.

Humic acid also may have increased chlorophyll production. Humic acid speeds respiration and photosynthesis by changing mitochondrial and chloroplast processes (Orlov et al. 2005). Hidangmayum and Sharma (2017) found that 0.55% seaweed extract produced the maximum carotenoid content in onion. Additionally, micronutrients like Cu, Mn, and Zn are cofactors of antioxidant enzymes and play a role in metabolic processes (Grotz and Guerinot, 2006).

Carotenoids, essential for photoprotection and antioxidant activity, showed a similar trend, with T11 achieving the highest levels (1.391 mg/g) (Fig.7d). The second-best treatment was T10 (1.183 mg/g). The control (T9) had the lowest carotenoid content (0.550 mg/g), indicating the inability of untreated plants to enhance their photoprotective mechanisms. Biostimulants like seaweed and humic acid have been shown to boost carotenoid content, improving stress tolerance and enhancing overall plant health (Sherinlincy et al., 2020).

The pH of plant tissues is an important indicator of metabolic processes, nutrient uptake, and health of the plant as a whole. The highest leaf pH was recorded in T3 (seaweed extract at 6%) with a pH of 5.37 compared to the control (T9-4.54) (Fig.8a). Higher pH recorded in T3 reveals that seaweed extract helps in keeping a balanced cellular environment, which is necessary for enzyme activities and nutrient absorption. Higher pH will increase the uptake of nutrients like nitrogen, phosphorus, and potassium that are essential for plant growth and yield. Moreover, seaweed extract contains bioactive compounds and minerals, which may help to regulate pH by neutralizing acidity in plant tissues (Whapham et al., 1993). Maintenance of

an optimal pH level also strengthens the plant to cope with environmental stresses, hence promoting overall plant health. The results indicate that the T3 treatment optimally adjusts the physiological conditions in *Amaranthus* that assure maximum nutrient uptake and metabolic processes, which in turn increase the yields and quality.

Oxalic acid is one of the important anti-nutritional factors in *Amaranthus*, which reduces the bioavailability of essential minerals such as calcium and magnesium (Shyfa & Dewi, 2021). The treatments in this study showed a significant influence on oxalic acid content under different biostimulant applications. The lowest amount of oxalic acid was found in the treatment T10 at 0.0218 mg/g (Fig.8b), which proves that the combination of these biostimulants helps to reduce the accumulation of oxalic acid in plant tissues. This reduction is very vital in improving the nutritional quality of the leaves to make them safer for consumption.

Compared to the control (T9-0.0368 mg/g), T10 has a reduced content of oxalic acid by approximately 41%. The other treatments that also exhibited a lower oxalic acid content are T7, which was a seaweed extract @ 8%, and T2, which was 2% MLE at 0.0237 mg/g and 0.0254 mg/g, respectively. The reduction of oxalic acid is due to the facts that the biostimulants applied to the plants causes a higher metabolic rate and efficient nutrient adsorption as well as better photosynthesis, stress tolerance absorptions give rise to a decreased concentration of oxalates as a stress product (Lestari & Dewi, 2020).

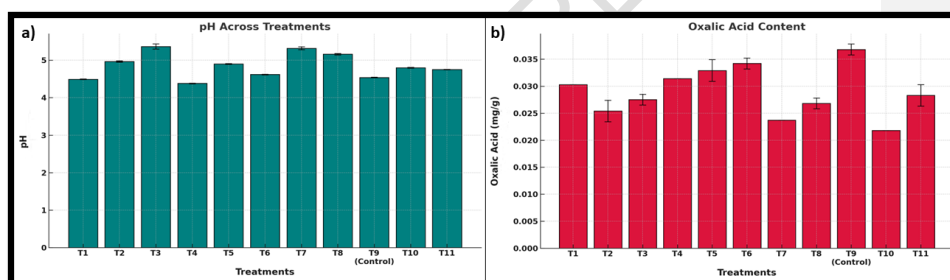


Fig. 8 Influence of various treatments (T1 to T11, including control) on pH and oxalic acid content in *Amaranthus* plants. (a) pH levels across treatments and (b) Oxalic acid content (mg/g). Data are shown as mean $\pm$ standard error (SE).

The reduction in oxalic acid is particularly important because high oxalic acid levels can form insoluble complexes with calcium, reducing its bioavailability and leading to calcium deficiency in humans. Therefore, T10 appears to be the most effective treatment in reducing oxalic acid content, improving the nutritional quality and edibility of *Amaranthus* leaves. This aligns with the findings of Halliwell and Gutteridge (2015), who reported that reducing oxalate levels can enhance the mineral content of leafy vegetables. In summary, the biostimulant treatments significantly reduced oxalic acid levels, with T10 being the most effective, followed by T7 and T2. These results highlight the potential of biostimulants in improving the health benefits of *Amaranthus* by reducing anti-nutritional factors.

Specifically, reducing oxalic acid levels has major benefits because high oxalic acid levels form insoluble complexes with calcium rendering it bioavailable to humans and leaving them susceptible to calcium deficiency. T10 is therefore the most effective reducing oxalic acid content, improving the nutritional quality and edible of *Amaranthus* leaves. This is in line with what Halliwell and Gutteridge (2015) found, who said that in some cases reducing the oxalate level of leafy vegetables might boost their mineral composition. Overall, biostimulant

treatments significantly reduced oxalic acid levels with the lowest at T10, then T7 and finally T2. These results suggest that biostimulants may enhance the health benefits of *Amaranthus* by removing anti-nutritional factors.

3.4. Cost-Benefit ratio

Table 5 highlights the economic performance of various biostimulant treatments applied to *Amaranthus dubius* cultivation over 5 cents, showing variation in cost, yield, gross income, and the Benefit-Cost Ratio (B:C ratio). T2 (3% MLE) emerged as the most profitable treatment with the highest yield (163.73 kg) and a B:C ratio of 2.44, followed by T8 (0.4% Humic Acid) with a B:C ratio of 2.30. In contrast, T4 (8% Seaweed Extract) and T11 (Combination Treatment) had the lowest B:C ratios of 1.11 and 1.35 respectively, due to high input costs and relatively lower yields.

Table 5 Cost of benefit ratio of the treatments

Treatment	Cost of Cultivation (Rs.) for 5 cents	Yield Obtained (kg) in 5 cents	Price of 1 kg (Rs.)	Gross Income (Rs.)	Benefit-Cost Ratio (B:C Ratio)
T1	2005	126.97 ^b	30	3808.98 ^b	1.90 ^b
T2	2010	163.73 ^a	30	4911.81 ^a	2.44 ^{a*}
T3	2307.5	135.91 ^b	30	4077.42 ^b	1.77 ^b
T4	2615	96.83 ^c	30	2904.85 ^c	1.11 ^d
T5	2034.5	100.20 ^c	30	3005.89 ^c	1.48 ^c
T6	2069	110.45 ^b	30	3313.41 ^b	1.60 ^b
T7	2012	113.00 ^b	30	3390.03 ^b	1.68 ^b
T8	2024	155.23 ^a	30	4656.84 ^a	2.30 ^a
T9 (Control)	2000	91.22 ^c	30	2736.60 ^c	1.37 ^c
T10	2359	147.66 ^a	30	4429.71 ^a	1.88 ^b
T11	2718	121.91 ^b	30	3657.25 ^b	1.35 ^c

Values in the table are average of two seasons. Values followed by the same letter within a column are not significantly different at $p < 0.05$. Asterisks (*) denote treatments with the best result for a given parameter.

The control treatment (T9) resulted in the lowest gross income and a B:C ratio of 1.37, emphasizing the economic benefits of biostimulant applications. T2 and T8 show the most optimal cost effectiveness. Therefore, these are better candidates for larger scale application, while expensive ones such as T4 and T11 need optimization further.

4. CONCLUSION

The foliar spray of biostimulants effectively influences *Amaranthus*'s growth, yield, and biochemical attributes. The seaweed extract @ 6% was seen to surpass all other treatments, while the moringa leaf extract @ 2% influenced most of the parameters after the treatment (T3).

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