

Assessment of a Polycomponent Biostimulant for Improving Growth, Yield, and Salinity Tolerance in Spinach (*Spinacia oleracea*)

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Abstract: Salinity stress is a major abiotic factor limiting agricultural productivity, necessitating eco-friendly strategies to enhance crop resilience. This study evaluates the effectiveness of microbial and polycomponent biostimulants in alleviating salt stress in spinach (*Spinacia oleracea*). Soil-derived *Bacillus subtilis* and freshwater algae (*Chlorella* sp. and *Diatoms*) were isolated and morphologically characterized as potential biostimulants. Their monocomponent, dual-component, and polycomponent formulations were assessed along a gradient of NaCl concentrations ranging from 25 to 100 mM for their effects on seed germination, plantlet growth, and physiological performance.

Among monocomponent treatments, algae exhibited the highest growth-promoting activity under saline conditions. In dual-component combinations, Proline + Humic Acid outperformed Algae + Bacteria, at higher salinity levels. Notably, the polycomponent biostimulant formulation Poly6—comprising 0.01% each of humic acid, proline, algae, and bacteria—demonstrated the highest efficacy. Poly6 promoted superior seed germination, plantlet survival, and overall biomass accumulation under both normal and saline soil conditions.

Biochemical analysis revealed that Poly6-treated plants exhibited elevated total chlorophyll content (1.09 mg/g) and the highest antioxidant activity (Radical Scavenging Activity: 89.72%), indicating enhanced photosynthetic capacity and improved oxidative stress tolerance. These findings suggest that polycomponent biostimulants Poly6, offer a promising and sustainable approach for improving salinity stress. Further field validation and mechanistic studies are recommended to optimize its application in diverse agricultural systems.

Index terms: Abiotic stress tolerance, Biostimulants, *Bacillus subtilis*, *Chlorella*, Humic acid, Proline, Polycomponent formulations, RSA (Radical Scavenging Activity), Sustainable agriculture, Soil salinity

I. INTRODUCTION

With the increasing severity of soil salinization and the broader impacts of climate change, modern agriculture faces major challenges in sustaining crop productivity. Salinity impairs plant physiological functions, disrupts ion balance, causes osmotic stress, and reduces nutrient uptake, ultimately limiting arable land and reducing crop yields. Consequently, there is an urgent need for sustainable, eco-friendly strategies that mitigate stress-induced damage and enhance plant growth (4).

Among such strategies, the use of plant biostimulants has emerged as a promising approach. Biostimulants are biologically derived substances or microorganisms that enhance plant growth, nutrient efficiency, and stress tolerance. Unlike fertilizers, which directly supply essential nutrients, biostimulants improve plant physiology by modulating metabolic pathways, strengthening stress responses, and promoting overall resilience. These include humic acids, protein hydrolysates, amino acids, seaweed extracts, and beneficial microbes, all of which stimulate root development, nutrient uptake, and hormonal regulation (10). While excessive fertilizer use can cause environmental problems, biostimulants complement fertilizers by enhancing nutrient use efficiency, improving enzymatic activity, and supporting beneficial rhizosphere interactions. Their integration into conventional agricultural practices boosts productivity while promoting sustainability (4,6).

Salinity and other abiotic stresses, such as drought and extreme temperatures, significantly hinder crop productivity. In crops like spinach (*Spinacia oleracea*), which is particularly sensitive to salt stress, these impacts result in reduced growth, yield, and quality.

Biostimulants help mitigate salinity effects by enhancing antioxidant defenses, stabilizing cellular structures, improving root permeability, supporting microbial communities, and regulating water and ion uptake, thus maintaining plant vigor under adverse conditions (4,1).

Polycomponent biostimulants, which combine different functional agents such as humic acids, amino acids, algal extracts, and plant growth-promoting bacteria (PGPB), offer enhanced benefits compared to mono-component formulations (1). Their synergistic actions target multiple plant systems simultaneously, improving nutrient acquisition, boosting stress tolerance, stimulating microbial activity, and promoting higher biomass and yield under abiotic stress (1,4).

The functional roles of key biostimulant components are as follows:

- Humic acids: improve nutrient availability, stimulate root growth, and enhance stress tolerance (1);
- Proline: stabilizes proteins and membranes, scavenges reactive oxygen species (ROS), and maintains osmotic balance during stress (4);
- Freshwater algae extracts: enhance root development and boost antioxidant responses (1, 5);
- Beneficial bacteria (PGPB): promote nitrogen fixation, phosphorus solubilization, and growth hormone production, thereby improving plant vigor and stress resilience (2, 7).

This study investigates the potential of a polycomponent biostimulant in enhancing the growth, yield, and salinity tolerance of spinach (*Spinacia oleracea*), a high-value leafy vegetable known for its nutritional richness but sensitivity to salt stress (3). By exploring the synergistic interactions of multiple bioactive ingredients, this research supports the adoption of sustainable agricultural practices to enhance crop resilience under salt stress.

II. AIM AND OBJECTIVES

This study aims to evaluate the efficacy of a monocomponent, dual-component, and polycomponent biostimulant in enhancing the growth, yield, and stress resilience of spinach under saline stress conditions. The specific objectives include

determining the number of plantlets in both control and test conditions, analyzing the time required to reach the maximum number of plantlets, evaluating the chlorophyll content in spinach under control and biostimulant-treated conditions, measuring antioxidant activity in plants subjected to saline stress, and comparing overall growth and yield parameters between control and biostimulant-treated spinach plants.

III. MATERIALS AND METHODOLOGY

3.1. Experimental Setup to assess Salinity stress in Spinach

To assess the salinity stress sensitivity or resistance towards spinach, the experiment was conducted wherein spinach seeds (obtained from Namdeo Umaji Agritech Pvt. Ltd., Byculla, Mumbai, India) were exposed to varying salt (NaCl) concentrations.

Seed Sample and Treatment Conditions

- Sample Size: Each treatment was performed in triplicate, with 10 seeds per replicate to ensure statistical reliability and reproducibility of the results.
- Salt Concentrations: Seeds were exposed to 25 mM, 50 mM, 75 mM, and 100 mM NaCl.
- Experimental Setup: Seeds were sown in paper cups treated with different salt concentrations.

3.2. Treatment of Garden Soil
Garden soil (approximately 100 kg) was divided into four parts, each weighing 25 kg, and treated with NaCl solutions of 25 mM, 50 mM, 75 mM, and 100 mM. The soil was dried in sunlight and treated twice to ensure uniform salinity. The treated soil was used for the experiment.

3.3. Procurement of Biostimulants

Biostimulants such as Humic acid and Proline were obtained from Namdeo Umaji Agritech Pvt. Ltd., Byculla, Mumbai, India. The other two biostimulants, bacteria and algae were isolated from natural sources such as soil samples and pond water, respectively. Isolation of bacteria was carried out using serial dilution technique and the genus of isolated organism was confirmed by performing suitable Biochemical tests. Enrichment of freshwater water algae was carried out in Allen No. 3 and Chu 10 media at room temperature.

3.4. Set up of Control Treatments

The control setup in this methodology was essential to establish baseline comparisons and to assess the specific effects of saline stress and biostimulant treatments on spinach.

- Control 1: No salt stress and no biostimulant.
- Control 2: Salt stress applied at 25 mM, 50 mM, 75 mM, and 100 mM NaCl concentrations.
- Control 3: Biostimulant treatment without salt stress, using proline, humic acid, freshwater algae, and bacteria at 0.01%, 0.025%, 0.05%, and 0.1% concentrations.

3.5. Experimental setup to assess the Biostimulant effect on salinity stress conditions

The experiment was conducted under three different conditions to evaluate salinity effects:

- Salty Soil - Distilled water treated Seeds: Seeds were sown in soil treated with salt solutions without pre-soaking.
- Normal Soil - Distilled water treated Seeds: Seeds were planted in untreated soil with distilled water.
- Normal Soil - Salty Seeds: Seeds were pre-soaked in salt solutions before planting.

3.6. Study of Biostimulant Effect

- Monocomponent Biostimulant Effect: Each biostimulant (proline, humic acid, algae, and bacteria) was tested at 25 mM, 50 mM, 75 mM, and 100 mM salt concentrations.
- Dual-component Biostimulant Effect: The combined effect of two biostimulants was studied at varying concentrations.

a) Proline + Humic acid

Treatment	Biostimulant Concentration (%)
Combination 1	0.025% Proline + 0.01% H.A
Combination 2	0.01% Proline + 0.01% H.A.
Combination 3	0.005% Proline + 0.005% H.A.

b) Algae + Bacteria

Treatment	Biostimulant Concentration (%)
Combination 1	0.025% Algae + 0.01% Bacteria
Combination 2	0.01% Algae + 0.01% Bacteria

Combination 3	0.005% Algae + 0.005% Bacteria
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- Polycapnent Biostimulant Study: Six polycapnent biostimulant combinations were prepared based on monocomponent and dual-component biostimulant results. These included various concentrations of humic acid, proline, algae, and bacteria. Two combinations (Combination 3 and Combination 6) were selected for mass production and chemical testing.

Treatment	Biostimulant Concentration (%)
Combination 1	0.005% H.A. + 0.005% Proline + 0.005% Algae + 0.005% Bacteria
Combination 2	0.005% H.A. + 0.005% Proline + 0.01% Algae + 0.005% Bacteria
Combination 3	0.0025% H.A. + 0.0025% Proline + 0.0025% Algae + 0.0025% Bacteria
Combination 4	0.01% H.A. + 0.025% Proline + 0.05% Algae + 0.05% Bacteria
Combination 5	0.025% H.A. + 0.025% Proline + 0.025% Algae + 0.025% Bacteria
Combination 6	0.01% H.A. + 0.01% Proline + 0.01% Algae + 0.01% Bacteria

3.7. Biochemical Analysis

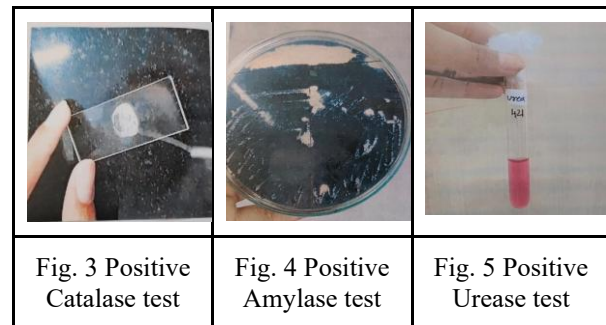
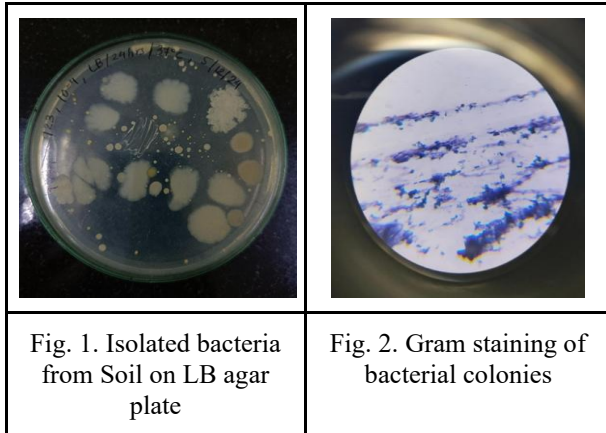
Biochemical analyses was performed to assess the stress response and the impact of polycapnent biostimulants (9).

- Chlorophyll Estimation: Leaf samples were ground and treated with 80% acetone. Absorbance was measured at 660 nm and 645 nm to estimate chlorophyll content (8).
- DPPH Antioxidant Assay: A 25 µL plant extract was incubated with DPPH solution and measured for absorbance at 517 nm to assess antioxidant activity (8).

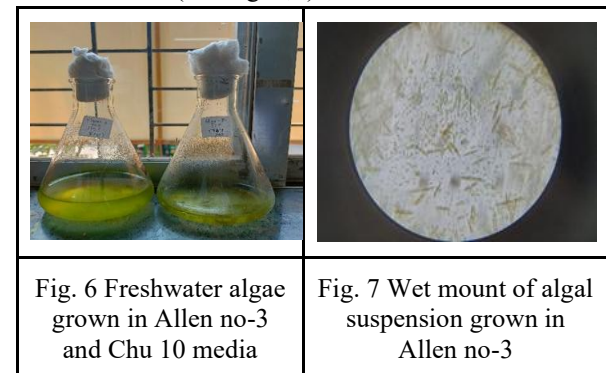
IV. RESULTS

4.1. Isolation of Biostimulant (Bacteria and algae)

Isolation of bacteria was carried out using soil sample and LB agar plate. Gram-staining revealed isolated bacterium was Gram positive bacilli. Upon performing Biochemical tests, it could be suggested that isolated organism is *Bacillus subtilis*. (See Fig. 1, 2, 3,4,5)



Allen no. 3 and Chu no. 10 medium was used to isolate Freshwater algae, and upon microscopic examination, it was revealed that the given sample contained *Chlorella* and *Diatoms*, both can be used as potential biostimulants (see Fig. 6,7).



4.2. Experimental Setup to assess Salinity stress in Spinach and Control setup

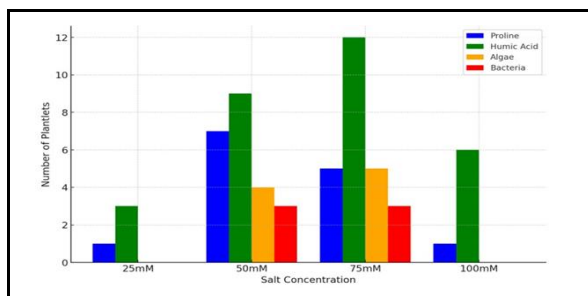
Experimental setups were carried out to evaluate how different concentrations of salt affect the plant's growth, physiological processes, and stress responses (see Fig. 8). The control setup in this methodology was crucial for establishing baseline comparisons and assessing the effects of saline stress and biostimulant treatments on spinach. Control 1 (No Salt Stress & No

Biostimulant) provided a baseline for natural plant growth under normal conditions, while Control 2 (Salt Stress Only) allowed for the evaluation of spinach growth under saline conditions without biostimulants, helping to assess the impact of salt stress alone. Control 3 (Biostimulant Treatment Without Salt Stress) ensured that any observed effects could be attributed to the biostimulants, independent of salt stress. Together, these controls enabled a clear understanding of how saline stress and biostimulant treatments interact to influence spinach growth and stress resilience (see Fig. 9).

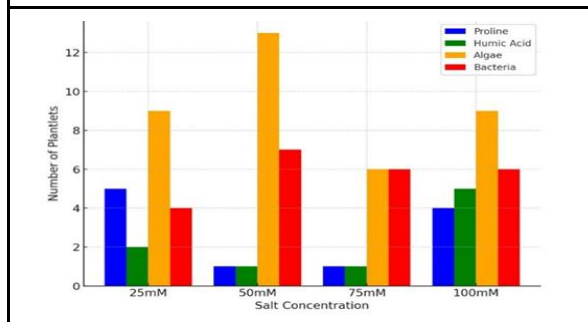


4.3. Study of Individual Biostimulant Effect in Saline soil

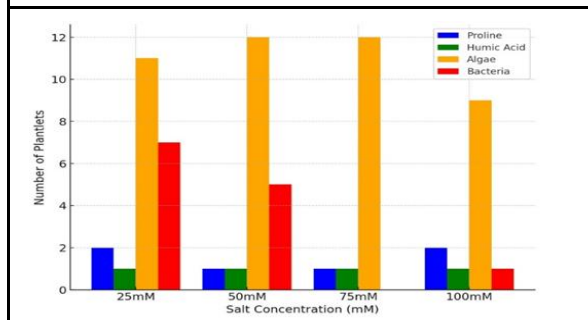
The results demonstrate that biostimulant concentrations significantly influence plantlet growth under salt stress. At lower concentrations (0.01% and 0.025%), algae and bacteria positively impacted growth, with algae promoting the highest number of plantlets, particularly at a 50mM salt concentration. Proline and humic acid had comparatively weaker effects at these concentrations. As the concentration increased to 0.05% and 0.1%, algae continued to enhance plantlet growth substantially across all salt concentrations, while the effect of bacteria diminished. Proline and humic acid exhibited moderate effects, with proline showing better results at higher salt concentrations. Overall, algae emerged as the most effective biostimulant for promoting plantlet growth under salt stress, particularly at higher concentrations.



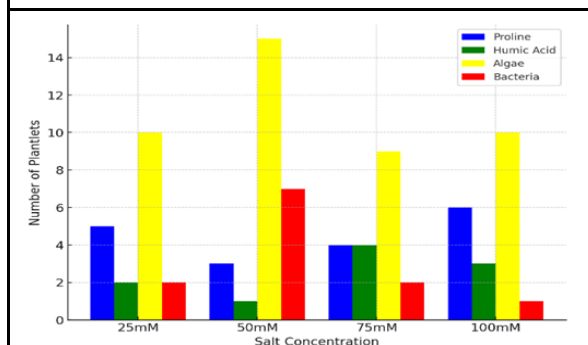
Graph 1. 0.01% concentration of monocomponent Biostimulant



Graph 2. 0.025% concentration of monocomponent Biostimulant



Graph 3. 0.05% concentration of monocomponent Biostimulant

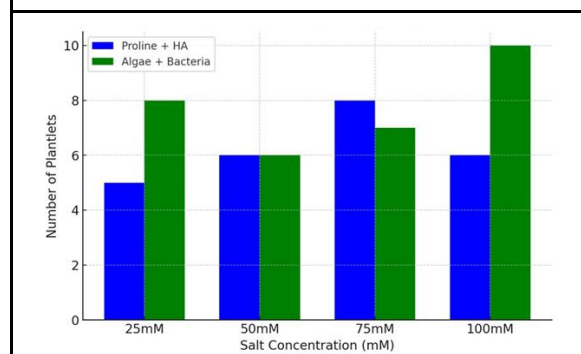


Graph 4. 0.1% concentration of monocomponent Biostimulant

4.4. Study of Dual-component Biostimulant

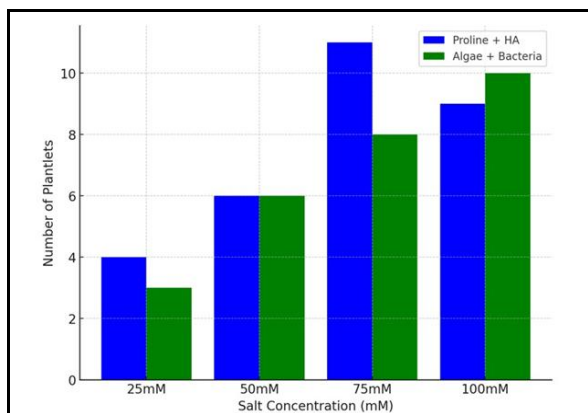
The results from the graphs illustrate the effects of various bivalent biostimulant combinations under different salt concentrations. Two treatment groups were compared: Proline + Humic Acid (HA) and Algae + Bacteria. In Combination 1, Algae + Bacteria showed higher plantlet numbers at lower salt concentrations (25mM and 50mM), but plantlet numbers decreased at higher salt levels (75mM and 100mM). In contrast, the Proline + HA treatment exhibited a more consistent plantlet count across all salt concentrations. For Combination 2, Proline + HA consistently outperformed Algae + Bacteria, especially at 50mM and 100mM, where Algae + Bacteria showed a decline in plantlet growth, indicating reduced effectiveness under increasing salt stress. In Combination 3, Proline + HA maintained higher plantlet numbers across all salt concentrations, whereas Algae + Bacteria showed moderate effectiveness at lower salt concentrations but a significant decline at higher salt levels, indicating lower tolerance to high salinity. Overall, the results suggest that Proline + Humic Acid is more effective in promoting plantlet growth under salt stress, particularly at higher salt concentrations, where it better sustains plantlet numbers and enhances plant resilience to salinity.

a) Combination 1- 0.025% Proline + 0.01% H.A.,
Combination 1- 0.025% Algae + 0.01% Bacteria



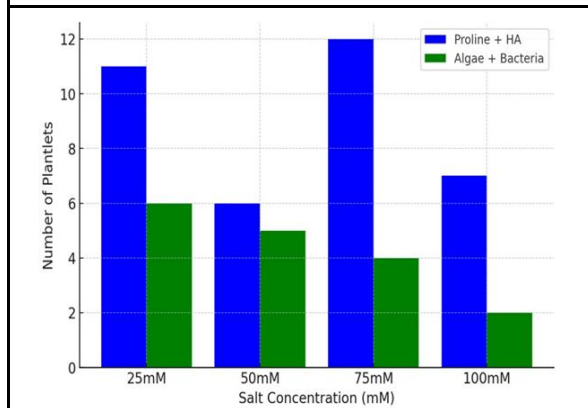
Graph 5. Effect of Bivalent biostimulant on seed germination in salinity stress using Combination 1

b) Combination 2- 0.01% Proline + 0.01% H.A.,
Combination 2- 0.01% Algae + 0.01% Bacteria



Graph 6. Effect of Bivalent biostimulant on seed germination in salinity stress using Combination 2

c) Combination 3- 0.005% Proline + 0.005% H.A.),
Combination 3- 0.005% Algae + 0.005% Bacteria



Graph 7. Effect of Bivalent biostimulant on seed germination in salinity stress using Combination 3

4.5. Study of Polycomponent Biostimulant

The experimental results highlight the impact of polycomponent biostimulants on spinach growth under two conditions: normal soil with distilled water used for irrigation (Condition 1) and saline soil with intermittent saline water used for irrigation (Condition 2). In Condition 1, Poly 6 emerged as the most effective, consistently producing the highest number of plantlets. Poly 3 followed closely, maintaining stable growth. Poly 5 showed moderate effectiveness, while Poly 1 and Poly 2 yielded average results; Poly 4 was the least effective. In Condition 2, where salt stress was more pronounced, overall growth declined, but Poly 6 again led in performance, especially at

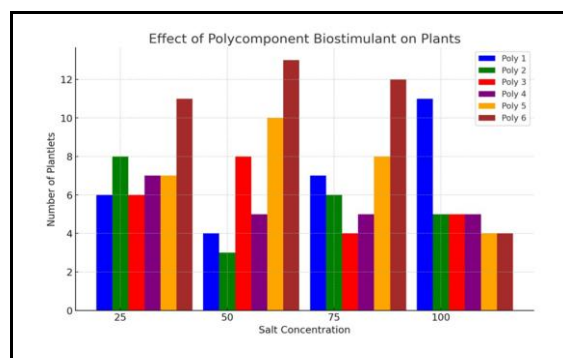
100mM salt concentration. Poly 3 remained the second-best, while Poly 5 had a moderate effect. Poly 1 and 2 showed limited response, and Poly 4 continued to be the least effective. Overall, Poly 6 consistently outperformed all combinations in enhancing plant resilience under both normal and saline conditions.



Fig. 10 Effect of Combination 6 of Polycomponent Biostimulant on Spinach growth in the normal soil



Fig. 11 Effect of Combination 6 of growth in the presence of various concentrations of Salts



Graph 8. Effect of Polycomponent biostimulant on seed germination in salinity stress using Poly Combination 6 - 0.01% H.A. + 0.01% Proline + 0.01% Algae + 0.01% Bacteria

4.6. Biochemical analysis

a) Chlorophyll estimation

The chlorophyll estimation revealed notable differences among the samples. Using absorbance values at 645 nm

and 660 nm, chlorophyll a, b, and total chlorophyll contents were calculated. The Poly6-treated sample exhibited the highest total chlorophyll content (1.09 mg/g), with Chl-a at 0.55 mg/g and Chl-b at 0.76 mg/g, indicating a strong potential to enhance chlorophyll synthesis and photosynthetic efficiency. Poly3 showed a moderate increase with a total chlorophyll content of 0.78 mg/g (Chl-a: 0.33 mg/g, Chl-b: 0.45 mg/g), while the normal untreated sample had the lowest total chlorophyll (0.70 mg/g). These results suggest that Poly6 is the most effective biostimulant, followed by Poly3, making them ideal candidates for plantlet mass production and further analysis.

b) Antioxidant activity measurement

The antioxidant activity of the tested samples, measured in terms of Radical Scavenging Activity (%RSA), revealed significant variation among treatments. Poly6 exhibited the highest antioxidant potential, recording an RSA value of 89.72%, indicative of strong free radical neutralization capacity. The Normal sample demonstrated a moderate RSA of 74.65%, while Poly3 showed the lowest activity at 34.23%, suggesting limited antioxidant efficacy. These results underscore the superior antioxidative efficacy of Poly6, which may contribute to enhanced cellular protection against oxidative stress. The markedly higher RSA observed in Poly6 warrants further investigation into its constituent bioactive compounds responsible for its potent antioxidant properties.

V. DISCUSSION

The present investigation demonstrates the significant potential of biostimulants in mitigating salinity-induced stress in *Spinacia oleracea*. Initial microbial isolation yielded promising candidates for biostimulant development: *Bacillus subtilis*, identified via Gram staining and biochemical tests, and freshwater algae, including *Chlorella* and diatoms, isolated using Allen No.3 and Chu No.10 media. These organisms, individually and in various combinations, were evaluated for their capacity to enhance seed germination, plantlet growth, and physiological performance under saline conditions. The evaluation of individual biostimulants revealed that algal extracts were the most effective in enhancing plantlet growth under increasing salt

concentrations, particularly at higher dosages. Bacterial biostimulants showed beneficial effects at lower concentrations but diminished performance at higher salinity. Proline and humic acid demonstrated moderate but stable growth-promoting effects, with proline showing enhanced effectiveness at higher salt concentrations.

Bivalent biostimulant combinations (e.g., Proline + Humic Acid and Algae + Bacteria) showed differential responses to salt stress. The Proline + Humic Acid combination consistently supported higher plantlet numbers across all salt concentrations, even at elevated salinity levels, making it more effective under prolonged salt stress. In contrast, Algae + Bacteria showed promising results at lower salt concentrations but decreased effectiveness with increasing salinity.

The polycomponent biostimulant analysis further reinforced the superiority of combination treatments. Among all, Poly6 (comprising Proline, Humic Acid, Algae, and Bacteria in equal concentrations) emerged as the most potent formulation. It significantly enhanced plant growth and stress tolerance under both normal and saline soil conditions, outperforming other polycomponent combinations. Poly3 also demonstrated good potential but was consistently less effective than Poly6.

Biochemical analyses corroborated these findings. Chlorophyll content and antioxidant activity were highest in plants treated with Poly6, indicating improved photosynthetic efficiency and cellular defense mechanisms against oxidative stress. Poly6-treated samples exhibited the highest total chlorophyll content (1.09 mg/g) and RSA value (89.72%), suggesting a strong physiological and biochemical basis for its enhanced performance.

Collectively, these findings highlight Poly6 as a robust, synergistic formulation capable of alleviating salinity stress and improving spinach performance through physiological and biochemical modulation.

While the outcomes of this study provide a strong foundation for biostimulant-based salinity stress management, several avenues demand further investigation: The efficacy of Poly6 should be assessed under diverse agro-climatic conditions through multi-location field trials to establish its practical relevance and consistency in real-world farming systems. Further research is necessary to refine the Poly6 formulation, including the development of delivery systems (e.g., encapsulation, foliar sprays) that enhance stability, shelf

life, and field applicability. Detailed phytochemical and metabolite profiling of the constituent biostimulants—particularly algal and bacterial components—may reveal key compounds responsible for the observed biological effects. Expanding the use of Poly6 to other economically important crops could offer insights into its versatility and contribute to crop-specific biostimulant development strategies. Long-term ecological assessments are essential to evaluate the impact of repeated biostimulant application on soil microbiota, nutrient dynamics, and overall agroecosystem health.

In conclusion, the integration of polycomponent biostimulants such as Poly6 into modern agronomic practices holds promise for enhancing crop resilience to abiotic stress, reducing chemical input dependency, and advancing sustainable agriculture.

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