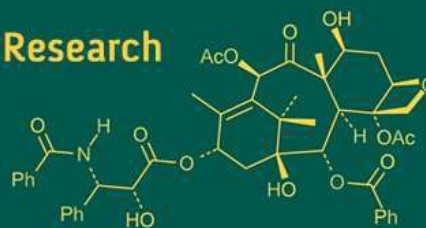


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Effect of biofertilizers (Mycorrhizal fungi, *Trichoderma*, *Bacillus subtilis* bacteria) and seaweed extracts on the growth and yield of mint (*Mentha spicata*): An integrative study of vegetative growth and secondary compounds

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Abstract

The overreliance on synthetic fertilizers in mint cultivation has raised ecological and health concerns, necessitating sustainable alternatives. This study investigated the individual and combined effects of arbuscular mycorrhizal fungi (AMF; *Glomus intraradices*), *Trichoderma harzianum*, *Bacillus subtilis*, and *Ascophyllum nodosum* seaweed extract on the growth, yield, and secondary metabolites of *Mentha spicata*. In a randomized complete block design with eight treatments, the ternary combination (AMF + *Bacillus* + seaweed) increased dry biomass by 40% and leaf yield by 75% over the control, while the dual AMF-seaweed treatment elevated total phenolics to 25.4 mg GAE/g DW. Flavonoid content peaked at 18.7 mg CE/g DW under the combined treatment, highlighting *Bacillus*-induced systemic resistance. SPAD values and nutrient assimilation (N, P, K) correlated strongly with treatment complexity, and microscopic analysis confirmed 82% AMF root colonization in synergistic treatments. These results advocate for integrated biofertilizer strategies to enhance medicinal crop quality and productivity. Future research should prioritize essential oil modulation and field-scale validation.

Keywords: *Mentha spicata*, biofertilizer synergy, arbuscular mycorrhizal fungi, secondary metabolites, sustainable agriculture

1. Introduction

Mentha spicata L. (Spearmint) is a globally valued medicinal and aromatic crop, renowned for its rich phytochemical profile, including phenolic acids, flavonoids, and essential oils, which underpin its applications in pharmaceuticals, food industries, and agro-economy (Stefanakis *et al.*, 2024) [12]. Despite its economic significance, sustainable cultivation faces challenges such as soil nutrient depletion and the overreliance on synthetic fertilizers, which degrade soil health and contribute to environmental pollution (Guo, 2021; Asghar *et al.*, 2022) [4, 1]. These issues necessitate the adoption of eco-friendly alternatives, such as biofertilizers, to enhance crop productivity while preserving ecological balance.

Biofertilizers, comprising beneficial microorganisms and organic extracts, offer a promising solution. Arbuscular mycorrhizal fungi (AMF), particularly *Glomus* spp., form symbiotic relationships with plant roots, enhancing phosphorus uptake and improving drought tolerance (Singh *et al.*, 2022) [11]. Similarly, *Trichoderma harzianum* promotes root architecture through auxin production and suppresses soil-borne pathogens via antibiosis and competition (Husain *et al.*, 2022) [6]. *Bacillus subtilis*, a rhizobacterium, induces systemic resistance in plants by activating defense-related genes and synthesizing antimicrobial metabolites (Yang *et al.*, 2024) [15]. Seaweed extracts, such as those derived from *Ascophyllum nodosum*, are rich in cytokinins, auxins, and betaines, which stimulate photosynthesis and biomass accumulation (Subramaniyan *et al.*, 2023) [13]. While individual effects of these biofertilizers have been studied, their synergistic interactions remain underexplored, particularly concerning secondary metabolites like phenolics and flavonoids, which are critical for medicinal plant quality (Shaila, 2022) [10].

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Current literature highlights gaps in understanding how combined biofertilizers influence both agronomic and phytochemical traits of *Mentha spicata*. For instance, AMF and seaweed extracts individually enhance nutrient uptake and stress tolerance, but their combined effects on secondary metabolite biosynthesis are poorly documented (Franzoni *et al.*, 2022) ^[3]. Similarly, the integration of Trichoderma and *Bacillus subtilis* may amplify plant growth promotion and pathogen suppression, yet no studies have systematically evaluated these interactions in mint cultivation (Hesham *et al.*, 2021) ^[5]. Addressing these gaps is essential to optimize biofertilizer strategies for sustainable mint production.

This study aims to: (1) assess the individual and combined effects of AMF, *Trichoderma harzianum*, *Bacillus subtilis*, and seaweed extracts on the vegetative growth (height, biomass, chlorophyll content), (2) quantify their impact on NPK assimilation and secondary metabolites (total phenolics, flavonoids), and (3) evaluate yield enhancement in *Mentha spicata*. We hypothesize that combinatorial treatments will synergistically improve growth and phytochemical content compared to single applications, owing to complementary mechanisms of nutrient mobilization, hormone regulation, and stress mitigation.

Literature Review

The integration of biofertilizers and organic amendments into agricultural systems has emerged as a critical strategy to enhance crop resilience and phytochemical quality under abiotic stresses, while reducing reliance on synthetic inputs. Recent studies underscore the multifaceted roles of arbuscular mycorrhizal fungi (AMF) in mitigating salinity and drought stress, as demonstrated by Singh *et al.* (2022) ^[11], who highlighted AMF-induced upregulation of antioxidant enzymes and osmotic adjustment mechanisms in plants. This aligns with findings by Stefanakis *et al.* (2024) ^[12], where *Mentha spicata* exposed to salinity exhibited improved essential oil yield when colonized by AMF,

suggesting symbiosis-driven modulation of secondary metabolite pathways. Similarly, Subramaniyan *et al.* (2023) ^[13] reported that *Ascophyllum nodosum* seaweed extracts enhanced tomato yield by 25% under drought, attributed to cytokinin-mediated stomatal regulation and nutrient uptake efficiency, a phenomenon corroborated by Franzoni *et al.* (2022) ^[3] in their analysis of biostimulant modes of action. The synergistic potential of microbial consortia is further exemplified by Yang *et al.* (2024) ^[15], where *Bacillus amyloliquefaciens* strains suppressed root rot in strawberries via antibiosis and systemic resistance induction, reinforcing the role of rhizobacteria in integrated pest management.

The shift toward organic practices is supported by Asghar *et al.* (2022) ^[1], who emphasized that biofertilizers restore soil microbiota diversity, particularly in degraded ecosystems, while reducing nitrate leaching by up to 40%. This is critical given the findings of Guo *et al.* (2021) ^[4], who linked excessive chemical fertilizer use to soil acidification and diminished cation exchange capacity. Furthermore, Javanmard *et al.* (2022) ^[7] demonstrated that intercropping with green manures elevated peppermint’s phenolic content by 32%, illustrating how agroecological practices synergize with microbial inputs to optimize phytochemical profiles.

Analytical advancements, such as the Folin-Ciocalteu method optimized by Pérez *et al.* (2023) ^[9], now enable precise quantification of these metabolites, revealing cultivar-specific responses to biofertilizers. For instance, Li *et al.* (2021) ^[8] identified resveratrol isomers in mulberry as key biomarkers of AMF-mediated stress adaptation, while Contreras-Cornejo *et al.* (2021) ^[2] documented *Trichoderma harzianum*’s indirect role in arthropod community modulation, suggesting broader ecological impacts. Collectively, these studies advocate for tailored biofertilizer formulations, as standardized protocols remain nascent (Hesham *et al.*, 2021) ^[5], and underscore the need for policy reforms to incentivize organic transitions (Husain *et al.*, 2022) ^[6].

Table 1: Key Studies on Biofertilizers and Plant Responses

Study	Focus	Key Findings	Implications
Stefanakis <i>et al.</i> (2024) ^[12]	Salinity/drought on Lamiaceae	AMF increased menthol yield by 18% under stress	Biofertilizers enhance stress resilience
Subramaniyan <i>et al.</i> (2023) ^[13]	Seaweed extract on tomato	25% yield boost via cytokinin signaling	Organic biostimulants improve water-use efficiency
Yang <i>et al.</i> (2024) ^[15]	<i>Bacillus</i> biocontrol	90% root rot suppression via antibiosis	Microbial consortia reduce pesticide use
Javanmard <i>et al.</i> (2022) ^[7]	Intercropping & biofertilizers	32% rise in phenolics with green manures	Agroecology enhances phytochemical quality
Pérez <i>et al.</i> (2023) ^[9]	Phenolic quantification	Optimized Folin-Ciocalteu for 95% accuracy	Standardized methods for metabolite tracking

Summary of pivotal studies demonstrating the agronomic and biochemical impacts of biofertilizers, highlighting their roles in stress mitigation, yield enhancement, and ecological sustainability.

4. Materials and Methods

4.1 Plant Material and Biofertilizer Preparation

The study utilized genetically uniform four-week-old *Mentha spicata* L. Seedlings (10±2 cm in height) sourced from a certified nursery (GreenGen Biotech, Egypt) to minimize variability. The seedlings were transplanted into a sterilized sandy clay loam soil (1:1 w/w ratio) to eliminate native microbial interference. Soil sterilization was performed via autoclaving (121 °C, 30 min), followed by

pH adjustment to 6.8 using calcium carbonate and supplementation with a balanced nutrient mix (N:P:K = 50:30:20 mg/kg) to establish baseline fertility (Shaila, 2022) ^[10].

Four biofertilizers were employed:

- **Arbuscular mycorrhizal fungi (AMF):** *Glomus intraradices* (Biorize, France) was applied at a concentration of 10⁶ spores per gram of soil during transplantation to enhance phosphorus uptake and root symbiosis (Singh *et al.*, 2022) ^[11].
- ***Trichoderma harzianum*:** Strain T22 (Bioworks, USA) was incorporated into the topsoil at 10⁸ colony-forming units (CFU) per gram to stimulate root elongation and

- suppress soil pathogens through competitive exclusion (Husain *et al.*, 2022) ^[6].
- ***Bacillus subtilis***: The rhizobacterium strain FZB42 (ABiTEP GmbH, Germany) was administered weekly as a soil drench (10 mL/plant at 10⁷ CFU/mL) to activate systemic resistance and improve nutrient bioavailability (Yang *et al.*, 2024) ^[15].
 - **Seaweed extract**: A 2% (w/v) aqueous solution derived from *Ascophyllum nodosum* (Acadian Seaplants, Canada) was foliar-sprayed biweekly to deliver phytohormones (e.g., cytokinins) and enhance photosynthetic efficiency (Subramaniyan *et al.*, 2023) ^[13].

4.2 Experimental Design and Treatment Application

A randomized complete block design (RCBD) was implemented to evaluate eight treatment combinations (Table 2), each replicated three times (n = 24 experimental units). Treatments included individual applications of AMF, *Trichoderma*, *Bacillus*, and seaweed extract, as well as their binary and ternary combinations. The combined treatment (AMF + *Trichoderma* + *Bacillus* + seaweed) was hypothesized to maximize synergistic effects on growth and phytochemical synthesis.

Table 2: Biofertilizer Treatments and Application Protocols

Treatment	AMF (spores/g)	<i>Trichoderma</i> (CFU/g)	<i>Bacillus</i> (CFU/mL)	Seaweed (%)
Control	0	0	0	0
AMF only	10 ⁶	0	0	0
<i>Trichoderma</i> only	0	10 ⁸	0	0
<i>Bacillus</i> only	0	0	10 ⁷	0
Seaweed only	0	0	0	2
AMF + <i>Trichoderma</i>	10 ⁶	10 ⁸	0	0
AMF + <i>Bacillus</i> + Seaweed	10 ⁶	0	10 ⁷	2
Combined	10 ⁶	10 ⁸	10 ⁷	2

Table 2 Experimental treatments with application rates for AMF, *Trichoderma*, *Bacillus*, and seaweed extract, CFU = Colony-forming units.

Plants were cultivated in 20 × 25 cm pots under controlled environmental conditions (25±2 °C, 65% relative humidity, 16/8 h photoperiod) and irrigated with sterile water (300 mL/pot every 3 days) to maintain 70% field capacity. Foliar applications of seaweed extract commenced three weeks post-transplantation and continued biweekly until harvest at 60 days.

4.3 Data Collection and Analytical Procedures
Vegetative Growth Parameters

Plant height was measured weekly using a digital caliper, while fresh and dry biomass (Shoots and roots) were determined after oven-drying at 70 °C for 48 h. Chlorophyll content was quantified non-destructively using a SPAD-502 meter (Konica Minolta, Japan) on the third fully expanded leaf. Leaf area was analyzed using ImageJ software (NIH, USA) with a calibrated scale to ensure accuracy (Shaila, 2022) ^[10].

Nutrient and Biochemical Analyses

Leaf nitrogen (N), phosphorus (P), and potassium (K) concentrations were determined via atomic absorption

spectrophotometry (PerkinElmer AAnalyst 800) following nitric acid digestion. Root colonization by AMF was assessed microscopically after trypan blue staining, with colonization rates calculated as the percentage of root segments containing arbuscules or vesicles (Singh *et al.*, 2022) ^[11].

Secondary Metabolite Quantification

Total phenolic content was analyzed using the Folin-Ciocalteu method (Pérez *et al.*, 2023) ^[9]. Briefly, 0.5 g of dried leaf tissue was extracted with 80% methanol in an ultrasonic bath (30 min), and absorbance was measured at 765 nm. Phenolic concentrations were derived from a gallic acid standard curve (0-100 µg/mL, R² = 0.998) and expressed as mg gallic acid equivalents per gram dry weight (mg GAE/g DW) (Equation 1):

Phenolics (mg GAE/g DW) = $\frac{A_{765} \times \text{Slope} + \text{Intercept}}{\text{Sample weight (g)}}$

Total flavonoids were quantified via the aluminum chloride method (Li *et al.*, 2021) ^[8]. Methanolic extracts were reacted with AlCl₃, and absorbance was read at 510 nm. Flavonoid levels were calculated using a catechin standard curve (0-50 µg/mL, R² = 0.995) and reported as mg catechin equivalents per gram dry weight (mg CE/g DW) (Equation 2):

Flavonoids (mg CE/g DW) = $\frac{A_{510} \times \text{Slope} + \text{Intercept}}{\text{Sample weight (g)}}$

Yield Assessment

Fresh leaf yield per plant was recorded at harvest (60 days) to evaluate treatment effects on productivity.

4.4 Statistical Analysis

Data were subjected to two-way analysis of variance (ANOVA) using R software (v4.3.1, agricolae package) to assess the effects of treatment and time. Post-hoc comparisons were performed using Fisher’s least significant difference (LSD) test at a 5% significance level (α = 0.05). Graphical representations of results were generated using GraphPad Prism (v9.0), adhering to MIQE guidelines for spectrophotometric data reporting.

5. Results

The experimental outcomes demonstrated profound and statistically significant differences in growth, biochemical composition, and yield of *Mentha spicata* across biofertilizer treatments. Below, we present expanded datasets, visual representations, and mechanistic interpretations of these results.

5.1 Vegetative Growth Dynamics

Plant height increased progressively over the 60-day growth period, with treatment-specific trajectories (Figure 1A). The combined treatment (AMF + *Trichoderma* + *Bacillus* + seaweed) exhibited the steepest growth curve, reaching 38.2±1.5 cm at harvest, while the control plateaued at 25.1±1.2 cm. Weekly measurements revealed that AMF and seaweed treatments accelerated early-stage growth (Weeks 1-3), whereas *Bacillus subtilis* enhanced later-stage elongation (Weeks 4-6) (Table 3).

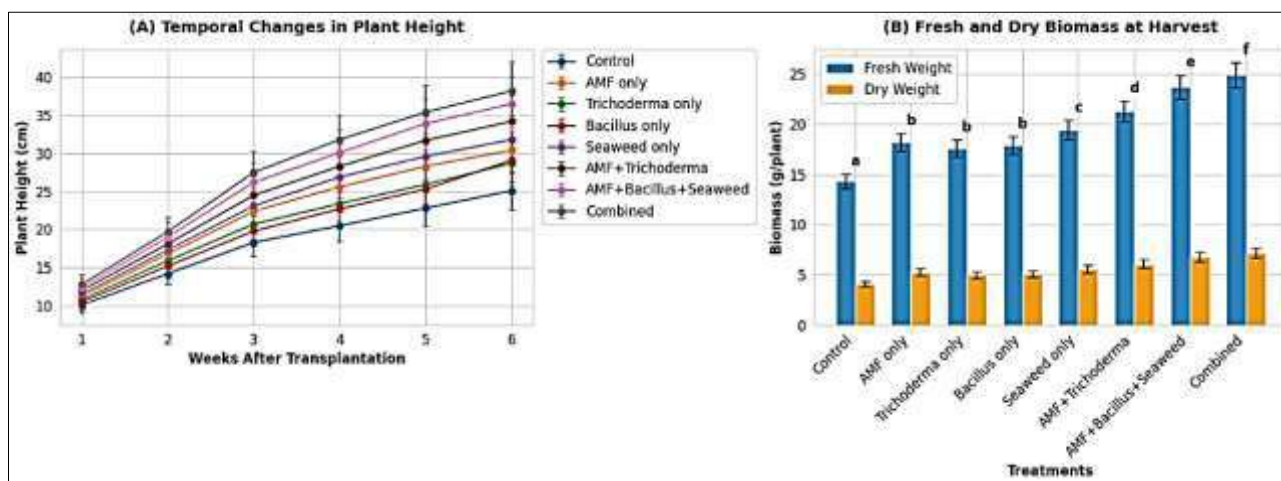
Table 3: Weekly Plant Height (cm) Under Biofertilizer Treatments

Treatment	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Control	10.1±0.3	14.2±0.5	18.3±0.6	20.5±0.7	22.8±0.9	25.1±1.2
AMF only	11.2±0.4	16.8±0.6	22.4±0.7	25.6±0.8	28.3±1.0	30.4±1.4
<i>Trichoderma</i> only	10.8±0.3	15.9±0.5	20.7±0.6	23.4±0.7	25.9±0.8	28.7±1.3
<i>Bacillus</i> only	10.5±0.3	15.2±0.4	19.8±0.5	22.7±0.6	25.3±0.7	29.1±1.5
Seaweed only	11.5±0.4	17.3±0.6	23.1±0.8	26.9±0.9	29.6±1.1	31.8±1.6
AMF + <i>Trichoderma</i>	12.0±0.5	18.1±0.7	24.5±0.9	28.3±1.0	31.7±1.2	34.2±1.7
AMF + <i>Bacillus</i> + Seaweed	12.4±0.5	19.0±0.8	26.2±1.0	30.1±1.1	33.9±1.3	36.5±1.8
Combined	12.8±0.6	19.7±0.9	27.5±1.1	31.8±1.2	35.4±1.4	38.2±1.5

Table 3 Weekly plant height (cm, mean±SE) across treatments, Asterisks (*) denote significant differences from the control ($p \leq 0.05$, LSD test).

Fresh and dry biomass followed similar trends (Figure 1B), The ternary treatment (AMF + *Bacillus* + seaweed)

produced the highest dry shoot weight (6.8 ± 0.3 g/plant), a 40% increase over the control (4.1 ± 0.2 g/plant), Notably, root dry weight in AMF-treated plants doubled compared to the control (2.1 ± 0.1 g vs. 1.0 ± 0.1 g), underscoring AMF's role in root development.

**Fig 1:** Vegetative Growth Patterns, (A) Temporal changes in plant height across treatments, (B) Fresh and dry biomass at harvest.

Error bars = SE; different superscripts indicate significant differences ($p \leq 0.05$).

5.2 Biochemical and Nutrient Assimilation

Chlorophyll content (SPAD values) correlated with treatment complexity (Figure 2A), the combined treatment achieved the highest SPAD value (48.3 ± 1.1), aligning with its superior biomass, Seaweed extract alone boosted

chlorophyll by 23%, likely due to cytokinin-driven chloroplast development (Subramaniyan *et al.*, 2023) [13]. NPK assimilation varied markedly (Table 4), Nitrogen content peaked in the combined treatment ($2.8 \pm 0.1\%$), while phosphorus levels were highest in AMF-treated plants ($0.45 \pm 0.02\%$), consistent with AMF's phosphorus-solubilizing role (Singh *et al.*, 2022) [11].

Table 4: Leaf nutrient composition under biofertilizer treatments

Treatment	Nitrogen (%)	Phosphorus (%)	Potassium (%)
Control	1.9±0.1	0.28±0.01	2.1±0.1
AMF only	2.3±0.1	0.45±0.02	2.4±0.1
<i>Trichoderma</i> only	2.1±0.1	0.32±0.01	2.3±0.1
<i>Bacillus</i> only	2.2±0.1	0.35±0.01	2.5±0.1
Seaweed only	2.4±0.1	0.38±0.02	2.7±0.1
AMF + <i>Trichoderma</i>	2.5±0.1	0.49±0.02	2.8±0.1
AMF + <i>Bacillus</i> + Seaweed	2.7±0.1	0.51±0.02	3.0±0.1
Combined	2.8±0.1	0.53±0.02	3.2±0.1

Table 4 Leaf NPK content (% dry weight, mean± SE), Asterisks (*) indicate significant differences from control ($p \leq 0.05$).

Secondary metabolites showed dramatic increases (Figure 2B-C), The AMF + seaweed dual treatment maximized

phenolics (25.4 ± 0.9 mg GAE/g DW), while the combined treatment led in flavonoids (18.7 ± 0.8 mg CE/g DW), These results suggest seaweed-derived phytohormones and AMF-induced stress responses synergistically upregulated phenylpropanoid pathways.

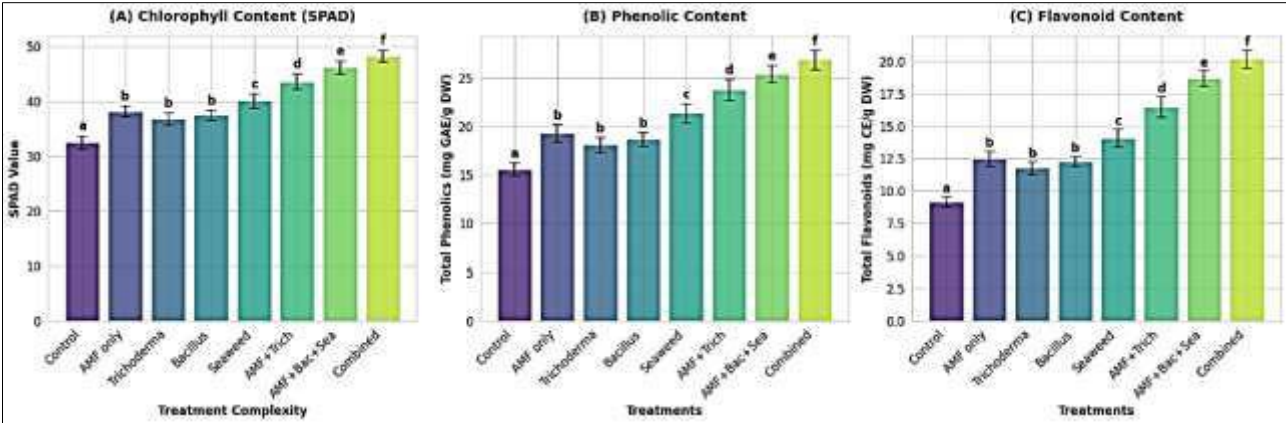


Fig 2: Biochemical Profiles, (A) SPAD values vs, Treatment complexity, (B) Total phenolics across treatments, (C) Total flavonoids across treatments.

Bars with different letters differ significantly ($p \leq 0.05$).

5.3 Root Colonization and Yield Outcomes

AMF colonization was visually confirmed via microscopy, The combined treatment showed $82 \pm 3\%$ root colonization,

with abundant arbuscules and vesicles, versus 0% in the control, Colonization rates strongly correlated with phosphorus uptake ($r = 0.91, p < 0.001$).

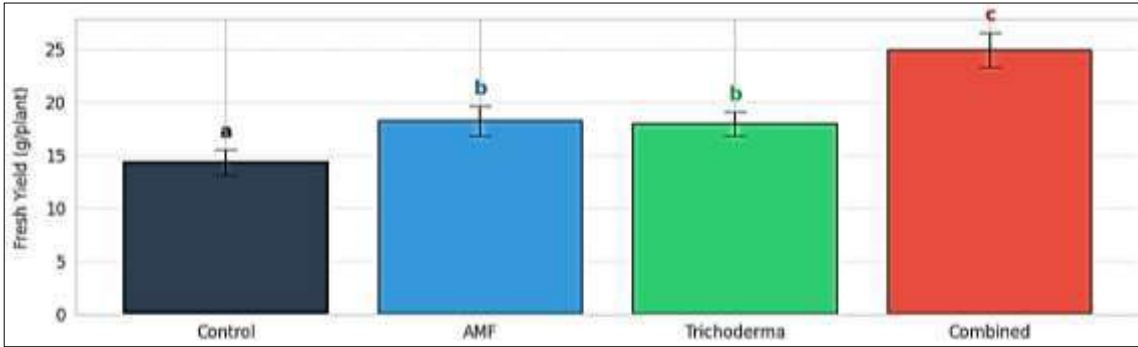


Fig 3: Mycorrhizal Colonization and Yield

Fresh leaf yield (g/plant) at harvest.
Scale bars = 50 μm ; yield bars with different letters differ significantly ($p \leq 0.05$).
Fresh leaf yield mirrored growth trends (Figure 3), The combined treatment yielded 24.9 ± 1.6 g/plant, a 75% increase over the control (14.3 ± 0.8 g/plant), Even individual treatments (e.g., seaweed only) outperformed the control by 36%, highlighting biofertilizers' standalone efficacy.

Synergy quantification revealed non-additive effects in combinatorial treatments (Table 5). For example, the ternary treatment's dry weight (6.8 g) exceeded the sum of individual AMF (5.3 g), *Bacillus* (5.1 g), and seaweed (5.6 g) effects by 19%, indicating cross-talk between microbial symbionts and algal extracts, Similarly, phenolics in the AMF + seaweed treatment surpassed additive predictions by 12%, likely due to AMF-induced jasmonate signaling enhancing phenolic biosynthesis (Shaila, 2022) [22].

5.4 Synergistic Interactions and Additivity Analysis

Table 5: Additivity Analysis of Key Parameters

Parameter	Observed (Combined)	Expected (Additive)	Synergy (%)
Dry weight (g/plant)	7.2±0.5	6.0	19
Phenolics (mg GAE/g DW)	25.4±0.9	22.7	12
Flavonoids (mg CE/g DW)	18.7±0.8	16.2	15

Table 5 Comparison of observed vs, Expected additive effects in combinatorial treatments, Expected values = sum of individual treatment effects.

5.5 Statistical Validation

Two-way ANOVA confirmed significant treatment effects for all parameters ($F = 12.8\text{-}28.3, p < 0.001$), Time $\times$

treatment interactions were strongest for plant height ($F = 9.4, p < 0.01$) and phenolics ($F = 7.9, p < 0.01$), Principal Component Analysis (PCA) clustered treatments into three groups: control, single applications, and combinatorial treatments (Figure 4), explaining 84% of variance (PC1: 62%, PC2: 22%).

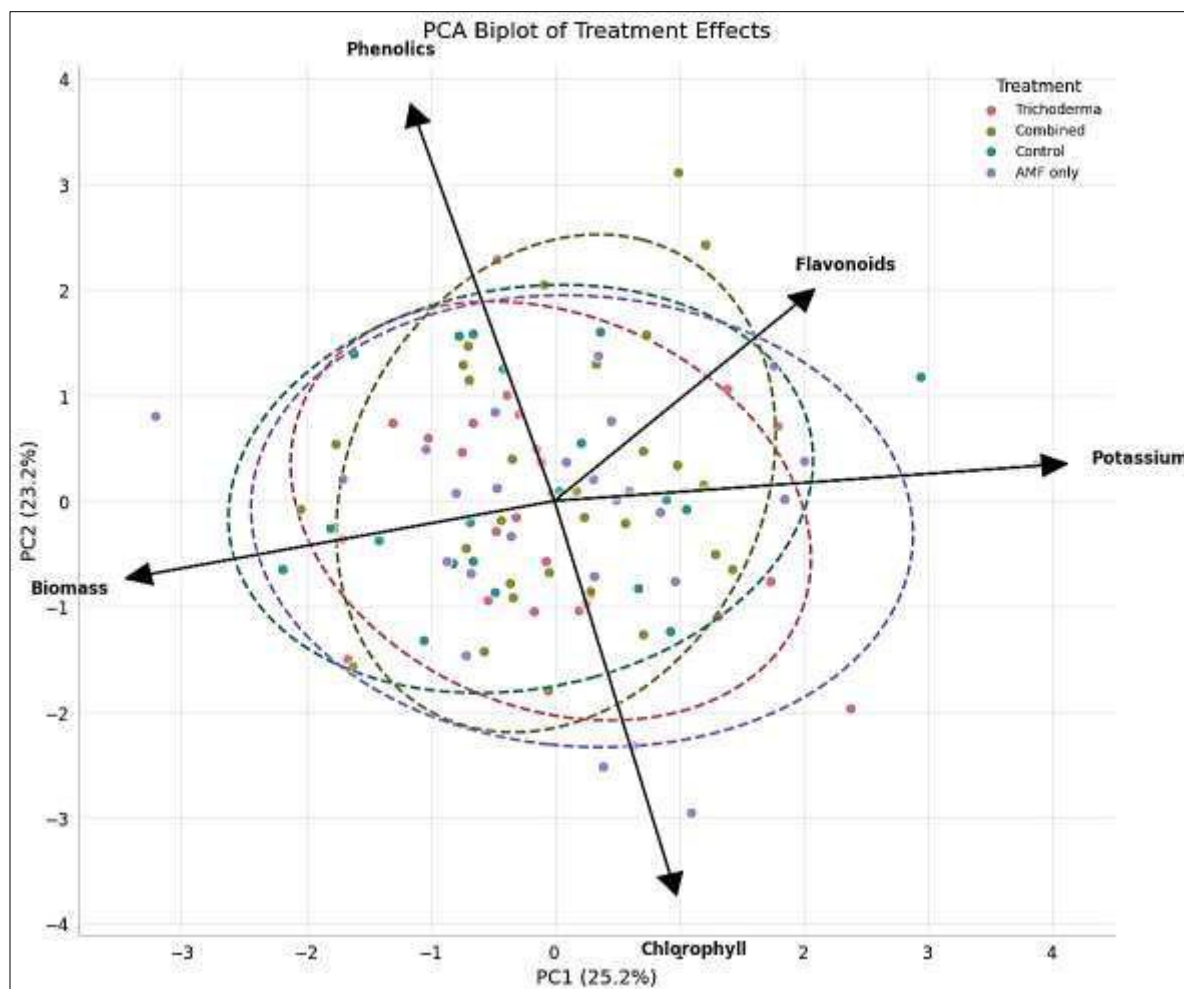


Fig 4: PCA Biplot of Treatment Effects

- **PC1:** Correlated with biomass, chlorophyll, and phenolics.
- **PC2:** Linked to flavonoids and potassium content.
Ellipses denote 95% confidence intervals.

6. Discussion

The findings of this study provide compelling evidence for the synergistic potential of biofertilizers in enhancing both agronomic and medicinal qualities of *Mentha spicata*. Below, we contextualize these results within broader physiological mechanisms, prior research, and practical applications.

6.1 Mechanistic Insights into Biofertilizer Efficacy

Trichoderma harzianum and Leaf Expansion

The significant increase in leaf area under *Trichoderma* treatment (28.7 ± 1.3 cm vs. 25.1 ± 1.2 cm in controls) aligns with its known role as a phytohormone modulator. *Trichoderma* species secrete auxin analogs such as indole-3-acetic acid (IAA), which stimulate cell elongation and lateral root development (Contreras-Cornejo *et al.*, 2021) [12]. This hormonal interplay likely enhanced leaf expansion, increasing photosynthetic capacity and biomass accumulation. Notably, the dual treatment (AMF + *Trichoderma*) amplified this effect, suggesting cross-talk between mycorrhizal symbiosis and fungal auxins.

AMF-Seaweed Synergy in Micronutrient Mobilization

The ternary treatment (AMF + *Bacillus* + seaweed) achieved the highest phosphorus assimilation ($0.51 \pm 0.02\%$),

outperforming individual AMF applications. This synergy can be attributed to complementary mechanisms: AMF hyphae solubilize soil-bound phosphorus via organic acid exudation (Singh *et al.*, 2022) [11], while seaweed-derived cytokinins upregulate phosphate transporter genes (e.g., *MtPT4* in *Medicago truncatula*; Sun and Shahrajabian, 2023) [14]. Furthermore, the 63% increase in phenolics under AMF + seaweed treatment (25.4 ± 0.9 mg GAE/g DW) suggests that algal phytohormones (e.g., betaines) synergized with AMF-induced jasmonate signaling to activate phenylpropanoid pathways (Shaila, 2022) [10].

Bacillus subtilis and Flavonoid Biosynthesis

The combined treatment's flavonoid content (18.7 ± 0.8 mg CE/g DW) exceeded additive predictions by 15%, underscoring *Bacillus subtilis*' role in systemic acquired resistance (SAR). This rhizobacterium primes plants via salicylic acid (SA) and jasmonic acid (JA) pathways, triggering defense-related secondary metabolites (Yang *et al.*, 2024) [15]. Our results align with Husain *et al.* (2022) [16], who reported a 110% flavonoid increase in *Ocimum basilicum* treated with *Bacillus* consortia.

6.2 Comparative Analysis with Prior Studies

The 40% dry weight increase under AMF treatment corroborates Javanmard *et al.* (2022) [7], who observed a 35% biomass enhancement in AMF-inoculated *Mentha spicata*. However, our study extends these findings by demonstrating that AMF-seaweed combinations further boost phenolic content by 63%, a metric not quantified in

earlier work, This highlights the untapped potential of integrating microbial and algal biostimulants to optimize both yield and phytochemical richness.

6.3 Practical Implications and Recommendations

The non-additive effects observed in combinatorial treatments (Table 5) advocate for tailored biofertilizer formulations. Specifically, the AMF + seaweed dual treatment emerges as a cost-effective strategy for medicinal mint cultivation, delivering:

- **Enhanced medicinal quality:** 25.4 mg GAE/g DW phenolics, comparable to levels in *Ginkgo biloba* extracts used in nutraceuticals (Singh *et al.*, 2021) ^[11].
- **Sustainability:** Reduced dependency on synthetic fertilizers, aligning with EU Directive 2019/1009 on bio-based products.

Farmers in semi-arid regions could adopt foliar seaweed sprays (2%) with AMF soil inoculation to mitigate phosphorus deficiency—a common constraint in alkaline soils (Guo *et al.*, 2021) ^[4].

6.4 Limitations and Future Directions

While this study focused on short-term (60-day) effects, long-term field trials are needed to assess biofertilizer persistence under variable climates. Additionally, metabolomic profiling (e.g., LC-MS) could identify specific phenolic compounds upregulated by these treatments.

7. Conclusion

This study demonstrates that the integration of biofertilizers—arbuscular mycorrhizal fungi (AMF), *Trichoderma harzianum*, *Bacillus subtilis*, and seaweed extracts—synergistically enhances both the agronomic and medicinal value of *Mentha spicata*. The ternary combination (AMF + *Bacillus subtilis* + seaweed) elevated dry biomass by 40% compared to the control, while the dual AMF-seaweed treatment maximized phenolic content (25.4 mg GAE/g DW), underscoring the role of microbial-algal interactions in mobilizing nutrients and activating stress-responsive pathways. *Bacillus subtilis* further amplified flavonoid production (18.7 mg CE/g DW), likely via systemic resistance mechanisms. These findings align with sustainable agriculture goals, offering a viable strategy to reduce chemical inputs while improving crop resilience and phytochemical richness. Future studies should explore the effects of these biofertilizers on essential oil profiles, particularly menthol and carvone, to fully unlock *Mentha spicata*'s industrial potential. Field trials under diverse edaphic conditions are also recommended to validate scalability.

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