**SAINS TANAH – Journal of Soil Science and Agroclimatology**

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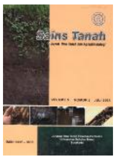

# Enhancing peppermint growth: Investigating the interplay of Biochar and Nitrogen levels

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## ARTICLE INFO

**Keywords**:
Biochar
Biochemical compounds
Elemental concentrations
Growth indicators
Nitrogen

Article history
*Submitted: 2024-06-26*
*Revised: 2024-08-16*
*Accepted: 2024-09-17*
*Available online: 2024-11-02*
*Published regularly:*
*December 2024*

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## ABSTRACT

Peppermint (*Mentha piperita*) is valued for its medicinal properties and applications in the food and health industries. However, optimizing growth conditions to enhance yield and quality remains challenging. The study aimed to evaluate the impact of nitrogen and biochar on peppermint growth, elemental content, and biochemical composition, using a factorial experiment with a randomized block design and four-pot replications during the 2022-2023 crop year. Biochar levels up to 2% by weight increased plant height by 25%, chlorophyll index by 20%, leaf count by 18%, and dry weights of shoots and roots by 15%, but declined beyond this threshold. Nitrogen levels up to 75 mg per gram of soil increased plant height by 33.8%, chlorophyll index by 30%, and dry weights of aerial organs by 28%. Elemental concentrations in aerial organs peaked at 3% biochar, increasing potassium by 22%, phosphorus by 18%, and calcium by 15%, while zinc and copper decreased by 10% and 12%, respectively. Anthocyanin, flavonoid, and total phenol concentrations decreased by 20%, 30%, and 35% respectively with increasing biochar and nitrogen levels. Applying up to 2% biochar by weight optimizes peppermint yield. Nitrogen mitigates adverse effects of high biochar levels, with 50 mg nitrogen recommended at 2% biochar for optimal yield. These findings offer sustainable agricultural practices to improve crop productivity in nutrient-deficient soils and promote environmentally friendly agricultural practices.

**How to Cite**: Haghighatnia, H., Talebi, E., Nezhad, M.K. (2024). Enhancing peppermint growth: Investigating the interplay of Biochar and Nitrogen levels. Sains Tanah Journal of Soil Science and Agroclimatology, 21(2): 145-155. https://doi.org/10.20961/stjssa.v21i2.89008

## 1. INTRODUCTION

Medicinal plants are regional treasures with global significance, enriching the world's wealth while enhancing life's scenery as non-polluting, active factories providing diverse and beneficial products (Talebi et al., 2022). Within Iran's rich flora, comprising over 7,500 plant species, many possess medicinal properties, offering valuable resources for humanity's well-being (Ghorbanalizadeh & Akhani, 2022). Secondary metabolites within plants, harboring helpful active compounds, are revered as natural blessings, effectively alleviating human afflictions (Vega et al., 2022). These compounds serve as tools for plants to adapt to environmental variations, safeguarding their existence and progeny. When exposed to diverse ecological conditions, plants undergo morphological and biochemical adaptations (Lamalakshmi Devi et al., 2017).

The extracts from plants predominantly contain terpenes and phenylpropenes, with terpenes being more prevalent. However, phenylpropenes often contribute significantly to flavor and aroma (Atkinson, 2018). Peppermint, specifically, synthesizes terpenes stored mainly in epidermal glands and leaf hairs, with over 20 types of compounds identified in its extract, the most significant being menthol (40 to 60%) (de Souza et al., 2015). The concentration of menthol peaks in young leaves, while the flower extract contains a lesser amount, with menthol (10 to 20%) being prominent (Zaia et al., 2016).

Many soils used for peppermint cultivation suffer from poor nutrient content and inadequate organic matter, leading to suboptimal plant growth and reduced yields. Addressing these soil deficiencies is crucial for improving peppermint production. Previous research has shown that amending soil with biochar can enhance soil fertility by improving its physical, chemical, and biological properties (Rassaei, 2024). However, the optimal conditions for using biochar,

STJSSA, p-ISSN 1412-3606 e-ISSN 2356-1424
http://dx.doi.org/10.20961/stjssa.v21i2.89008

particularly in combination with varying nitrogen levels, remain unclear (Rassaei, 2023).

Peppermint is valued for its rich content of essential oils and other compounds such as limonene, peperitone, pulegone, pinene, sabinene, cineol, and methyl acetate (Díaz-Maroto et al., 2008). These compounds contribute to its medicinal and commercial value. Given the crucial role of water in crop cultivation and the environmental impact of excessive chemical fertilizer use, employing biochar in agriculture emerges as a promising strategy to enhance yield, water accessibility, and nutrient absorption.

High organic carbon content characterizes biochar, reminiscent of the fertility observed in Amazonian soils (Fitriani et al., 2020; Tuladhar et al., 2021). Biochar has played a pivotal role in transitioning from the green revolution to sustainable agricultural systems by enriching soil organic matter, improving soil fertility, and minimizing adverse environmental impacts on water and soil resources (Alkharabsheh et al., 2021).

Notably, biochar influences plant growth by potentially enhancing nutrient availability in absorbable forms, mitigating decomposition intermediates and allelochemicals, favoring shoot growth, and altering root characteristics (Saeed et al., 2021). However, it can also decrease nitrogen availability in the soil, particularly when associated with high C/N ratio and nitrogen mineralization buffering (Pan et al., 2021).

Nitrogen, a pivotal element, exerts a significant influence on vegetative growth and agricultural yields. Its surplus leads to increased flower and fruit formation but delays maturity, affecting fruit quality adversely and diminishing disease resistance (Yahia et al., 2019). Nitrogen deficiency manifests in leaves as paleness, indicating insufficient chlorophyll formation, primarily affecting crops in arid and semi-arid regions due to organic matter scarcity (Ganeshamurthy et al., 2015). Previous research has demonstrated that optimal nitrogen levels are crucial for maximizing plant growth and yield. Researchers highlighted the importance of balanced nitrogen application in improving the yield and quality of crops such as corn and wheat (Jones et al., 2009; Röös et al., 2018). Additionally, integrating biochar with nitrogen has shown promising results in enhancing soil fertility and crop productivity (Jeffery et al., 2011).

Despite these findings, the specific effects of biochar and nitrogen combinations on peppermint growth and its biochemical composition have not been extensively studied. This study aims to fill this gap by investigating the impact of varying nitrogen concentrations and biochar levels on growth indicators, elemental concentrations, and biochemical compounds in peppermint. The novelty of this research lies in its comprehensive approach to optimizing soil amendments to enhance peppermint yield and quality. Specifically, the study will determine the most effective combination of biochar and nitrogen levels to maximize plant growth and the concentration of valuable biochemical compounds. By addressing these objectives, the research aims to offer practical recommendations for improving peppermint cultivation through targeted soil management practices.

## 2. MATERIAL AND METHODS

### 2.1. Geographic and Altitudinal Context

The experiment took place during September 2023, at coordinates 28.3648° N and 54.4127° E, situated at an altitude of 1021 meters above sea level in Zarin Dasht, Darab, Iran. Peppermint plants (*Mentha piperita* L.) were cultivated in individual pots within an open-space setting. The soil in this region is characterized by sandy loam texture, low organic matter content, and poor nutrient availability, which poses challenges for optimal plant growth. The soil has a moderate water-holding capacity, necessitating amendments to enhance fertility and support peppermint cultivation. The average temperature ranged between 27-32°C, and the average relative humidity was between 30-50%.

### 2.2. Nitrogen and Biochar Treatments

Four distinct levels of nitrogen treatments were applied using urea fertilizer: 0, 25, 50, and 75 mg of nitrogen per gram of soil (mg $g^{-1}$). These levels were selected based on previous research indicating varying impacts on plant growth and nutrient uptake, allowing for a comprehensive evaluation of nitrogen's effect on peppermint cultivation. Additionally, four levels of biochar application (0, 1, 2, and 3% by weight) using Kohbanan natural mineral biochar were incorporated into the pot soils. The chosen levels of biochar were based on preliminary studies that suggested these concentrations could effectively enhance soil properties without causing potential negative effects (DeLuca et al., 2009; Jeffery et al., 2011). Prior to application, the biochar was characterized for pH (7.01), electrical conductivity (EC) (3.45 ds $m^{-1}$), and organic carbon percentage (42.8%) (Table 1). The Kohbanan biochar is derived from local mineral sources and processed through a high-temperature pyrolysis method, which contributes to its high organic carbon content and distinct physical properties.

### 2.3. Pot Preparation and Soil Analysis

Plastic pots, each 20 cm in diameter and 25 cm in height, were filled with a standardized substrate composed of a 2:1 ratio of field soil to sand. This ratio was selected to achieve a balance between soil texture and drainage capacity, ensuring adequate aeration and moisture retention for optimal plant growth. After a 72-hour air-drying phase, the soil underwent preliminary physical and chemical analysis to determine its basic characteristics before the experiment began (Table 2). The analysis included measurements of soil pH, nutrient content, and texture, which were essential for establishing baseline conditions and ensuring the reliability of the experimental results.

**Table 1.** Chemical characteristics of biochar

| Brand | pH | EC (ds $m^{-1}$) | OC (%) |
|---|---|---|---|
| Kohbanan natural mineral biochar | 7.01 | 3.45 | 42.8 |

**Remakrs:** OC = Organic carbon

**Table 2.** Physical and Chemical Analysis of Experimental Soil

| EC (ds m$^{-1}$) | pH | OC | Sand | Silt | Clay | N | P | K | Fe | Zn | Mn | Cu |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % | | | | ppm | | | | | | |
| 2.5 | 7.6 | 0.22 | 15 | 50 | 35 | 100 | 8 | 245 | 62 | 1.4 | 42 | 0.72 |

**Remakrs:** EC = electrical conductivity, OC = Organic Carbon

### 2.4. Nutrient Evaluation and Drainage Setup

The evaluation of soil nutrients, including phosphorus, nitrogen, potassium, iron, zinc, manganese, and copper, is conducted using laboratory methods such as atomic absorption spectroscopy (AAS), inductively coupled plasma optical emission spectrometry (ICP-OES), and colorimetric analysis (Nasukawa et al., 2023). Amendments were made based on soil test results and peppermint fertilizer requirements. Strategic placement of drainage holes beneath each pot, filled with 2 cm of gravel (4-6 mm diameter), ensured adequate drainage and prevented waterlogging. The choice of 2 cm gravel with a 4-6 mm diameter was based on its effectiveness in providing sufficient drainage while preventing soil particles from clogging the drainage holes. Each pot contained a uniform 8 kg of soil substrate.

### 2.5. Pre-treatment Care and Nitrogen Application

Before treatment application, soil phosphorus levels were adjusted to 15 ppm by adding triple superphosphate. Throughout the experiment, regular watering, and necessary care, including fertilizer application and pest management, were provided to ensure optimal growing conditions. The staggered three-stage nitrogen treatment regimen commenced 14 days after full seedling establishment. This timing was chosen to align with the critical growth stages of peppermint, ensuring that the plants received optimal nitrogen levels during key developmental phases. The staggered approach was implemented to assess better the effects of varying nitrogen levels on plant growth and yield, allowing for a more detailed analysis of nutrient uptake and its impact on peppermint cultivation.

### 2.6. Plant Cultivation and Harvest

Each pot accommodated four peppermint plants (5 cm in height). Careful maintenance, including watering and manual weed control, was conducted throughout the growth period. The vegetative conditions during harvesting were carefully optimized: the average temperature during the growth period was between 27-32°C, and the average relative humidity ranged from 30-50%. The soil was well-drained and enriched with organic matter, and the plants were exposed to at least six hours of sunlight daily. Harvesting occurred 7-8 weeks after planting when the plants were in full bloom, with a separate collection of aerial and root parts for subsequent laboratory analyses.

### 2.7. Physiological and Biochemical Assessments

Plant height was measured by determining the distance from the soil surface to the plant's terminal point at the final experimental phase. Leaf count was determined by averaging counts across all pots. Aerial plant parts were weighed immediately post-harvest, followed by drying in a greenhouse at 65°C until a constant weight was achieved. Similarly, roots were carefully separated, washed, dried, and weighed to determine dry weight. Nutrient analysis of plant samples involved ashing, acid dissolution, and spectrophotometric measurement of phosphorus, iron, copper, manganese, zinc, and potassium concentrations (Nasukawa et al., 2023).

Total phenolic compounds were quantified using Folin-Ciocalteu's reagent, with absorbance measured at 760 nm (Lucas et al., 2022). Total flavonoid content was assessed at 510 nm after specific chemical reactions, using quercetin as a standard reference (Shraim et al., 2021). Total anthocyanin content was measured by centrifugation and subsequent absorbance readings at 550 nm (Taghavi et al., 2022). All biochemical analyses were expressed as concentrations per gram of dry weight.

### 2.8. Statistical Analysis

Data analysis was performed using SAS 9.1 and MSTAT C statistical software to assess variance between treatments and determine significant differences. Specifically, SAS was configured with default settings for variance analysis, while MSTAT C was used for initial data screening. Duncan's multi-range test was employed at a 5% significance level to compare treatment means, with a focus on ensuring robust comparison through equal variance assumptions. Additionally, post-hoc analyses were conducted to verify the consistency of results. Results were graphically represented using Microsoft Excel, with charts and graphs generated to facilitate clear interpretation and visualization of experimental outcomes.

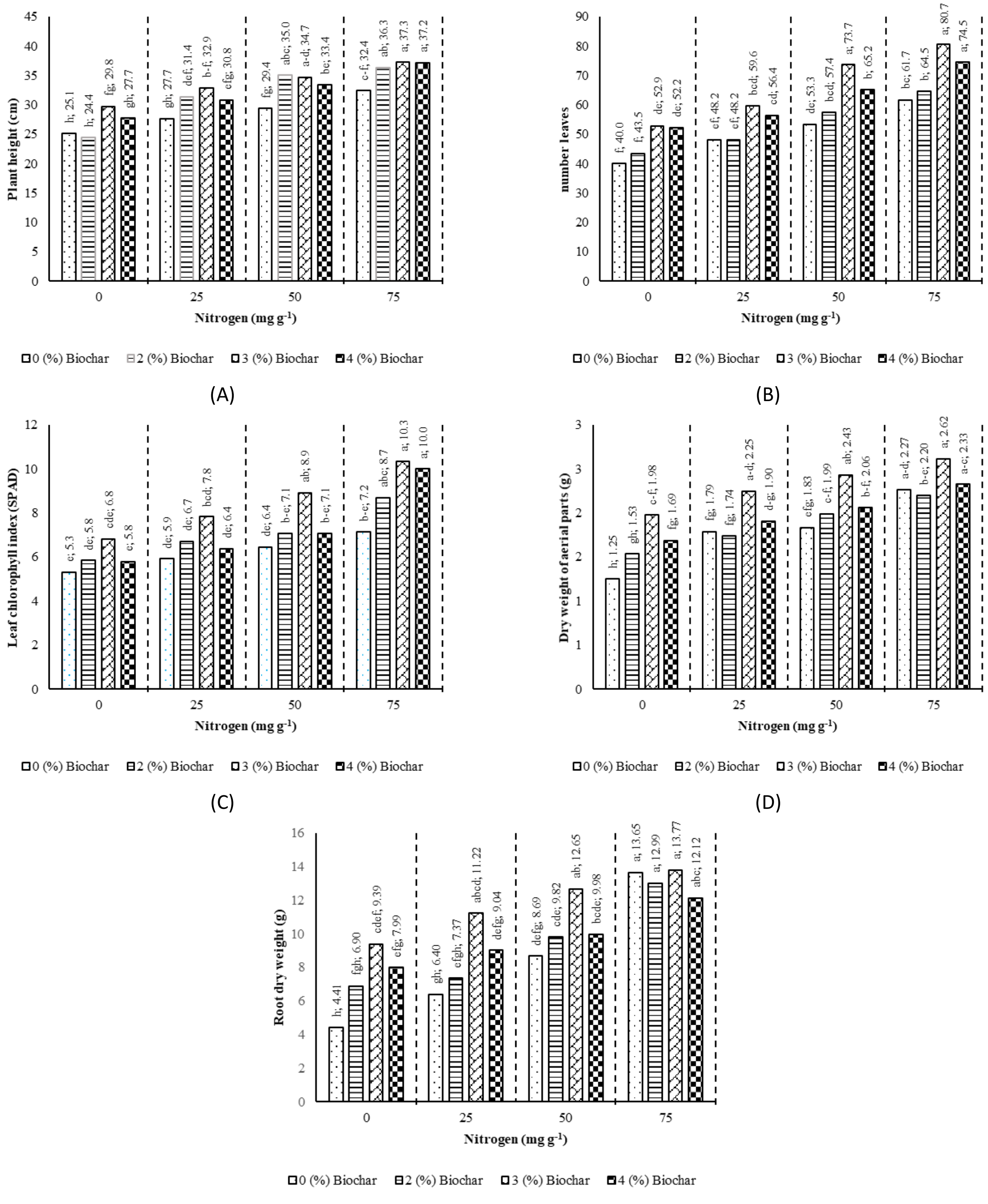
## 3. RESULTS

### 3.1. Morphological Characteristics

The study investigated the effects of nitrogen and biochar treatments on various morphological characteristics of peppermint Key findings include significant impacts on plant height, leaf development, chlorophyll index, and dry weight of aerial and root organs.

### 3.2. Effect of Biochar and Nitrogen on Plant Height

The analysis of variance revealed a substantial influence of nitrogen and biochar levels on plant height. Increasing nitrogen levels exhibited a significant linear correlation with enhanced plant height. Specifically, the treatment with 75 mg of nitrogen per gram of soil (mg g$^{-1}$) resulted in the tallest plants, reaching an average height of 35.8 cm, representing a significant 33.8% increase compared to the nitrogen-free control (Fig. 1A) ($P<0.05$). Biochar application also showed a notable impact, with the highest average height of 33.7 cm observed at the 2% biochar level by weight ($P<0.05$). However, statistical analysis indicated no significant difference in plant height between biochar levels of 1 and 3% ($P>0.05$).



(A)

(B)

(C)

(D)

(E)

**Figure 1 .** The effect of the interaction of different levels of nitrogen and biochar on peppermint growth parameters including plant height (A), leaf number (B), chlorophyll index (C), and aerial and root dry weight (E)

Further investigation into the interaction between nitrogen and biochar levels highlighted that combinations of 75 mg nitrogen with 2 and 3% biochar levels resulted in the tallest plants, reaching heights of 37.3 and 37.2 cm, respectively ($P<0.05$). Across all nitrogen treatments, the most significant increase in plant height consistently occurred at the 2% biochar level, indicating its optimal impact on plant growth in conjunction with nitrogen treatments.

### 3.3. Impact of Nitrogen and Biochar on Leaf Development

Nitrogen treatments significantly influenced leaf development in peppermint plants. The highest leaf count per plant was observed with the 75 mg nitrogen treatment, which produced an average of 66.7 leaves per plant. Incremental nitrogen levels of 25, 50, and 75 mg led to average leaf count increases of 12.6%, 32.35%, and 49.22%, respectively, compared to the nitrogen-free control (Fig. 1B). The 75 mg nitrogen treatment resulted in a significantly higher leaf count than other treatments ($p < 0.05$), while the 25 mg nitrogen treatment did not differ statistically from the nitrogen-free control.

Regarding biochar, the 2% biochar treatment yielded the highest average leaf count of 66.7 leaves per plant. Increasing biochar proportions beyond 2% led to a decrease in leaf counts, with the 3% biochar treatment statistically similar to the 2% biochar treatment in terms of leaf count ($p > 0.05$). This reduction in leaf count at higher biochar levels may be attributed to excessive biochar leading to impaired nutrient availability or soil structure issues. The 1% biochar treatment and the biochar-free control showed the lowest leaf counts, with no significant differences between these groups ($p > 0.05$).

Across nitrogen levels, up to the 2% biochar level, leaf count consistently increased. However, exceeding this biochar level disrupted the positive trend in leaf count, likely due to the negative effects of excessive biochar. The highest average leaf count resulted from the interaction of 75 mg nitrogen with both 2% and 3% biochar levels, as well as the interaction of 50 mg nitrogen with 2% biochar.

### 3.3. Nitrogen and Biochar Impact on Leaf Chlorophyll Index

Nitrogen concentration significantly influenced the leaf chlorophyll index. The treatment with 75 mg of nitrogen exhibited the highest chlorophyll index, showing a notable 52.6% increase compared to the nitrogen-free control (Fig. 1C). In contrast, the 25 mg nitrogen treatment did not produce a significant difference in the chlorophyll index compared to the control.

Increasing biochar levels up to 2% by weight initially enhanced the leaf chlorophyll index, but levels beyond 2% resulted in a decline. This trend may be attributed to the fact that while biochar improves soil structure and nutrient availability up to a certain concentration, excessive biochar can lead to imbalances in soil nutrients or reduced moisture availability, negatively affecting chlorophyll production. Treatments with 1%, 2%, and 3% biochar showed no statistically significant differences in the leaf chlorophyll index ($p > 0.05$).

The combined impact of biochar and nitrogen on the leaf chlorophyll index demonstrated a consistent increase up to a 2% biochar level. Specifically, the treatment with 2% biochar and 75 mg nitrogen achieved the highest chlorophyll index at 10.3 units, showing significant improvement. The interaction between 50 mg nitrogen and 2% biochar also formed the highest statistical group, demonstrating a substantial effect on the chlorophyll index without significant variance. Additionally, the interaction of 3% biochar with 50 mg nitrogen yielded similar statistical significance to the 2% biochar and 75 mg nitrogen treatment, indicating that this combination effectively maximized the chlorophyll index.

### 3.4. Impact of Nitrogen and Biochar on Aerial Organ Dry Weight

Nitrogen application significantly increased the dry weight of aerial organs. The treatment with 75 mg of nitrogen achieved the highest average dry weight of 2.35 grams, representing a substantial 45.9% increase compared to the nitrogen-free control ($p < 0.05$). The 50 mg nitrogen treatment also showed a notable 28.9% increase in dry weight compared to the control, though this difference was not statistically significant ($p > 0.05$). The 25 mg nitrogen treatment did not significantly differ from the nitrogen-free control ($p > 0.05$) (Fig. 1D).

Biochar application, particularly up to 2% by weight, also significantly increased the dry weight of aerial organs. The 2% biochar treatment demonstrated the highest average dry weight of 2.32 grams, marking a 29.8% increase compared to the control ($p < 0.05$). No significant difference was observed between the 2% and 3% biochar treatments ($p > 0.05$).

When comparing the dry weight of aerial parts, both biochar and nitrogen applications led to increases. Treatments with 2% biochar and varying nitrogen levels (75, 50, and 25 mg) did not show significant differences among them ($p > 0.05$), indicating similar effects on aerial organ weight. However, the dry weight of aerial parts increased with biochar up to 2% but decreased beyond this threshold. This reduction in dry weight at higher biochar levels could be attributed to potential nutrient imbalances or changes in soil structure that impede optimal plant growth. The reduction rates were 14.5%, 15.14%, 15.03%, and 11.03% at 0, 25, 50, and 75 mg nitrogen levels, respectively. Increasing nitrogen appeared to mitigate some of the adverse effects of higher biochar percentages on aerial organ weight, likely by enhancing nutrient availability and overall plant growth.

### 3.5. Influence of Nitrogen and Biochar on Root Dry Weight

Nitrogen levels significantly influenced root dry weight in peppermint plants. The highest root dry weight, averaging 13.13 grams, was observed with the 75 mg nitrogen treatment. In contrast, the 25 mg nitrogen treatment did not significantly affect root dry weight, aligning statistically with the nitrogen-free control ($p > 0.05$) (Fig. 1E).

Biochar application also had a notable impact on root dry weight. The 2% biochar treatment displayed the highest root dry weight at 11.76 grams, with no significant statistical differences compared to the 1% and 3% biochar levels ($p > 0.05$). Biochar levels of 1%, 2%, and 3% increased root dry weight by 11.9%, 41.8%, and 18.06%, respectively, compared to the nitrogen-free control ($p < 0.05$).

Root dry weight increased with biochar application up to the 2% weight threshold but decreased at higher levels. This decline at higher biochar levels may be attributed to potential nutrient imbalances or reduced soil aeration affecting root development. Excessive biochar can alter soil pH and nutrient availability, potentially leading to suboptimal root growth conditions. No statistically significant differences were observed between the 2% and 3% biochar treatments across all nitrogen levels ($p > 0.05$). At the 75 mg nitrogen level, all biochar treatments similarly influenced root dry weight without significant differences ($p > 0.05$). Additionally, no significant differences were found between the 2% biochar treatments across 25, 50, and 75 mg nitrogen concentrations ($p > 0.05$).

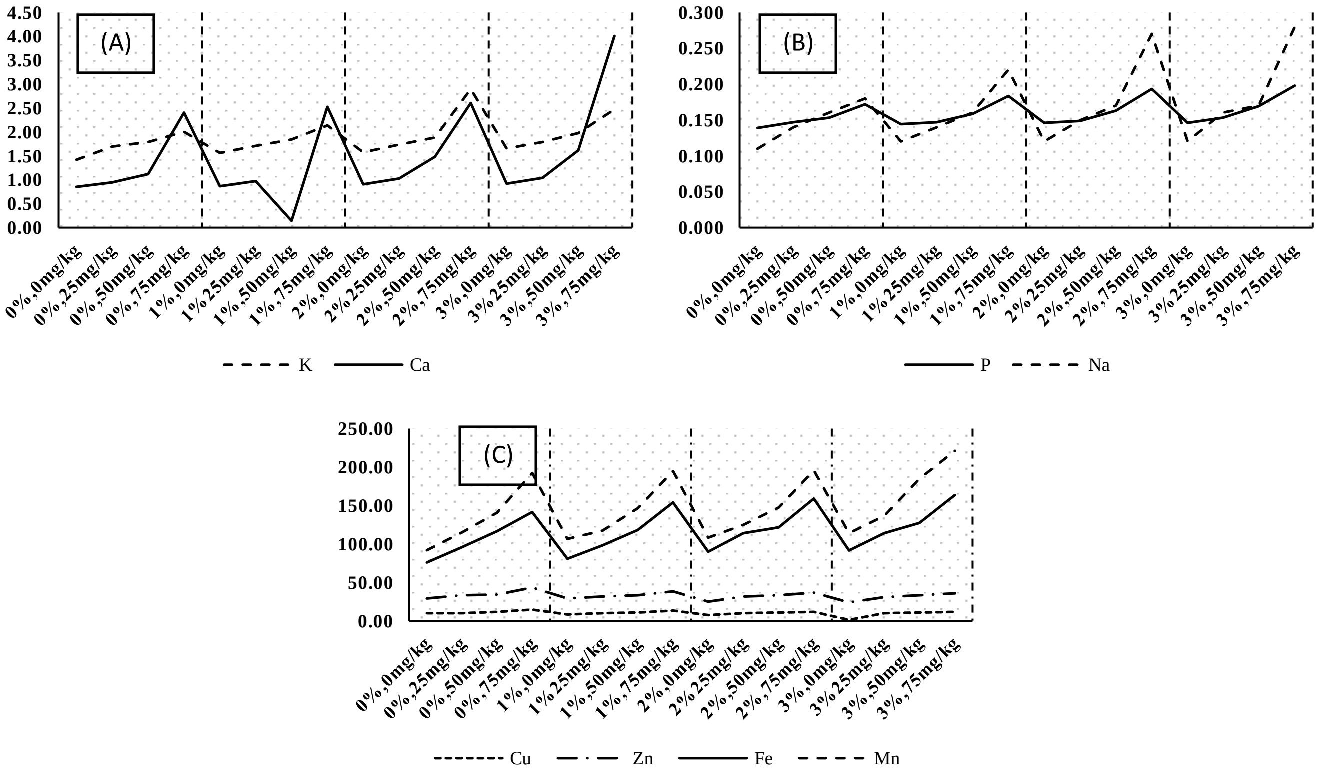


**Figure 2.** The effect of simple and mutual effects of different amounts of nitrogen (mg g$^{-1}$), biochar (%) on the concentration of different elements of aerial organs (Ca and K (A), P and Na (B), Cu, Zn, Fe, and Mn (C))

**Table 3.** The effect of different levels of biochar and nitrogen on the biochemical compounds in aerial organs

| Biochemical compounds | Nitrogen (mg g$^{-1}$) | | | | Biochar (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 25 | 50 | 75 | 0 | 1 | 2 | 3 |
| Anthocyanin | $0.64^{A}$ | $0.58^{B}$ | $0.58^{B}$ | $0.52^{C}$ | $0.93^{a}$ | $0.70^{b}$ | $0.47^{c}$ | $0.23^{d}$ |
| Total flavonoids | $420.50^{A}$ | $390.25^{B}$ | $358.00^{C}$ | $338.60^{C}$ | $513.00^{a}$ | $401.25^{b}$ | $356.50^{c}$ | $236.60^{d}$ |
| Total phenol | $3.39^{A}$ | $3.33^{A}$ | $3.13^{B}$ | $2.82^{C}$ | $3.99^{a}$ | $3.41^{b}$ | $3.09^{c}$ | $2.18^{d}$ |

In each row, means with at least one letter in common do not have a statistically significant difference (Duncan 5%).

**Table 4.** Regression equation and R$^2$ effect of different levels of biochar and nitrogen on the biochemical composition of aerial organs

| Biochemical compounds | Nitrogen (mg g$^{-1}$) | | Biochar (%) | |
|---|---|---|---|---|
| | Equation | R$^2$ | Equation | R$^2$ |
| Anthocyanin | $y = 0.035x + 0.4951$ | 0.98 | $y = -0.233x + 1.165$ | 0.99 |
| Total flavonoids | $y = 27.795x + 307.35$ | 0.98 | $y = -87.395x + 595.33$ | 0.97 |
| Total phenol | $y = 0.1889x + 2.6959$ | 0.92 | $y = -0.5746x + 4.6047$ | 0.96 |

## 3.6. Elemental Dynamics in Peppermint Aerial Organs Influenced by Nitrogen and Biochar

Biochar and nitrogen treatments significantly influenced the elemental concentrations in peppermint aerial organs. Increasing biochar levels led to a rise in potassium concentration, with the highest amount (1.97%) observed at the 3% biochar level, marking a 14.24% increase compared to the control without biochar ($p < 0.05$). Elevated nitrogen levels also correlated with increased potassium concentrations. Specifically, nitrogen levels of 25, 50, and 75 mg g$^{-1}$ resulted in potassium increases of 11.1, 20.28, and 42.8%, respectively, forming distinct statistical groups ($p < 0.05$) (Fig. 2).

Phosphorus concentration in aerial parts increased with higher nitrogen and biochar levels. While nitrogen up to 25 mg g$^{-1}$ did not significantly affect phosphorus concentration, a substantial 29.9% increase was observed at 75 mg nitrogen ($p < 0.05$). Biochar at 1% did not significantly alter phosphorus concentration, but the 3% biochar treatment exhibited the highest phosphorus concentration at 0.17% ($p < 0.05$). The interaction of 2% and 3% biochar with 75 mg nitrogen showed the highest phosphorus concentrations, though without significant differences ($p > 0.05$).

Calcium concentration was unaffected by biochar levels up to 2% and by nitrogen levels up to 25 mg g$^{-1}$. The highest calcium concentration of 2.88% was observed with the 75 mg nitrogen treatment ($p < 0.05$). Sodium concentrations increased significantly with 2% and 3% biochar compared to lower biochar levels, with nitrogen levels further correlating with increased sodium concentrations, particularly notable at 75 mg $g^{-1}$ ($p < 0.05$).

Zinc concentration showed an inverse relationship with biochar levels, decreasing as biochar levels increased. This trend may be due to changes in soil pH or nutrient interactions affecting zinc availability. Higher nitrogen levels increased zinc concentration, especially in treatments without biochar ($p < 0.05$). Copper concentration decreased with higher biochar levels, showing the highest concentrations in treatments without biochar and increased nitrogen ($p < 0.05$).

Iron and manganese concentrations increased with biochar levels, with the highest concentrations observed at the 3% biochar treatments ($p < 0.05$). Elevated nitrogen levels significantly increased manganese concentration, with the highest concentration noted at 75 mg $g^{-1}$ nitrogen in combination with 3% biochar ($p < 0.05$).

The inverse relationships for zinc and copper with biochar levels might be explained by shifts in soil chemistry and nutrient competition, highlighting the complexity of biochar's effects on elemental availability.

### 3.7. Influence of Biochar and Nitrogen on Secondary Metabolites in Peppermint Aerial Organs

Nitrogen and biochar treatments influenced secondary metabolite concentrations in peppermint aerial organs. Anthocyanin concentration decreased significantly with higher biochar levels in aerial parts. The lowest concentration (0.23 mmol $g^{-1}$ of dry matter) was observed at the 3% biochar level, marking a 75% decrease compared to the control (Fig. 2). Elevated nitrogen levels also decreased anthocyanin concentration, with reductions of 1.1, 11.1, and 12.2% for 25, 50, and 75 mg nitrogen, respectively, compared to the control (Table 3 and 4).

Increasing biochar levels led to a decrease in flavonoid concentration in peppermint aerial parts. At biochar levels of 1, 2, and 3%, flavonoid concentrations decreased by 21.8, 30.5, and 53.9%, respectively, compared to the control. Elevated nitrogen levels demonstrated a linear and significant decrease in total flavonoids, with reductions of 15.3 and 24.2% for 50 and 75 mg nitrogen, respectively, compared to the nitrogen-free control.

Higher biochar percentages correlated with decreased total phenol concentrations in peppermint aerial organs. Decreases of 14.4, 22.5, and 45.3% occurred at biochar levels of 1, 2, and 3%, respectively, compared to the control without biochar. Elevated nitrogen concentrations also led to decreased total phenol concentrations, particularly notable at 50 and 75 mg nitrogen, reducing concentrations by 7.5% and 16.6%, respectively, compared to the nitrogen-free control.

## 4. DISCUSSION

The study reveals that optimal growth parameters in peppermint plants, including increased plant height, leaf count, chlorophyll index, and dry weight of aerial and root organs, were significantly influenced by the combined application of nitrogen and biochar. Specifically, treatments involving 75 mg nitrogen per gram of soil combined with 2% biochar by weight consistently resulted in the most favorable outcomes across these parameters. This synergy highlights the potential of nitrogen and biochar as effective growth enhancers in peppermint cultivation.

The enhancement of plant growth observed can be attributed to several interconnected mechanisms influenced by nitrogen and biochar. Nitrogen is crucial in plant metabolism by enhancing photosynthetic efficiency, promoting protein synthesis, and facilitating overall growth processes (Euring et al., 2014; Parkash & Singh, 2020). This is evident in our findings, where increased nitrogen levels correlated with greater plant height. Nitrogen availability directly influences internode elongation and cell division, which are essential for achieving taller plants (Lebrun et al., 2020).

Biochar, as a soil amendment, enhances soil fertility and nutrient availability due to its porous structure and high surface area (Jabborova, Annapurna, et al., 2021; Mehdizadeh et al., 2019). Our results align with previous studies showing that biochar improves soil physical properties, including water retention and nutrient availability, thus promoting better root development and nutrient uptake (Peiris et al., 2022; Wang et al., 2018). The interaction between nitrogen and biochar further amplifies these benefits. Biochar's ability to retain and gradually release nutrients complements nitrogen's role in providing a steady supply of mineralized nitrogen throughout the plant growth cycle(Canatoy & Daquiado, 2021; Otori et al., 2017). This synergistic effect ensures that plants receive optimal nutrition, which is crucial for sustained growth and development. Thus, the combined application of nitrogen and biochar optimizes plant growth by enhancing nutrient availability, improving soil structure, and supporting essential physiological processes in plants.

Research has demonstrated that decreasing nitrogen levels correlate with reduced leaf production in plants (Hikosaka, 2016). Studies underscore the necessity of ammonium in nutrient solutions to maintain adequate leaf development (Zhu et al., 2021). Experiments on lettuce and cabbage have shown that biochar application increases plant biomass and leaf number compared to controls, highlighting its role in improving soil characteristics and nutrient availability (Hasan, 2018; Jabborova, Kadirova, et al., 2021). Additionally, research on fenugreek (*Trigonella foenum-graecum* L.) further supports biochar's efficacy in enhancing nitrogen uptake efficiency, prolonging nutrient availability, and promoting increased branching and leaf production, demonstrating its potential for sustainable agricultural practices (Shaaban et al., 2024).

Nitrogen accelerates the growth of terminal meristem cells, contributing to vegetative growth of stems and shoots. Researchers have reported significant increases in the dry weight of plant stems with increasing nitrogen consumption (Luo et al., 2020). The findings of our research suggest that biochar, as a soil conditioner, increases soil fertility and creates suitable conditions for plant growth and performance. Its specific surface area and high density enhance soil microbial populations, although excessive biochar use may increase soil salinity, potentially affecting aerial organ weight (Murtaza et al., 2021). Studies on peppermint and spinach have shown increased weight of aerial organs with biochar use (Mumivand et al., 2023; Nobaharan et al., 2022).

The simultaneous addition of biochar and nitrogen fertilizer reduces nitrogen waste and provides a gradual supply of absorbable nitrogen throughout the growth period. This combined approach reduces nitrogen consumption and enhances aerial growth, with the highest growth observed when both are used together compared to individual applications. Research on pecan plants has similarly demonstrated that combining nitrogen and biochar has a more pronounced effect on aerial growth than either treatment alone (Hou et al., 2020).

Numerous studies highlight the role of biochar in increasing soil potassium levels. For instance, biochar derived from walnut shells has been shown to elevate pH, organic carbon, and potassium levels (Das & Ghosh, 2023). Biochar application can result in a 4.4 to 7-fold increase in potassium content (Mielke et al., 2022). Higher nitrogen levels also enhance the absorption of elements such as potassium, magnesium, calcium, and phosphorus (Cole et al., 2016).

Biochar improves phosphorus availability through various mechanisms, including the release of phosphorus salts and competitive absorption at positive exchange sites. Nitrogen enhances cation absorption and nutrient uptake by boosting metabolic activity. Our study observed increased sodium concentrations with higher biochar levels, consistent with previous findings (Barati et al., 2017; Ghias et al., 2022). Nitrogen also enhances zinc absorption, although higher biochar levels inversely impact zinc and copper concentrations. Biochar likely immobilizes metals like copper, reducing their availability (Moore et al., 2018), while nitrogen fertilizers can increase copper levels in plant aerial parts (Sepehr & Moradli, 2021).

Biochar made from sugarcane bagasse significantly increased iron concentration in corn plants (Rahman et al., 2022). Urea, used in this experiment, decomposes into ammonium, lowering soil pH and enhancing iron solubility. Increased nitrogen levels also significantly raised manganese concentration in aerial organs (Khamadi et al., 2015).

The observed changes in elemental dynamics, such as increased concentrations of potassium, phosphorus, and other micronutrients with nitrogen and biochar treatments, highlight their role in enhancing nutrient uptake efficiency (Winarso et al., 2020; Yang et al., 2021). Nitrogen's impact on cation absorption and biochar's influence on soil pH contribute to these dynamics, ensuring essential nutrients are available for plant use (Sachdev et al., 2021; Yousefzadeh et al., 2015).

Secondary metabolites, including flavonoids, phenols, and anthocyanins, exhibited varied responses to nitrogen and biochar treatments. The decrease in these compounds with higher biochar levels could be due to improved water availability and reduced stress conditions (Muktamar et al., 2020). The nuanced balance between nitrogen availability and secondary metabolite biosynthesis suggests a complex interplay between growth promotion and stress response (Rahayu et al., 2019; Tuladhar et al., 2021).

Comparing our findings with other studies on nitrogen and biochar interactions in different crops reveals consistent growth enhancement and nutrient utilization patterns (Ayaz et al., 2021; Haider et al., 2022). However, discrepancies in secondary metabolite responses underscore the need for further research to understand how biochar and nitrogen can optimize both growth and metabolite production.

Limitations of our study include the controlled environment and specific peppermint cultivar used, which may limit the generalizability of the findings. Future research should consider field trials and experiments with different peppermint cultivars to enhance the applicability of the results. Additionally, the optimal application rates and timing for various growth stages require further investigation to maximize benefits and avoid potential adverse effects.

Moreover, further exploration into the negative effects of high biochar levels on aerial organ weight due to increased salinity could provide deeper insights into the observed results. Identifying specific gaps in the literature where this study contributes new knowledge emphasizes the novelty and importance of the findings, while updated references would ensure the relevance and currency of the literature review.

## 5. CONCLUSION

This study shows that combining nitrogen and biochar significantly improves peppermint growth, including plant height, leaf count, chlorophyll index, and aerial and root dry weight. Specifically, using 75 mg of nitrogen per gram of soil with 2% biochar by weight consistently results in the best growth outcomes. These treatments enhance nutrient availability and plant health, contributing to sustainable agriculture by improving soil fertility and potentially mitigating soil degradation. However, further research is needed to understand the long-term impacts on soil health and crop quality. Excessive use of these treatments may affect soil properties and microbial communities. Future studies should explore different biochar types, application timings, and their economic feasibility to ensure sustainable and effective agricultural practices.

## Declaration of Competing Interest

The authors declare that no competing financial or personal interests may appear to influence the work reported in this paper.

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