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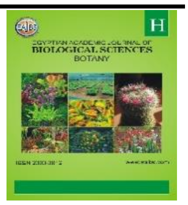

Enhancing Growth, Biochemical Traits, and Stress Resilience of *Mentha pulegium* L. in Calcareous Soil Using PGPR

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ABSTRACT

This study conducted to evaluate the influence of plant growth-promoting rhizobacteria (PGPR), specifically *Paenibacillus polymyxa* and *Bacillus halotolerans*, on the growth, biochemical, and antioxidant parameters of *Mentha pulegium* cultivated in calcareous soil. PGPR inoculation significantly enhanced plant growth parameters, including branch number, shoot and root lengths and fresh and dry weights. The combined inoculation of *P. polymyxa* and *B. halotolerans* achieved significant increase, with the highest number of branches (17.7), shoot length (30 cm), root length (31 cm), and fresh/dry weights (14.7/2.2 g/plant)as compared with the control plants. Chlorophyll content, nitrogen (N) and phosphorus (P) uptake, and protein content were significantly improved with inoculated treatments. Dual inoculation recorded the highest chlorophyll reading (42.2 SPAD units), N uptake (0.279%), P uptake (0.533%), and protein content (1.744%). Proline and total protein levels in leaves increased significantly, with dual inoculation achieving the highest values at 60 and 110 days. Antioxidant enzyme activities (CAT, POD, SOD, and PPO) were significantly elevated, with the combined inoculation yielding the most substantial enrichment. Additionally, the levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), markers of oxidative stress, were significantly reduced in inoculated plants. The dual inoculation treatment exhibited the lowest MDA (0.13/1.051 µM g^{-1} FW) and H_2O_2 (0.173/0.215 µM g^{-1} FW) levels, indicating its superior ability to mitigate oxidative stress. These findings highlight the potential of PGPR in promoting the growth and stress resilience of *Mentha pulegium* under calcareous soil conditions.

INTRODUCTION

Mentha is a genus comprising 61 species from the Lamiaceae family (Tacherfiout *et al*., 2022), renowned for its aromatic herbs, including thyme, oregano, and mint (Uritu *et al*., 2018). *Mentha pulegium* L., one of the *Mentha* species, is native to Europe, North Africa, Asia Minor, and the Middle East (Amtaghri *et al*., 2024a). It serves as a source of essential oils and is widely utilized in cuisine for flavoring, traditional medicine, and the pharmaceutical industry (Hajian *et al*., 2011). Commonly known as pennyroyal, *Mentha pulegium* is also referred to as squaw mint, mosquito plant, and pudding grass (Amtaghri *et al*., 2024b) . This fragrant, perennial herbaceous plant has been the focus of several studies

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emphasizing the strong biological efficacy of its extracts, which demonstrate powerful antioxidant, anticancer, antiviral, antiallergenic, and antibacterial properties (Soukaina *et al*., 2024).

Plants are subjected to a wide range of environmental stresses that reduce and limit the productivity of agricultural crops. Abiotic stresses, including radiation, salinity, floods, drought, extreme temperatures, high lime content, and heavy metals, are major contributors to global crop losses (Gull *et al*., 2022). Calcareous soils, characterized by more than 15% calcium carbonate ($CaCO_3$), may manifest in various forms, such as powder, nodules, concretions, or crusts (Taalab *et al*., 2019). These soils significantly affect plant growth due to their impact on soil properties, including limited availability of essential nutrients like phosphorus (P) and trace metals such as copper (Cu), zinc (Zn), and iron (Fe), which are critical for plant development (Kaiser *et al*., 2020). With a pH often exceeding 7 and sometimes surpassing 8 when enriched with free $CaCO_3$, calcareous soils can occasionally form a unique impermeable layer known as caliche *(Wahba et al*., 2019). Ghahrie *et al.* (2024) highlighted the increasing prevalence of micronutrient deficiencies in *Mentha* plants, particularly in calcareous soils.

The rhizosphere, often referred to as the "heart of the soil," is the zone of direct interaction between plant roots and active microorganisms. Plant roots deposit photosynthates into the rhizosphere, which stimulate microbial colonies, enhancing plant growth, development, and nutrient use efficiency through mechanisms such as organic matter mineralization, biological control of soil-borne pathogens, mineral solubilization, and root growth promotion (Olanrewaju *et al*., 2019). Among these microorganisms, plant growth-promoting rhizobacteria (PGPR) play a vital role. (Widnyana, 2018). These rhizobacteria colonize the rhizosphere, forming synergistic and antagonistic interactions with soil bacteria, and contribute to plant growth by mitigating biotic and abiotic stresses and improving nutrient availability (Behera *et al*., 2024; Ranjan *et al*., 2022; Valdez-Nuñez *et al*., 2019). Certain PGPR, such as *Paenibacillus* species, are known for their plant growth promotion, bioremediation, and biocontrol properties (Patowary & Deka, 2020).

This study aimed to examine the effects of PGPR, specifically *Paenibacillus polymyxa*, *Bacillus halotolerans*, and their combination, on the development, physiological, and biochemical characteristics of *Mentha pulegium* grown in calcareous soil.

MATERIALS AND METHODS

Site Setup:

The study was conducted in a greenhouse at the Faculty of Agriculture, Saba Basha, Alexandria University, Egypt during season 2023 to study the effects of PGPR strains and their combination, on the development, physiological, and biochemical characteristics of *Mentha pulegium* grown in calcareous soil.

Experimental Setup:

The experimental design followed a randomized complete block design (RCBD) with four treatments: control (no bacterial inoculation), inoculation with *Paenibacillus polymyxa* (1MF), inoculation with *Bacillus halotolerans* (3MF), and combined inoculation with both strains (1MF+3MF) in the three replicates.

Soil Analysis:

The soil used in the experiment was calcareous, characterized by a pH of 8.32, organic matter content of 0.94%, $CaCO_3$ content of 32.3%, total nitrogen of 321 mg/kg, total phosphorus of 480 mg/kg, and available phosphate of 6.3 mg/kg. Each treatment had three replicates, and pots were irrigated daily to maintain optimal growth conditions. The plant samples were collected at different time points: samples for vegetative parameters and chemical analysis were collected at 110 days, while those for physiological parameters and enzymatic activity assays were collected at 60 and 110 days.

Microorganisms and Inoculum Preparation:

The bacterial strains *Paenibacillus polymyxa* (1MF) and *Bacillus halotolerans* (3MF) were obtained from the Agricultural Microbiology Laboratory, Faculty of Agriculture, Saba Basha, Alexandria University, Egypt. A bacterial suspension containing 1.3×10^6 viable cells/mL was prepared and applied to the soil 15 days after transplanting. The bacterial inoculum was cultured in nutrient broth to ensure a sufficient microbial population density for the experiment.

The Studied Characteristics:

Vegetative Parameters Analysis:

Plant samples were collected to estimate vegetative growth parameters. Two plants per pot were selected randomly to measure plant height (cm), the number of branches, fresh weight (g/plant), and dry weight (g/plant). Dry weights were determined after oven-drying samples at 65 °C for 72 h.

Chemical Analysis:

Chemical analyses were conducted on the collected plant tissue samples. The samples were washed with tap water followed by distilled water, dried, milled, and stored for further analysis. For nutrient analysis, 0.5 g of plant powder was wet-digested using an H_2SO_4-H_2O_2 mixture (Lowther, 1980). Nitrogen uptake was determined using the micro-Kjeldahl method (Paech, 1956), and phosphorus uptake was quantified using the vanadomolybdate yellow technique (Jackson, 1973). Absorbance for phosphorus analysis was measured at 405 nm using a spectrophotometer.

Physiological Parameters:

Physiological parameters, including protein, chlorophyll, and proline content, were assessed. Total protein content was evaluated using the Kjeldahl method, with results expressed as percentages of dry weight (Ortiz *et al*., 2006). The chlorophyll index was measured using a SPAD 502 meter (Arjenaki *et al*., 2012) , and proline content was determined following the method described by (Bates *et al*., 1973)

Enzymatic Activity Assays:

Enzymatic activities were analyzed to evaluate the antioxidant defense system in *Mentha pulegium*. Fresh leaf tissues (1 g) were homogenized in liquid nitrogen and extracted with 3 mL of chilled 50 mM potassium phosphate buffer (pH 7.5). The homogenate was centrifuged at 12,000 rpm for 30 minutes at 4 °C, and the supernatant was stored at -80 °C for subsequent enzymatic analysis. Enzymatic activity assays were conducted for peroxidase (Doley & Jite, 2014; Rached-Kanouni & Alatou, 2013), superoxide dismutase (Beauchamp & Fridovich, 1971), catalase (VG & Murugan, 2013), polyphenol oxidase (Srivastava & Huystee, 1973). Lipid peroxidation and hydrogen peroxide content were assessed using the methods described by Junglee *et al*. (2014), respectively. Total protein was measured following Shams Moattar *et al*. (2016).

Statistical Analysis:

Statistical analysis was performed using one-way analysis of variance (ANOVA) to determine the significance of treatments. Tukey's multiple range test at a 5% significance level was employed for mean comparisons. Data were analyzed using CoStat (Cardinali & Nason, 2013) software version 6.4 to ensure accurate and reliable results.

RESULTS

Plant Growth Parameters of *Mentha pulegium* in Calcareous Soil:

The results showed the impact of PGPR inoculation on growth parameters of *Mentha pulegium* grown in calcareous soil, focusing on the number of branches, shoot and root lengths, fresh and dry weight per plant (Table 1). The combined inoculation with *Paenibacillus polymyxa* and *Bacillus halotolerans* yielded the highest number of branches (17.7), significantly exceeding all other treatments, while the control had the lowest count (7.7). Shoot length increased significantly in all inoculated treatments compared to the control (12.6 cm), with no significant differences among inoculated groups (25–30 cm). Root length also improved in inoculated plants (29.6–31.0 cm) compared to the control (24.0 cm), with *Paenibacillus polymyxa* achieving the longest roots (31.0 cm). Dual inoculation recorded the highest fresh and dry weights (14.7 g and 2.2 g/plant, respectively), significantly surpassing all other treatments, whereas the control had the lowest values (7.65 g and 1.2 g / plant).

Table 1. Effects of inoculation with *Paenibacillus polymyxa* and *Bacillus halotolerans* on growth parameters in *Mentha pulegium* grown in calcareous soil

Inoculation	Number of branches	Shoot length (cm)	Root length (cm)	Fresh weight (g/plant)	Dry weight (g/plant)
Control	*7.7^{d}	12.6^{b}	24^{b}	7.65^{d}	1.2^{d}
1MF	13.7^{b}	27^{a}	31^{a}	13.5^{b}	2.1^{b}
3MF	11^{c}	25^{a}	30^{a}	10.9c	1.85^{c}
1MF +3MF	17.7^{a}	30^{a}	29.6^{a}	14.7^{a}	2.2^{a}

* Values represent the mean of three replicates ± SD per treatment. Means were compared using one-way ANOVA followed by Tukey's post-hoc analysis at $p < 0.05$. Control: uninoculated plants; 1MF: *Paenibacillus polymyxa*; 3MF: *Bacillus halotolerans*.

Chlorophyll Index, Nitrogen and Phosphorus Uptake, and Protein Content:

The obtanied results in Table 2, illustrated the data of the chlorophyll index, nitrogen and phosphorus uptake, and protein content. The combined inoculation resulted in the highest chlorophyll index (42.2 SPAD units), outperforming the control (30.2 SPAD units). Nutrient uptake was also significantly enhanced in inoculated plants, with the dual inoculation achieving the highest P (0.533%) and N uptake (0.279%). Similarly, protein content was highest in dual inoculation (1.744%), contrasting with the control, which recorded the lowest values for nitrogen (0.069%), phosphorus (0.0716%), and protein (0.428%).

Table 2. Effects of inoculation with *Paenibacillus polymyxa* and *Bacillus halotolerans* on chlorophyll content, nitrogen, phosphorus uptake, and protein content in *Mentha pulegium* grown in calcareous soil

Inoculation	Chlorophyll index (SPAD unit)	Nitrogen uptake (%)	Phosphorus uptake (%)	Protein content (%)
Control	30.2^{b}	0.069^{b}	0.0716^{d}	0.428^{b}
1MF	36.8^{a}	0.258^{a}	0.202^{c}	1.163^{a}
3MF	39.1^{a}	0.186^{a}	0.390^{b}	1.163^{a}
1MF +3MF	42.2^{a}	0.279^{a}	0.533^{a}	1.744^{a}

* Values are the mean of 3 replicates ± SD per treatment. Means were compared using one-way ANOVA followed by Tukey's post-hoc analysis at $p < 0.05$. Control: uninoculated plants; 1MF: *Paenibacillus polymyxa*; 3MF: *Bacillus halotolerans*. Means sharing the same letter in the same column show no significant difference.

Proline Content and Total Protein Concentration:

The proline content of *Mentha pulegium* was substantially higher in the combined treatment of *Paenibacillus polymyxa* and *Bacillus halotolerans* at both 60 and 110 days (75.2 and 156.7 µmol g^{-1} FW, respectively), compared to the control (44.4 and 35.0 µmol g^{-1} FW). At 60 days, *Paenibacillus polymyxa* inoculation resulted in the highest total protein concentration (541.8 µg mL^{-1}), followed by *Bacillus halotolerans* (489.3 µg mL^{-1}), whereas the control gave the lowest (299.5 µg mL^{-1}). This trend persisted at 110 days, with *Paenibacillus polymyxa* recording 611.2 µg mL^{-1}, far exceeding the control (337.9 µg mL^{-1}) (Table 3).

Table 3. Effects of inoculation with *Paenibacillus polymyxa* and *Bacillus halotolerans* on proline content and total protein in *Mentha pulegium* grown in calcareous soil

Inoculation	Proline (µmol g^{-1} FW)		Total protein (µg mL^{-1})	
	60 d**	110 d	60 d	110 d
Control	*44.4^{c}	35^{d}	299.5^{d}	337.9^{d}
1MF	66.2^{ab}	74.4^{c}	541.8^{a}	611.2^{a}
3MF	59.8^{b}	101.1^{b}	489.3^{b}	552.1^{b}
1MF +3MF	75.2^{a}	156.7^{a}	444.7^{c}	501.8^{c}

* Values are the mean of 3 replicates ± SD per treatment. Means were compared using one-way ANOVA followed by Tukey's post-hoc analysis at $p < 0.05$. Control: uninoculated plants; 1MF: *Paenibacillus polymyxa*; 3MF: *Bacillus halotolerans*. Means sharing the same letter in the same column show no significant difference.

Antioxidant Enzyme Activities:

Antioxidant enzyme activities, including catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and polyphenol oxidase (PPO), were significantly higher in inoculated plants. The dual inoculation treatment demonstrated superior results, with increases in CAT (48.8% and 48.8%), POD (42.4% and 31.4%), SOD (79.2% and 688%), and PPO (33% and 18.7%) at 60 and 110 days, respectively. This indicates enhanced stress resistance compared to individual inoculations or the control (Table 4).

Table 4. Effects of inoculation with *Paenibacillus polymyxa* and *Bacillus halotolerans* on antioxidant activities in *Mentha pulegium* grown in calcareous soil

Inoculation	CAT		POD		SOD		PPO	
	60 days	110 days	60 days	110 days	60 days	110 days	60 days	110 days
Control	*2.467^{c}	4.729^{c}	2.963^{c}	3.82^{c}	0.607^{c}	0.186^{c}	0.267^{d}	0.459^{d}
1MF	3.457^{b}	6.626^{b}	3.497^{b}	4.153^{b}	0.851^{b}	1.15^{b}	0.350^{b}	0.536^{b}
3MF	3.665^{a}	7.026^{a}	3.436^{b}	4.08^{b}	0.778^{b}	1.05^{b}	0.325^{c}	0.500^{c}
1MF +3MF	3.670^{a}	7.035^{a}	4.222^{a}	5.02^{a}	1.088^{a}	1.466^{a}	0.355^{a}	0.545^{a}

Means were compared using one-way ANOVA followed by Tukey's post-hoc analysis at $p < 0.05$. Control: uninoculated plants; 1MF: *Paenibacillus polymyxa*; 3MF: *Bacillus halotolerans*; CAT: Catalase; POD: Peroxidase; SOD: Superoxide Dismutase; PPO: Polyphenol Oxidase. All enzyme activities are expressed in μM g^{-1} fresh weight (FW).

Malondialdehyde (MDA) and Hydrogen Peroxide (H_2O_2) Levels:

MDA and H_2O_2 levels were markedly lower in inoculated plants compared to the control, indicating reduced oxidative stress. The control recorded the highest levels at 60 and 110 days (0.273/1.865 μM g^{-1} FW for MDA and 0.344/0.803 μM g^{-1} FW for H_2O_2) (Figs. 1 and 2). In contrast, combined inoculation with *Paenibacillus polymyxa* and *Bacillus halotolerans* resulted in the lowest levels (0.13/1.051 μM g^{-1} FW for MDA and 0.173/0.215 μM g^{-1} FW for H_2O_2), demonstrating its effectiveness in alleviating stress during later growth stages (Figs. 1 and 2).

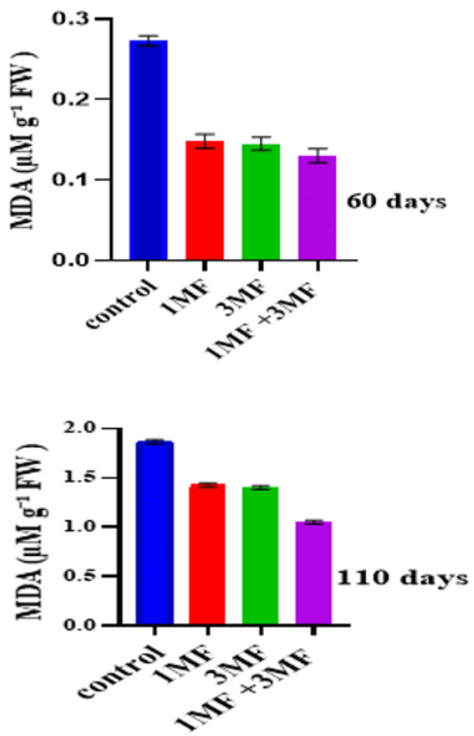

Fig. 1. Effect of inoculation with *Paenibacillus polymyxa* and *Bacillus halotolerans* on malondialdehyde (MDA) levels in *Mentha pulegium* grown in calcareous soil. This figure illustrates the impact of inoculating *Mentha pulegium* with *Paenibacillus polymyxa* (1MF) and *Bacillus halotolerans* (3MF) on MDA content in plants at 60- and 110-days post-planting. The control treatment refers to uninoculated plants. The data are presented as the mean of three replicates ± standard deviation (SD) for each treatment.

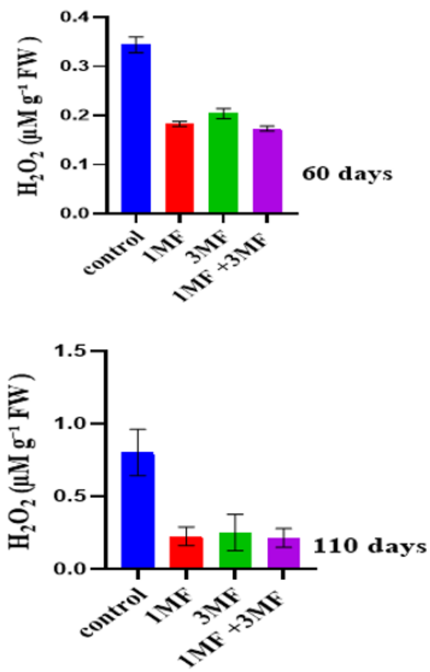

Fig. 2. Impact of PGPR inoculation on hydrogen peroxide (H_2O_2) levels in *Mentha pulegium* grown in calcareous soil. The figure illustrates the effects of inoculating *Mentha pulegium* with *Paenibacillus polymyxa* (1MF) and *Bacillus halotolerans* (3MF) on H_2O_2 levels at 60 and 110 days after planting. The control represents uninoculated plants. Bars indicate the mean values of three replicates ± standard deviation (SD) per treatment.

DISCUSSION

The objective of this study was to evaluate the physiological, morphological, and biochemical changes in *Mentha pulegium* cultivated in calcareous soil following inoculation with plant growth-promoting rhizobacteria (PGPR). Soil health is intrinsically linked to its microbial community. The plant rhizosphere serves as a complex and dynamic ecological environment that facilitates diverse plant-microbe interactions, enriching soil texture and optimizing the availability of essential nutrients from a limited nutrient pool (Liu-Xu *et al*., 2024). Beneficial microorganisms in the rhizosphere enhance plant vigor, bolster disease resistance, mitigate environmental stresses, and improve overall productivity.

The results indicate that the combined inoculation with *Paenibacillus polymyxa* and *Bacillus halotolerans* was the most effective treatment, significantly enhancing all growth parameters compared to the control. While individual inoculations with either *Paenibacillus polymyxa* or *Bacillus halotolerans* also improved plant growth, the dual treatment yielded superior values. These results align with the work of Kaymak *et al*. (2008) , who demonstrated that bacterial treatments (*Pseudomonas putida*, *Bacillus subtilis*, and *Bacillus megaterium*) markedly improved the growth of mint (*Mentha piperita* L.) under calcareous soil conditions. Similarly, (Ribaudo *et al*., 2006; Xia *et al*., 1990) reported that inoculation with *Azospirillum brasilense* enhanced root biomass in tomato plants. Moreover, Liu *et al*. (2017) observed that siderophore-producing strains of *Paenibacillus illinoisensis* and *Bacillus* promoted peanut growth in calcareous soils. Ipek *et al*. (2021a) further highlighted the efficacy of PGPR (*Staphylococcus*, *Bacillus*, and *Pantoea*) in enhancing the growth of Braeburn apples in lime-rich soils.

The experimental findings results also revealed that dual inoculation significantly increased chlorophyll content, phosphorus and nitrogen uptake, and protein levels at both growth stages. Individual treatments also showed significant improvements over the control, though the combined inoculation was most effective. These results recommended that dual inoculation synergistically enhances nutrient acquisition, photosynthetic efficiency, and protein synthesis in *Mentha pulegium* under calcareous conditions. Similarly, Ghahrie *et al*. (2024) highlighted micronutrient deficiencies in *Mentha* plants cultivated in calcareous soils and demonstrated that inoculation with PGPR (*Staphylococcus*, *Bacillus*, and *Pantoea*) improved nutrient uptake and chlorophyll synthesis (Ipek *et al*., 2021b). Desoky *et al*., (2022) who demonstrated that *Bacillus licheniformis*, *Bacillus megaterium*, and *Bacillus subtilis* inoculation significantly improved chlorophyll, carotenoid levels, and photosynthetic efficiency in wheat under similar soil conditions.

Dual inoculation led to the highest proline content in both growth stages, indicating improved stress tolerance in calcareous soil conditions. The single inoculation with *Bacillus halotolerans* was particularly effective in increasing total protein content, reflecting enhanced metabolic activity. These findings align with studies demonstrating that PGPR inoculation upregulates proline biosynthesis pathways, leading to higher osmoprotectant levels and better stress management (Ahmed *et al*., 2019; Hasanuzzaman *et al*., 2022).

The highest enzymatic activities (CAT, POD, SOD, and PPO) were detected in treatments involving *Paenibacillus polymyxa*, *Bacillus halotolerans*, or their combination. This underscores their potential to enhance antioxidative responses in *Mentha pulegium*. Control plants consistently showed the lowest enzyme activity, emphasizing the pivotal role of PGPR in bolstering plant stress tolerance. Dual inoculation also significantly reduced malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) levels in *Mentha pulegium* leaves, corroborating findings by Desoky *et al*. (2022) that bacterial inoculation mitigates oxidative damage and boosts antioxidant enzyme activity in wheat. The reduction of H_2O_2, mediated by antioxidant enzymes such as catalase (CAT) and ascorbate peroxidase (APX), reflects the effective management of reactive oxygen species (Feierabend, 2005). The bacterial inoculant increased the activity of antioxidant enzymes, thereby mitigating ROS-induced oxidative damage in plants, and elevated proline quantities in plant tissues, which are crucial for plant stress tolerance (Egamberdieva *et al.*, 2017).

In conclusion, this study highlights the efficacy of dual inoculation with *Paenibacillus polymyxa* and *Bacillus halotolerans* in improving growth, nutrient uptake, photosynthesis, stress tolerance, and antioxidative defense mechanisms in *Mentha pulegium* cultivated in calcareous soils. These findings underscore the potential of PGPR as a sustainable approach for enhancing agricultural productivity under nutrient-limited soil conditions.

CONCLUSION

This study highlighted the significant impact of PGPR inoculation with *Paenibacillus polymyxa* and *Bacillus halotolerans* on the growth, nutrient uptake, and stress tolerance of *Mentha pulegium* in calcareous soil. Dual inoculation in comparison with single treatments, improving growth parameters, chlorophyll content, protein %, and antioxidant enzyme activities while reducing oxidative stress markers like MDA and H_2O_2. These results underscore the potential of PGPR as an eco-friendly, sustainable approach to improving crop productivity and resilience in challenging soils, contributing to the advancement of sustainable agricultural practices.

Declarations:

Ethical Approval: No animal model(s) or human subjects were recruited directly for the current study. Consequently, no ethical considerations are necessary.

Conflict of interest: The authors declare no conflict of interest.

Authors Contributions: I hereby verify that all authors mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

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Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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