

Evaluating the influence of *Bacillus* spp. mediated growth enhancement and antioxidative defence activation in *Mentha piperita* L

Vagmi Singh

CSIR-Central Institute of Medicinal and Aromatic Plants

Shivani Chandra

CSIR-Central Institute of Medicinal and Aromatic Plants

Ram Kishor

CSIR-Central Institute of Medicinal and Aromatic Plants

Nidhi Maurya

Central Institute of Medicinal and Aromatic Plants

Suaib Luqman

Central Institute of Medicinal and Aromatic Plants

Rajesh Kumar Verma

Central Institute of Medicinal and Aromatic Plants

Anju Kumari Yadav

Central Institute of Medicinal and Aromatic Plants

Birendra Kumar

b.kumar@cimap.res.in

CSIR-Central Institute of Medicinal and Aromatic Plants

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Abstract

Peppermint (*Mentha piperita* L.) is a valuable medicinal and aromatic herb cultivated globally for its essential oil, prized for therapeutic and flavoring properties. Despite increasing global demand projected to rise from USD 230.3 million in 2024 to USD 444.6 million by 2034, its cultivation remains limited due to comparatively lower herb and oil yields than menthol mint (*Mentha arvensis* L.). To address this, the present study evaluated the plant growth-promoting potential of rhizobacteria isolated from the menthofuran-rich peppermint variety CIM-Patra, endowed with phosphate solubilization, ammonia production, siderophore production, and Indole acetic acid production efficiencies. The surface sterilized, uniform sized suckers of a menthol-rich peppermint variety CIM-Suras were inoculated with five isolated PGPRs as *Bacillus tequilensis* (T1), *Bacillus subtilis* (T2), Bacterium strain BS0393 (T3), *Bacillus thuringiensis* (T4), and *Bacillus cereus* (T5), in triplicate manner. The results of the study revealed that T2 (*Bacillus subtilis*) showed maximum improvement in almost all the studied morphological, physiological, and biochemical parameters, followed by T1 (*Bacillus tequilensis*), and T4 (*Bacillus thuringiensis*). The application T2 with the suckers of CIM-Suras depicted to significantly improve the photosynthetic process, contents of chlorophyll (64.71%), accumulation of proline (43.17%), SOD (24.89%), and catalase (56.33%), over the control, in peppermint suggests the potential of the isolate to abdate the oxidative damage of the cells during altering environmental conditions. These findings underscore the significance of the administration of *Bacillus* spp. based PGPRs as a sustainable approach to escalate the peppermint cultivation through a climate-conscious solution.

1. Introduction

Mentha piperita L., having great significance in the food, flavouring, cosmetic, ornamental, and pharmaceutical industries (Hudz et al. 2023). The extracts and essential oil of peppermint majorly contain steroids, terpenoids, phenolic acids, flavonoids, and their glycosides which contribute to its markedness in various biological activities like antioxidant, antimicrobial, biopesticidal, anticancer, antiviral, antiallergic, anti-inflammatory, antihypertensive, urease inhibitory activities and many more (Wu et al. 2019; Hudz et al. 2023). The essential oil of peppermint is majorly extracted through steam or hydro-distillation, complex compositions of various chemical compounds, primarily monoterpenoids stored specifically in specialized structures known as glandular trichomes, found on the aerial part of the plants (Turner et al. 2000; Croteau et al. 2005; Hassani, 2020). A major constituent of the essential oil of peppermint is *L*-menthol along with α -pinene, β -pinene, sabinene, myrcene, cis-ocimene, β -caryophyllene, γ -terpinene, germacrene, carvone, *L*-menthone, iso-menthone, 1,8-cineole, menthofuran, neo-menthol, menthyl-acetate, and pulegone (Rayan et al. 2023; Prasad et al. 2021). Tepecik et al. (2022) reported that the quality of peppermint oil is determined by the proportion of its chemical constituents, especially higher levels of menthol, menthone, and lower levels of menthofuran and pulegone are treated as the admirable quality of its essential oil. Endowing various aromatic and therapeutic potentials, the peppermint oil market in 2024 is valued at 232.4 million USD and is expected to reach a height of 458.4 million USD by 2034 (Choudhury, 2024). The high demand for peppermint essential oil in the sectors of pharmaceuticals as well as Fast-moving Consumer Goods (FMCG) is in contrast with its lower popularity among growers due to its low biomass, resulting in the lower oil yield of peppermint. Therefore, improving the agro-economic traits of peppermint along with holding the essential oil quality on the mark of globally accepted US-type peppermint having *L*-menthol (36–43%), *L*-menthone (15–25%), 1,8-cineole (4.0–6%), iso-menthone (2.0–4.5%), neo-menthol (2.5–4.5%), menthofuran (1.5–6%), menthyl acetate (3.0–6.5%), and pulegone (0.5–2.5%), is the pressing priority of the current time (Hudz et al. 2023; Prasad et al. 2021). As peppermint is a vegetatively propagated crop with very low seed viability, conventional breeding practices in this crop become very tough (Prasad et al. 2021). Therefore, the implementation of plant growth-promoting rhizobacterial isolates to positively modulate the herb and oil yield of peppermint is suggested as the best alternative to overcome the discussed issue.

Plant growth-promoting rhizobacteria (PGPR) are well known to establish harmonious relationships with the host plant to mitigate abiotic stress and provide a potential to host for combating pathogen pressure (Singh and Kumar, 2023). The PGPRs are also reported to improve the health and growth of the host plant by mobilizing nutrients from the soil (Goswami and Suresh, 2020; Mathur and Roy, 2021), regulating and synthesizing plant growth promoting signal molecules (Zhang et al. 2021), enhancing photosynthetic activities of plants along with facilitating attenuation against oxidative damage (Batool et al. 2020). There are several studies available discussing abiotic stress diminution as well as growth and yield promotion through the application of PGPRs in various crops like tomatoes (Abbasi et al. 2020; Brilli et al. 2019), potatoes (Batool et al. 2020), lettuce (Ikiz et al. 2024), paddy (Giri et al. 2023), soybean (Jabborova et al. 2021), *Ocimum basilicum* L. (Yilmaz and Karik, 2022) and many other staples as well as medicinal crops (Santoro et al. 2011; Yilmaz and Karik, 2022). However, the application of PGPRs as growth modulators along with the exploration of their efficiency in regulating the antioxidant capacities in peppermint has yet to be explored. Therefore, the current study is designed to investigate the potential of *Bacillus tequilensis* strain CC1, and *Bacillus subtilis* strain BAB-2857, Bacterium strain BS0393, *Bacillus thuringiensis* strain YGd22-03, and *Bacillus cereus* strain FORC_005, to estimate their morpho-chemical, physiological, and biochemical response over a menthol rich variety, CIM-Suras of *Mentha piperita* L.

2. Materials and methods

The study was conducted at CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India (26° 48' 0" N, 80° 54' 0" E) from January to May months of the year 2023. The soil texture used for the experimentation was sandy loam with a pH of 7.7 and electrical conductivity of 0.341 mS/cm. The climatic condition of the experimental site was semi-arid subtropical with temperatures ranging average maximum from 45°C during

June to an average minimum of 4°C during January of the experimentation year. The concentration of available P in the soil before the onset of the experiment was recorded at 37.8 Kg/ha, extractable potassium 190.7 Kg/ha, the total nitrogen was recorded as 379.2 Kg/ha, and the organic carbon concentration was 5.2 mg/L.

2.1 Cultivation and maintenance of isolates

The PGPR isolates used for the study were cultured from the rhizospheric soil region of the menthofuran-rich peppermint variety CIM- Patra during the year 2023 and were examined for their plant growth-promoting efficiencies (IAA production, Ammonia production, phosphate solubilization, and siderophore production) under invitro conditions at Seed quality laboratory, Plant Breeding & Genetic Resource Conservation Division of CSIR-CIMAP, Lucknow, India. Further, the genomic DNA of the five isolates showing maximum plant growth-promoting efficiencies under in vitro conditions were amplified with 16Sr DNA primers 8F: 5'AGA GTT TGA TCC TGG CTC AG'3 and 1492R: 5'GGT TAC CTT GTT ACG ACT T'3 for their molecular identification under the Proflex PCR system from Applied Biosystems. The consensus sequence of about 1400bp was resolved by using Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) at Biokart India Private Ltd., a sequencing service at Banglore, India. The results of the received genomic sequences were analyzed using BLAST to compare them with the already available genomic sequences at NCBI (Xing et al. 2023; Jamalzadeh et al. 2021; our paper). Further, for experimentation bacterial cultures were grown over Nutrient Agar Medium (HiMedia Laboratories Pvt. Ltd.). A single colony of pure cultures was picked as inoculum into 100ml autoclaved Luria Bertini broth (HiMedia Laboratories Pvt. Ltd.) into three separate 250ml Erlenmeyer flasks at a rotatory shaker of speed 200 rpm for 48hours and at the temperature of 28 ± 2°C. Now these aerobically grown bacterial suspensions were centrifuged and diluted at 10⁶cfu/ml with the help of sterile distilled water which was used to dip the surface disinfected and distilled water washed 5-10cm rhizomes of peppermint variety CIM-Suras for 2 hours at 30 ± 2°C.

2.2 In vivo study to evaluate PGP efficiencies of isolates

The pot experiment was conducted in a completely randomized design (CRD) with 3 replications of each treatment. A 75% (v/v) ethanol and 0.1% (v/v) sodium hypochlorite solution was used to disinfect the rhizomes of size 5–7 cm from a newly released peppermint variety CIM-Suras. These surface sterilized and distilled water rinsed (3 times) rhizomes of mentioned variety were inoculated with the *Bacillus tequilensis* strain CC1 (T1), *Bacillus subtilis* strain BAB-2857 (T2), Bacterium strain BS0393 (T3), *Bacillus thuringiensis* strain YGd22-03 (T4), and *Bacillus cereus* strain FORC_005 termed as (T5), isolated and identified in this study, of concentration 10⁶cfu/ml. The non-inoculated propagules of the variety CIM-Suras were considered as control. Those inoculated rhizomes of uniform size were incubated at 30°C for 2 hours with cultures and 2 rhizomes of each variety/genotype of the same length were potted into each pot of diameter 30.48cm (12inches), filled with autoclaved soil and watered at every alternate day after planting throughout the experiment. The rhizomes of variety CIM-Suras, treated with the mentioned bacterial isolates were inoculated again near their root zone with 10⁶cfu/ml of their respective treatments, before flowering initiation or after 45 days of planting (DAP). Details of the experiment are given in Table 1. Further, the data for morphological, physiological, and biochemical experiments of each treatment along with the control was recorded at the time of maturity i.e., after 90 DAP. However, 100g herb of each treatment in a triplicate manner was harvested and steam distilled after 110 days of planting for analyzing the essential oil content and quality of treated as well as control plants by performing GC-MS analysis.

Table 1
Details of treatments applied over peppermint variety CIM-Suras to analyse their effect over its growth improvemet

SL No.	Treatment names	Treatment details
1	Control	surface sterilized suckers of CIM-Suras without any PGPR application
2	T1	surface sterilized suckers of CIM-Suras with <i>Bacillus tequilensis</i> strain CC1
3	T2	surface sterilized suckers of CIM-Suras with <i>Bacillus subtilis</i> strain BAB-2857
4	T3	surface sterilized suckers of CIM-Suras with Bacterium strain BS0393
5	T4	surface sterilized suckers of CIM-Suras with <i>Bacillus thuringiensis</i> strain YGd22-03
6	T5	surface sterilized suckers of CIM-Suras with <i>Bacillus cereus</i> strain FORC_005

2.3 Measurement of morphological parameters of plants

2.3.1 Plant height

The plant height of the treatments along with the control of each genotype/accession of peppermint were recorded by measuring the length of the aerial part of the plants from the base to the tip of the main stem with the help of a measuring scale.

2.3.2 Leaf length and width

The length and width of the leaves of each treatment along with the control were recorded by using a measuring scale from the base to the tip of the petiole.

2.3.3 Number of nodes

Nodes are those points over the stem of plants from where the leaves or branching originates. The number of nodes of each treatment and the control was recorded by visually counting them from the base to the top of the main stem.

2.3.4 Internodal length

The length between two nodes is termed as internodal length and was recorded by using a measuring scale.

2.3.5 Leaf / Stem ratio

The weight of the leaves per plant divided by the weight of the stem of that plant provides an estimation of the leaf to stem ratio.

2.4 Chlorophyll estimation

The total chlorophyll content in treated and non-treated plants was assessed in a triplicate manner by performing the method described by Kizhedath and Sunitha (2011) with few modifications. The fresh leaf samples (1000 mg) of each treatment as well as the control were crushed with 20 ml of 80% acetone with an autoclaved pestle mortar and centrifuged at 5000rpm for 5 minutes. The supernatants were transferred into separate tubes marked with respective treatments and the pellet was resuspended with 20 ml of acetone and centrifuged again to collect the supernatant. This step was repeated until the residue became colorless. The volume of collected supernatants for each treatment and control was made up to 100ml with the help of 80% acetone and the absorbance of 1 ml of this collected supernatant was taken at 645nm and 663nm (Muthuraja et al. 2023; Kizhedath and Sunitha, 2011). The estimation of milligrams of chlorophyll content per gram of tissue (mg/g) was calculated by the formula described by Sadasivam and Manickman, 1991 (Kizhedath and Sunitha, 2011).

$$\text{Chlorophyll } a = [12.7(A \text{ at } 663) - 2.69(A \text{ at } 645)] \times V / (1000 \times W)$$

$$\text{Chlorophyll } b = [22.9(A \text{ at } 645) - 4.68(A \text{ at } 663)] \times V / (1000 \times W)$$

$$\text{Total chlorophyll} = [20.2(A \text{ at } 645) + 8.02(A \text{ at } 663)] \times V / (1000 \times W)$$

Where,

A = absorbance;

V = final volume of supernatant i.e., 100ml;

W = fresh weight of the tissue used for extraction i.e., 1g or 1000mg;

Therefore, $\frac{V}{1000 \times W} = \frac{100}{1000} \times 1 = 0.1$

2.5 Antioxidant and non-antioxidant enzyme assay

Fresh leaf samples at the age of maturity i.e., after 90 DAP of the treated and non-treated (control) plants were used to determine the enzymatic and non-enzymatic antioxidant parameters by following the method described by Venkidasamy et al. (2019). Fresh leaves of treatments and control were plucked and immediately stored at -80°C for further extraction.

2.5.1 Proline

Proline content was estimated with few modifications in the calorimetric method described by Bates et al. 1973. Measuring 100mg of stored leaf samples were crushed with liquid nitrogen using an ice-cold pestle mortar and transferred into priorly autoclaved and marked microcentrifuge tubes. This crushed sample was mixed with sulfosalicylic acid (5µl/mg fresh weight of sample) and centrifuged at 3000×g for 10 minutes at room temperature. After centrifugation, 100µl of supernatant was mixed with a reaction mixture containing 1% ninhydrin (w/v), 60% glacial acetic acid, and 20% ethanol, in a ratio of 1:2 (plant extract: reaction mixture) and incubated under water bath at 95°C for 60 minutes. This reaction was terminated on ice for cooling and was vortexed for 20 seconds by adding 1 ml of toluene. After giving 5 minutes of rest to this mixture the chromophore containing upper layer was collected and the absorbance was recorded at 520nm, using toluene as a reference.

2.5.2 Malonaldehyde (MDA)

MDA content was determined by using the Thiobarbituric acid test (TBA), by homogenizing 100mg of fresh leaf samples with 200µl of 10% Trichloroacetic acid (TCA). It was then centrifuged at 5000×g for 5 minutes at 4°C and the supernatants were collected into fresh tubes. This collected supernatant was mixed with an equal volume of 5% TBA (Sigma) and incubated at 96°C for 30 minutes. The mixture was spun at 10000×g for 10 minutes and the absorbance of supernatants was recorded at 532nm and 600nm (Venkidasamy et al. 2019).

2.5.3 Super oxide dismutase (SOD)

The estimation of SOD activity into the leaves of treated and non-treated peppermint samples was accessed by measuring their ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) through the method described by Beauchamp and Fredovich (1971). 100mg of leaf samples were homogenized with extraction buffer containing 50mM phosphate buffer, 1 mM EDTA, 0.05% Triton X-100, 1 mM polyvinylpyrrolidone and 1mM ascorbate. It was centrifuged at 10000×g for 15 minutes at 4°C and the supernatant was collected into fresh tubes. The absorbance of collected and diluted supernatant at different concentrations was read by mixing it with extraction buffer and 2M pyrogallol at 426nm under UV-visible spectrophotometer in kinetic of 60 seconds up to 5 minutes. The activity of the enzyme was calculated by comparing it with the curve formed from the standard (extraction buffer with pyrogallol) and was expressed as units/mg protein.

2.5.4 Catalase (CAT)

The activity of catalase is majorly seen inside cytosol and peroxysomes which catalyzes the conversion of H_2O_2 into H_2O and O_2 (Venkidasamy et al. 2019). The estimation of CAT activity was calculated using the protocol described by Aebi and Lester (1984). 100 mg of leaf tissues were homogenized with 50 mM potassium phosphate buffer and 30 mM H_2O_2 and the absorbance was read at 240 nm in kinetics for 5 minutes at the interval of 60 seconds. The unit of catalase activity was estimated as the amount of catalase needed to degrade 1 mol H_2O_2 /mg protein/min.

2.6 Extraction of essential oil

After 90 days of the onset of the experiment 200grams of fresh herb of potted plants of peppermint variety CIM-Suras, treated with the *Bacillus tequilensis* strain CC1 termed as (T1), *Bacillus subtilis* strain BAB-2857 termed as (T2), Bacterium strain BS0393 (T3), *Bacillus thuringiensis* strain YGd22-03 (T4), and *Bacillus cereus* strain FORC_005 termed as (T5) along with the control were harvested and hydro-distilled by using Clevenger apparatus (Clevenger, 1928) and estimated in content (%). The extracted essential oil was treated with anhydrous sodium sulfate to remove any traces of water and stored under the refrigerator in a dark condition. The qualitative analysis of these extracted essential oils was performed on Perkin Elma Auto XL Gas Chromatograph, equipped with a DB-Wax (30 m ×0.32 mm internal diameter, film thickness 0.25 µm) fused silica capillary column and flame ionization detector with oven temperature ranging from 40°C to 170°C with a ramp of 3°C/min then programmed at 40°C to 120°C at 3°C (9 minute hold), 120°C to 140°C at 2°C (2 minute hold) then finally 140°C to 230°C at 5°C with 2 minute hold time. The injector and detector temperature were set at 240°C, and hydrogen was used as carrier gas at 7.5 psi with a split ratio of 1:40 (Prasad et al. 2021). The identification of essential oil constituents was performed based on the retention index (RI), further determined regarding homologous series of n-alkanes (C7–C30), MS Library search (NIST and WILEY), and by comparing RI and mass spectral data with the literature published (Adams, 2007).

2.7 Statistical analysis

All data were recorded in triplicate and at the end of the experiment, data were subjected to analysis of variance (ANOVA). The mean table CD at a 5% level ($p \leq 0.05$) and CD at a 1% level ($p \leq 0.01$) was used for comparing treatment means according to Katsenios et al. (2022).

3. Results & discussion

Plant growth-promoting rhizobacteria (PGPRs) is an assembly of microorganisms, mainly bacteria, potentially involved in improving the growth and health of the host plants by secreting various hormones/enzymes (Singh and Kumar, 2023; Hyder et al. 2023). The administration of PGPRs is reported to increase nutrient uptake in plants, by solubilizing insoluble phosphate, assimilating nitrogen, and synthesizing auxin, cytokinin, siderophores, and HCN (Shahrajabian and Sun, 2022). *Bacillus* spp., is a gram-positive, rod-shaped, spore-forming, famed genus, forming excellent colonization with plant roots, efficient in phosphate solubilization, nitrogen fixation, siderophore production, and synthesizing various phytohormones (Sun and Shahrajabian, 2025). The employment of *Bacillus* spp. as a plant growth promoter is a well-known practice to achieve sustainability in agriculture (Prakash and Arora, 2019). The application of four different *Bacillus* spp. along with a Bacterium strain BS0393, isolated from the rhizospheric region of peppermint variety CIMAP- Patra was assessed over peppermint variety CIM-Suras and reported to ameliorate the crop's development (Table 1). All the studied morpho-chemical parameters were influenced positively by the application of isolated plant growth-promoting rhizobacteria, *Bacillus tequilensis* (T1), *Bacillus subtilis* (T2), Bacterium strain BS0393 (T3), *Bacillus thuringiensis* (T4) and *Bacillus cereus* (T5) (Table 1). A highly significant increase in the plant height of the crop was recorded with the application of T1, T2, and T4, where increments of 67.84%, 125.0%, and 98.21% respectively were reported over the control (Table 2). Prakash and Arora (2019) have also reported the positive influence of *Bacillus* spp. over plant height in menthol mint (*Mentha arvensis* L.). The plant growth is directly dependent on the availability of nutrients like nitrogen, phosphorous, and IAA. The increase in plant height of variety CIM-Suras due to the implementation of *Bacillus* spp. (T1, T2, and T4) with its suckers, may be suggested because of the secretion of cytokinin (zeatin glycoside, zeatin, zeatin riboside, isopentyl adenine, etc.) and other volatile organic carbons (VOCs), which may have induced the cell division and plant growth of the tested variety (CIM-Suras) of peppermint (Tsotetsi et al. 2022). The maximum improvement in plant height (125.0%) of peppermint was reported with the administration of T2 (*Bacillus subtilis*) is similar to the findings of Xie et al. (2014), Zhang et al. (2007), Karadeniz et al. (2006), and Ryu et al. (2003). *Bacillus subtilis* induces plant growth by releasing two most special VOCs, 3-hydroxy-2-butanone and 2,3-butanediol which is suggested to regulate the homeostasis between ethylene and cytokinin (Tsotetsi et al. 2022). Kudoyarova et al. (2014) have reported that the release of

zeatin and zeatin ribosides through *Bacillus subtilis* in wheat increases the exudation of amino acids, responsible for enriching the rhizobacterial niche of the tested plant which eventually influences the plant growth. Total essential oil content ranged from 0.20% (control) to 0.42% (for treatments). The study depicted a highly significant improvement in oil content (%) for the peppermint variety CIM-Suras at treatments T1 (60.0%) and T2 (110.0%), over the control. Besides increasing oil content, the application of these two treatments, T1 (7.23%), and T2 (9.51%), has also altered the content of menthol highly significantly, in comparison with the control (Table 2). This finding of the experiment is in accordance with the study conducted by Prakash and Arora (2019) and Khan et al. (2015), with the application of *Bacillus* spp. over menthol mint and peppermint, respectively. Walpoli and Yoon (2013), have introduced the IAA-producing ability of *Bacillus* spp. influences the lateral root formation, cell division, and elongation in the implemented crop (Goswami et al. 2016). The IAA production efficiency of *Bacillus tequilensis* (T1), and *Bacillus subtilis* (T2), might be the reason for better growth and development of roots and shoots, associated with improved oil content (%) and menthol content (%) in peppermint variety CIM-Suras. Also, the phosphate solubilization efficiency of *Bacillus* spp. with the mechanism of nutrient plant growth enhancement is responsible for inducing the synthesis of secondary metabolites in medicinal and aromatic plants (Malusa et al. 2006; Nell et al. 2009). The levels of other major essential oil constituents of peppermint i.e., pulegone (%) and menthone (%) depicted their varying levels, over the control in to all five applied treatments. This indicates that the application of administered plant growth promoting bacteria may have significantly altered the terpene biosynthesis pathway in peppermint, although the elaborated study of this prospect is yet to be performed. Similar findings have been reported earlier to other medicinal and aromatic crops viz., *Mentha arvensis*, *Origanum* sp., *Ocimum basilicum* etc. (Cappellari et al. 2015).

Table 2
Effect of various treatments on morpho-chemical parameters of peppermint variety CIM-Suras

Treatment	Plant Height (cm)	Leaf length (cm)	Leaf width (cm)	No. of nodes	Internodal length (cm)	L/S ratio	Oil content (%)	Menthone (%)	Menthofuran (%)	Pulegone (%)	Menthol (%)
Control	18.67	1.47	1.00	12.33	0.93	0.78	0.20	7.23	0.51	1.32	70.10
T1	31.33**	1.90**	1.30*	19.00**	0.87	1.62**	0.32**	4.67**	1.84**	0.81**	75.17**
T2	42.00**	2.50**	1.50**	23.67**	1.27*	1.17*	0.42**	2.13**	1.29**	2.16**	76.77**
T3	23.33	1.50	0.80	14.33	1.00	0.54	0.27*	6.29**	0.86**	6.23**	59.22**
T4	37.00**	2.07**	1.23	22.33**	1.33*	1.00	0.24	4.62**	0.77**	3.68**	69.17**
T5	24.33	2.30**	1.30*	14.00	1.53**	0.54	0.21	4.91**	0.58	0.52**	70.46
Min	18.67	1.47	0.80	12.33	0.87	0.54	0.20	2.13	0.51	0.52	59.22
Max	42.00	2.50	1.50	23.67	1.53	1.62	0.42	7.23	1.84	6.23	76.77
CD 5%	6.28	0.21	0.24	4.09	0.30	0.35	0.04	0.16	0.10	0.10	0.37
CD 1%	8.94	0.31	0.34	5.82	0.43	0.50	0.06	0.23	0.15	0.15	0.53
*p < .05 and **p < .01											

The contents of chlorophyll a, chlorophyll b, and total chlorophyll were recorded to increase highly significantly for all the applied treatments in comparison to control (Table 3 & Fig. 5). All three values of chlorophyll content were reported to be highest for T2 (*Bacillus subtilis*), followed by T1 (*Bacillus tequilensis*), and T4 (*Bacillus thuringiensis*). These findings of the study may be supported by the fact that the application of plant growth promoting rhizobacteria may have improved the water absorption as well as mineralization of soil nutrients which facilitated the treated plants against the damage caused to their photosynthetic apparatus by the oxidative stress (Cappellari et al. 2015).

Table 3
Effect of various treatments on contents of chlorophyll *a*, chlorophyll *b* and total chlorophyll content in peppermint variety CIM-Suras

Treatments	Chlorophyll <i>a</i> (mg/g FW)	Chlorophyll <i>b</i> (mg/g FW)	Total chlorophyll (mg/g FW)
Control	1.5650	3.6116	5.2092
T1	2.8535**	5.22818**	8.0724318**
T2	2.90497**	5.380317**	8.278534**
T3	1.8098**	3.992251**	5.96896**
T4	2.6758**	4.87360**	7.54623**
T5	2.45223**	4.211047**	7.1239857**
CD 5%	0.083	0.077	0.138
CD 1%	0.085	0.110	0.197
*p < .05 and **p < .01			

During various life stages, plants undergo several biotic and abiotic stresses, which stimulate oxidative stress and accumulation of toxicity in cells. However, administration of PGPRs to stressed plants is reported to mitigate the damage caused to their plasma membrane (Neshat et al. 2022). The osmolytes synthesised by PGPRs interplay synergistically with the osmoregulators of host plants, responsible for their overall growth and development (Chieb and Gachomo, 2023). This study of plant biochemicals majorly focuses on estimating proline due to its significant relevance in biotic and abiotic stress tolerance. Proline is suggested to produce cationic polyamines, responsible for the growth and development of plants, further forming an association with the anionic phospholipids of the cell membrane, which protects the bilayer membrane from the injury caused by stress (Bailly et al. 2014). The results of the study demonstrated a significant increase in the proline content ($\mu\text{mol/mg}$ fresh weight) for all five studied treatments over the control. The treatment T2 resulted to cause the most significant improvement (46.6%) in proline accumulation in leaves on a fresh weight basis, followed by T4 (30.5%) and T1 (28.7%), over the control (Fig. 1 & Fig. 5). Similar findings of plant growth-promoting rhizobacteria (PGPR) assisted priming were stipulated by the study on crops like rice and wheat (Bhattacharyya et al. 2020; Upadhayay et al. 2012). Superoxide dismutase (SOD) carries out oxidation as well as reduction (dismutation) of superoxide, releasing O_2 with higher oxidation state and H_2O_2 with lower oxidation state, preventing the generation of reactive oxygen species (ROS) via Haber Wiess reaction (Pan and Yau, 1992). Catalase is another enzyme working in the close proximity with SOD, synchronized to preclude the formation of reactive oxygen species (ROS). Results revealed a significant increase in superoxide dismutase and catalase activity levels for all the treatments when compared with the control (Fig. 2, Fig. 3 & Fig. 5). These findings indicate that the PGPR treatment upregulates the antioxidant enzyme cascade in treated plants to overcome the biotic and abiotic stresses (Noreen et al. 2024). This finding was in accordance with the study conducted by Li and Jiang (2017) over maize, Santos et al. (2018) over cowpea, Khan et al. (2020) over soybean, and Luqman et al. (2025) over cotton plants. The increment in the catalase enzyme activity in treated plants suggests the efficiency of treatments to neutralize H_2O_2 , produced during stress (Fatima et al. 2024). Malondialdehyde (MDA) is a cytotoxic aldehyde that accumulates after lipid peroxidation, responsible for the damage of the cell membrane of the plant. The results of the study revealed that the application of PGPRs with peppermint has decreased the levels of MDA in treated plants, over the control (Fig. 4 & Fig. 5). Similar findings of decreased MDA activities in potato were reported by Batool et al. (2020). The increased activities of proline, SOD and catalase with reduced MDA activities in PGPR treated peppermint variety CIM-Suras might be supported by the fact the plant growth promoting rhizobacterial treatment may have influenced the redox defence status to abduct the damage through ROS by mitigating the peroxidation of cell membrane lipids (Batool et al. 2020). However, the increased accumulation of proline, SOD, and catalase in PGPR-administered plants is suggested by the fact that this accumulation subsidizes the osmotolerant activity of the plants, preparing them to overcome the biotic or abiotic stress exposures (Etesami and Maheshwari, 2018).

Conclusion

Under this study a menthofuran rich peppermint variety CIM-Patra was used to isolate the rhizobacteria which further assessed for their plant growth promotion efficiencies under laboratory conditions, and the best performers were selected for their implementation with the suckers of peppermint by performing pot experiments. In the present study, a newly released, menthol-rich peppermint variety, CIM-Suras, was tested against five plant growth-promoting rhizobacteria to estimate their growth-promoting and oil content-improving efficiencies. All five implemented rhizobacteria were found to ameliorate the evaluated morpho-chemical as well as biochemical parameters in the tested variety of peppermint, probing their potential to be explored as bioformulants. The application of *Bacillus subtilis* (T2), *Bacillus tequilensis* (T1), and *Bacillus thuringiensis* (T4) was recorded to maximally increase the morpho-chemical variables along with influencing the accumulation of antioxidant enzymes in the leaves of treated plants of peppermint, suggesting their efficiency in activating the antioxidative defence mechanism. This indicates the significant role of administered *Bacillus* spp. in mitigating the cell damage caused by the ROS burden over host plants. The current study gives insight into the potential of isolated plant growth-promoting *Bacillus* spp. in enhancing morphological, physiological, and biochemical traits of

peppermint. Thus, this study suggests that the implementation of plant growth-promoting rhizobacteria to improve the yield and essential oil quality of peppermint would be a great strategy for sustainable agriculture, facilitating the ecologically powered economy of the country, aligning with the United Nations Sustainable Goal (SDGs) of climate resilient agriculture.

Declarations

Author Contribution

V.S. wrote the main manuscript; S.C., R.K., and N.M. helped in data collection, S.L., R.K.L., and B.K. critically reviewed the manuscript and supervised throughout the study. All authors reviewed the manuscript.

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Declaration of competing interests

The authors declare no competing financial interests or personal relationships.

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Figures

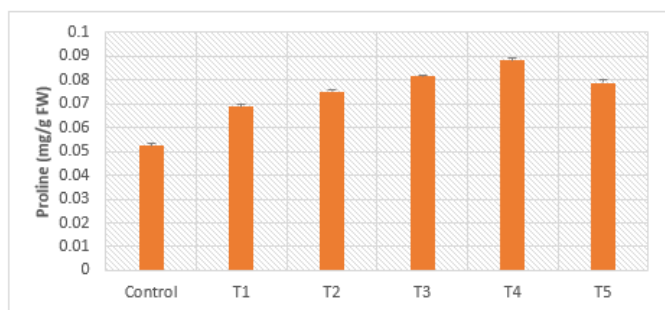


Figure 1

Impact of different rhizobacterial treatments on shoot proline of peppermint variety CIM-Suras.

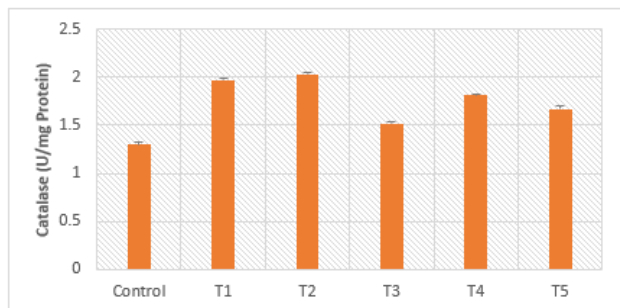


Figure 2

Impact of different rhizobacterial treatments on shoot catalase of peppermint variety CIM-Suras.

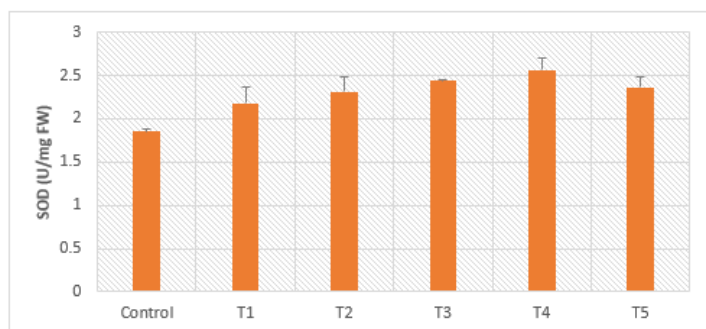


Figure 3

Impact of different rhizobacterial treatments on shoot superoxide dismutase (SOD) of peppermint variety CIM-Suras.

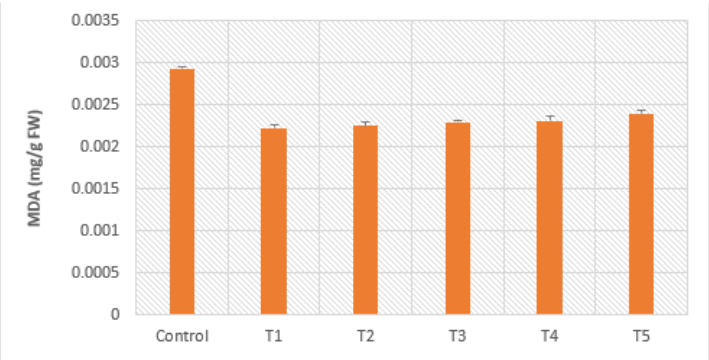


Figure 4

Impact of different rhizobacterial treatments on shoot Malondialdehyde (MDA) of peppermint variety CIM-Suras.

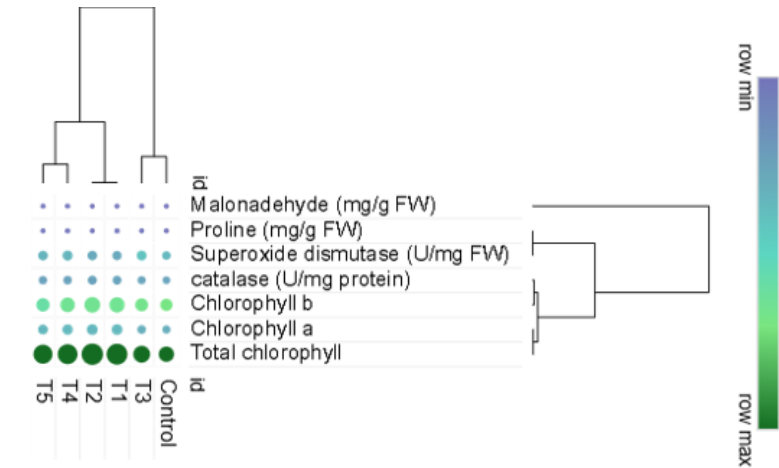


Figure 5

A Pearson's correlation heatmap of different rhizobacterial treatments for physiological and biochemical parameters in the peppermint variety CIM-Suras.