

BIOPROSPECTING THE POTENTIAL OF PLANT GROWTH PROMOTING RHIZOBACTERIA-ASSISTED CULTIVATION PRACTICES TO REDEFINE THE PRODUCTIVITY OF PEPPERMINT (*MENTHA PIPERITA* L.)

Vagmi Singh^{1,2}, Rajesh Kumar Verma^{2,3}, Rakesh Kumar³, Akanksha Singh^{2,3}, Neelam³, Ram Kishor^{1,2}, Akancha Gupta^{1,2}, Nashra Aftab^{1,2}, Birendra Kumar^{1,2*}

¹Seed Quality Lab on MAPs, Genetics & Plant Breeding Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India;

²Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, India;

³Crop Production & Protection division, CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India.

*Corresponding Author: Email: bkumar.cimap@csir.res.in; birendrak67@rediffmail.com

ABSTRACT

Aim To evaluate the potential of the plant growth-promoting rhizobacterium (PGPR) *Agrobacterium radiobacter*, isolated from the rhizosphere of peppermint genotype MPS-3637, in enhancing biomass, essential oil yield, and menthol content of peppermint (*Mentha piperita* L.) variety CIM-Suras under field conditions.

Methods and results *Agrobacterium radiobacter* was aseptically isolated from the rhizospheric soil of peppermint genotype MPS-3637 and applied to suckers of peppermint variety CIM-Suras under field conditions, either alone or in combination with vermicompost (@ 5 kg ha⁻¹). Compared with untreated control plants, inoculation with *A. radiobacter* alone increased biomass by 1.5-fold, resulting in an 80.70% increase in essential oil content and a 13.78% increase in menthol content. The combined application of *A. radiobacter* and vermicompost further enhanced biomass by 2.5-fold, increased essential oil content by 54.39%, and improved menthol content by 11.08% over the control.

Conclusion The application of *Agrobacterium radiobacter*, either alone or in combination with vermicompost, significantly enhanced biomass production, essential oil yield, and menthol content in peppermint variety CIM-Suras. These findings demonstrate the potential of this PGPR as a sustainable and environmentally friendly approach for improving peppermint productivity and profitability while reducing reliance on conventional agricultural inputs.

Impact statement Plant growth-promoting rhizobacteria offer a sustainable alternative to conventional inputs, but their effectiveness depends on the selection of compatible host-microbe combinations. This study demonstrates that *Agrobacterium radiobacter* can substantially enhance peppermint biomass, essential oil yield, and menthol content under field conditions. These findings support the development of targeted microbial bioinoculants for medicinal and aromatic crops, contributing to sustainable peppermint cultivation, improved farmer profitability, and reduced reliance on chemical inputs.

KEYWORDS: *Agrobacterium* spp., biopriming, essential oil, *Mentha* spp., menthol, PGPR.

INTRODUCTION

Peppermint (*Mentha piperita* L.), a member of the family Lamiaceae is a medicinally important aromatic herb, widely grown in the temperate to sub-temperate areas of the world (Prasad et al. 2021). It is mainly cultivated in Europe, China, Brazil, America, and India as one of the most important essential oil-bearing crops (del Rosario Cappellari et al. 2015, Hayes et al. 2007). The essential oil (EO) of the crop, rich in terpenoids, flavonoids, polyphenols, carotene, alpha-tocopherol, betaine, and choline, is extracted by steam distilling its fresh herbs (Farnad et al. 2014). Other secondary metabolites of peppermint, extracted from this crop are hesperidin, didymin, rosmarinic acid, and diosmin which are reported by Rizvi et al. (2022). Its essential oil along with dried and fresh herbs are used in the sectors of pharmaceuticals as well as Fast-moving Consumer Goods (FMCG). EOs of peppermint are extensively used in the food and confectionary industries for adding flavours to chewing gums, candies, toothpaste, mouthwashes, etc. (del Rosario Cappellari et al. 2015). Along with contributing to aromatherapy, peppermint is also used as an antipyretic, antispasmodic, analgesic, carminative, diaphoretic, anti-diarrhea, and antimicrobial agent (Chiappero et al. 2019, Zheljazkov et al. 2010). The extracts of *M. piperita* are well known to cure various diseases in humans like the inflammation of the pharynx and liver, gastrointestinal tract disorders, nausea, vomiting, cramps, and dyspepsia (Shalayel et al. 2017). The production of EO from peppermint is chiefly associated with the abundance of peltate and capitate types of glandular trichomes present over the upper as well as lower surface of the plant (Werker 2000). The accumulation of monoterpenes in peppermint is reported to occur only in the peltate type of glandular trichomes and EO gets secreted into the cavity formed by the separation of layers of cuticular material (del Rosario Cappellari et al. 2015). The globally accepted major constituents of its essential oil include menthol (36–46%), menthone (15–25%), iso-menthone (2.0–4.5%), 1,8-cineole (4.0–6%), menthofuran (1.5–6%), neo-menthol (2.5–4.5%), menthyl acetate (3.0–6.5%), and pulegone (0.5–2.5%) (Prasad et al. 2020). Even after being highly demanded in the food, pharma, and aroma industries the cultivation of peppermint is not that much popular among its growers as compared to menthol mint (*Mentha arvensis* L.). This comparably lower popularity of the crop is

due to its lower oil yield, generating lower profit for its growers. As it is a vegetatively propagated crop its genetic improvement is a major area of concern among breeders (Prasad et al. 2021). Although, there are numerous proficient varieties with improved morpho-chemical characteristics are being released by the breeding experts but genetic improvement of peppermint through the application of plant growth-promoting rhizobacteria (PGPR) is anticipated as a sustainable solution to the problem.

Bacteria inhabiting the rhizospheric region of root system of plants, assisting via directly or indirectly to improve the yield or quality of the crop is termed as plant growth promoting rhizobacteria (PGPR) (Singh and Kumar 2023). Solubilization of minerals like N, P, K, and increasing their availability for plant uptake is mediated through direct mechanisms while regulation of phytohormones and release of plant defensive chemical molecules/ biocontrol molecules to improve plant health, and growth is facilitated via indirect mechanisms of PGPRs (Singh et al. 2020). Inoculating aromatic plants with helpful microorganisms frequently improves plant growth indices and secondary metabolic responses. There are numerous reports available depicting the positive influence of PGPR inoculation with medicinal and aromatic crops (Banchio et al. 2009, Santoro et al. 2011, del Rosario Cappellari et al. 2013; Naik et al. 2020, Arora and Fatima, 2022, Singh and Kumar, 2023). Therefore, the present study is designed to harness the role of PGPR in improving growth and quality attributing traits of peppermint.

MATERIALS AND METHODS

Soil sample collection

The sampling of rhizospheric soil with intact root system was performed during October 2022 from the rhizospheric region of a morphologically distinct, menthofuran-rich line/genotype MPS36-37 of peppermint, growing at the experimental farm of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India (25.89°N latitude, 80.98°E longitude). The topsoil containing dry matter was removed with the help of sterile equipment and plants with their upper and underground stems were collected by digging up to 5-10cm of depth. Sterile plastic bags were used to carry the sample from the experimental farm to the Seed Quality Lab, Plant Breeding & Genetic Resource Conservation Division of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India.

Isolation and morphological characterization of rhizobacteria

The isolation of bacteria inhabiting the rhizospheric region of peppermint line/genotype SP36-37 was performed over Nutrient Agar medium (NA), and Kings B medium (KB) (HiMedia Laboratories Pvt. Ltd) at 28±2°C by following the technique of serial dilution up to six dilutions (10⁻⁶). All the dilution plates, spread with 1ml aliquotes of each dilution over 2 selected growth mediums were examined for typical bacterial colonies. The well-defined single colonies were selected for streaking over fresh plates of their respective media for 3-5 days (Ashrafuzzaman et al. 2009, Saharan and Shuchita. 2015). Loopful cultures of purified single colonies from these freshly grown plates were streaked again over KB medium and incubated for 3 days at 28±2°C and examined under UV light of 365nm to observe the fluorescence production ability of the isolates (Nepali et al. 2018). Further, the morphology of the isolates was observed based on the shape, size, color, margin, elevation, and pigmentation of the colonies. Gram staining of the isolates was performed, using Gram Stains Kit (HiMedia Laboratories Pvt. Ltd.) following the manufacturer's instructions. After examining and recording the morphological description of isolates they were stored in glycerol stock at – 80 °C until further analysis.

Screening of isolates for their plant growth promotion activities

All the bacterial isolates from 2 different media (NA and KB) were screened for Indole acetic acid production, NH₃ production, phosphate solubilization, and siderophore production to access the plant growth-promoting potentials of isolates.

IAA production assay

IAA production of isolates was estimated by following the calorimetric method (Okon et al. 1977). Bacterial cultures were inoculated with Luria Bertini broth (7.0 ± 2 pH) amended with 2% L-tryptophan and incubated under the orbital shaking incubator with the speed of 150 rpm and at the temperature of 28 ± 2°C for 5 days. Salkowski reagent was prepared by using the steps described by (Bric et al. 1991). The five days old cultures were centrifuged at 10000rpm for 10 minutes under a cold centrifuge and cell-free supernatant was mixed with Salkowski reagent in a 1:2 ratio. This mix was immediately placed in dark for 30 minutes and the absorbance was recorded at 530nm under spectrophotometer (Lebrazi et al. 2020a). The standard curve was prepared by using varying concentrations of IAA and results were recorded in µg/ml over control (Gordon and Weber, 1951).

NH₃ production test

The assessment of isolates for NH₃ production was performed by inoculating the bacterial isolates into 10ml peptone water (7.2 ± 2 pH) and incubated for 5 to 7 days at 28 ± 2°C under orbital shaking incubator of speed 150rpm (Iwata et al. 2010). The analysis of NH₃ production was done by adding 1.0ml of Nessler's reagent into each tube, producing yellow to brown colour (Cappuccino and Sherman 2007). Afterwards, spectrophotometric estimation was done at 450nm for the quantitative analysis of ammonia production by each isolate. The standard was prepared by using different concentrations of ammonium sulphate ranging from 2µM to 10µM and the ammonia production was estimated in ppm (mg/L).

Inorganic phosphate solubilization

Invitro evaluation of all the isolates for phosphate solubilization was performed by point inoculating the bacterial cultures over Pikovaskaya's agar medium (HiMedia Laboratories Pvt. Ltd), enriched with 0.4% tricalcium phosphate, and the plates were incubated at 32 ± 2°C (Singh, 2015) for 5 days. The formation of a clear halo zone around the point inoculated culture represents the phosphate solubilization activity index (PSI) of the isolates (Premono et al., 1996). The formula calculated the efficiency of isolates to solubilize phosphate:

$$\text{PSI} = \frac{\text{Colony diameter (cm)} + \text{Halo zone diameter (cm)}}{\text{Colony diameter (cm)}}$$

Siderophore production

The siderophore production assay of isolates was performed by the protocol described by (Louden et al. 2011). Firstly, Chrome Azurol S (CAS) reagent was prepared and autoclaved by following the steps elaborated by (Schwyn and Neilands 1987). Afterward, 100ml of CAS reagent (pH 6.8) was mixed with sterilized 900ml of King's B agar medium and plates were incubated at $28 \pm 2^\circ\text{C}$ for 3-5 days and were analyzed after incubation (Alexander and Zuberer 1991). The formation of yellow to orange zone around the culture over blue-coloured CAS media, confirms the siderophore production ability of isolates (Saharan and Shuchita 2015).

Identification of potential PGPR

On the basis of plant growth promotion efficiency tests single isolate, giving positive results for NH_3 production, K solubilization, IAA production and siderophore production along with giving fluorescence under UV light when inoculated over King's B Agar medium (Nepali et al. 2018) was selected for identification. The genomic DNA of the PGPR was isolated with the help of Xploreagen™ Bacterial DNA extraction Kit by following the manufacturer's instructions. Thereafter, the isolated DNA was amplified at V3-V4 regions of the 16S rRNA gene by using the universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG- 3') and 1492R (5'- CGGT TAC CTT GTT ACG ACT T- 3') by making 20 μl of reaction mixture. The reaction mixture was prepared by adding, 2 μl of PCR assay buffer (10X), 2 μl of (2mM) dNTPs, 0.6 μl of forward and reverse primers (10pmol), 2 μl of Taq DNA polymerase (3U/ μl) (Takara Bio India Pvt Ltd.) along with 1.5 μl of DNA template (50ng/ μl) and rest of the volume is made up with Millipore distilled water. The amplification of the reaction mixture was carried out under the Proflex PCR system from Applied Biosystems with initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 1 minute, extension at 65°C for 2 minutes, followed by a final extension at 72°C for 5 minutes. Further, this amplified PCR product was sent to a sequencing service (Anuvanshiki (OPC) Private Ltd., Kishangarh, India). The 16S rDNA gene of approximately 1400bp consensus sequence was resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The identification of received sequences was analyzed by comparing their nucleotide homology with the genomic sequences available in the NCBI database (Jamalzadeh et al. 2021, Kumar et al. 2017). Afterward, the gene sequence was also submitted to GeneBank and the accession number was assigned.

In vivo analysis of plant growth promotion efficiency of isolate

The field experiment was carried out in Randomized Block Design (RBD) in a triplicate manner at the experimental farm of CSIR-CIMAP, Lucknow. A menthol-rich, high-yielding, newly released variety of peppermint, CIM-Suras was selected to access the plant growth promotion efficiencies of potential PGPR strain. A bacterial culture SPK-8, growing in Luria Bertini broth (adjust the pH to 7 ± 2) with a cell density of about 106cfu/ml was used to inoculate/bioatize the 20-day-old vegetative propagules of peppermint variety CIM-Suras. The suckers of CIM-Suras of size 5-10cm were surface sterilized with 70% ethanol and 0.5% sodium hypochlorite and were used to treat with different treatment conditions (Table 1). All the seven treatments, T1: surface sterilized suckers of CIM-Suras, biotized with isolate SPK-8; T2: surface sterilized suckers amended with vermicompost of 5 tons/ha on dry weight basis; T3: surface sterilized suckers amended with Farm Yard Manure (FYM) of 25 tons/ha on dry weight basis; T4: surface sterilized suckers amended with required dose of fertilizer (RDF) (120:60:60); T5: Suckers of CIM-Suras biotized with SPK-8 and amended with sterilized vermicompost (5 tons/ha); T6: Suckers of CIM-Suras biotized with SPK-8 and amended with sterilized Farm Yard Manure (FYM); T7: Suckers of CIM-Suras biotized with SPK-8 and amended with recommended dose of fertilizer (RDF). The surface sterilized suckers of peppermint variety CIM-Suras without any biotization and amendment were used as control. All seven sets of treatment along with control were transplanted end to end in 4-5cm deep furrows into eight rows of equal spacing (50cm) into 8 different beds with bed size $3 \times 4 \text{ m}^2$ and with 20cm plant to plant distance, during the mid-January month of 2023 (Table 1). The irrigation was provided once a week up to 20 days of transplanting after that the fields were irrigated as and when required to maintain the humidity of soil up to 60-70% throughout the experiment. A booster dose of the bacterial culture of the same strength (10⁶cfu/ml) was diluted with distilled water (1:100) and sprayed again, into the soil near the root zone of plants, before the flowering initiation stage of the crop i.e., 45 days after transplantation. The metrological data of daily temperature fluctuation, precipitation, and average relative humidity were recorded during the experimentation period from January to May 2023. Also, the physiochemical properties of the field soil were analyzed and recorded during the onset of the experiment. The morphological data including plant height (cm), number of branches, number of leaves/branch and canopy diameter (cm) were recorded at the maturity stage of plants (90 DAP) and the plants were harvested during the first week of May 2023, after 110 days of transplanting to record the herb yield per bed (kg/bed). The collected herb was steam distilled to evaluate the oil content (%) as well as chemical constituents of the extracted essential oil by performing GC analysis.

Data collection and Statistical analysis

A one-way Analysis of Variance (ANOVA) was analyzed with the help of recorded data of treatments (T1, T2, T3, T4, T5, T6, T7), and control to assess the effect of plant growth promoting bacterial (PGPB) application over peppermint variety CIM-Suras. In the mean table CD at a 5% level ($p \leq 0.05$) was used for comparing treatment means according to Katsenios et al. (2022). The estimation of genotypic and phenotypic correlations (Dewey and Lu, 1959) and D² analysis (Mahalanobis, 1936) was performed using the software OriginPro 9.1 and Microsoft Excel program.

RESULTS AND DISCUSSION

Morphological characterization of isolates

A total of 14 bacterial isolates growing over both Nutrient Agar medium (NA), and Kings B medium (KB) were isolated from the line/genotype MPS-36-37 of peppermint and named according to the medium used for their isolation (Table 2). Fluorescence production by the isolates was also examined and recorded with other morphological characters of the isolates under Table 2. Isolates from SPN-1 to SPN-6 were pure cultured from the dilution plates of Nutrient Agar medium

(NA) and from SPK-1 to SPK-8 were pure cultured from Kings B medium (KB) plates. Out of 14 isolates, 8 bacterial isolates were gram-positive and 6 were gram-negative. However, only 4 out of 14 were fluorescence-producing, SPN-4 and SPK-8 were producing green pigment whereas SPK-1 and SPK-5 were producing yellow pigment when analyzed under 365nm of UV illumination (Table 2).

Plant probiotic activities of isolates

IAA production

All the morphologically characterized isolates were examined for their IAA production efficiency as the ability of microbes to produce IAA, an auxin-derived crucial phytohormone, is suggested as an effective measure for the screening of plant probiotics (Maharana 2019). Matsuda et al. (2018) describe IAA as a phytohormone directly influencing plant growth by interacting with plants and microbes. Cell division initiation, elongation, and enlargement in root cells are the properties of IAA described by Lebrazi et al. (2020a), increasing the surface area of the root thereby improving the absorption of soil nutrients and ultimately influencing plant health. In general, various organic compounds including L-Trp have been reported to be utilized by soil bacteria for the biosynthesis of IAA by involving processes like decarboxylation, deamination and/or hydrolysis reactions (Lebrazi et al. 2020b). The most likely pathway involved for the biosynthesis of IAA through rhizobacteria is the Indole-3-pyruvic acid pathway whereas, several other pathways such as Indole-3-acetonitrile pathway, tryptamine pathway, and Indole-3-acetamide pathway are also reported to occur in some species (Lee et al. 2004). The qualitative evaluation of collected rhizospheric samples for IAA production ability of isolates depict that out of 14 isolates only 6 (42.8%) were positive for IAA production test (Table 2). Those 6 isolates were quantitatively accessed further for their IAA production efficiency and results revealed that SPK-8 (24.8µg/ml) was highest IAA producer followed by SPK-2 (16.5µg/ml), SPN-2 (13.5µg/ml), SPK-4 (12.4µg/ml), SPK-7 (11.4µg/ml) and SPN-4 (10.8µg/ml) (Table 2). A significantly higher production of IAA, than our findings were recorded by Vasant et al. (2023), ranging from 20.7-133µg/mL. Whereas, during the study conducted by Singh et al. (2020), IAA production by the isolates ranged between 7.01-35.56µg/mL which was almost similar to the results we have obtained. The maximum IAA production in the study conducted by Bessai et al. (2022) was 67.14µg/mL and was about 37% higher than the concentration of IAA recorded in our study.

Ammonia production

The cellular synthesis of plant's enzymes is chiefly dependent on biologically fixed nitrogen which also promotes the synthesis of chlorophyll, proteins, DNA and RNA in plants (Hayat et al. 2010). The puissant plant growth-promoting microflora assists in fixing atmospheric nitrogen for plants either by associating symbiotically with them or by being present in free-living state. In the current study morphologically characterized rhizobacterial population was accessed for their ammonia production efficiency and results revealed that only 5 (35.7%) bacterial isolates out of 14 were able to produce ammonia in a range from 19.5mg/L to 49.4mg/L (Table 2). Similar findings of ammonia production through isolates were suggested by the study conducted by Vasant et al. (2023). A comparatively lower range of ammonia (5.0µM/ml-12.5µM/ml) produced through plant growth promoting isolates was recorded by Alotaibi et al. (2022).

Phosphate solubilization

Phosphorous is suggested as an essential macronutrient involved in proliferation and development of new tissues, production of nucleic acids, and regulation of protein synthesis as well as energy regulation in plants (Elhaissofi et al. 2022). It is reported to increase the yield of crop by inducing the development of roots and root hairs formation, cell signalling and redox homeostasis (Elhaissofi et al. 2020, Siedliska et al. 2021). According to Wang et al. (2022) most of the phosphate solubilizers induces IAA production along with plying their role in P cycling. Under the current study, the phosphate solubilization efficiency by isolates was estimated and only 3 out of 14 isolates, SPN-2, SPK-1 and SPK-8 were giving positive results. The phosphate solubilization index (PSI) recorded for potential phosphate solubilizers was in the range from 2.05 to 3.63 (Table 2) which was in accordance with the findings of Manzoor et al. (2017) and Rfaki et al. (2015). A slightly higher range of PSI, between 2.9 to 4.1 was recorded during the study conducted by Janati et al. (2022). However, the phosphate solubilization index by plant growth-promoting isolates ranged from 1.2 to 2.3, as reported by Mohamed et al. (2019).

Siderophore production

Siderophores are the low molecular weight (200-2000 Da) secondary metabolites produced from the rhizo-microbial niche of the plants during iron stress conditions, composed of small peptides with low molecular weight and high iron chelating potentials (Arora and Verma 2017). The iron-binding ligands, known as siderophores, released from the rhizobacteria facilitate the binding of ferric ions and transport them through the membrane receptor molecules into the cell thereby increasing the availability of iron to host plant (Niehus et al. 2017). It is reported to improve the health and growth of plants either by improving the iron availability to crop or by reducing its accessibility to phytopathogens (Santoro et al. 2015). After 7 days of incubation all of the isolates were accessed for siderophore production efficiency and results revealed that only one (SPK-8) out of 14 was giving positive results for the siderophore production test (Table 2).

Identification of potential PGPR

Out of 14 bacterial isolates cultured from the rhizospheric soil of line/genotype MPS36-37 of peppermint; single isolate SPK-8 was selected based on its strong ability to depict plant growth promotion activities. The 16S rRNA sequence identification of strain SPK-8 showed that the selected isolate belonged to the species *Agrobacterium radiobacter* DSM 30147 with 99.7% similarity and was assigned with accession no. PV682772. The selected isolate was applied further to assess its plant growth-promoting efficiency with the runners of variety CIM-Suras of peppermint in field conditions.

Field Experiment

A field experiment was established at CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India with the temperature fluctuation from a low range of 4°C in January to a high temperature of 45°C in June during the year 2023. A significant variation in relative humidity was recorded to range from 26% to 95% from January to May 2023. The mean

average precipitation during these five months was recorded 20.69mm. The soil type used for the field trial was sandy loam with pH 7.5 (soil to water ratio 1:2.5), electrical conductivity of 0.344mS/cm with medium range of Organic C (5.4 mg/L), medium concentration of available N₂ (398.27 Kg/ha), high amount of available phosphorous (41.9 Kg/ha) and moderate K⁺ (193.5 Kg/ha).

The surface sterilized suckers of CIM-Suras of size 5-10cm without any treatment and any amendment (control) were transplanted into the first bed of size 3×4 m² and with 20cm plant-to-plant distance. Further, the surface-sterilized suckers administered with selected PGPB (SPK-8) without any amendment (T1) was transplanted into the second bed. For treatment, T2, vermicompost with the rate of 5 tons/ha was used to amend the surface sterilized suckers of CIM-Suras, transplanted in the third bed. The surface-sterilized suckers of variety CIM-Suras of the same size i.e., 5-10cm were transplanted into fourth bed with the amendment of Farm Yard manure (FYM) of rate 25 tons/ha (T3). Similarly, for T4 surface sterilized suckers were amended with N:P:K taken in ratio the 120:60:60, T5 (PGPR+vermicompost@5tons/ha), T6 (PGPR+FYM@25tons/ha) and T7 (PGPR+RDF (N:P:K)@120:60:60) were transplanted in the fifth, sixth, seventh, and eighth beds respectively. Regrowth/leaf initiation into the transplanted suckers was initiated after 15 days of transplanting. The morphological data of the plants was collected after 90 days of transplanting and their mean was recorded under Table 4. The analysis of the variance of the morpho-chemical results shows that the mean square of all treatments along with the control was found to be highly significant ($p < 0.01$) for all the studied qualitative and quantitative parameters (Table 3). A similar trend of highly significant variability within morpho-chemical traits with PGPR treatments in peppermint (Chiappero et al., 2019) has been reported previously. The estimations of genotypic and phenotypic variance, genotypic and phenotypic coefficient of variation, along with heritability and genetic advance for all the morphometric characters were calculated and recorded under Table 5. The phenotypic coefficient of variation for the studied characters like number of branches/plant, number of leaves/branch, canopy diameter, biomass (kg/bed) and oil content (%) were found to be slightly higher than the genotypic coefficient of variation depicting that the environmental effects have minorly influenced the studied traits up to some extent. the difference between GCV and PCV was found to be maximum for the trait: number of leaves/branch, showing the maximum influence of the environmental factors over this specific trait. The values of PCV in comparison to GCV recorded to be slightly higher in peppermint was reported earlier by Prasad et al. (2020) and similar results were depicted for different spp of *Mentha* by Mishra et al. (2016) and Venkatesha et al. (2019). However, the values of genotypic coefficient of variation and phenotypic coefficient of variation for the traits: the content of menthol (%), menthofuran (%), pulegone (%), and menthone (%), were recorded to be almost similar, suggesting that these traits were nearly unaffected by the influence of the environmental factors. The maximum value for the genotypic coefficient of variation was recorded for menthofuran (%) (36.81%), followed by menthone (%) (25.44%) and biomass (kg/bed) (21.87%) indicating the scope of further improvement of these traits through treatments (Prasad et al. 2021). A high heritability (>75%) was recorded for all the studied characters and high genetic advance as a percentage of the mean (>20%) was recorded for all the morphometric characters except for plant height, number of branches/plant, number of leaves/branch, canopy diameter and menthol content (Table 5). The highest genetic advance as a percentage of the mean was recorded for the menthofuran content (75.78%). The high heritability for all characters and high genetic advance for characters like biomass (kg/bed), oil content (%), menthofuran (%), pulegone (%) and menthone (%), suggests that these traits are highly influenced by the treatments used for the study.

The study of correlation determines the magnitude and nature of the association between different studied characters and reveals about the character responsible for selecting the most favourable treatment (Aftab et al. 2024). However, the relationship between phenotypic and genotypic correlation becomes important when the results of studied traits are contemplated to choose the best-applied treatment of the study (Toth et al. 2020). Table 6 shows that for almost all the characters except for canopy diameter for oil content, menthofuran, and biomass, the genotypic correlation was found higher than the phenotypic correlation, showing prominent inherent interconnection among the characters (Table 6, Fig.1). An Array of correlations among morpho-chemical parameters shows that plant height (cm) is positively and significantly correlated with biomass (kg/bed), oil content (%), menthol, and menthofuran (%). Oil content (%) was positively and significantly correlated with menthol (%), menthofuran (%), menthone (%), and biomass (kg/bed). The content of menthol showed a positive correlation between menthofuran (%) along with biomass (kg/bed) and menthofuran was correlated with biomass (kg/bed) (Table 6). The segregation of correlations into direct and indirect effects depicts the complex inter-relationship among different studied traits. Path coefficient analysis is simply standardized partial regression coefficient which split the correlation coefficient into direct and indirect effects on predicted variable (Neelam et al. 2016). A highly positive direct effect on biomass (kg/bed) was imparted by the content of pulegone (1.309), followed by menthofuran (1.135), menthol (0.352), and plant height (0.104) (Table 7). The residual effect of 0.077 indicates that the experimental model was reasonably fit (MacCallum et al. 1994). The study, therefore determines that including traits like pulegone, menthofuran, menthol, and plant height are important parameters for selecting treatments to improve the biomass (kg/bed) of the peppermint variety CIM-Suras.

The clustering of applied treatments using Mahalanobis D² analysis and Tocher's method was performed and the results of the study grouped all eight treatments including control into five different clusters (Fig.1). The findings indicate that cluster I, cluster II, cluster III had the highest number of genotypes i.e., two followed by cluster IV, and cluster V with one genotype in each cluster. Cluster I, comprised of two treatments T5 and T7 with favourable improvement in plant height, biomass (kg/bed), oil content (%), and menthol content (%) were grouped together. Cluster II was comprised of T4 along with control, showing almost similar for the studied traits like number of leaves/plant, oil content (%), menthol (%), and menthofuran content (%) (Fig.2). The third cluster comprised treatments like T3 and T6 showing almost similar values for traits like plant height, number of branches/plant, and the content of menthofuran, pulegone, and menthone. The first treatment (T1) was solely lying under cluster IV, showing maximum oil content (%) and menthol content (%). Whereas, cluster V contained a single treatment T2, having the highest values for the content of pulegone. As per Singh

(1981), the trait with the highest contribution to treatmental effect was number of branches/plant with contribution of 207.7381 % followed by number of leaves/branch with contribution of 132.4405 %. The inference regarding the means of the traits for clusters depicts that all clusters performed differently for various studied treatments in peppermint variety CIM-Suras (Table 8). Cluster I showed the highest mean performance for plant height, oil content, menthol content, and menthofuran (%). Cluster II showed the maximum mean performances for the number of leaves/branch, pulegone, and menthone contents. Cluster III had the highest intracluster distance (470.42) followed by Cluster II (327.52) and Cluster I (307.54) (Table 8, Fig.2). Among five clusters the largest (7385.55) inter-cluster distance was within Cluster IV and Cluster III, followed by Cluster III and Cluster II (4985.91), and the minimum inter-cluster distance (833.4) was within Cluster I and Cluster IV (Table 8, Fig.2). This inter-cluster distance signifies the proportion of divergence in treatments, clubbed between different clusters and eases down the process of selecting the most favorable treatment. The treatments grouped under the clusters showing minimum inter-cluster distance are considered to exhibit more similarity in traits. Out of a total of five clusters, during this study T1, lying under Cluster IV, and T5 and T7 lying under Cluster I showed maximum inter-cluster difference or divergence from other clusters signifies that selecting these treatments (T1, T5 & T7) would be more favorable for improving the studied traits in peppermint variety CIM-Suras.

The deliberate analysis of mean data of morpho-chemical traits for various treatments revealed that a highly significant improvement throughout the experiment, over the control was recorded for plant height, biomass (kg/bed), and content of menthol (%). The maximum improvement (42.72%) in plant height and biomass (kg/bed) was recorded for T5 (SPK-8+vermicompost@5tons/ha) which was in accordance with Singh et al. (2007) and Padmavathiamma et al. (2008) when crops like sugarcane and chili were implemented with vermicompost and growth promoting bacterial inoculum (Gopinath et al. 2010). Similar results have been obtained for *Mentha arvensis* L. also with the same treatment conditions (Lothe et al. 2021). The suckers of peppermint variety CIM-Suras, amended with vermicompost (T2) was recorded to show maximum improvement (27.50%) in number of leaves/branch along with increasing the oil content (%) by 28.75% and menthol content by 7.43%, over the control (Table 4). Ram et al. (2012) reported that the amendment of vermicompost with peppermint increases the morphometric parameters of the crop. The experiment conducted by Pourhosseini et al. (2024) suggests that implementing vermicompost with peppermint significantly increases the number of leaves/plant and doubles the essential oil content along with increasing its menthol content. Implementing the isolate SPK-8 (T1) with the suckers of CIM-Suras was recorded to maximumly improve the content of essential oil (80.70%) and menthol (13.81%), over the control which was in accordance with the results derived from the study conducted by Pourhosseini et al. (2024) where growth promoting bacteria, employed with peppermint have increased the content of essential oil and menthol. The treatment of vegetative propagules of CIM-Suras with isolate SPK-8 amended with FYM @ 25tons/ha was recorded to double the biomass (kg/bed) and improve the plant height by 28.49%, oil content by 35.09% and 6.92% improvement in menthol content, over the control (Table 4). Verma et al. (2016) have reported that the application of Farm Yard Manure (FYM) with *Mentha piperita* was found to increase the biomass and essential oil yield of the crop significantly. The overall analysis of the results determines that T1 was most suitable for improving oil content (%) along with T5 showing the most suitability in improving biomass (kg/bed) by keeping the essential oil quality of CIM-Suras up to the level of globally accepted essential oil quality of peppermint.

CONCLUSIONS

This study was designed with an interest of improving the growth and yield of peppermint variety CIM-Suras without deflecting its essential oil quality. To achieve this objective a plant growth-promoting bacterial inoculum of SPK-8 (*Agrobacterium radiobacter* DSM 30147), isolated from the rhizospheric region of a peppermint line/genotype MPS-36-37 was introduced with the suckers of newly released, menthol rich peppermint variety CIM-Suras. This study compared the performance of the implementation of this plant growth-promoting bacterial inoculum solely as well as in combination with various organic and inorganic additives like vermicompost @5tons/ha, farm yard manure @25tons/ha, and recommended dose of NPK (120:60:60) over the variety CIM-Suras. The results of the study revealed that the application of different treatments showed improvement in plant height ranging from (80.83cm to 89.77cm); biomass (kg/bed) ranged from (3.57-5.00); oil content (%) from (0.55-1.03); menthol (%) from (69.98-80.85); and menthofuran (%) from (1.43-3.41). The treatment of suckers solely with SPK-8 (T1) showed maximum improvement in oil content (80.70%) with the highest improvement in menthol content (13.78%) along with keeping the menthofuran content in the acceptable range of US-type peppermint. The T1 was also found to be the most divergent from other applied treatments, interpreted from the clustering analysis. However, the highest improvement in plant height (42.72%), biomass (kg/bed) (150.0%), and content of menthofuran (122.88%) in peppermint variety CIM-Suras was analyzed for the suckers treated with SPK-8, amended with vermicompost (T5). Whereas, the administration of the same bacterial isolate (SPK-8) with vermicompost amendment is most favorable to improve the biomass as well as plant height of the variety CIM-Suras. Therefore, on the basis of designed experiment, T1 i.e., implementation of bacterial isolate *Agrobacterium radiobacter* with the suckers of CIM-Suras is the best-suited treatment condition to improve oil content of CIM-Suras with essential oil quality lying in the range of globally acceptable oil quality of peppermint. Thus, it may be suggested to peppermint growers to cultivate variety CIM-Suras with the implementation of plant growth-promoting bacterial isolate *Agrobacterium radiobacter* to harness its maximum growth potential. However, the cultivation of variety CIM-Suras amended with SPK-8 (*Agrobacterium radiobacter*), at varying locations and climatic conditions is essential to analyse the accurate performance for its growth and yield attributes.

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Author contributions

Vagmi Singh: Writing - original draft, Writing - review & editing, Formal analysis. Rajesh Kumar Verma: Conceptualization, Visualization, Methodology, Investigation. Rakesh Kumar: Visualization, Methodology, Investigation. Akanksha Singh: Conceptualization, Visualization, Investigation. Neelam: Visualization, Data curation. Ram Kishor: Visualization, Data curation. Akancha Gupta: Visualization, Data curation. Nashra Aftab: Visualization, Data curation. Birendra Kumar: Conceptualization, Visualization, Supervision, Writing - review & editing, Validation.

Conflicts of interest

The authors have declare no competing financial interests or personal relationships.

Data availability

The 16S rRNA gene sequences of the bacterial isolates have been deposited in the NCBI GenBank database under accession number PV682772 (<https://submit.ncbi.nlm.nih.gov/subs/genbank/>).

Ethical approval statement

This study did not involve human participants, human tissues, vertebrate animals, or endangered species. All experimental procedures were conducted using plant materials and microbial isolates in accordance with the institutional guidelines and applicable national regulations. Therefore, formal ethical approval and informed consent were not required for this study.

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

SL No.	Treatment names	Treatment details
1	Control	surface sterilized suckers of CIM-Suras without any treatment
2	T1	surface sterilized suckers administered with <i>Agrobacterium radiobacter</i> (SPK-8)
3	T2	surface sterilized suckers amended with vermicompost of 5 tons/ha on dry weight basis
4	T3	surface sterilized suckers amended with Farm Yard Manure (FYM) of 25 tons/ha on dry weight basis
5	T4	surface sterilized suckers amended with required dose of fertilizer (RDF) (120:60:60)
6	T5	surface sterilized suckers of CIM-Suras biotized with SPK-8 and amended with sterilized vermicompost (5 tons/ha)
7	T6	surface sterilized suckers of CIM-Suras biotized with SPK-8 and amended with sterilized Farm Yard Manure (FYM)
8	T7	surface sterilized suckers of CIM-Suras biotized with SPK-8 and amended with recommended dose of fertilizer (RDF)

Table 2. Details of rhizospheric bacteria isolated from rhizospheric soil of MPS-36-37 genotype of *Mentha piperita* L.

Isolates	Shape	Size	Colour	Surface	Margin	Florescence Production	Gram stain	Siderophore Production	IAA production (µg/ml)	Ammonia Production (mg/L)	Phosphate solubilization Index
SPN-1	Round	Small	Yellowish	Smooth	Entire	-	+	-	-	-	-
SPN-2	Irregular	Medium	Creamish	smooth	Irregular	-	+	-	13.510	-	2.771
SPN-3	Round	Small	Yellowish	Smooth	Irregular	-	+	-	-	-	-
SPN-4	Round	Small	White	Smooth	Entire	Green	-	-	10.833	23.217	-
SPN-5	Round	Small	Yellowish	Smooth	Entire	-	+	-	-	-	-
SPN-6	Irregular	Small	Creamish	Smooth	Irregular	-	-	-	-	-	-
SPK-1	Round	Small	White	Shiny	Entire	Yellow	+	-	-	38.922	2.053
SPK-2	Round	Small	Yellowish	Shiny	Entire	-	-	-	16.477	-	-
SPK-3	Round	Small	White	Smooth	Irregular	-	+	-	-	19.513	-
SPK-4	Round	Small	Creamish	Smooth	Entire	-	-	-	12.423	2.160	-
SPK-5	Round	Small	Creamish	Smooth	Entire	Yellow	-	-	-	-	-
SPK-6	Irregular	Medium	White	Rough	Irregular	-	+	-	-	-	-
SPK-7	Round	Small	Yellowish	Smooth	Entire	-	-	-	11.432	-	-

SPK-8	Round	Small	White	Smooth	Entire	Green	+	+	24.792	4.941	3.632
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Table 3. Analysis of Variance of morphochemical traits of peppermint variety CIM-Suras undergone through various treatments.

Source of Variation	D F	Plant Height (cm)	No. of Branch/plant	No. of Leaf/branch	Canopy Diameter (cm)	Biomass (kg/b ed)	Oil yield (%)	Menthol (%)	Menthofuran (%)	Pulegone (%)	Menthone (%)
Replication	2	2.57	0.04	0.13	0.53	0.016	0.010	0.306	0.004	0.005	0.0012
Treatments	7	189.78**	20.18**	38.55**	29.80**	2.272**	0.081**	43.18**	1.452**	0.018**	5.6078**
Error	14	3.37	2.47	6.17	1.60	0.019	0.003	0.146	0.0007	0.0004	0.0016
Total	23										

*p<.05 and **p<.01

Table 4. Effect of various treatments on morpho-chemical parameters of peppermint variety CIM-Suras

Treatments	Plant Height (cm)	No. of Branch/plant	No. of Leaf/branch	Canopy Diameter (cm)	Biomass (kg/be d)	Oil content (%)	Menthol (%)	Menthofuran (%)	Pulegone (%)	Menthone (%)
Control	62.90	28.67	30.33	48.00	2.00	0.57	71.06	1.53	0.61	5.73
T1	86.40**	24.67**	30.67	46.50	4.53*	1.03*	80.85*	2.34**	0.60	5.16**
T2	82.00**	22.67**	38.67**	47.80	4.17*	0.80*	76.34*	1.43**	1.97**	6.15**
T3	81.50**	16.00**	26.33	45.73*	4.27*	0.55	72.48*	1.49	0.55**	6.67**
T4	83.55**	16.33**	30.67	44.33*	4.00*	0.63	69.98*	1.58*	0.48**	5.30**
T5	89.77**	23.33**	35.67*	45.50*	5.00*	0.88*	78.93*	3.41**	0.37**	4.93**
T6	80.83**	22.00**	28.67	38.10*	4.00*	0.77*	75.98*	1.50	0.45**	5.78
T7	82.27**	17.00**	22.33**	43.73*	3.57*	0.75*	76.35*	1.44**	0.51**	6.27**
SEm	1.061	0.907	1.434	0.730	0.079	0.030	0.221	0.016	0.012	0.023
CD (5%)	3.22	2.75	4.351	2.214	0.239	0.0906	0.669	0.047	0.037	0.069
CD (1%)	4.46	3.82	6.039	3.073	0.332	0.1258	0.928	0.066	0.051	0.096

T1: surface sterilized suckers administered with SPK-8; T2: surface sterilized suckers amended with vermicompost of 5 tons/ha on dry weight basis; T3: surface sterilized suckers amended with Farm Yard Manure (FYM) of 25 tons/ha on dry weight basis; T4: surface sterilized suckers amended with required dose of fertilizer (RDF) (120:60:60); T5: Suckers of CIM-Suras biotized with SPK-8 and amended with sterilized vermicompost (5 tons/ha); T6: Suckers of CIM-Suras biotized with SPK-8 and amended with sterilized Farm Yard Manure (FYM); T7: Suckers of CIM-Suras biotized with SPK-8 and amended with recommended dose of fertilizer (RDF).

Table 5. Estimates of genetic parameters for various morpho-chemical traits of peppermint variety CIM-Suras undergone through different sets of treatments

Sl no.	Characters	σ^2g	σ^2p	GCV	PCV	h^2	GA as % of mean
1	Plant Height (cm)	62.134	65.507	9.713	9.974	94.85	19.487
2	No. of Branch/plant	5.905	8.375	10.322	12.293	79.259	17.854
3	No. of Leaf/branch	10.792	16.964	10.512	13.180	82.587	17.272

4	Canopy Diameter (cm)	9.400	10.997	6.819	7.376	85.469	12.986
5	Biomass (kg/bed)	0.751	0.770	21.872	22.141	96.858	44.507
6	Oil content (%)	0.026	0.029	21.600	22.651	90.649	42.277
7	Menthol (%)	14.343	14.489	5.033	5.059	98.992	10.316
8	Menthofuran (%)	0.484	0.485	36.814	36.841	97.565	75.784
9	Pulegone (%)	0.006	0.006	15.012	15.513	99.655	29.922
10	Menthone (%)	1.869	1.870	25.440	25.451	99.563	52.385

Table 6. The genotypic and phenotypic correlation coefficient among ten morpho-chemical traits on biomass (kg/bed) of peppermint variety CIM-Suras

Characters		No. of Branches	No. of Leaf/plant	Canopy Diameter (cm)	Oil content (%)	Menthol (%)	Menthofuran (%)	Pulegone (%)	Menthone (%)	Biomass (kg/bed)
Plant Height (cm)	G	-0.485*	0.170	-0.235	0.641**	0.603**	0.529**	-0.042	-0.294	0.978**
	P	-0.428*	0.147	-0.227	0.585**	0.583**	0.508*	-0.047	-0.287	0.931**
No. of Branch/plant	G	1	0.494*	0.337	0.338	0.295	0.312	0.168	-0.447*	-0.385
	P		0.438*	0.296	0.255	0.252	0.282	0.146	-0.393	-0.324
No. of Leaf/branch	G		1	0.421*	0.365	0.235	0.426*	0.629**	-0.457*	0.297
	P			0.369	0.285	0.199	0.381	0.574**	-0.402	0.292
Canopy Diameter (cm)	G			1	-0.023	-0.051	0.142	0.442*	-0.011	-0.178
	P				-0.037	-0.050	0.144	0.407*	-0.007	-0.180
Oil content (%)	G				1	0.959**	0.618**	0.106	-0.571**	0.614**
	P					0.897**	0.565**	0.099	-0.543**	0.552**
Menthol (%)	G					1	0.599**	0.090	-0.340	0.597**
	P						0.587**	0.090	-0.339	0.585**
Menthofuran (%)	G						1	-0.306	-0.741**	0.571**
	P							-0.300	-0.728**	0.553**
Pulegone (%)	G							1	0.313	0.017
	P								0.310	0.021
Menthone (%)	G								1	-0.274
	P									-0.270

Table 7. The direct (in bold letters) and indirect effect of 10 morpho-chemical traits on biomass (kg/bed) of peppermint variety CIM-Suras.

Characters	Plant Height (cm)	No. of Branches/plant	No. of Leaf/branch	Canopy Diameter (cm)	Oil content (%)	Menthol (%)	Menthofuran (%)	Pulegone (%)	Menthone (%)
Plant Height (cm)	0.104	0.300	-0.125	0.089	-0.322	0.212	0.600	-0.055	0.175
No. of Branches/plant	-0.050	-0.618	-0.363	-0.127	-0.170	0.104	0.354	0.220	0.266
No. of Leaf/branch	0.018	-0.306	-0.735	-0.159	-0.183	0.083	0.484	0.823	0.272
Canopy Diameter (cm)	-0.024	-0.208	-0.309	-0.377	0.012	-0.018	0.162	0.579	0.006
Biomass (kg/bed)	0.066	-0.209	-0.268	0.009	-0.502	0.338	0.701	0.139	0.341
Menthol (%)	0.062	-0.183	-0.172	0.019	-0.481	0.352	0.680	0.118	0.202
Menthofuran (%)	0.055	-0.193	-0.313	-0.054	-0.310	0.211	1.135	-0.401	0.442

Pulegone (%)	-	0.004	-0.104	-0.462	-0.167	-0.053	0.032	-0.347	1.309	-0.187
Menthone (%)	-	0.030	0.276	0.336	0.004	0.287	-0.120	-0.840	0.410	-0.596

Table 8. Cluster means of different clusters for the morpho-chemical traits in peppermint variety CIM-Suras

Trait	cluster I	cluster II	cluster III	cluster IV	cluster V
Plant Height (cm)	88.08333	82.13333	73.22333	81.5	80.83333
No. of Branches/plant	22.66667	23.5	25.66667	25.66667	21.66667
No. of Leaf/branch	31.66667	33.66667	30.5	26.33333	32
Canopy Diameter (cm)	46	45.76667	46.16667	45.73333	38.1
Biomass (kg/bed)	4.333333	4	3	4.533333	4
Menthol (%)	0.958333	0.775	0.6	0.55	0.766667
Menthofuran (%)	79.88833	76.345	70.52167	72.48333	75.98
Pulegone (%)	2.876667	1.433333	1.556667	1.49	1.503333
Menthone (%)	0.488333	1.236667	0.541667	0.546667	0.453333
Plant Height (cm)	5.045	6.208333	5.515	6.67	5.78

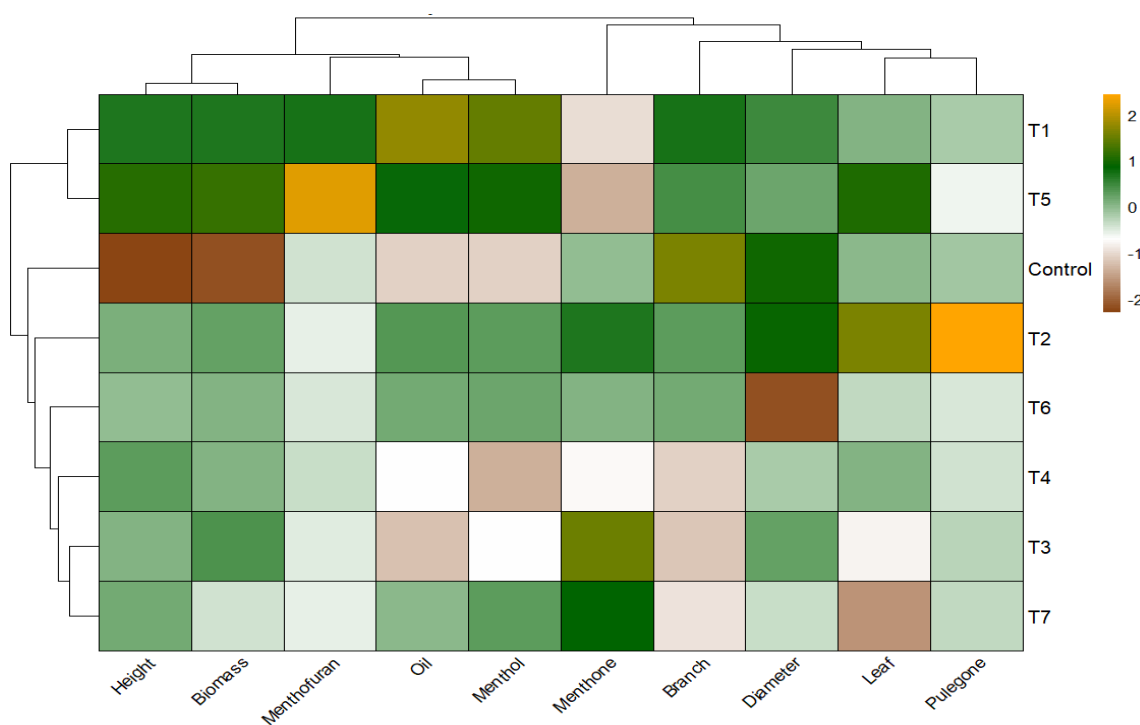


Figure 1. Heat map showing correlation between morpho-chemical attributes along with applied treatments over peppermint variety CIM-Suras.

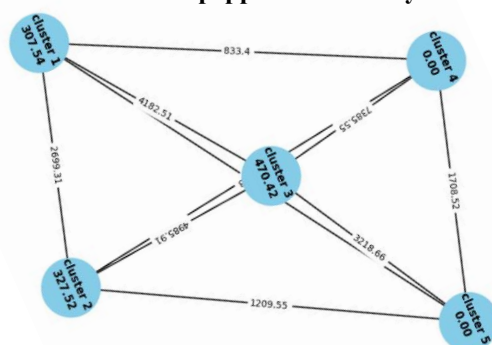


Figure 2. Average inter and intra cluster distances (Mahalanobis Euclidean distance) among 8 treatments of peppermint variety CIM-Suras.