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Research Article

Rhizospheric Microflora Escalating Aroma Constituents and Yield Attributes in *Ocimum tenuiflorum* (L.) cv. CIM-Ayu

Shilpi Khare Saikia and Rakesh Pandey

Microbial Technology and Nematology Department, CSIR-Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow 226015, India

Correspondence should be addressed to Rakesh Pandey; r.pandey@cimap.res.in

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The exploration of rhizospheric microbial flora for crop yield enhancement is well established. Rhizospheric microbes influence the plant physiology by imparting several beneficial effects, namely, Nitrogen fixation, increased nutrient uptake, and secondary metabolites production on their host plants. The present study investigates the response of *Bacillus megaterium* ATCC No. 13525, *Pseudomonas fluorescens* ATCC No. 14581, and *Trichoderma viride* MTCC No. 167 in alone and combined treatments for their effect on growth and yield parameters in a commercially important *Ocimum tenuiflorum* L. cv. CIM-Ayu. The plant is therapeutically important for its essential oil constituents, namely, eugenol, β-caryophyllene, and various monoterpenes. The combination treatments, T7 (*B. megaterium* + *P. fluorescens*) and T8 (*B. megaterium* + *P. fluorescens* + *T. viride*), showed maximum enhancement (27.27%) of percentage essential oil as compared to untreated control. Nutrient uptake especially N_2 content was significantly increased (43%) with the treatment T8 (*B. megaterium* + *P. fluorescens* + *T. viride*). Amongst major essential oil constituents, eugenol content was maximally increased by 58.5% as compared to 42.9% (control) indicating a cumulative role of microbial inoculants for crop yield boost-up.

1. Introduction

The members of genus *Ocimum* (Gk. ozo = smell) from family Lamiaceae are medicinally important plants owing to their therapeutic potentials as antiseptics, antioxidants, antistressors, antipyretics, antimicrobials, and insecticidal [1]. *Ocimum tenuiflorum* is an imperative species from the genus, widely cultivated for its high essential oil yields (0.5 to 0.7%), rich in eugenol, methyl chavicol, and linalool content [2]. The commercial importance of essential oil depends on its constituents and hence methods are required to enhance these valuable phytomolecules.

Rhizospheric microorganisms are recognized as an economic and sustainable input for increasing the productivity of several agricultural, horticultural, forestry, and medicinal crops [3–5]. Numerous microbes such as *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus,* and *Serratia* have been reported to enhance the plant growth [6, 7]. These microorganisms coexist in the rhizosphere, which is a thin soil layer immediately surrounding plant roots. Rhizospheric microbial wealth is widely acclaimed in agricultural practices for enhancement of crop yield attributes as they share their environment with the host plants and thus exhibit better adaptation [8]. Rhizospheric microbes play significant role/s in improving the growth and yield of host plant [9] by imparting several beneficial effects, namely, N_2 fixation, increased nutrient uptake, siderophores, and secondary metabolite/s production [8, 10]. This interest is linked to environmental concerns for reduced use of chemicals as well as an appreciation for utilization of biological and organics in agriculture.

Therefore, a proper understanding of microbial species associated with the host plants is essential in improving the quality and yield in a desired crop. In this regard, the present experiment was designed to study the influence of selected rhizospheric microbial inoculants (previously proven as effective biocontrol agents), namely, *Bacillus megaterium* ATCC No. 13525, *Pseudomonas fluorescens* ATCC No. 14581, and *Trichoderma viride* MTCC No. 167 under greenhouse conditions on the agronomical attributes of *O. tenuiflorum*

cv. CIM-Ayu. The study also validates the role of microbial inoculants for plant growth promotion and alterations in aromatic oil symphony due to them.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions. Seeds of high yielding cultivar of *O. tenuiflorum* "CIM-Ayu," obtained from the National Gene Bank for Medicinal and Aromatic Plants at the Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, India, were surface-sterilized by soaking in 10% (v/v) sodium hypochlorite solution for 5 minutes, washed with distilled water, and soaked for 4 h. After sterilization and soaking, healthy looking uniform sized seeds were sown in content plug plates filled with sterilized soil in a greenhouse under natural light conditions, a daytime temperature of about 28°C and relative humidity of 65–70%. Twenty-one days after sowing, four healthy leafed stage seedlings were transplanted into 7.0 kg soil capacity clay pots containing a mixture of autoclaved soil (76% sand, 8% silt, and 16% clay, pH 7.7) and composted farm manure in 5 : 1 ratio. Soil mineralizable nitrogen, phosphorus, and potassium content were estimated [11–13]. Plants were irrigated manually at alternate days to ensure adequate soil moisture throughout the experimentation.

2.2. Microbial Inoculants (Culture and Maintenance). The rhizospheric microbes *Bacillus megaterium* (ATCC-13525), *Pseudomonas fluorescens* (ATCC No. 14581), and *T. viride* (MTCC-167) are continuously maintained in the Microbial Technology and Nematology Department, CSIR-CIMAP, Lucknow. These microbes have a proven role in biocontrol experiments [14–16] and thus were selected for observing plant growth promotion effects. The fungus *T. viride* was cultured using sand maize media while the bacterial strains were cultured in Luria broth. The fungal seed culture was incubated at 30 ± 1°C for 96 h. After mass-multiplication, the mycelial mat with conidia was homogenized and suspended in 500 mL of 0.1 M phosphate buffer (K_2HPO_4; KH_2PO_4) adjusting the colony forming units (CFU) at 1.2×10^6 per mL. The bacterial cultures were incubated at 28 ± 1°C for 48 h under shaking conditions (200 rpm). After proper multiplication, the cultures were centrifuged at 6000 g for 10 min. The supernatants were discarded and the pellets containing bacterial cells were suspended in 500 mL of 0.9% saline, adjusting the CFU as 2.5×10^8 per mL^{-1} for *B. megaterium* and 1.8×10^8 for *P. fluorescens*. Bacterial cell suspensions were inoculated as 10 mL of 10^8 CFU/pot whereas the fungal seed culture was inoculated as 10 mL of 10^6 CFU/pot. Nine microbial treatments were considered (T1 = uninoculated control, T2 = *B. megaterium,* T3 = *P. fluorescens,* T4 = *T. viride,* T5 = *B. megaterium* + *T. viride,* T6 = *P. fluorescens* + *T. viride,* T7 = *B. megaterium* + *P. fluorescens,* and T8 = *B. megaterium* + *P. fluorescens* + *T. viride*). Nitrogenous fertilizers were added to all the treatments according to the soil test. Shoot height, root length, shoots and root weight (fresh and dry), and N, P, and K content were determined after 90 days of microbial inoculation, at the harvest of the crop.

2.3. Chemical Composition of Essential Oil (GC-Analysis). Essential oil from the aerial biomass was hydrodistilled using Clevenger apparatus for 4 h. The oil was dried over anhydrous sodium sulphate (Na_2SO_4) and then analysed for gas chromatography (GC) on an Agilent Perkin Elmer GC Instrument (Model) fitted with FID (flame ionization detector) and electronic integrator with (30 m × 32 mm i.d., 0.25 μm film thickness) fused-silica capillary column. Nitrogen (at 0.4 mL/min) was used as a carrier gas. Samples were injected in the split mode at a ratio of 1 : 10–1 : 100. The injector was kept at 250°C and the transfer line at 280°C. The column was maintained at 50°C for 2 min and then programmed to 220°C at 5°C/min and held for 10 min at 300°C. The relative quantities of individual components of essential oil were calculated based on software computed GC peak area percentage without applying correction for FID response factor. The identification of the compounds was performed by comparing their retention indices (RI), determined with reference to a homologous series of n-alkanes (C9–C24, Polyscience Corp.) under identical experimental conditions in both polar and nonpolar columns, coinjection with standards. The relative quantities of the individual components were calculated based on computer calculated GC peak areas without correction for flame ionization detection response factors.

2.4. Nutrient Uptake Analysis. Soil mineralizable nitrogen, phosphorus, and potassium content were estimated in various treatments for assessing growth promotion effects. Initial soil samples were analyzed for available N (kg ha^{-1}), available P (Olsen's P_2O_5, kg ha^{-1}), and available K (exchangeable K_2O, kg ha^{-1}) following Jackson [17]. The estimations were made through FOSS application notes AN5222, ENISO 11732:1997, and AN5249 ISO 6878: 1996 with slight modifications in flame photometer 128, Cistronic model.

2.5. Statistical Analysis. The plant treatment was conducted in a random complete block design with three replicates per treatment. The data were subjected to analysis of variance (ANOVA) by using Duncan's multiple test, and treatment means were compared using least significant difference at $P \leq 0.05$. Least significant difference (LSD) was calculated at 5% probability level ($P = 0.05$) for comparing the significance of difference between any two treatment means [18]. The statistical analyses were performed using ASSISTAT 2012 software (version 7.6, Brazil). When the main effects were significant, differences among factor levels were tested for significance using the mean sum of square statement at $P = 0.05$.

3. Results

3.1. Growth Promoting Parameters. The results reveal that the microbial treatments not only enhanced the growth parameters in *O. tenuiflorum* but also significantly modulated aromatic oil's quality. The physical parameters of soil indicate good soil health with a neutral pH (Table 1). Electrical conductivity is the ability of a material to transmit an electrical current and is expressed as milli-Siemens per meter (mS/m). In the present experiment, the pH and conductivity of the

Table 1: Physical parameters of the soil.

Physical parameters	Values
Electrical conductance (dsm. sup.$^{-1}$)	135 μS/cm
N	182.74 Kg/h
K	84 Kg/h
P	52 Kg/h
Na	205 Kg/h
Organic carbon	0.68%
pH	7.53

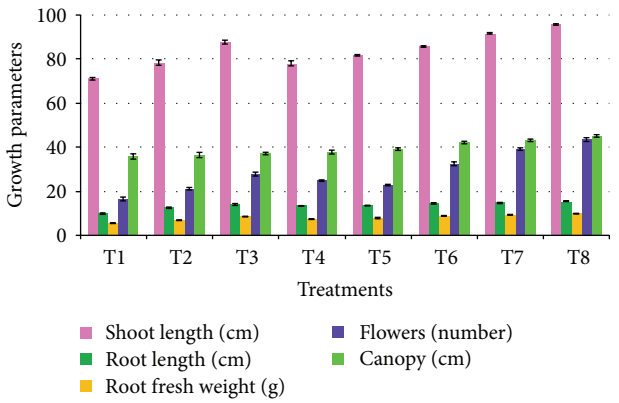

Figure 1: Growth parameters as influenced by the microbial treatments in *Ocimum tenuiflorum* cv. CIM-Ayu. a. Standard error of means (SEM) is indicated by the error bars.

initial soil sample were 7.53 and 135 μScm^{-1}, respectively. According to USDA criteria, EC was lower than 400 μSm^{-1} indicating neutral stress of soil against the existing flora signifying for the good quality of the soil. The attributes like shoot height and weight, root length and weight, and so forth are not considered as an important character determining essential oil extraction but are momentous for growth promotion decision. The traits are also desirable as the flowering tops and young shoots of *O. tenuiflorum* are also used for herbal tea preparations. All of inoculation treatments increased shoot length which were significantly different ($P < 0.05$) when compared with uninoculated control. All single and coinoculation treatments enhanced shoot length ranging from 88.33 cm to 95.67 cm (Figure 1). In the single inoculation, *P. fluorescens* (T3) treatment was observed with maximum effects as the plants depicted a 16.67 cm increase in shoot length over the control plants. The treatments had a lower variation coefficient of 1.52 (Table 2(a)) depicting the significant impact of all the treatments in enhanced growth. *P. fluorescens* intervention also resulted in the maximum increase in root length (13.8 cm) in alone treatments with an overall mean root length augmentation of 5.6 cm (9.7 to 15.3 cm). Fresh root weight was enhanced chiefly due to the combined treatment of *P. fluorescens* and *B. megaterium* (9.2 g). In the alone treatments, *T. viride* treatment depicted an augmentation of 2 cm over the control plants while the maximum shift of approximately 10 cm was observed in the combined treatment (T8) of all the bioinoculants, reflecting

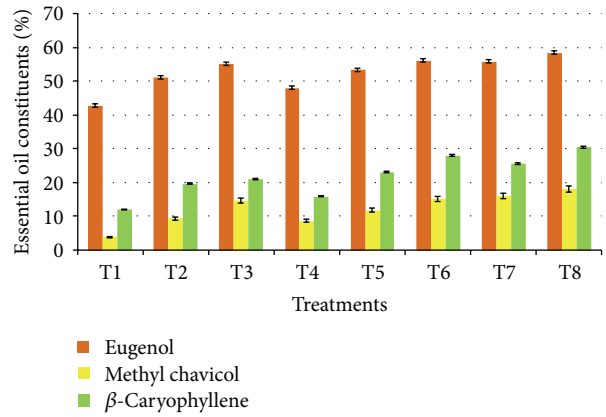

Figure 2: The major essential oil constituents influenced by different microbial treatments in *Ocimum tenuiflorum* cv. CIM-Ayu.

the growth promotional aspect of the organic treatments (Figure 1).

3.2. Leaf Yield and Essential Oil Content (%w/v). Leaf yield that is leaf fresh weight per plant is an important character in determining the aromatic oil yield. The microbial inoculants can vary the constituents of essential oil and thus determine its quality. Observations for leaf biomass depicted a maximum of 49.17 g increase (T8) against the control (T1) plants. In the alone treatments, the highest leaf and oil yield were recorded in *P. fluorescens treatment* with an overall increase of 15.6% and 11%, respectively, against the control plants (Table 2(a)). Essential oil content showed a minimum of 21% enhancement in the combined treatments (T7 and T8) of microbial inoculants. Table 2(b) depicts a quick glance of ANOVA analysis for biomass and percentage essential oil with highly significant results as is indicated by the P values. Means were compared using the least significant difference (LSD) at probability level $P = 0.05$ and revealed significant mean differences against the control plants. CV% values for leaf yield and percentage essential oil were 1.1 and 1.88, respectively, which showed greater variation among various treatments.

3.3. Identification of Essential Oil Components. To identify the mixture components, calculated retention indices (RI) were used. Retention indices of "neutral" components were compared with those reported (Adams 1995). When not less than three RI values are given for the same compound in different sources they were randomized. The results of Head Space analysis indicated that essential oil is rich in mono- and sesquiterpenoids. The major essential oil components as quantified through gas chromatography showed much variability. In alone treatments, the maximum increase (12.34%) in eugenol content was recorded in *P. fluorescens* treatment followed by *B. megaterium* treatment, showing 8.33% increase over the control plants (Figure 2). The combined treatment of *P. fluorescens* and *T. viride* enhanced eugenol content up to 56.22% as compared to 42.86% in control plants.

Table 2: (a) Relation between plant biomass and percentage essential oil in *O. tenuiflorum* cv. CIM-Ayu. (b) ANOVA table for biomass and percentage essential oil.

(a)

Treatments	Shoot length (cm)	Root length (cm)	Root fresh weight (g)	Leaf yield (g)	Essential oil yield (%)
T1	71.00^{f}	9.67^{e}	5.33^{f}	160.83^{f}	0.56^{e}
T2	78.33^{e}	12.33^{d}	6.67^{e}	178.33^{e}	0.61^{d}
T3	87.67^{c}	13.83^{bc}	8.33^{c}	185.83^{d}	0.67^{c}
T4	78.00^{e}	13.17^{cd}	7.167^{de}	181.50^{e}	0.60^{d}
T5	81.67^{d}	13.33^{c}	7.67^{d}	187.17^{d}	0.67^{c}
T6	85.67^{c}	14.33^{b}	8.67^{b}	196.50^{c}	0.71^{b}
T7	91.67^{b}	14.50^{ab}	9.17^{ab}	203.50^{b}	0.77^{a}
T8	95.67^{a}	15.33^{a}	9.67^{a}	210.00^{a}	0.77^{a}
LSD ($P = 0.05$)	2.21	0.88	0.59	3.59	0.02
CV%	1.52	3.83	4.32	1.10	1.88

[a]Mean in each column followed by the same letters does not differ significantly ($P < 0.05$) according to Duncan's multiple range test.
[b]Values followed by different small letters within columns are significantly different ($P < 0.05$) according to the LSD test.

(b)

Source of variation	Degree of freedom (df)	Shoot length (cm)	Root length (cm)	Root fresh weight (g)	Leaf yield (g)	Essential oil yield (%)
Treatment	7	1358.96^{**}	63.24^{**}	42.50^{**}	5026.79^{**}	0.09^{**}
Error	16	26.00	4.17	1.83	68.67	0.003
Total	**23**	**1384.96**	**67.41**	**44.33**	**5095.46**	**0.09**

**Significance at a level of 5% of probability ($P < 0.05$).

Other essential oil constituents responsible for the characteristic aroma of *O. tenuiflorum,* namely, β-caryophyllene and methyl chavicol, were also appreciably enhanced by microbial treatments. A quick insight into major essential oil constituent for three best treatments is depicted through their chromatograms (Figure 3). The peaks for eugenol, methyl chavicol, and β-caryophyllene were identified at retention index (RI) of 26.6, 28.2, and 29.5 minutes, respectively.

3.4. Nutrient Uptake. Nitrogen uptake was drastically enhanced in all the treatments with *B. megaterium* showing the maximum uptake of 2.83% followed by *P. fluorescens* as 2.62% in the alone treatments. The combined treatment of *B. megaterium, P. fluorescens,* and *T. viride* showed maximum uptake (2.93%) for nitrogen which was much higher than the control (1.67%) values. An enhanced uptake for nitrogen in all the treatments clearly shows that these microbial inoculants have an unambiguous role in growth promotion of plants (Table 3(a)). The phosphorus uptake results were quite contrasting to N_2 uptake as the phosphorus content declined in most of the alone treatments. However, the combined treatments showed a slight rise in phosphorus uptake with the highest phosphorus content recorded in the plants inoculated with the consortia of *B. megaterium, P. fluorescens,* and *T. viride.* An increased potassium uptake was also little influenced by the microbial treatments as is evident from the least significant difference and *P* values (Table 3(b)). These inoculants also depicted an augmented uptake when combined with *T. viride*.

Table 3: (a) Effect of rhizospheric microbes on nutrient uptake in *O. tenuiflorum* cv. CIM-Ayu. (b) ANOVA analysis for nutrient uptake in *O. tenuiflorum* cv. CIM-Ayu.

(a)

Treatments	Nutrient uptake (%)		
	N	P	K
T1	1.67^{g}	0.32^{d}	2.27^{a}
T2	2.83^{b}	0.28^{ef}	2.30^{a}
T3	2.62^{c}	0.36^{c}	1.62^{d}
T4	2.23^{d}	0.31^{de}	1.93^{c}
T5	2.13^{e}	0.25^{f}	1.85^{c}
T6	2.07^{f}	0.33^{d}	2.08^{b}
T7	2.11^{e}	0.41^{b}	2.24^{a}
T8	2.93^{a}	0.44^{a}	2.33^{a}
LSD ($P = 0.05$)	0.04	0.03	0.11
CV%	0.98	5.50	2.96

[a]Mean in each column followed by same letters does not differ significantly ($P < 0.05$) according to Duncan's multiple range test.

(b)

Source of variation	Degree of freedom (df)	N%	P%	K%
Treatment	7	3.99^{**}	0.085^{**}	1.37^{**}
Error	16	0.01	0.01	0.06
Total	**23**	**3.99**	**0.09**	**1.43**

**Significance at a level of 5% of probability ($P < 0.01$).

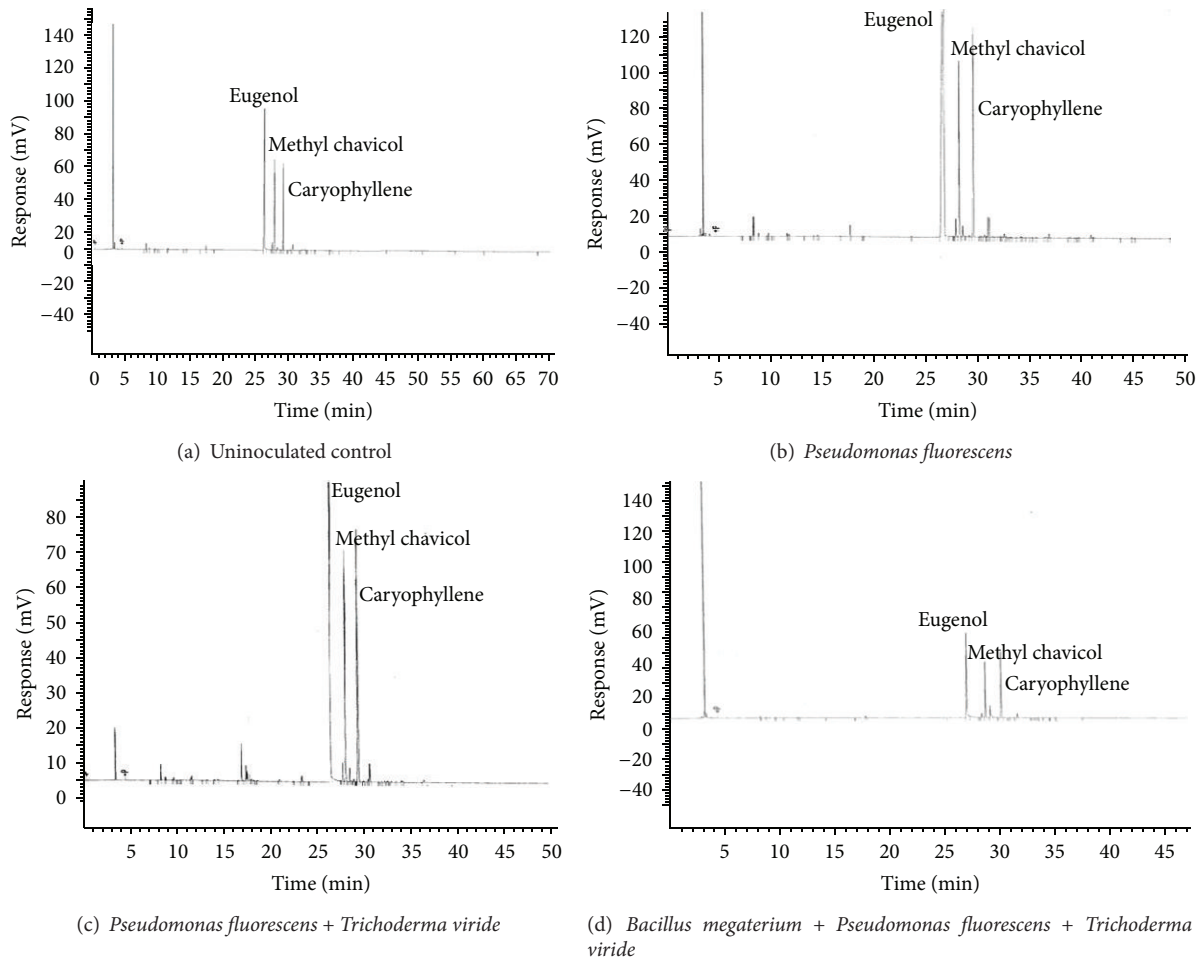

Figure 3: GC chromatograms of selected microbial treatments depicting major essential oil constituents.

4. Discussion

Medicinal plants constitute a large segment of the flora, which provide raw materials for pharmaceutical, cosmetic, and fragrance industries. Noteworthy increases in growth and yield of vital crops can be the outcome of plant growth promoting rhizobacteria (PGPR) inoculation. PGPRs actively colonize plant roots and increase plant growth and yield [19]. In the present study, growth parameters were significantly enhanced by the microbial treatments with an improved oil quality and quantity.

Glick et al. [20] considered plant growth promoting rhizobacteria as a better alternative to the established chemical strategies for facilitating plant growth. Rhizospheric microbes influence plant growth through the release of several antibiotics such as celastramycins A-B [21], kakadumycins [22], and demethylnovobiocins [23]. Furthermore PGPRs are also known to secrete a number of bioactive metabolites, for example, Taxol, maytansinoids. PGPRs facilitate the production of plant growth promotion compounds, such as auxins, cytokinins, and gibberellins, or by producing siderophore to bind Fe^{3+} from the environment and help to improve nutrient uptake, supply of plant nutrients (nitrogen, phosphate, and other mineral nutrients), or suppression of stress ethylene production by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity [24].

The present experimentation evaluates the effect of plant growth promoting rhizobacteria (PGPR) on nutrient uptake, growth, and yield attributes in Basil (*O. tenuiflorum*). The estimated impacts of microbial treatments on plant growth indices, mineral content, and essential oils in combined treatments validate the consortia inoculation to be a better option for enhancing growth parameters and yield attributes. Nitrogen and phosphorus are the two major plant nutrients responsible for influencing vegetative and reproductive phase of the plant growth, respectively. The colonization of root by PGPRs stimulates increased nutrient uptake at the root interface which results in better absorption of water and nutrients from the soil [25]. In the present study, nitrogen content in the biomass of the inoculated plants was much higher and it was significantly high in *B. megaterium* treatments. *Bacillus* spp. being a free living N_2-fixer supplements more nitrogen to biomass during the growth. In addition to the improvement of plant growth, these microbes also improve the soil fertility by aggregation and adding nutrients to soil. Similarly better response of *O. basilicum* plants inoculated with consortia of

Glomus fasciculatum, *P. fluorescens,* and *B. megaterium* has been reported [26]. Thus it is suggested that microbial treatment improves the plant growth, biomass, and yield by supplementing plant nutrients and producing growth hormones.

Increased nutrient uptake by plants inoculated with PGPRs has been attributed to the production of plant growth regulators at the root interface, which stimulated root development and resulted in better absorption of water and nutrients from the soil as PGPRs are able to solubilise "unavailable" forms of minerals by excreting organic acids which dissolve or chelate mineral ions to soluble form, thereby improving the competitiveness and external stress responses of the plant. The results in our experiment indicated a higher response with all the microbial treatments especially in combined microbial treatments. Essential oils are commonly extracted from aromatic crops through steam distillation. Since the method requires huge amounts of biomass, the microbial treatment proves to be beneficial for improved biomass yield. The GC-analysis of the oil having a combined treatment of *B. megaterium, P. fluorescens,* and *T. viride* revealed a major compound (58.5%) with a Kovat's index of 1356. The compound was identified as eugenol, a monoterpene having antimicrobial, insecticidal, antihelminthic, and nematicidal properties. Methyl chavicol also possesses antifungal and antibacterial activities.

5. Conclusions

The results reveal that microbial association not only enhanced the growth parameters in *O. tenuiflorum* but also significantly modulated aromatic oil's quality. The observations explained appreciable correlations between functional rhizospheric microbes and growth promoting traits, namely, plant height, shoot and root fresh weight, N, P, and K uptake, leaf yield and essential oil content, and major oil constituents. The study also emphasises that *P. fluorescens* greatly alters the growth parameters in *O. tenuiflorum*. With this experiment as primary step, further study is needed to develop biofertilizers consortium for commercially grown medicinal plants.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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