Ha QT *et al*. (2024)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 52, Issue 3, Article number 13895
DOI:10.15835/nbha52313895
Research Article

Changes in composition, yield, antimicrobial and antioxidant activities of the *Ocimum tenuiflorum* L. essential oils as affected by fertilizers

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Abstract

The purpose of this study is to evaluate the impact of multifunctional microbial fertilizer and inorganic macronutrient fertilizers N, P, K on plant biomass, essential oil yield, chemical composition, antimicrobial and antioxidant activities of tulsi (*Ocimum tenuiflorum* L.) grown in Hanoi, Vietnam. Among the four formulas tested, F2 emerged as the most favourable, comprising a basal fertilization of 10 tons of decomposed manure, 15 kg of multifunctional microbial fertilizer, 150 kg of P fertilizer, and 75 kg of K fertilizer, together with top dressing of 200 kg of N fertilizer and 75 kg of K fertilizer per hectare. This formula yielded the highest tulsi dry biomass (4.73 ton/ha), essential oil yield (0.831%), and essential oil production (39.29 L/ha), which were significantly different ($p < 0.001$) from those of the remaining formulas. In addition, the concentration of the essential oil main compound - eugenol (63.1%) of tulsi in F2 was also the highest, which may be the cause of the strongest antibacterial activity against *Bacillus subtilis*, *Escherichia coli* (IC_{50} of 320, 273 µg/mL, respectively), and antifungal activity against *Candida albicans* (IC_{50} of 400 µg/mL), as well as antioxidant activity (IC_{50} of 0.165 µg/mL) compared to the other formulas tested. The results suggested the role of both multifunctional microbial fertilizer and inorganic macronutrient fertilizers N, P, K on growth and essential oil biosynthesis in tulsi. These findings are important in identifying the most efficient fertilization formula for cultivating tulsi, serving the needs of the cosmetic and medicinal industries.

Received: 08 May 2024. Received in revised form: 18 Jun 2024. Accepted: 31 Jul 2024. Published online: 20 Aug 2024.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Keywords: gas-chromatography; eugenol; (*E*)-*β*-caryophyllene; essential oil composition; fertilizers; *Ocimum tenuiflorum* L.; tulsi

Introduction

In recent years, there has been a notable surge in the trend of utilizing natural products derived from medicinal plants and essential oils, due to increased consumer awareness of potential side effects and environmental impacts associated with synthetic chemicals (Amberg and Fogarassy, 2019; Tsitlakidou *et al.*, 2023). Essential oils extracted from plants have attracted widespread popularity due to their extensive traditional uses and perceived health benefits. Many individuals are turning to herbal remedies and aromatherapy to address a variety of health concerns, from stress and anxiety to common illnesses. Furthermore, the integration of natural ingredients in skin care, personal care products, and household cleaners reflects a growing demand for sustainable and nontoxic alternatives. The global essential oil market reached $23.74 billion in 2023 and is expected to reach $40.12 billion by 2030 (Grand View Research, 2023).

Ocimum tenuiflorum L., commonly known as holy basil or tulsi, belongs to the Lamiaceae family and is of significant cultural, medicinal, and culinary importance in various Asian countries, including Vietnam, India, China, and other countries. In Vietnam, tulsi is highly valued for its traditional medicinal properties and is often included in herbal remedies to treat a variety of diseases (Do *et al.*, 2004; Vo, 2012). Many other countries also integrate tulsi into their traditional medicine and cuisine. Its leaves and essential oil are used in formulations to address respiratory issues, aid digestion, alleviate stress, protect the liver, reduce inflammation, treat malaria, arthritis, and other diseases on skin (Chiu *et al.*, 2012; Stefan *et al.*, 2013; Aggarwal and Mali, 2015; Verma, 2016; Dharsono *et al.*, 2022; Gupta *et al.*, 2002).

Previous studies have demonstrated the biological activities of tulsi essential oil, including antimicrobial properties against bacteria and fungi (Joshi, 2013; Bugayong *et al.*, 2019), as well as antioxidant activity (Trevisan *et al.*, 2006; Bunrathep *et al.*, 2007). The widespread use of tulsi emphasizes its cultural importance and recognition of its potential health benefits in various regions. Furthermore, its adaptability to different climates has contributed to its cultivation on a global scale, making it a valuable and versatile herb in both traditional and modern applications worldwide.

The growth, biomass, and essential oil quality of plant species, particularly tulsi, are profoundly influenced by diverse environmental factors such as geography, weather, season, and genetic traits. It is important to note the type and quantity of chemical, organic, and microbial fertilizers applied, as they provide essential nutrients important for biosynthesis and establishment of symbiotic relationships with mycorrhizal fungi, thus enhancing nutrient uptake and overall plant health (Zheljazkov *et al.*, 2008; Sims *et al.*, 2014; Verma *et al.*, 2014; Fuller *et al.*, 2018; Rastogi *et al.*, 2019). Previous studies highlighted the impact of fertilizers on tulsi, with findings indicating that inorganic fertilizers N, P, K influenced growth, essential oil content, and concentration of the main oil compound - eugenol (Pooja *et al.*, 2018), while the combination of organic and microbial fertilizers affect dry biomass, essential oil content, and yield (Smitha *et al.*, 2019). Additionally, microbial fertilizers were shown to influence essential oil content and concentration of the main oil compound - eugenol (Saikia and Pandey, 2014). The complex interactions between environmental variables and essential oil-bearing plants significantly shape the quality, fragrance, and medicinal properties of the resultant essential oils. Therefore, it is necessary to understand and optimize these factors, especially fertilizers, to ensure sustainable and high-quality production of essential oils.

The present study aimed to evaluate the impact of different fertilizer formulas on tulsi essential oil production, composition, antimicrobial, and antioxidant activities. Our findings provide valuable insights to improve cultivation techniques, thereby enhancing the production of high-quality essential oils and boosting the potential of tulsi for diverse applications in traditional medicine, aromatherapy, and the fragrance industry.

Materials and Methods

Plant materials and growth conditions

The experiment was performed at the experimental station in Hanoi, Vietnam (N21°04′08″, E105°45′50″) with soil characteristics as follows: pH: 5.96, N_{total}: 0.25%, P_{total}: 0.19%, K_{total}: 1.54%, Organic-$matter_{total}$: 2.66%. These parameters were determined according to Vietnam Standard Methods (Vietnam Standard Methods 5979:2007; Vietnam Standard Methods 6498:1999; Vietnam Standard Methods 8660:2011; Vietnam Standard Methods 8940:2011; Vietnam Standard Methods 8941:2011). The tulsi seeds, purchased from Duc Thang Company in Hanoi, were sown in March 2022, and the resulting seedlings were transplanted in May of the same year. The fertilizer experiment lasted 90 days, conducted from May to August 2022. All experiments were carried out using a completely randomized design method with 3 replications. Harvesting of all tulsi plants took place at the full bloom stage.

Details of the type and dosage of applied fertilizers are given in Table 1. Each fertilizer formula was repeated three times, for a total of 45 plants. Formula F1 (control) was devised based on previous literatures (La *et al*., 2001; Do *et al*., 2004). On the other hand, formulas F2 to F4 involved reducing the amounts of inorganic fertilizers and increasing the amounts of multifunctional microbial fertilizer, aiming to explore the potential for partially substituting inorganic fertilizers with multifunctional microbial alternatives. Simultaneously, the main objective was to identify the formula that gives the highest quality and quantity of tulsi.

Table 1. The type and amounts of fertilizers applied in cultivation of *O. tenuiflorum*

Formulas	Basal fertilized (kg/ha)				Top dressed (kg/ha)	
	Decomposed manure	Multifunctional microbial fertilizer	P fertilizer	K fertilizer	N fertilizer	K fertilizer
F1 (Control)	10,000	0	150	75	200	75
F2	10,000	15	150	75	200	75
F3	10,000	20	100	50	100	50
F4	10,000	25	50	50	50	50

Note: Time of basal fertilizing: At the beginning of planting seedlings. Time of top dressing: Divided into 3 times and mixed with water for irrigation: When the plant takes root; When the plant branches; When the plant is about to flower. Information on fertilizers in the experiments included:
+ N fertilizer: Ntotal 46.3%.
+ P fertilizer: Effective phosphorus (P_2O_5) 16%.
+ K fertilizer: Effective potassium (K_2O) 61%.
+ Multifunctional microbial fertilizer: >10^8 CFU/g of each strain of *Bacillus* sp., *Azotobacter chrococcum, A. vinelandii*, *Azospirillum brasilense*, *Acetobacter diazotrophicus*, *Aspergillus awamori, A. tubingensi*, *A. niger*, *Bacillus thuringiennsis*, and *Pseudomonas pitida*.

Plant sample harvest and water content determination

After the end of 90-day experiment, the entire aerial biomass of tulsi was harvested. Each tulsi sample was measured for its water content using A&D Weighing AD-4714A General purpose moisture determination balance at 105 °C for 30 minutes.

Essential oil isolation

Each fresh tulsi aerial biomass sample (1.3 kg) was shredded and hydrodistilled in triplication for 4 h using a Clevenger type apparatus according to the previously published procedure (Ministry of Health of Vietnam, 2017). The essential oil was then separated and stored at -5 °C for further analysis.

Determination of essential oil physical properties

Three physical properties of essential oils: relative density, refractive index, and optical rotation, were evaluated according to the methods stated in ISO standards (ISO 279:1998; ISO 280:1998; ISO 592:1998).

GC-MS and GC-FID analysis

The essential oils were analysed using GC/MS-FID via an Agilent GC7890A system equipped with a Mass Selective Detector (Agilent 5975C). A fused silica capillary column, HP-5MS (60 m × 0.25 mm i.d. × 0.25 μm film thickness), was used, with helium serving as the carrier gas at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 250 °C, while the oven temperature followed a programmed increment from 60 °C to 240 °C at a rate of 4 °C/min. The split ratio was set at 100:1, with the detector operating at 280 °C and an injection volume of 1 μL. MS analysis occurred with the interface temperature at 280 °C, in MS mode, using an E.I. detector voltage of 1258 V, and scanning the mass range from 35 to 450 Da at a rate of 4.0 scans/s. FID analysis followed identical chromatographic conditions, with the FID temperature set at 250 °C. Essential oil components were identified by their relative retention indices, determined through co-injection of a series of homologous *n*-alkanes (C7-C30), and by comparing their mass spectral fragmentation patterns with those stored in the MS library NIST08, Wiley09, and HPCH1607 (Adams, 2017; Linstrom and Mallard, 2021). MassFinder 4.0 software (König *et al*., 2022) was utilized for data processing, with component relative concentrations calculated based on the peak areas of FID chromatograms without standardization.

Microbial strains

The antimicrobial activities of the oils were evaluated using three Gram (+) bacteria strains, including *Staphylococcus aureus* (ATCC 13709), *Bacillus subtilis* (ATCC 6633) and *Lactobacillus fermentum* (VTCC N4), along with three Gram (–) bacteria strains, including *Salmonella enterica* (VTCC), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 15442), as well as one yeast strain - *Candida albicans* (ATCC 10231). The ATCC strains were sourced from the American Type Culture Collection, while the VTCC strains were obtained from the Vietnam Type Culture Collection at the Institute of Microbiology and Biotechnology, Vietnam National University, Ha Noi.

Screening of antimicrobial activity

The MIC and IC_{50} of the essential oils were determined in triplicate using the broth microdilution susceptibility testing (Hadacek and Greger, 2000; Cos *et al*., 2006). Stock solutions of the oil were prepared in dimethylsulphoxide (DMSO), and dilution series ranging from 8,192 μg/mL to 2 μg/mL (2^{13}, 2^{12}, 2^{11}, 2^{10}, 2^9, 2^7, 2^5, 2^3, 2^1 μg/mL) were prepared in sterile distilled water in micro-test tubes, which were then transferred to 96-well microplates. Bacteria grown in double-strength Mueller-Hinton broth or double-strength tryptic soy broth, and fungi grown in double-strength Sabouraud dextrose broth, were standardized to 5×10^5 and 1×10^3 CFU/mL, respectively. The last row, containing only the serial dilutions of the sample without microorganisms, served as a negative control, while sterile distilled water and medium were used as a positive control. After incubation at 37 °C for 24 h, the MIC values were determined at the well with the lowest concentration of agents that completely inhibited microbial growth. The IC_{50} values were determined based on the percentage of growth inhibited microorganisms, using turbidity measurement data obtained from the EPOCH2C spectrophotometer (BioTeK Instruments, Inc Highland Park Winooski, USA) and Rawdata computer software (Intercity Business Park Mechelen Noord, Zone L Mechelen, 2800 Belgium) according to the following equations:

$$\% \text{ Inhibition} = \frac{OD_{\ control(+)} - OD_{\ Test\ agent}}{OD_{\ control(+)} - OD_{control\ (-)}} \times 100\%$$

$$IC_{50} = High_{Conc} - \frac{(High_{Inh\%} - 50\%)\ (High_{Conc} - Low_{Conc})}{(High_{Inh\%} - Low_{Inh\%})}$$

Where: OD: optical density; control (+): only cells in medium without antimicrobial agent; test agent: corresponding to a known concentration of antimicrobial agent; control (–): culture medium without cells. $High_{Conc}/Low_{Conc}$: concentration of test agent at high concentration/low concentration; $High_{Inh\%}/Low_{Inh\%}$: % inhibition at high concentration/% inhibition at low concentration.

Reference materials: Ampicillin for Gram (+) bacteria with IC_{50} and MIC values in the ranges of 0.02-3.62 μg/mL and of 0.125-32.0 μg/mL, Cefotaxime for Gram (–) bacteria with IC_{50} and MIC values in the range of 0.07-4.34 μg/mL and of 0.5-32.0 μg/mL, Nystatin for fungal strains with IC_{50} and MIC values of 1.32 μg/mL and 8.0 μg/mL.

Screening of antioxidant activity

The antioxidant capacities of the essential oils were evaluated in triplicate using the DPPH radical-scavenging assay (Duan *et al*., 2007) with slight modifications. In brief, 0.1 mL of essential oil, diluted in methanol at concentrations ranging from 0.05-5 μg/mL, was thoroughly mixed with 2.9 mL of 0.1 mM DPPH solution. The mixture was then incubated at room temperature for 30 minutes in the dark. The control sample was prepared by replacing the essential oil with 0.1 mL of methanol. Then, the absorbance (A) of each sample was measured at 517 nm. The DPPH radical inhibition of the sample was calculated according to the formula:

DPPH free radical scavenging activity (%) = $100 * (A_{control} - A_{sample}) / A_{control}$.

A standard curve ($y = ax + b$) was generated using the percentage inhibition of DPPH at different concentrations. Based on this curve, the IC_{50} value was determined. The reference material, Trolox, exhibited an IC_{50} average value of 16.805 ± 0.060 μg/mL.

Statistical analysis

The data of physiological and essential oil parameters of tulsi were analysed using a single factor completely randomized analysis of variance (ANOVA) to compare the fertilization treatments. Significance levels were determined, and means were separated using the least significant difference (LSD) test at $p \leq 0.05$. The analysis was performed using IRRISTAT ver. 5.0 (International Rice Research Institute, Philippines).

Results and Discussion

The effect of fertilizers on biomass and essential oil production of O. tenuiflorum

Differences in aboveground biomass and essential oil yield of tulsi were observed among the four fertilization conditions after 90-day experiment (Table 2). Formula F2 gave the highest fresh plant weight (581.0 g/plant), significantly different ($p < 0.001$) compared to the other three treatments (311.7 g/plant in F4, 351.2 g/plant in F3, and 460.5 g/plant in F1).

The water content of fresh tulsi varied depending on the fertilization conditions, with the highest value recorded in formula F2 (79.13%). On the contrary, significant reductions ($p < 0.001$) in tulsi water content were observed in the remaining three formulas (74.27% in F1, 75.37% in F3, and 75.80% in F4). Notably, the water content and essential oil yield of tulsi in formulas F2-F4, supplemented with multifunctional microbial fertilizer, increased compared to those in the control formula F1, suggesting the fertilizer's role in water absorption and organic compound biosynthesis in tulsi. Previous research showed that beneficial microorganisms with many different functions contained in microbial fertilizers help increase concentrations of digestible nutrients in the soil, through the following processes: decomposing organic matter, dissolving potassium and phosphate (phosphate-solubilizing bacteria - PSB), fixing nitrogen (nitrogen fixers), producing phytohormone and antagonism of plant pathogens, thereby promoting better plant growth (Bhattacharyya and Jha, 2012; Glick, 2012; Sehrawat and Sindhu, 2019; Sharma *et al.*, 2019). Specifically, beneficial microorganisms in multifunctional microbial fertilizers impact plant metabolism, thereby changing the composition of root exudates as well as affecting the solubility and availability of nutrients, while enhancing interactions with other soil microorganisms. Notably, bacterial strains (such as *Pseudomonas*, *Bacillus*, *Azotobacter*, *Azospirillum* species, *etc.*) often included in microbial fertilizers have the ability to fix nitrogen and produce phytohormones necessary for fine root growth, increasing surface area to help absorb nutrients and water effectively. These microorganisms promote cell division, elongation and differentiation, leading to enhanced plant growth, increased biomass and production of secondary metabolites (Adesemoye *et al.*, 2009; Glick, 2012; Egamberdieva *et al*., 2017).

In formula F2, the average tulsi fresh biomass (22.66 ton/ha) and dry biomass (4.73 ton/ha) increased compared to the control treatment (17.96 ton/ha and 4.62 ton/ha). However, these values decreased in the F3 and F4 treatments due to reduced amounts of macronutrients N, P, and K applied.

A significant difference ($p < 0.001$) was found in the essential oil yields of plants among the four treatments. Formula F2 yielded the highest value (0.831%, v/w), while the lowest essential oil content was observed in tulsi from formula F1 (0.425%, v/w), increasing to 0.588% in F3 and 0.524% in F4, calculated on a dry weight (DW) basis.

Consistent with the trend observed in fresh plant weight, estimated biomass, and essential oil yield data, the highest tulsi essential oil production was observed in F2 (39.29 L/ha). This value was significantly higher than those of the other formulas at the 0.05 level ($p < 0.001$), which ranged from 15.41 to 19.85 L/ha. Inorganic macronutrients N, P, and K played an important role in tulsi essential oil production, as oil production decreased significantly when the amounts of N, P, K fertilizers decreased, even when the amount of multifunctional microbial fertilizer increased (in F4). The minimum requirement of N, P, K fertilizers needs to be equal to the amount added in formula F3, along with the addition of multifunctional microbial fertilizer, so that tulsi produces the same level of essential oils as the control formula F1. Thus, partially replacing these inorganic fertilizers with multifunctional microbial fertilizer could maintain production quality under safe agricultural conditions, while reducing environmental pollution.

The role of multifunctional microbial fertilizer in tulsi growth and essential oil biosynthesis is also significant. In F2, applying multifunctional microbial fertilizer (15 kg/ha) while maintaining the amount of inorganic fertilizers N, P, K equal to F1 led to significant increases in fresh biomass, essential oil yield, and essential oil production compared to those in F1.

Table 2. Biomass and essential oil production of *O. tenuiflorum* cultivated under different fertilization conditions

Formulas	Fresh weight of plant (g)	Water content (%)	Fresh biomass (ton/ha) *	Dry biomass (ton/ha) *	Essential oil yield (%, v/w, DW)	Essential oil production (L/ha) *
F1 (Control)	460.5 ± 9.94 b	74.27 ± 0.22 c	17.96 ± 0.39 b	4.62 ± 0.10 a	0.425 ± 0.011 d	19.63 ± 0.42 bc
F2	581.0 ± 3.18 a	79.13 ± 0.24 a	22.66 ± 0.12 a	4.73 ± 0.03 a	0.831 ± 0.015 a	39.29 ± 0.21 a
F3	351.2 ± 1.68 c	75.37 ± 0.25 b	13.70 ± 0.07 c	3.37 ± 0.02 b	0.588 ± 0.010 b	19.85 ± 0.09 b
F4	311.7 ± 9.17 d	75.80 ± 0.19 b	12.16 ± 0.36 d	2.94 ± 0.09 c	0.524 ± 0.016 c	15.41 ± 0.45 d
LSD 5%	15.78	0.52	0.62	0.15	0.025	0.75

Note: Mean values followed by the same letter within a column are not statistically different for 0.05 significant level;
*Estimated values (cultivation distance of tulsi: 40 × 50 cm, estimated number: 39,000 plants per ha)

The noteworthy point of this study lies in the harmonious combination of multifunctional microbial fertilizer and inorganic macronutrient fertilizers, satisfying the plant's growth and developmental needs to achieve high production. The appropriate application of multifunctional microbial fertilizer (15 kg/ha) along with inorganic fertilizers (N:P:K at 200:150:150 kg/ha) based on decomposed manure (10,000 kg/ha) resulted in the highest tulsi essential oil and biomass production in F2, indicating the optimal fertilization formula for tulsi cultivation.

In a previous study, fertilizing N, P, K at doses of 150, 85, 70 kg/ha, respectively, helped tulsi achieve the highest fresh biomass and essential oil yield values of 15.18 ton/ha and 0.40%, respectively (Pooja *et al*., 2018).

It was demonstrated that supplementing a mixture of several rhizobacteria strains had a more beneficial effect on tulsi yield than the addition of a single microorganism strain (Saikia and Pandey, 2014; Harishkumar *et al.*, 2019). The findings of the present study are consistent with previous research that highlighted the combined effect of macronutrients with bio-fertilizers in increasing dry biomass, essential oil yield, and essential oil production of tulsi in India (Smitha *et al.*, 2019). This may be because the multifunctional microbial fertilizer providing rhizobacteria that facilitate nutrient availability and absorption, accelerate the synthesis of organic compounds containing volatile essential oils in plants, enhance plant growth, and ultimately boost production.

The effect of fertilizers on essential oil composition of O. tenuiflorum

Hydrodistillation of the aboveground biomass of tulsi, harvested from four different fertilization formulas, yielded pale yellow essential oils with the relative densities d^{20} ranging from 0.993 to 1.009 g/mL, refractive indices n^{20} ranging from 1.514 to 1.519 and optical rotations $[\alpha]D^{20}$ ranging from [-]3.52° to [-]5.00° (Table 3). The values of oil densities were roughly comparable to those of previously reported tulsi stem oil, whereas the refractive indices were slightly higher (Hikmawanti and Nurhidayah, 2019). However, research by Khair-ul-Bariyah (2013) revealed different values for the relative density d^{20}, refractive index n^{20}, and optical rotation $[\alpha]D^{25}$ of tulsi, which were reported to be 1.552 g/cm^3, 1.6362 and -0.044°. Another study noted slight variations in the physical properties of tulsi essential oil depending on regional conditions in Pakistan, with refractive indices n^{25} ranging from 1.515 to 1.530 and relative densities d^{25} ranging from 0.920 to 0.921 g/cm^3 (Hussain, 2009).

Table 3. Some physical properties of essential oil of *O. tenuiflorum* cultivated under different fertilization conditions.

Parameters	F1(control)[a]	F2[a]	F3[a]	F4[a]
Relative density d^{20}	1.000	1.009.	0.997	0.993
Refractive index n^{20}	1.514	1.519	1.515	1.515
Optical rotation $[\alpha]D^{20}$	[-]5.00	[-]3.96	[-]4.01	[-]3.52

Note: [a]Standard deviation were insignificant and excluded from the Table to avoid congestion

In addition to the essential oil yield and production of tulsi, the chemical composition of the essential oil, which is one of the parameters for assessing its quality, is also important in evaluating the impact of environmental conditions on this plant species. The identification of compounds that are present in the essential oils of tulsi harvested from four fertilization formulas after 90 days of experiment was conducted using mass spectral (MS) and retention index (RI) data. Table 4 presents the identified compounds in their elution order on the HP-5MS column used for the GC-MS analysis. A total of 15 to 18 compounds, representing from 99.9% to 100% (by mass intensity) of the compositions, were identified in the essential oils of tulsi cultivated under 4 different fertilization formulas. Among these, oxygenated monoterpenes predominated from 55.1% to 64.1%, including the benzenoid aromatics ranging from 54.6% to 63.7%, which accounted for the total content of two phenylpropanoids (eugenol and methyl eugenol) in the tulsi essential oils. Sesquiterpene hydrocarbons accounted for 34.3-42.3%, while monoterpene hydrocarbons and oxygenated sesquiterpenes were present at very low concentrations, consistent with previous studies (Joshi, 2013).

The most significant difference in the composition of tulsi essential oils from the four fertilization conditions was observed in the content of the two main compounds, eugenol and (*E*)-*β*-caryophyllene. The concentration of eugenol, ranging from 53.5% to 63.1%, with the order of F2 > F3 > F4 > F1, indicated that the combination of multifunctional microbial fertilizer and macronutrient N, P, and K fertilizer dosage in formula F2 was most suitable for the biosynthesis of eugenol in tulsi essential oil. In contrast, the concentration of (*E*)-*β*-caryophyllene, ranging from 31.7% to 39.1%, followed the reverse order: F1 > F4 > F3 > F2 (Table 4). It is evident that the essential oil of tulsi in treatments with multifunctional microbial fertilizer had higher concentrations of eugenol and lower concentrations of (*E*)-*β*-caryophyllene compared to the control formula F1. This suggested that the addition of multifunctional microbial fertilizer may influence the biosynthesis and accumulation of these main compounds in tulsi essential oil due to the function of beneficial microorganisms to decompose cellulose and organic compounds, fix nitrogen and dissolve phosphorus, resist pest and insect, *etc*. contained in that type of fertilizer.

Specifically, the process of decomposing organic matter enriches digestible nutrients in the soil, helping plants absorb these nutrients faster, thereby enhancing plant growth and metabolic activities (Verma *et al.*, 2010). Nitrogen-fixing bacteria convert atmospheric nitrogen into ammonia, a form of nitrogen that plants can assimilate. Nitrogen is an important component of amino acids, proteins and nucleic acids, which are essential for plant growth and the production of secondary metabolites (Bhattacharyya *et al.*, 2012). Phosphate-solubilizing bacteria convert insoluble phosphorus compounds into a soluble, easily digestible form that helps plants quickly absorb. Phosphorus is important for ATP production and various metabolic processes in plants (Rodriguez & Fraga, 1999). Some beneficial microorganisms can protect plants from pests by producing antibiotics, siderophores and other bioactive compounds or by inducing systemic resistance in plants. A specific example is that *Bacillus subtilis* can secrete volatiles activating an induced systemic resistance pathway in *Arabidopsis* seedlings challenged with the soft-rot pathogen *Erwinia carotovora* subsp. *carotovora* (Compant *et al*., 2005). The combination of these microbial activities can lead to enhance nutrient availability in soil, improve plant health, growth, metabolic activity, proliferation, synthesis and accumulation of compounds in plants including essential oils. However, the effect is not simply proportional to the dose of multifunctional microbial fertilizer but also depends on the dose of N, P, and K fertilizers applied in combination in the present study. Other compounds with relatively high concentration in tulsi essential oil included *α*-humulene (1.9-2.3%), methyl eugenol, and caryophyllene oxide with values ranging around 1%. The remaining compounds in the essential oil were present at very small concentrations ranging from 0.1% to 0.6% (Table 4).

Table 4. Composition of essential oils (%) of *O. tenuiflorum* cultivated under different fertilization conditions

N°	RI[a]	RI[b]	Compounds[c]	F1 (control)[d]	F2[d]	F3[d]	F4[d]
1	847	850	(3*Z*)-Hexen-1-ol	0.3	ND	0.3	ND
2	931	932	*α*-Pinene	0.2	0.1	0.2	0.2
3	947	946	Camphene	0.2	0.1	0.2	0.2
4	976	974	*β*-Pinene	0.1	ND	0.1	0.2
5	1042	1044	(*E*)-*β*-Ocimene	0.3	0.3	0.2	0.4
6	1063	1063	1-Octanol	0.2	0.2	0.1	0.3
7	1169	1165	Borneol (= *endo*-Borneol)	0.5	0.4	0.6	0.6
8	1365	1356	**Eugenol**	**53.5**	**63.1**	**56.0**	**54.7**
9	1396	1390	*β*-Elemene	0.3	0.3	0.3	0.3
10	1402	1403	Methyl eugenol	**1.1**	0.6	0.9	0.8
11	1411	1407	*α*-Barbatene	0.2	0.1	0.2	0.2
12	1418	1417	**(*E*)-*β*-Caryophyllene**	**39.1**	**31.7**	**37.2**	**38.5**
13	1446	1440	*β*-Barbatene	0.2	0.2	0.2	0.2
14	1453	1452	*α*-Humulene	2.3	1.9	2.2	2.2
15	1518	1522	*δ*-Cadinene	0.1	0.1	0.1	0.1
16	1523	1529	(*E*)-*γ*-Bisabolene	0.1	ND	0.2	0.1
17	1545	1548	Elemol	0.3	0.3	0.3	0.2
18	1586	1582	Caryophyllene oxide	1	0.6	0.7	0.7
Total				**100.0**	**100.0**	**100.0**	**99.9**
Number of compounds identified				18	15	18	17
Monoterpene hydrocarbons				0.8	0.5	0.7	1.0

Nº	RI[a]	RI[b]	Compounds[c]	F1 (control)[d]	F2[d]	F3[d]	F4[d]
Oxygenated monoterpenes				55.1	64.1	57.5	56.1
Sesquiterpene hydrocarbons				42.3	34.3	40.4	41.6
Oxygenated sesquiterpenes				1.3	0.9	1.0	0.9
Others				0.5	0.2	0.4	0.3
Benzenoids				54.6	63.7	56.9	55.5

Note: [a]RI: retention index of compounds on the HP-5MS column; [b]According to Adams, 2017; [c]Order of compounds eluted on the HP-5MS column; ND: Not detected; [d]Standard deviations were insignificant and excluded from the Table to avoid congestion.

The main components in tulsi essential oil were reported to vary depending on the growth stage of the plant (Sims *et al*., 2014), weather (Fuller *et al*., 2018), light (Saikia and Pandey, 2014; Chutimanukul *et al*., 2022), plant cultivar (Bunrathep *et al*., 2007), and geographical area with different climatic and soil conditions (Fikadu *et al*., 2022). Specifically, after the first 30 days of growth, high (*E*)-*β*-caryophyllene concentration was obtained, and at the reproductive stage after 60 days of growth, eugenol became the predominant component of tulsi essential oil (for accession PI 652057 among 3 accessions of tulsi planted) (Sims *et al*., 2014). In addition, the eugenol concentration increased in the low rainy season and prolonged hot weather (Fuller *et al*., 2018). Research by Bunrathep *et al.* (2007) showed that the main components in the essential oil of red and blue tulsi in Thailand were methyl eugenol (47.18% and 53.67%) and (*E*)-*β*-caryophyllene (37.83% and 35.2%). The study of Chutimanukul *et al.* (2022) also recorded two main compounds in the essential oil of red and blue tulsi in Thailand as follows: methyl eugenol (78.76-80.77% and 78.19-87.52%) and (*E*)-*β*-caryophyllene (12.01-14.53% and 7.77-13.11%), depending on different additional lighting conditions. Meanwhile, Indian tulsi had methyl eugenol (92.4%) as the main compound in the essential oil, while eugenol (2.4%) and (*E*)-*β*-caryophyllene (1.3%) accounted for very small amounts (Joshi, 2013). Another study showed that the main components of tulsi essential oil in India included eugenol, methyl chavicol and (*E*)-*β*-caryophyllene, with the percentages varying depending on light conditions (Saikia and Pandey, 2014). Recently, the main components of tulsi essential oil in two different areas in Ethiopia, were reported to include: *β*-bisabolene (31.38% and 24.45%), (*Z*)-*α*-bisabolene (25.56% and 19.61%), and 1,8-cineole (17.12% and 13.42%) (Fikadu *et al*., 2022).

Eugenol is a substance well known for its diverse applications in various fields such as pharmaceuticals, food, aromatherapy, cosmetics, agriculture, and many other industries. Compared to the control formula F1 without adding multifunctional microbial fertilizer, the eugenol concentrations in tulsi essential oils in the remaining formulas supplemented with multifunctional microbial fertilizers were higher from 1.20% to 9.60%, which ranged from 54.7% to 63.1% compared to 53.5% in F1 (Table 4). A previous study conducted in India demonstrated the role of microorganisms in increasing the eugenol content in tulsi essential oil by 13.36% higher than that of the control after 90 days of experiments (Saikia and Pandey, 2014). In addition, another study in India showed that the appropriate dosage of N, P, K fertilizers also changed the eugenol content in tulsi, leading to a 17.59% increase compared to that of the control (Pooja *et al*., 2018). (*E*)-*β*-Caryophyllene showed promising applications in treating various medical conditions. The (*E*)-*β*-caryophyllene concentrations in tulsi essential oils in formulas F2-F4 fluctuated compared to the F1 control in the inverse direction to the eugenol content. Specifically, they were lower from 0.6% to 7.4% than the control formula F1, which ranged from 31.7% to 38.5% compared to 39.1% in F1 (Table 4).

The effect of fertilizers on antimicrobial activity of essential oil of O. tenuiflorum

The tulsi essential oils from four fertilization formulas were evaluated for antimicrobial activity using the broth microdilution susceptibility testing. IC_{50} and MIC of essential oils were conducted on six bacterial strains and one yeast strain. The essential oil from formula F1 showed the strongest inhibitory activity against

S. aureus and *S. enterica* strains, as indicated by the lowest mean values of IC_{50} (569 and 592 µg/mL). While in formulas F2-F4, the respective IC_{50} for these two strains ranged from 2365-2539 µg/mL and from 666-2339 µg/mL. Tulsi essential oil in formula F2 exhibited the strongest inhibitory activity against *B. subtilis*, *E. coli* and *C. albicans* strains with the lowest mean values of IC_{50} (320, 273 and 400 µg/mL). In the remaining formulas, the respective mean values of IC_{50} ranged from 349-574 µg/mL, 316-574 µg/mL and 417 to over 8192 µg/mL. The strongest inhibitory activity against *L. fermentum* and *P. aeruginosa* strains with the lowest mean values of IC_{50} (3260-3275 µg/mL and 3079-3143 µg/mL) were found in the essential oil of tulsi in formulas F3 and F4. While in formulas F1 and F2, both values exceeded 8192 µg/mL. The MIC values of essential oils for the tested microbial strains generally ranged from 2048 to over 8192 µg/mL, except for the two most sensitive strains to tulsi essential oil in this study, *B. subtilis* and *E. coli* (1024-4096 µg/mL and 512-2048 µg/mL) (Table 5).

Table 5. Antimicrobial activity of essential oils of *O. tenuiflorum* cultivated under different fertilization conditions

The concentration of essential oil inhibiting the tested microorganisms								
Formulas	**Values (µg/mL)**	**Gram (+) bacteria**			**Gram (-) bacteria**			**Yeast**
		Staphylococcus aureus	***Bacillus subtilis***	***Lactobacillus fermentum***	***Salmonella enterica***	***Escherichia coli***	***Pseudomonas aeruginosa***	***Candida albicans***
F1 (control)	IC_{50}	569 ± 15	391 ± 15	>8192	592 ± 23	316 ± 5.5	>8192	417 ± 9.0
	MIC	4096	1024	>8192	8092	2048	>8192	2048
F2	IC_{50}	2539 ± 6.7	320 ± 5.6	>8192	666 ± 11	273 ± 3.3	>8192	400 ± 6.4
	MIC	4096	1024	>8192	8192	512	>8192	2048
F3	IC_{50}	2365 ± 34	349 ± 3.9	3260 ± 10	2339 ± 71	417 ± 3.1	3143 ± 38	627 ± 18
	MIC	8192	1024	8092	8192	2048	8192	4096
F4	IC_{50}	2439 ± 50	574 ± 9.8	3275 ± 31	2287 ± 75	574 ± 6.9	3079 ± 31	>8192
	MIC	8192	4096	8192	8192	1024	8192	>8192
Ampicillin	IC_{50}	0.02 ± 0.005	3.62 ± 0.15	1.03 ± 0.07				
	MIC	0.125 ± 0.0	32 ± 0.0	32 ± 0.0				
Cefotaxime	IC_{50}				0.43 ± 0.05	0.007 ± 0.002	4.34 ± 0.15	
	MIC				32 ± 0.0	0.5 ± 0.0	8 ± 0.0	
Nystatin	IC_{50}							1.32 ± 0.05
	MIC							8 ± 0.0

The antimicrobial activity of tulsi essential oil may be affected by some of the main components or by interactions between the essential oil components. In particular, the high concentration of eugenol in essential oil may have an inhibitory effect on *B. subtilis*, *E. coli* and *C. albicans* strains. Previous studies demonstrated the effects of eugenol in the prevention and treatment of *C. albicans* vaginal and oral thrush in rat (Chami *et al.*, 2004; Garg and Singh, 2011). Another study evaluated the antimicrobial activity of tulsi essential oil containing main compounds including *m*-eugenol (69.1%) and caryophyllene (19.2%), showing MIC values for strains of *S. aureus*, *P. aeruginosa* and *E. coli* were 1200, 2500 and 2000 µg/mL, respectively (Bugayong, 2019). On the other hand, a study on tulsi essential oil containing methyl eugenol (92.4%) as the main compound revealed the stronger antimicrobial activity with MIC values against *S. aureus*, *B. subtilis*, and *P. aeruginosa* of 0.41, 0.22, and 0.39 mg/mL, but weaker activity against *E. coli* with MIC value of 2.29 mg/mL (Joshi, 2013). The mechanism of inhibiting microbial activity of eugenol was also reported to be by inducing cell lysis through protein and lipid content in *E. coli* (Oyedemi *et al.*, 2009), preventing exotoxin production by *S. aureus* (Qiu *et al.*, 2010), and changes in membrane integrity of *C. albicans* (Braga *et al.*, 2007; Zore *et al.*, 2011). In general, eugenol at low concentrations has antioxidant and anti-inflammatory effects, while at high concentrations it acts as a pro-oxidant, resulting in tissue damage due to enhanced generation of free radicals (Chogo and Crank, 1981). (*E*)-*β*-Caryophyllene, the second most abundant compound in tulsi essential oil in the present study, may also contribute to microbial inhibition. This compound was reported to have antibacterial activity against many strains (Yang *et al*., 2015; Dahham *et al*., 2015). From the research results, it was found that the combination of decomposed manure, N, P, K and multifunctional microbial fertilizers in this study showed an impact on the ratio of active compounds in tulsi essential oil, thereby changing the antimicrobial activity compared to the control formula. And a notable result in the present study is that tulsi essential oil in formula F2 had the strongest inhibition against 3 strains of microorganisms including *B. subtilis*, *E. coli* and *C. albicans*. This may be because the highest content of eugenol in tulsi essential oil in formula F2 compared to the other formulas.

The effect of fertilizers on antioxidant activity of essential oil of O. tenuiflorum

The antioxidant activity of the essential oils was measured using the DPPH radical scavenging test. Data in Table 6 show variability in antioxidant activity of essential oils of tulsi from four fertilization conditions. Particularly, tulsi essential oil of formula F2 exhibited the strongest scavenging activity against DPPH, indicated by the lowest mean value of IC_{50} (0.165 μg/mL), demonstrating the optimal fertilization condition for enhancing its antioxidant capacity. The essential oils from other fertilization conditions also exhibited strong antioxidant activities in the order F3 > F4 > F1 with mean values of IC_{50} ranging from 0.194 to 0.309 μg/mL. These antioxidant activities were similar to that observed for tulsi essential oil from Brazil, which contained eugenol (59.4%), (*E*)-*β*-caryophyllene (29.4%) and germacrene D (8.1%) as the main compounds (Trevisan *et al*., 2006).

Table 6. Antioxidant activity of essential oils of *O. tenuiflorum* cultivated under different fertilization conditions

Formulas	F1 (control)	F2	F3	F4	Trolox
IC_{50} (μg/mL)	0.309 ± 0.13	0.165 ± 0.011	0.194 ± 0.012	0.209 ± 0.015	16.805 ± 0.060

The antioxidant activity of the essential oils was indicated to be correlated with the main compounds in the oils. In the present study, the antioxidant capacities of the tulsi essential oils were consistent with the eugenol concentrations in the oils, which aligns with previous studies (Joshi, 2013). In contrast, the weaker antioxidant activity of tulsi essential oil containing methyl eugenol (92.4%) and eugenol (2.4%) was attributed to the inactive characteristics of methyl eugenol (Joshi, 2013). Thus, the combination of decomposed manure, N, P, K, and multifunctional microbial fertilizers in this study showed an impact on the antioxidant capacities of tulsi essential oils. The F2 fertilization regimen is probably the most suitable to enhance that property.

Conclusions

Fertilization regimen clearly affected the growth, biosynthesis, accumulation, and main compound eugenol concentration, as well as the antimicrobial and antioxidant activities of tulsi essential oil. The highest positive effect was recorded in fertilization formula F2 (1 ha was basal fertilized with 10 tons of decomposed manure, 15 kg of multifunctional microbial fertilizer, 150 kg of P fertilizer, 75 kg of K fertilizer, and top-dressed with 200 kg of N fertilizer, 75 kg of K fertilizer) among the four fertilization conditions investigated. The appropriate combination of these fertilizers improved tulsi biomass, essential oil production and essential oil quality. Tulsi essential oil production increased to 39.29 L/ha in F2, which was more than 2 times higher than the control. The quality of the essential oil was improved through an increase in the content of the main compound - eugenol, as well as antimicrobial activity of essential oils against *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans*, and antioxidant activity of tulsi essential oil in F2 compared to the control sample. The results of this study can be applied with proper effectiveness in cultivating tulsi for the purpose of developing traditional medicine, aromatherapy, and the fragrance industries. Harnessing the power of beneficial microorganisms provides a sustainable and eco-friendly approach to agriculture and herbal medicine, contributing to improve soil health, reduce environmental impact and improve the production and quality of medicinal plants containing essential oils.

Authors' Contributions

QTH: Funding acquisition, conceptualization, methodology, investigation, review, and editing; H.T.T.C: Conceptualization, methodology, investigation, formal analysis, data curation, writing original draft, review, and editing; N.T.V: Conceptualization, investigation, review, and editing; T.T.T.D: Formal analysis, data curation, P.T.D: Resources, review, and editing; H.D.C: Review, and editing; P.L.H and U.N.L.P: Investigation; W.N.S: Supervision, review and editing. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

The research has been done under the research project QG.22.60 "Research and application of LED lighting technology in combination with fertilizers to boost the yield and quality of essential oils of *Ocimum gratissimum* L. and *Ocimum tenuiflorum* L." of Vietnam National University, Hanoi.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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