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IMPS

Optimizing Organic Fertilizer and Planting Density for Enhanced Biomass and Essential Oil Production in *Thymus kotschyanus* Boiss. & Hohen. Genotypes under Rainfed Cultivation

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ABSTRACT

This study investigated the effects of organic fertilizer (0 and 50 t/ha farmyard manure) and planting density (4, 6, and 8 plants/m) on biomass production, essential oil (EO) yield, and chemical composition of four *Thymus kotschyanus* genotypes under rainfed conditions in western Iran over two growing seasons. A factorial split-plot design with three replications was employed. Analysis of variance revealed highly significant effects of genotype, planting density, and their three-way interaction with fertilizer on most measured traits. The medium planting density (6 plants/m) combined with manure application consistently optimized productivity. The highest fresh biomass (15,323 kg/ha), dry biomass (5,308.8 kg ha^{-1}), and EO yield (188.6 kg/ha) were obtained from genotype 2 under this optimal treatment. Thymol (48.99–65.44%) and carvacrol (4.20–10.91%) were the dominant EO constituents. Other major compounds included *p*-cymene, borneol, α-terpineol, and γ-terpinene, whose concentrations varied significantly with treatments. Pearson correlation analysis indicated strong positive associations among fresh biomass, dry biomass, and EO yield, while major EO components showed reciprocal trends in response to density and fertilizer levels. Principal component and cluster analyses distinctly separate genotypes based on their EO profiles and yield performance. In conclusion, integrating superior genotype selection (genotype 2) with sustainable organic nutrition and optimized planting density (6 plants m^{-2} with 50 t/ha manure) provides an effective strategy for simultaneously enhancing both the yield and quality of *Thymus kotschyanus* essential oil under rainfed cultivation systems.

Keywords: Farmyard manure, Rainfed agriculture, Thymol, Carvacrol, Genotype × management interaction

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INTRODUCTION

The Importance of Medicinal and Industrial Plants

Medicinal plants are a vital source of bioactive compounds with diverse structures and therapeutic potential [1]. Different species of Lamiace family including Thymus, Satureja, Nepeta, Salvia, etc., are the major source of bioactive compounds with broad use in pharmaceuticals and food industries [2, 3]. These species produce biologically active compounds such as flavonoids, terpenoids, phenolics and alkaloids, that are characterized by antioxidant, anti-inflammatory, and antibacterial properties [4-7].

Thymus, because of its traditional uses and high content of bioactive compounds, is one of the most important lamiae plants, which is extensively used in medicine and food and hygienic industries [8]. Thyme oil contains phenolic compounds with sedative, antiseptic, antioxidative, expectorant, antispasmodic, antifungal, antibacterial, and anti-inflammatory properties [9-11]. The main components of thyme essential oil vary in order to compounds and contents depend on genetic variation and ecological conditions; however, it generally contains monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes including thymol, *p*-cymene, *γ*-terpinene, linalool, carvacrol, geraniol, and nerol, nerolidol, caryophyllene oxide, myrcene, and germacrene D [12-16].

Thymus kotschyanus Boiss., a wild thyme species, is a small semi-shrub valued for its aromatic and medicinal properties, grows throughout the temperate biome in Eastern Anatolia (S. & E. Turkey) Middle East, Caucasus and Iran, and it adapts to various ecological conditions [17, 18]. The essential oil of this plant contains many important phenolic compounds with antibacterial and antioxidant activity. In a research, the thirty-eight compounds including thymol (6.8-66.15%), carvacrol (2.38-20.06%), linalool (1.39-22.23%), α-terpineol (0.16-11.64%), and geraniol (0.36-11.37%) as major compounds were identified in EO extracted from different populations of *Thymus kotschyanus* [19]. Also, in another study, ten chemicals were identified in the essential oil of wild-growing *Thymus kotschyanus* in Iran, and the main constituents were thymol (89.08%) and γ-Terpinene (4.62%) [20]. The prevalent use of this species in the production of herbal cheese, spices, and medicines has severely decreased its populations in nature, highlighting the importance of conserving this natural resource and its cultivation [21]. In recent years, some experiments were conducted to domestication of this economic plant in Iran, especially its cultivation under dry farming.

Achieving the highest performance on the farm requires optimal and accessible levels of nutritional elements. Although the chemical fertilization is a pronounced approach to crop production improvement, however the extreme usage of chemical fertilizers causes detrimental effects on the soil structure, microorganisms, water holding capacity, imbalances in soil nutrients, and soil fertility as well as environmental contamination [22-25]. Therefore, it is necessary to take aid from sustainable and environmentally friendly agricultural systems, such as the use of organic fertilizers that support and provide optimal plant nutrition and mitigates adverse effects of industrial fertilizers [26-29]. Organic fertilizers, containing plant- or animal-based residuals, can improve soil fertility by influencing several chemical and biochemical parameters, having positive effects on plant nutrition and on consequential biomass production, as well as conserving the soil health [30-35]. Application of animal manure increases soil organic matter and uptake of trace elements, as well as gradually turns NPK nutrients into absorbable form and enhances nutrient use efficiency [36-38]. Additionally, increasing organic matter in the soil helps water infiltration into the soil and retain soil moisture, especially during dry conditions [37].

Intraspecific competition for environmental growth factors causes a decrease in plant yield and its economic productivity [39-41]. Planting density is a major factor that affects plant competition for the absorption of nutrients, water, and sunlight [42, 43]. The lower row spacing than optimal upsurges the competition between plants, limiting accessibility to the required resources and growth [42]. On the other hand, higher row spacing than optimum, while enhancing growth of individual plants, overall can reduce total yield [44-46]. Results of studies in some Lamiaceae plants confirmed that by increasing plant density, up to the optimal point, total biomass and EO production would increase linearly, while as the density increases beyond the optimum, total plant yield and essential oil yield will decrease [2, 47]. However, moving away from the optimum planting density, either increasing or decreasing plant density, will result in reduced yield. Therefore, achieving optimum planting density for any plant species under special environmental conditions plays a pivotal role in its maximum production [43].

Considering the economic value of the *Thymus kotschyanus* and its industrial and medicinal importance, the need to develop dryland cultivation of plants adapted to water scarcity and simultaneously reduce the use of chemical fertilizers. This study was conducted aim to domestication and evaluation of yield and EO production of this plant under rainfed cultivation.

MATERIALS AND METHODS

Experimental Site

The experiment was conducted at the Research Station of Kermanshah Agricultural and Natural Resources Research and Education center, Iran, at 46°50′ E longitude and 34°16′ N latitude, and an altitude of 1,380 m above sea level during 2020–2022. According to detailed soil survey studies, the soil of the experimental site is classified as Fine, Mixed, Thermic Vertic Calcixerepts based on the USDA soil taxonomy system. The soil texture ranges from heavy to very heavy, classified as silty clay to silty clay loam. The long-term (10-year) mean annual precipitation of the site is 335 mm. The ombrothermic diagram of the research station is presented in Fig. 1.

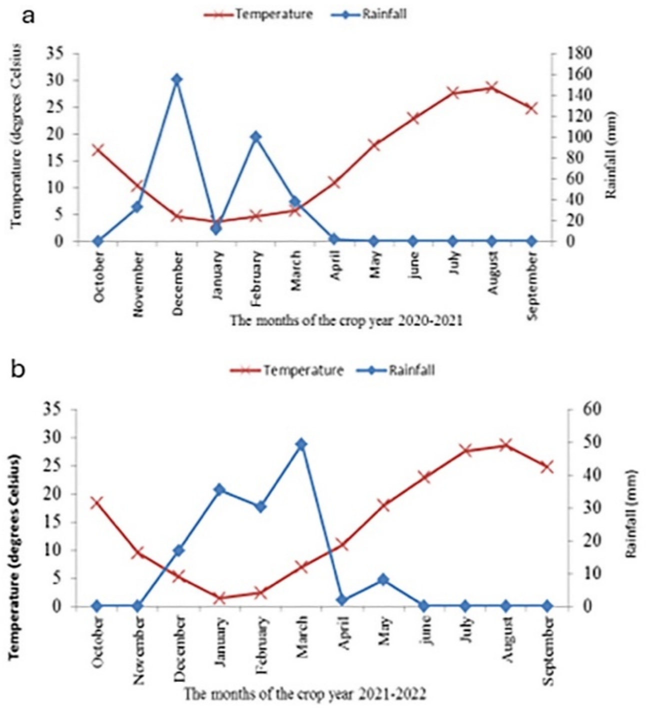

Fig. 1 The monthly ombrothermic diagram of the crop years 2020-2021 and 2021-2022

Plant Materials and Experimental Design

Four genotypes of *Thymus kotschyanus*, a wild perennial thyme species from Iran, were cultivated in a factorial experiment based on Randomized Complete Block Design (RCBD) with three replications under three planting densities (4, 6, and 8 plants m^{-2}) and two organic fertilizer levels (no manure application and application of 50 t ha^{-1} farmyard manure) under rainfed conditions. The seed of these population were obtained from the Gene Bank of the Research Institute of Forests and Rangelands, Tehran, Iran. The plant materials were evaluated during the 2019–2020 growing seasons to assess plant yield and phytochemical traits. Detailed information on the studied genotypes is provided in Table 1.

Land Preparation

The field was plowed in early autumn and subsequently disked twice in perpendicular directions prior to planting. Before transplanting, the field was leveled using a land leveler, and furrows and ridges were formed at 50 cm intervals. According to the experimental design, each plot received 7.5 kg of farmyard manure (corresponding to 50 t/ha). The manure was manually incorporated into the soil within the furrows. The total number of plots was 72.

Planting

Seeds of the four genotypes were sown in a 1:1 peat and coco peat bed under greenhouse conditions (24±2-18±2 °C and 60% humidity) in late September. After sufficient seedling establishment, transplants were transferred to the main field in mid-November. The results of soil analysis were presented in Table 2. The manure bed contains 138 ppm absorbable P, 6800 ppm absorbable K, and 1.75% organic carbon. Seedlings were planted within furrows in four rows with 3 m in length in each plot (3× 1.5 m) at a row spacing of 50 cm and intra-row spacings of 25 cm (8 plants/m), 35 cm (6 plants/m), and 50 cm (4 plants m^{-2}).

Table 1 the origin and specifications of *Thymus kotschyanus* seeds

Specious	Origin of seeds preparation	Gen bank number	Genotype Kode	Primary origin of seeds
Thymus kotschyanus	RIFR gen bank, Iran	27471	G1	Sardasht, West Azarbaijan, Iran
Thymus kotschyanus	RIFR gen bank, Iran	27800	G2	Sarain, Ardabil, Iran
Thymus kotschyanus	RIFR gen bank, Iran	13206	G3	Daylaman, Siahkal, Iran
Thymus kotschyanus	RIFR gen bank, Iran	27814	G4	Pars –Abad, Ardabil, Iran

Table 2 Physicochemical properties of experimental field soil

Available K (ppm)	Available P(ppm)	Total N (%)	O.C. (%)	EC (dS m^{-1})	pH	Sand (%)	Silt (%)	Clay (%)	Soil texture
462.0	23.6	0.22	1.2	0.92	7.8	7.4	45.6	47.1	Clay-silty

Crop Management

Weeding was carried out manually in three stages during the growing season to maintain weed-free conditions, which facilitated improved early seedling establishment. No chemical fertilizers were applied during the experiment. Due to the rainfed nature of the experiment, no irrigation was provided throughout the growing period. In the second growing season, at the 50% flowering stage, all plants within each plot were harvested at 5 cm above the soil surface, excluding 0.5 m from both ends of the plot to eliminate border effects.

Measuring Plant Weight

Fresh biomass was weighed using a balance with 0.5 g precision and recorded as fresh weight (g/m). Harvested plant material was shade-dried for one week and then weighed using a balance with 0.5 g precision. Dry weight was recorded as dry biomass (g/m). The dried aerial parts of plants were used for EO extraction.

Essential Oil Percentage

Dried aerial parts of plant material were ground, and a sample of 80 g was taken from each experimental unit (72 units in total) added to 1.5 L water in a clevenger balloon. Essential oil extraction was performed using a Clevenger apparatus by hydrodistillation for 2 h. The volume of water was 1.5 li. Essential oil content was expressed as percentage (w/w) based on dry matter.

Identification of Essential Oil Constituents

Essential oil constituents were identified using gas chromatography (GC) and gas chromatography–mass spectrometry (GC/MS). GC analysis was performed using a Shimadzu GC-9A equipped with a flame ionization detector (FID) and a Chromatopac C-R3A data processor, fitted with a DB-5 capillary column (30 m length, 0.25 mm internal diameter, 0.25 µm film thickness). The oven temperature program was set from 60 °C to 180 °C at a rate of 3 °C /min. Injector and detector temperatures were set at 300 °C and 280 °C, respectively. Helium was used as the carrier gas at a flow rate of 1.5 kg/cm.

GC/MS analysis was conducted using a Varian 3400 gas chromatograph coupled to an ion-trap mass spectrometer, equipped with a DB-5 column of identical specifications. The oven temperature program was the same as that used for GC analysis. The injector temperature was set 10 °C above the final oven temperature. Helium was used as the carrier gas at a flow rate of 1.5 kg cm^{-2}. The scan time was 1 s, and the ionization energy was 70 eV.

Compound identification was achieved by calculating Kovats retention indices using a homologous series of n-alkanes analyzed under the same conditions as the essential oil samples, and by comparison with published reference data. Mass spectra were further examined and matched against standard compound spectra and computerized spectral libraries to confirm compound identities [48].

Data Analysis

Data were analyzed using univariate and multivariate methods including ANOVA, Tukey's HSD test, GGE biplot, Pearson's Correlation Estimation, and Cluster Hierarchical Analysis with R software (version 4.3.1).

RESULTS

The analysis of variance revealed that organic fertilizer, plant density, genotype, and their interactions exerted significant effects on most essential oil constituents and yield-related traits. The significant main effects of organic fertilizer (OF) were detected for thymol ($p \le 0.05$), carvacrol ($p \le 0.05$), *α*-terpineol ($p \le 0.01$), γ-terpinene ($p \le 0.05$), *E*-caryophyllene ($p \le 0.05$), *cis*-sabinene hydrate ($p \le 0.05$), linalyl acetate ($p \le 0.01$), fresh yield ($p \le 0.05$), and dry yield ($p \le 0.05$). In contrast, the effects of OF were not significant for several minor constituents, essential oil percentage, or essential oil yield. Plant density exhibited a highly significant effect ($p \le 0.01$ or $p \le 0.001$) on nearly all evaluated traits, including thymol, carvacrol, *p*-cymene, borneol, *α*-pinene, *E*-caryophyllene, *1,8*-cineole, *α*-thujene, linalool, myrcene, terpinene-4-ol, linalyl acetate, methyl carvacrol, essential oil percentage, fresh yield, dry yield, and essential oil yield, indicating that plant density was a principal factor influencing both biomass production and essential oil composition. The fertilizer × density interaction was found to be significant for several compounds, including *α*-pinene ($p \le 0.05$), *α*-thujene ($p \le 0.01$), *cis*-sabinene hydrate ($p \le 0.05$), linalyl acetate ($p \le 0.001$), methyl carvacrol ($p \le 0.05$), as well as for essential oil percentage ($p \le 0.05$) and essential oil yield ($p \le 0.05$), suggesting that the response to manure application was dependent on planting density. Highly significant genotype effects ($p \le 0.001$) were observed for nearly all essential oil constituents, including thymol, carvacrol, *p*-cymene, borneol, *α*-terpineol, *γ*-terpinene, *α*-pinene, *E*-caryophyllene, camphene, *1,8*-cineole, *α*-thujene, *cis*-sabinene hydrate, linalool, myrcene, terpinene-4-ol, linalyl acetate, and methyl carvacrol, as well as for fresh and dry yield, reflecting substantial genetic variability. However, the main effect of genotype was not significant for essential oil percentage. Also, significant genotype × organic fertilizer interactions were observed for several essential oil constituents, including thymol, *p*-cymene, borneol, *α*-terpineol, *α*-pinene, *E*-caryophyllene, camphene, *1,8*-cineole, *α*-thujene, linalool, myrcene, linalyl acetate, and methyl carvacrol, whereas these interactions were generally not significant for yield-related traits.

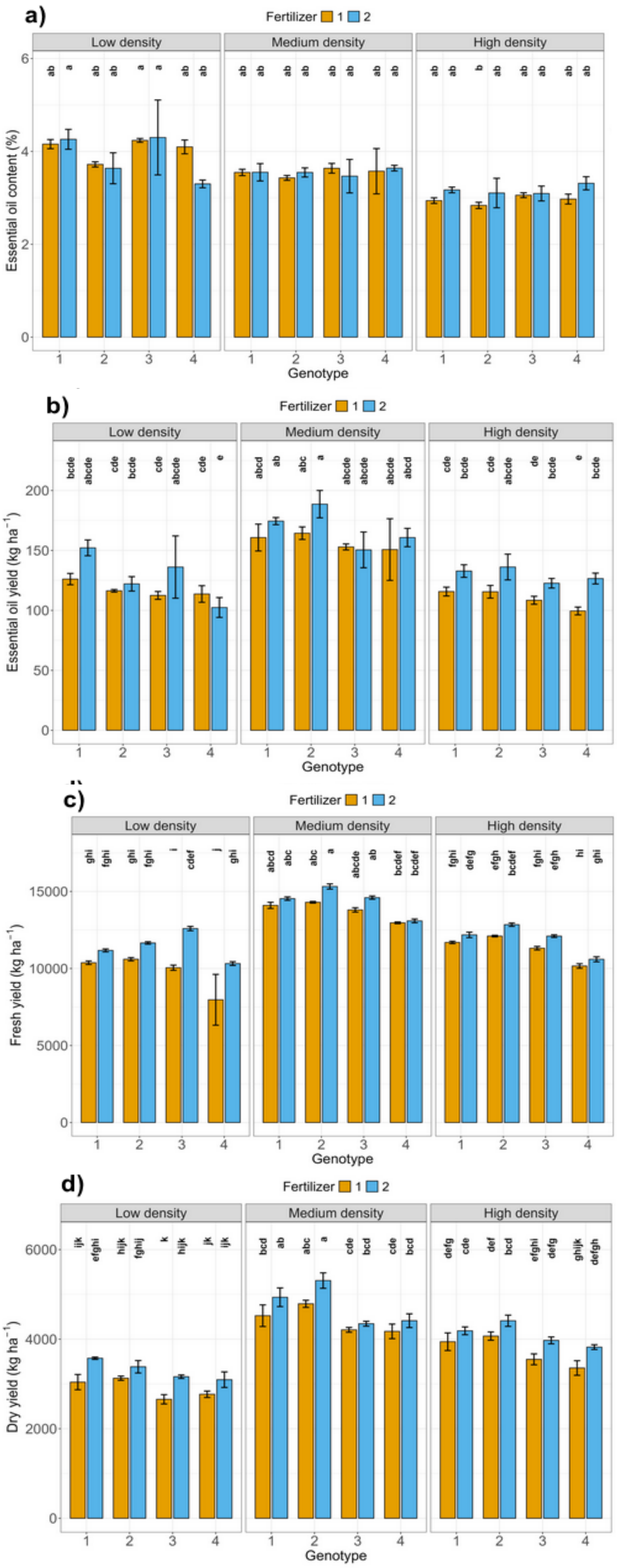

Fig. 2 The effect of different organic fertilizers × plant density on essential oil content (a), essential oil yield (b), plant fresh yield (c), and dry yield (d) in four genotypes of *Thymus kotschyanus* (Tukey's Test, α = 0.05, r = 3, n = 10). Genotypes 1 – 4: G1 – G4; Fertilizer 1: non-manure application and 2: manure application; low, medium, and high plant density: 4, 6, and 8 plant/m, respectively.

Likewise, the genotype × plant density interaction was highly significant for most essential oil constituents but was not significant for fresh yield, dry yield, essential oil percentage, or essential oil yield. Furthermore, the three-way interaction (genotype × organic fertilizer × plant density) was significant for many essential oil constituents, frequently at $p \leq 0.01$ or $p \leq 0.001$, indicating a complex interactive regulation of secondary metabolite accumulation. In contrast, this interaction was not significant for yield traits, suggesting that biomass production was comparatively less responsive to interactions between genetic, plant density and organic fertilizer application than essential oil composition.

Essential Oil Production and Plant Biomass

Essential oil percentage showed limited but significant variation among treatments, with most combinations clustering in the same Tukey group (ab), indicating moderate interaction effects. The highest EO contents were recorded in genotype 1 cultivated under low density and application of OF (4.26%), genotype 3 in response to low density without manure (4.24%), and genotype 3 under application of manure at low density (4.30%), all belonging to the top significance group (a) (Fig. 2a). In contrast, the lowest EO value was observed in genotype 2 cultivated under high plant density witout manure application (2.84%), which formed a distinct lower group (b) Fig. 2a). Overall, genotype 3 and genotype 1 tended to produce higher EO percentages, particularly under low planting density, whereas increasing density slightly reduced EO concentration, especially in genotype 2.

Essential oil yield, which integrates biomass production and EO percent, showed pronounced interaction effects in response to fertilizer × density × genotype. The highest EO yield (188.62 kg ha^{-1}) was achieved in genotype 2 cultivated under medium density and application of manure (Fig. 2b), clearly separated in group a, followed by genotype 1 in response to the mentioned treatments and genotype 2 under medium density and no manure application, which formed the next highest groups (ab–abc). Conversely, the lowest EO yields were obtained in genotype 4 cultivated under high density and no fertilizer application (99.53 kg ha^{-1}) and genotype 4 in low density and manure application (102.42 kg ha^{-1}), both classified in the lowest group (e). These results indicate that medium planting density (D2: 6 plant/m) along with application of 50 t/ha manure is optimal for maximizing essential oil yield in *Thymus kotschyanus*, particularly genotype 2.

EO Chemical Compounds

In total, 18 compounds represented 97.86% of total compounds identified (Table 3), including six main compounds: thymol, carvacrol, *p*-cymene, α-terpineol, γ–terpinene, and borneol. Although, the intraction effect of fertilizer × density × genotype was not significant, however, the maximum fresh yield (15,323 kg ha^{-1}) was obtained from genotype 2 under medium density and manure application, which formed a distinct highest group (a), followed by genotype 3 under the aforementioned condition and genotype 2 at medium density without manure application (Fig. 2c), both belonging to high-ranking groups (ab–abc). In contrast, the minimum fresh yield was recorded for genotype 4 under low plant density without manure application (7,968 kg ha^{-1}), classified in the lowest group (j). Across genotypes, medium planting density (6 plant/m) consistently produced higher fresh biomass than lower or higher densities, while manure application enhanced yield more effectively, particularly in genotype 2.

Dry matter yield followed a pattern similar to fresh yield, with significant treatment-dependent variation. The highest dry yield (5,308.85 kg ha^{-1}) was recorded for genotype 2 in response to medium density and manure application (group a), followed by genotype 1 in response to medium density and manure application and genotype 2 in response to medium density without manure application, which were not significantly different (Fig. 2 d). The lowest dry yield (2,657.03 kg ha^{-1}) was observed in genotype 3 under low plant density without manure application (group k). In general, medium density combined with manure application maximized dry biomass accumulation, whereas low density resulted in reduced dry matter production across all genotypes.

Overall, medium planting density (6 plants/m^2) consistently outperformed low and high densities for fresh yield, dry yield, and EO yield, while application of 50 t/ha manure (fertilize level 2) significantly enhanced plant yield. Although EO percentage alone varied only slightly among treatments, its interaction with biomass resulted in large differences in EO yield. Genotype 2 showed the best overall performance, especially in response to manure application + medium density cultivation, which can be considered the most favorable combination for maximizing both biomass and essential oil production.

Major Chemical Compounds of Essential Oil

The interaction among fertilizer level, planting density, and genotype significantly affected the concentrations of the essential oil constituents except carvacrol [thymol ($p \leq 0.05$), *p*-cymene ($p \leq 0.001$), *α*-Terpineol ($p \leq 0.001$), *γ*–Terpinene ($p \leq 0.001$), and borneol ($p \leq 0.05$)].

The highest thymol concentration was recorded for genotype 2 under medium density and non-fertilizer application, with a mean value of 65.44% (group a). Other relatively high thymol values were observed in genotype 3 under medium density and manure application (64.30%) and genotype 4 cultivated in low density condition without manure (62.79%), which were not significantly different from the maximum (Fig. 3a). In contrast, the lowest thymol content was found in genotype 2 under high density cultivation and no fertilizer application (48.99%), followed by genotype 2 under medium density and non-manure application (50.23%), both grouped in the lowest significance classes (j and ij), indicating a strong suppressive effect of low nutrients combined with higher cultivation density in certain genotypes.

Regarding borneol, the treatment effects were particularly pronounced. The highest borneol concentration (6.63%) was obtained in genotype 4 under medium row spacing (6plant/ m2) and manure application, which was significantly higher than the other statistical groups (Fig. 3 b). Most other treatments clustered in intermediate groups with values around 3.5–4.9%. In contrast, extremely low borneol contents were recorded under high–density conditions, especially in genotype 3 under manure application (0.33%) and genotype 1 without manure application (0.37%), as well as genotype 4 under manure application (0.80%) (Fig. 3 b), all belonging to the lowest significance groups (f or ef), demonstrating the strong negative effect of high plant density on borneol accumulation.

Table 3 Specification of EO chemical compounds identified in *Thymus kotschyanus* under impact of organic fertilizers and different plant densities in dry farming *

Chemical classification	Chemical name	RT	RI	Formula
Monoterpene	Terpinene-4-ol	2.58	1146.53	$C_{10}H_{18}O$
	Myrcene	1.16	986.46	$C_{10}H_{16}$
	Terpinolene	1.65	1054.11	$C_{10}H_{16}$
	α-Terpinene	1.22	1018.0	$C_{10}H_{16}$
Terpene alcohol	Linalool	6.95	1112.3	$C_{10}H_{18}O$
Acetate ester	Linalyl acetate	1.02	1257. 2	$C_{12}H_{20}O_2$
Isomeric monoterpenoids	α-Terpineol	3.50	1189.0	$C_{10}H_{18}O$
Bicyclic monoterpenoids	1,8-Cineol	5.61	1039.3	$C_{10}H_{18}O$
	Cis-Sabinene hydrate	7.18	1056.4	$C_{10}H_{18}O$
Phenol monoterpenoids	Carvacrol	4.07	1289.96	$C_{10}H_{14}O$
	Thymol	3.96	1281.22	$C_{10}H_{14}O$
	Borneol	4.79	1165.10	$C_{10} H_{18} O$
	Methyl ether carvacrol	3.60	1250.81	$C_{11}H_{16}O$
Bicyclic monoterpene	*α*-Pinene	0.87	931.13	$C_{10}H_{16}$
	Trans-Caryophyllene	5.33	1376.02	$C_{15}H_{24}$
	Camphene	5.60	953.0	C10H16
Ketonic monoterpene	*α*-Thujone	0.84	924.38	$C_{10}H_{16}O$
Isometric monoterpene	*γ*-Terpinene	1.63	1051.59	$C_{10}H_{16}$
Benzene alkyl	*p*-Cymene	1.36	1014.15	$C_{10}H_{14}$

* RT = retention time; RI = retention index

The maximum carvacrol percentage (10.91%) was observed in the genotype 2 in response to medium row spacing without manure application which, together with its content for this genotype under high density cultivation without manure application (10.87%) and with manure application (10.81%), formed the highest statistical group (a) (Fig. 3c). These results indicate that moderate density strongly enhanced carvacrol accumulation in the genotype 2, regardless of fertilizer level. Also, the lowest carvacrol content was recorded in genotype 2 again, but under application of manure and low planting density (4.20%), which was significantly lower than all other treatments and belonged to the lowest group (e), suggesting an unfavorable interaction between higher fertilizer and low density for carvacrol accumulation in this genotype.

The mean values of *α*–Terpineol ranged from 2.28% to 6.28%, indicating substantial genotype and treatment-dependent variations. The highest *α*–Terpineol content (6.28%) was observed for genotype 1 under non manure and high-density cultivation, which belonged to the top significance group (a) (Fig. 3d). Similarly high values were recorded in genotype 1 under non application of manure and medium–density cultivation (6.24%) and genotype 3 under non manure and high-density cultivation (6.03%), which were not statistically different from the maximum. In contrast, the lowest *α*–terpineol concentration was detected in genotype 2 in response to manure application and low–density cultivation (2.28%), classified in the lowest Tukey group (g), followed by genotype 2 under non manure application and medium-density cultivation (2.98%) (Fig. 3d).

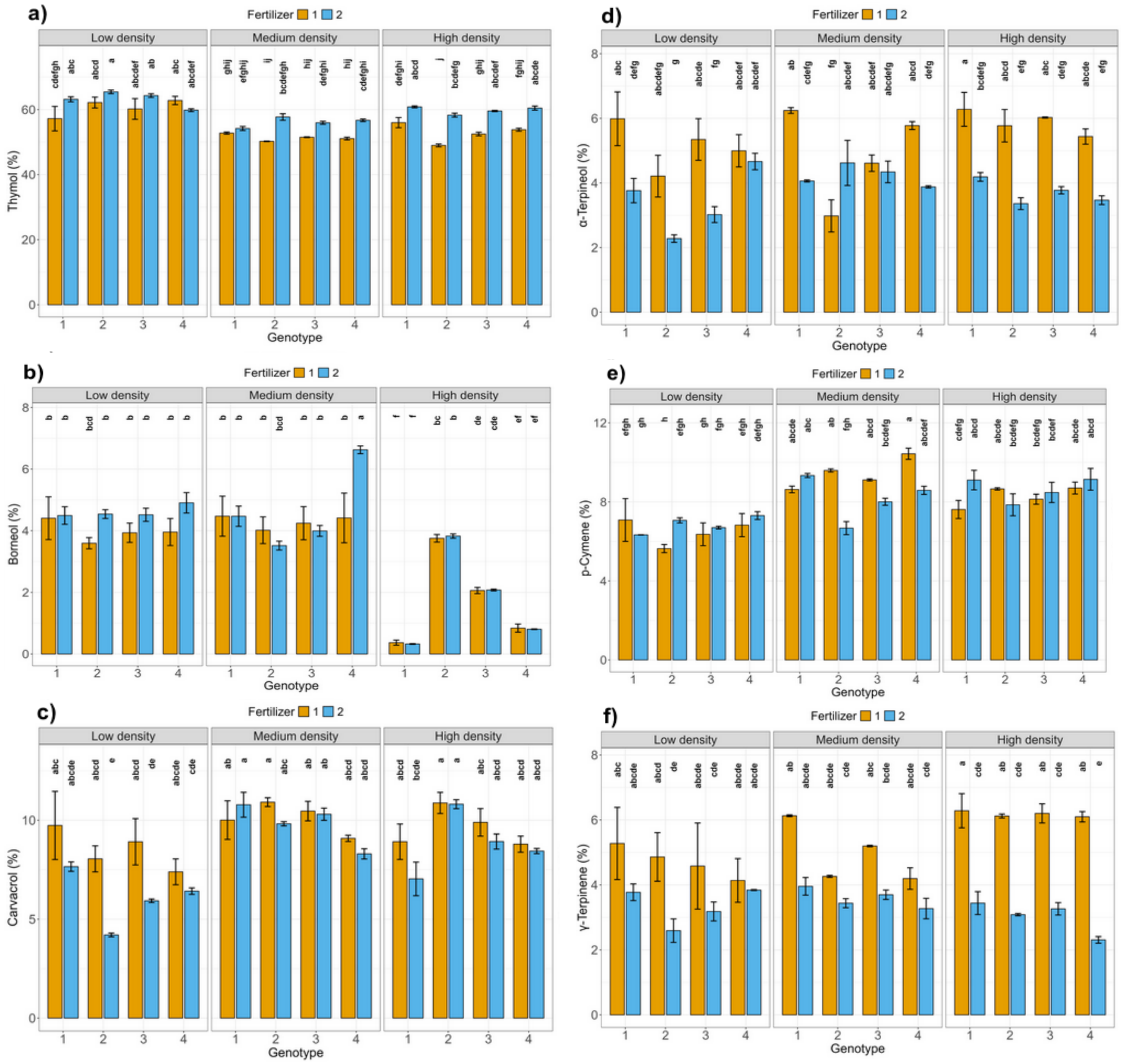

Fig. 3 The effect of different manure levels × plant dencity on essential oil compounds in four genotypes of *Thymus kotschyanus*: thymol (a), borneol (b), carvacrol (c), α –terpineol (d), ρ – cymene (e), and gamma – terpinene (f) (Tukey's Test, $\alpha = 0.05$, r = 3, n= 10). Genotypes 1 – 4: G1 – G4; Fertilizer 1: non-manure application and 2: manure application; low, medium, and high plant density: 4, 6, and 8 plant/m, respectively.

Overall, manure application generally resulted in lower α–terpineol contents, particularly under high density, while genotype 1 consistently exhibited superior terpineol accumulation compared with the other genotypes.

The concentration of *p*-cymene also differed significantly among treatments (Fig. 3e). The highest *p*-cymene content was achieved in genotype 4 under medium density without manure application (10.43%), clearly separated in the top significance group a (Fig. 3e). High values were also detected in genotype 1 under medium density with manure application (9.34%) and genotype 4 under high density with manure application (9.14%). In contrast, the lowest *p*-cymene level was observed in genotype 2 under low density without manure application (5.64%), which was statistically inferior to most other treatments (h), indicating that no manure application and low-density conditions were suboptimal for *p*-cymene production in genotype 2 (Fig. 3e).

The content of γ–terpinene varied between 2.31% and 6.28% (Fig. 3f), demonstrating a strong triple interaction effect between genotype, manure level and row spacing. The maximum γ–terpinene content was recorded in genotype 1 cultivated under high density (8 plant/m) without manure application (6.28%), followed closely by genotype 3 under high density planting without manure application (6.20%) and genotype 4 under high density planting without manure application (6.10%), all of which were grouped among the highest Tukey's classes (or ab). On the other hand, the minimum γ– terpinene value was obtained from genotype 4 under high-density planting plus manure application (2.31%), which was classified in the lowest group (e) (Fig. 3f). In general, γ– terpinene concentration was markedly lower under manure application, especially at high planting density.

In summary, high planting density maximized both α–terpineol and γ– terpinene concentrations, particularly in genotype 1, whereas application of 50 t/ha manure, especially under low or high density, led to significantly lower values. These results highlight the importance of optimizing fertilizer–density–

genotype combinations to enhance oxygenated monoterpene accumulation in aromatic crop production systems.

Non-major Chemical Compounds of Essential Oils

The *α*-Pinene content varied markedly among treatments, ranging from 1.78% to 3.67%. The highest value was observed in genotype 2 under medium density without manure application; 3.67% (group a). Similarly high values were recorded in genotype 2 under manure application and low-density planting and genotype 4 with manure application and low density, indicating a strong genotype effect under low to medium density (Fig. 4a). In contrast, the lowest *α*-pinene contents were recorded under high density in genotype 1 at high density condition without manure and also this genotype under high density and manure application (1.78–1.80%), both classified in the lowest group (h), demonstrating the negative effect of high density on *α*-pinene accumulation.

1,8-cineole exhibited pronounced treatment effects, with values ranging from 1.02% to 3.69%. The highest concentration of *1,8*-cineole (Fig. 4b) was found in genotype 2 under medium row spacing (6 plant/m) without manure application (3.69%, group a), followed by genotype 3 under medium row spacing without manure application and genotype 4 under medium row spacing without manure application, emphasizing the strong stimulatory effect of medium density across genotypes. The lowest value occurred in genotype 4 under high row spacing (4 plant/m) without manure application (1.02%, group h), indicating that low density limited cineole biosynthesis.

E-caryophyllene showed a wide range of variation (0.37–2.57%). The maximum concentration observed in genotype 2 in response to manure application and medium-density cultivation (2.57%), followed by genotype 2 under medium-density plantation without manure application (2.34%) (Fig. 4c), both belonging to the highest Tukey groups (a and ab), highlighting the strong positive response of genotype 2 under medium density, particularly with fertilizer 2. Conversely, the lowest value was detected in genotype 4 in response to manure application under low-density cultivation (0.37%, group j), indicating that genotype 4 did not respond to manure application under low density in order to accumulate *E*-caryophyllene.

Thujene content ranged from 0.71% to 1.78%. The maximum *α*-thujene content was recorded in genotype 2 in response to manure application under medium-density planting (1.78%, group a), closely followed by genotype 2 under high-density cultivation without manure application and genotype 3 in response to manure application and medium-density, highlighting the responsiveness of genotype 2 under medium to high density, particularly with manure application (Fig. 4d). In contrast, the lowest *α*-thujene content was observed in genotype 1 at high-density cultivation without manure application (0.71%, group i), showing a strong negative effect of high density on *α*-thujene production in genotype 1.

Camphene content ranged from 0.83% to 1.76% (Fig. 4e). The highest camphene level was recorded for genotype 4 under medium density planting without manure application (1.76%, group a), followed by genotype 4 under high density without manure application and genotype 2 under medium density without manure application, indicating that regardless manure application, medium to high density promoted camphene accumulation, especially in genotype 4. In contrast, the lowest camphene values were observed under low–density planting in genotypes 1, 2, and 4, particularly under non–application of fertilizer.

The content of *Cis*-sabinene hydrate showed relatively lower variation (0.37–0.93%). The highest value (0.93%, group a) was obtained in genotype 2 under low density plus manure application, followed by genotype 2 under medium–density cultivation without manure application (0.88%), indicating that genotype 2 responded positively to low and medium planting density regardless of the manure effect. The lowest concentration was recorded in genotype 4 under low-density planting (0.37%, group e), reflecting a strong genotype effect on *Cis*-sabinene hydrate production under low-density planting (Fig. 4f).

Collectively, these results demonstrate that medium planting density was generally optimal for maximizing *α*-pinene, *E*-caryophyllene, camphene, *1,8*-cineole, and *α*-thujene, whereas high density often reduced monoterpene hydrocarbons, particularly in genotype 1. Genotype 2 consistently showed superior performance for several compounds (*α*-pinene, *E*-caryophyllene, *1,8*-cineole, *α*- thujene, and *cis*-sabinene hydrate), especially under medium density. This confirms that fine-tuning planting density and genotype selection is critical for optimizing these essential oil compositions.

Linalool content was strongly affected by planting density and genotype, with a pronounced increase under high density (8 plant/m). The highest linalool concentration was recorded in genotype 1 under effect of manure and high-density condition (5.62%), followed by genotype 4 also under effect of manure and high-density condition (5.33%), genotype 1 in response to high density without manure application (4.95%), and genotype 4 at high density condition without manure (4.06%), all belonging to the top significance groups (a–abcd) (Fig. 5a). In contrast, low- and medium-density treatments (4 and 6 plant/m) across all genotypes produced very low linalool levels (0.5–0.8%), clustering in the lowest group (f) (Fig. 5a). These results indicate that high planting density is the dominant factor enhancing linalool accumulation, particularly in genotypes 1 and 4, with fertilizer application further amplifying this response in some genotypes.

Linalyl acetate exhibited very strong interaction effects, with a dramatic response to high density in genotype 4. The maximum concentration was observed in genotype 4 under high-density condition without manure application (2.05%), which formed a distinct top group (a), clearly separated from all other groups (Fig. 5b). In contrast, several genotypes including genotypes 2 and 3, and 4 met extremely low values of linalyl acetate (0.07–0.28%) under manure application at medium and high densities (Fig. 5b). This pattern demonstrates that non application of manure combined with high density is essential for maximizing linalyl acetate, particularly in genotype 4. Myrcene showed clear genotype-specific responses and strong sensitivity to fertilizer–density combinations. The maximum myrcene contents were observed in genotype 2 under low-density condition without manure application (0.92%), genotype 2 under medium-density condition with manure supplementation (0.84%), genotype 3 under low-density condition without manure application (0.81%), and genotype 4 under low-density condition with manure application (0.82%), all grouped in the highest significance classes (a–ab) (Fig. 5c). In contrast, genotype 2 under medium and high densities without manure application exhibited the lowest myrcene values (≤0.17%), forming distinct lowest groups (hi–i). Overall, genotype 2 exhibited strong myrcene accumulation only under manure and low–density combinations, while medium and high densities generally reduced myrcene content. Alfa–terpinene content varied moderately but showed a consistent enhancement under medium to high density, especially in genotype 2. The highest *α*-terpinene value was recorded in genotype 2 under medium-density condition without manure application (0.84%), followed by genotype 2 under high-density condition without manure application (0.71%) and genotype 2 under medium-density condition with manure application (0.71%), all grouped among the top higher classes (a–ab).

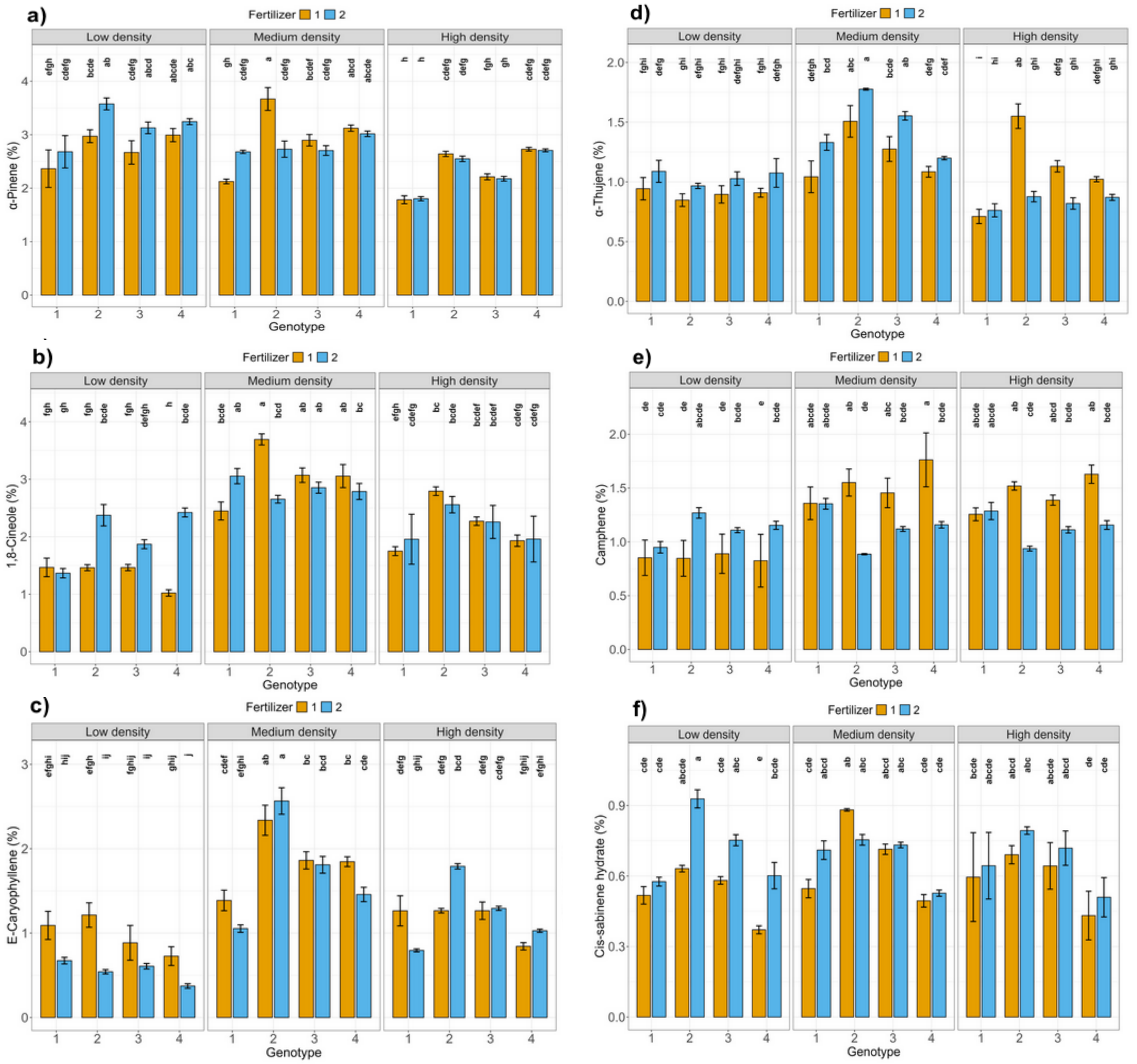

Fig. 4 The effect of different organic fertilizers × plant dencity on essential oil compounds in four genotypes of *Thymus kotschyanus*: *α*–pinene (a), 1,8–cineole (b), *E*–caryophyllene (c), *α*–α-thujene (d), camphene (e), and *Cis*–sabinene hydrate (f) (Tukey's Test, α = 0.05, r = 3, n= 10). Genotypes 1 – 4: G1 – G4; Fertilizer 1: non-manure application and 2: manure application; low, medium, and high plant density: 4, 6, and 8 plant/m, respectively.

In contrast, genotype 4 at low density condition without manure application produced the lowest *α*-terpinene levels (c) (Fig. 5d). Overall, genotype 2 showed the greatest capacity for *α*-terpinene accumulation, particularly under non–fertilization and increased density. Terpinen-4-ol content increased markedly under high density (8 plant/m), especially in genotype 4. condition without manure application (0.55%), followed by genotype 4 in the mentioned plant density with manure application (0.50%), which classified in the groups a and ab (Fig. 5e). Low- and medium-density treatments resulted the lower terpinen-4-ol concentration across genotypes 1, 2, and 3, mostly clustered in lower groups (fg–g), with the minimum value recorded in genotype 2 under medium-density condition and manure supplementation (0.12%). These results indicate that high planting density notably increased terpinen-4-ol accumulation, particularly in genotype 4.

Methyl carvacrol was primarily genotype-dependent, with genotype 2 clearly outperforming the others. The highest concentration was observed in genotype 2 at low-density condition plus manure application (0.86%), also followed by this genotype under low-density condition without manure supplementation (0.69%) (Fig. 5f). Genotypes 1, 3, and 4 showed moderate to low levels of methyl carvacrol (0.32–0.58%), especially in response to manure application and plantation under medium density, which clustered in groups f–h (lowest). These results indicate that genotype 2 is genetically predisposed to methyl carvacrol accumulation, with manure application at low density further enhancing its content. Inclusive, high planting density was the key factor increasing linalool, terpinen-4-ol, and linalyl acetate, particularly in genotypes 1 and 4, whereas genotype 2 showed superior performance for myrcene, terpinene, and methyl carvacrol.

Pearson's Correlation Estimation

Pearson's correlation analysis (Fig. 6) revealed clear and biologically meaningful association patterns among chemical constituents and yield-related traits. Thymol showed strong negative correlations with major monoterpenes, particularly carvacrol (r = −0.744**), *p*-cymene (r = −0.656**), and *γ*-terpinene (r = −0.623**), indicating a trade-off in their biosynthetic accumulation, while its positive association with myrcene (r = 0.601**) and essential oil content (r = 0.352**) highlights thymol-rich profiles as contributors to higher oil concentration. In contrast, carvacrol was positively correlated with several terpenes, notably *E*-caryophyllene (r = 0.615**), *γ*-terpinene (r = 0.384**), and *1,8*-cineole (r = 0.353**), and was also associated with increased fresh and dry biomass, suggesting its linkage with enhanced productivity.

The highest value was obtained in genotype 4 under high-density

P-cymene exhibited significant positive correlations with camphene and *1,8*-cineole, as well as with fresh and dry yields, underscoring its role as a central intermediate connected to oil composition biosynthesis, while its negative association with myrcene and methyl carvacrol reflects divergence among terpene pathways. Borneol displayed a pronounced negative correlation with linalool (r = − 0.836**) but positive correlations with *α*-pinene and essential oil content, indicating contrasting regulation between oxygenated monoterpenes. Notably, *E*-caryophyllene and *1,8*-cineole were among the strongest positive correlates of fresh and dry yields, highlighting these compounds as key indicators of high-yielding chemotypes.

Yield traits were tightly interrelated, with fresh and dry yields showing a strong positive correlation (r = 0.821**) and both exhibiting substantial positive associations with essential oil yield.

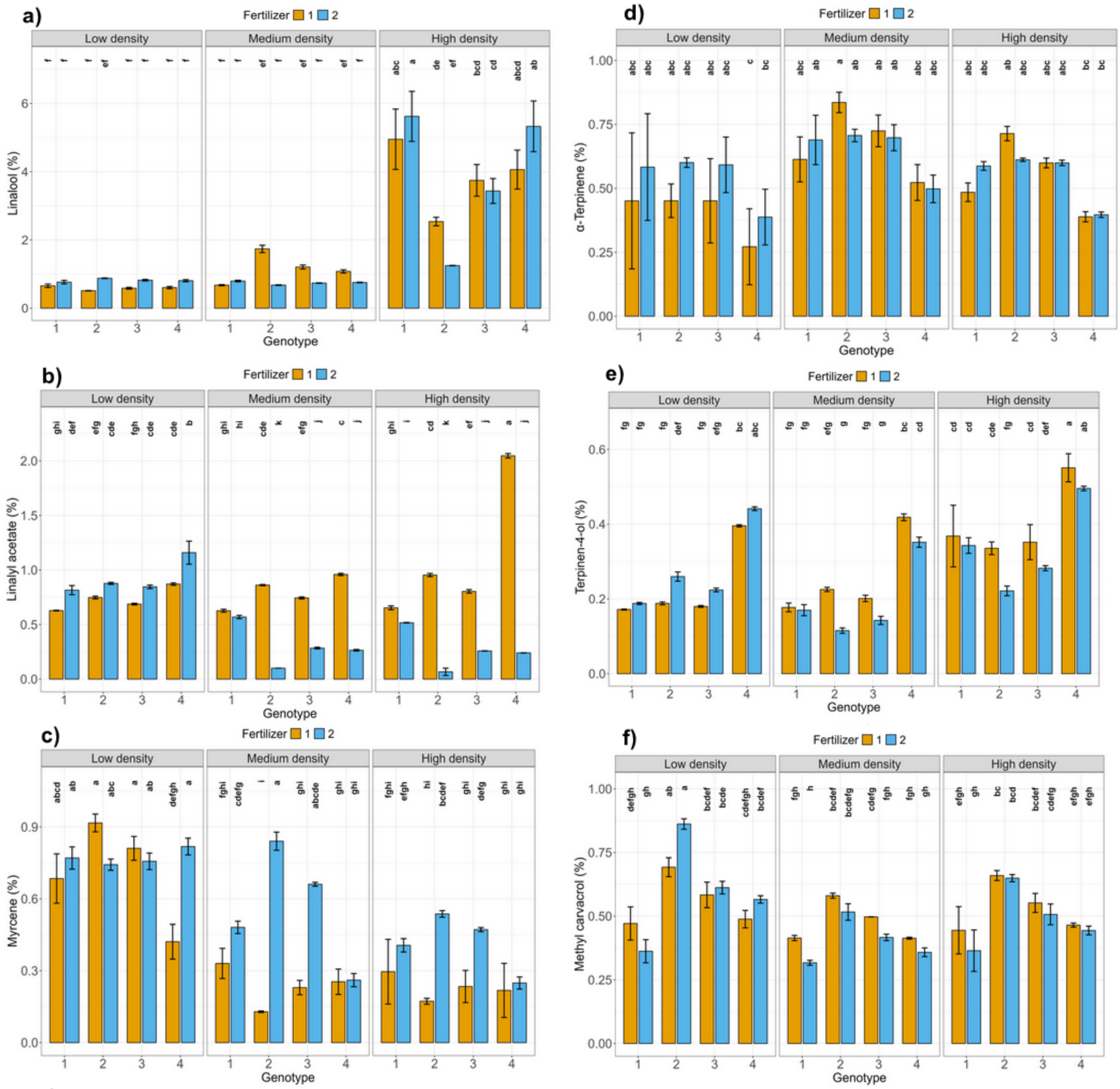

Fig. 5 The effect of different manure levels × plant dencity on essential oil compounds in four genotypes of *Thymus kotschyanus*: linalool (a), linalyl acetate (b), myrcene (c), *α*–terpinene (d), terpinene–4–ol (e), and methyl carvacrol (f) (Tukey's Test, α = 0.05, r = 3, n= 10). Genotypes 1 – 4: G1 – G4; Fertilizer 1: non-manure application and 2: manure application; low, medium, and high plant density: 4, 6, and 8 plant/m, respectively.

Collectively, these results emphasize coordinated relationships between biomass production and specific terpene profiles, and identify carvacrol, *E*-caryophyllene, and *1,8*-cineole as potential marker compounds for selecting high-yielding, oil-rich genotypes**.** *α*-terpineol exhibited a strong positive correlation with *γ*-terpinene (r = 0.764**) and negative correlations with *cis*-sabinene hydrate (r = −0.483**) and *α*-pinene (r = −0.387**). Furthermore, *E*-caryophyllene (%) exhibited strong positive correlations with fresh yield (r = 0.650**), dry yield (r = 0.679**), *1,8*-cineole (r = 0.575**), and *α*-thujene (r = 0.523**). Camphene was positively correlated with *p*-cymene (r = 0.544**) and *1,8*-cineole (r = 0.545**), but negatively correlated with myrcene (r = −0.668**). Also, *1,8*-cineole showed strong positive correlations with fresh yield (r = 0.724**), dry yield (r = 0.677**), and *α*-thujene (r = 0.564**). Finally, linalool was positively correlated with terpinen-4-ol (r = 0.564**) but negatively correlated with borneol (r = −0.836**).

PCA

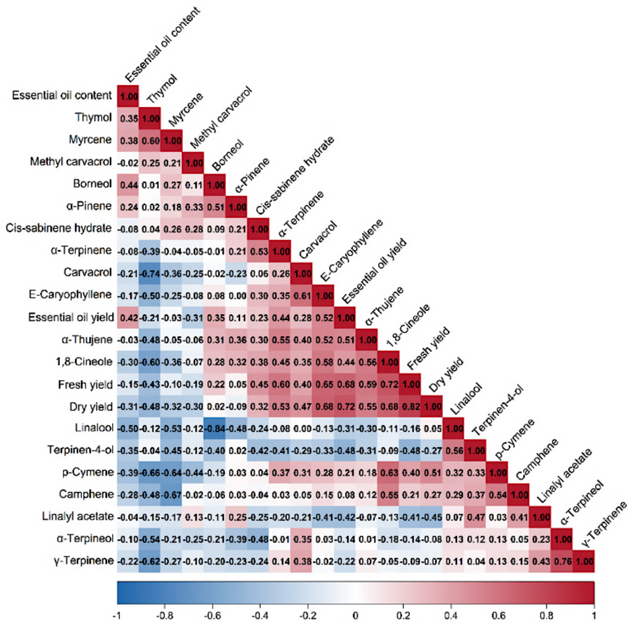

Fig. 6 The Pearson's correlation coefficients between different variables (yield and EO chemical compounds) of *Thymus kotschyanus* genotypes cultivated under different organic fertilizer × plant density

Principal component analysis (Fig. 7) revealed that the first two principal components (PC1 and PC2) together explained 49.86% of the total variation among essential oil constituents and yield-related traits, with PC1 accounting for 28.63% and PC2 for 21.24% of the variance (Table 4). PC1 was strongly and positively associated with fresh yield (0.868), dry yield (0.884), EO yield (0.674), *1,8*-cineole (0.832), *E*-caryophyllene (0.772), *α*-thujene (0.703), *α*-terpinene (0.658), carvacrol (0.636), and *p*-cymene (0.606), indicating that this component primarily represented overall productivity and major terpenoid accumulation. In contrast, thymol (−0.719), myrcene (−0.376), terpinen-4-ol (−0.331), linalyl acetate (−0.317), methyl carvacrol (−0.262), essential oil percentage (−0.231), and linalool (−0.094) loaded negatively on PC1, suggesting an inverse relationship between biomass-related traits and these compounds. PC2 was mainly characterized by strong positive loadings for myrcene (0.724), borneol (0.626), essential oil percentage (0.570), cis-sabinene hydrate (0.468), EO yield (0.413), and α-pinene (0.424), while strong negative loadings were observed for linalool (−0.739), terpinen-4-ol (−0.680), *γ*-terpinene (−0.553), *α*-terpineol (−0.557), *p*-cymene (−0.516), and camphene (−0.507). This pattern indicates that PC2 primarily separated treatments based on qualitative shifts in essential oil composition, particularly oxygenated monoterpenes versus hydrocarbon monoterpenes. The PCA clearly distinguished two major response gradients: PC1 reflected a yield–high-terpenoid axis, where higher biomass and EO yield were positively associated with *1,8*-cineole, *E*-caryophyllene, carvacrol, and *α*-thujene, whereas PC2 represented a compositional trade-off axis, contrasting myrcene-, borneol-, and cis-sabinene hydrate–rich profiles against linalool-, terpinen-4-ol–, and *γ*-terpinene–dominated profiles.

The PCA biplot revealed clear treatment-dependent clustering patterns. Treatments positioned in the first quadrant (F2 ×D2 ×G2; F2 ×D3 ×G2; F2 ×D2 ×G3; F2 ×D2 ×G4; F2 ×D2 ×G1; F1 ×D2 ×G2) were closely associated with higher fresh and dry yields, EO yield, and carvacrol-related compounds, suggesting that these fertilizer × density × genotype combinations improved both productivity and desirable essential oil profiles. Conversely, treatments of F2 ×D1 ×G1; F2 ×D1 ×G2; F1 ×D2 ×G2; F1 ×D1 × G3; F2 ×D1 ×G3; F1 ×D1 × G1; F1 ×D1 × G4; and F2 ×D1 ×G4 showed stronger associations with thymol, myrcene, and linalyl acetate, reflecting lower yield potential but distinct qualitative oil characteristics. Treatments in the fourth quadrant were mainly related to cymene and monoterpene hydrocarbons, indicating stress- or density-mediated shifts in secondary metabolism.

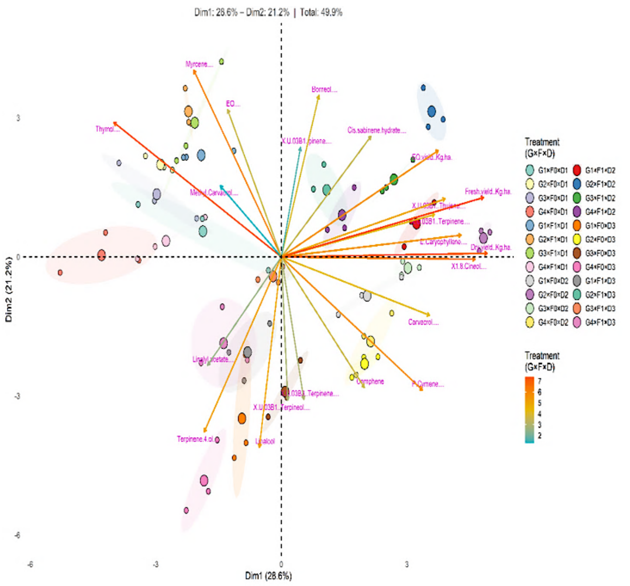

Fig. 7 The PCA diagram for PC1 and PC2 in the *Thymus kotschyanus* genotypes cultivated under different organic fertilizer × plant density. G1 – G4: genotypes 1 – 4, F1: non manure application, F2: manure application, D1 – D3: low, medium, and high plant density (4, 6, and 8 plant/m, respectively).

Clustering of Treatment and Variables

The clustered heatmap (Fig. 8), based on treatment means, illustrates clear multivariate differentiation among the combined fertilizer × plant density × genotype (F × D × G) treatments, confirming strong interactive effects on both agronomic and essential oil–related traits. Hierarchical clustering separated the traits into distinct functional groups and simultaneously grouped treatments according to their biochemical and yield responses. For traits, two major clusters were evident. The first cluster comprised yield-related traits and oxygenated sesquiterpenes, including essential oil yield, fresh yield, dry yield, *1,8*-cineole, *α*-thujene, and *E*-caryophyllene, which exhibited generally higher standardized values in several treatments, particularly under manure application (F1) combined with moderate to high plant density (D2–D3). This clustering indicates a close association between biomass production and enhanced accumulation of specific oxygenated terpenes. The second major trait cluster included monoterpene hydrocarbons and alcohols, such as thymol, myrcene, methyl carvacrol, *α*-pinene, camphene, *p*-cymene, linalool, and terpinen-4-ol, suggesting coordinated regulation of these compounds across treatments. Treatment clustering revealed a pronounced separation primarily driven by fertilizer application and plant density, with genotype-specific modulation within each group. Treatments combining fertilizer application (F1) with medium density (D2), particularly in G1 and G3 genotypes, clustered together and were characterized by elevated EO yield, fresh and dry biomass, and higher levels of carvacrol, *E*-caryophyllene, and *1,8*-cineole. In contrast, treatments under non-fertilized conditions (F0) and high density (D3), especially in G2 and G4, formed a separate cluster associated with lower biomass and EO yield, but relatively higher accumulation of some monoterpenes such as *p*-cymene, *γ*-terpinene, linalool, and terpinen-4-ol. This pattern suggests a density-induced metabolic shift toward precursor and stress-responsive compounds when nutrient availability is limited. Genotypic effects were evident within each fertilizer–density cluster. G3 and G1 generally showed stronger positive responses to fertilization, expressed as increased yield and oxygenated terpene content, whereas G2 and G4 exhibited more stable but lower overall responses, with a tendency toward higher monoterpene hydrocarbon accumulation.

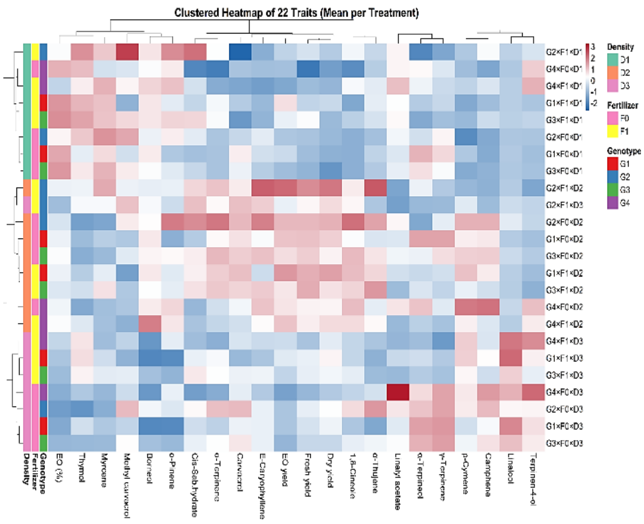

Fig. 8 The cluster diagram for different treatments (genotype× organic fertilizer × row spacing) and variables (yield and EO chemical compounds) of *Thymus kotschyanus.* G1 – G4: genotypes, F0: non manure application, F1: manure application, D1-D3: plant density 4,6, and 8 plant/m, respectively.

This indicates genotype-dependent plasticity in both growth and secondary metabolism. Overall, the clustered heatmap clearly demonstrates that fertilizer and plant density are the dominant drivers of trait variation, while genotype modulates the magnitude and direction of these responses.

The Contour Analysis for Relation between EO yield, EO Percentage, Plant Fresh Yield, and main EO Chemical Compounds

The trend between essential oil (EO) content as a function of carvacrol and thymol (Fig. 9a) showed that essential oil content is strongly structured by the combined proportions of carvacrol and thymol. The highest EO values (5.0–5.5%) are characterized by moderate carvacrol levels (approximately 6–8%) and higher thymol contents (62–66%). In contrast, higher carvacrol levels (8–9%) and lower thymol (56–58%) correspond to lower EO percentages (<4.0%). This pattern suggests a synergistic balance between carvacrol and thymol, where neither compound alone maximizes EO accumulation; instead, intermediate carvacrol combined with elevated thymol encouraged higher EO concentration. The relation between *p*-cymene percent as a function of carvacrol and thymol showed that the *p*-cymene concentration largely influenced by thymol content and vis versa, where higher *p*-cymene contents (9–10%), occurring at lower thymol levels (approximately 50–55%), particularly when carvacrol content is moderate to high (8–11%) (Fig. 9b).

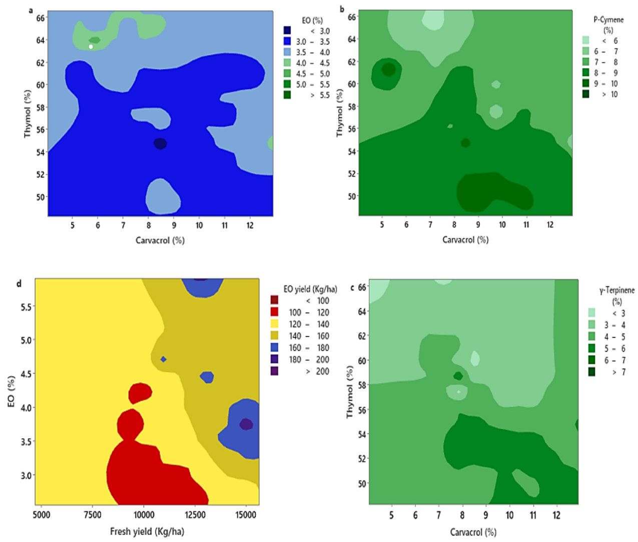

Fig. 9 The Contour Plot of EO percent vs thymol content and carvacrol content (a), *p*-cymene vs thymol and carvacrol content (b), γ-terpinene vs thymol and carvacrol content (c), and EO yield vs EO percent and plant fresh weight (d) in *Thymus kotschyanus* plants cultivated under different row spacing and manure application.

As thymol increases beyond 60%, *p*-cymene concentrations decrease (7–8%), regardless of carvacrol level. This inverse relationship is consistent with the biochemical role of *p*-cymene as a precursor in the biosynthetic pathway leading to thymol and carvacrol, indicating that greater conversion to phenolic monoterpenes is associated with reduced residual *p*-cymene. Also, *γ*-terpinene exhibits a distribution pattern similar to that of *p*-cymene, with higher concentrations (6–7%) observed at lower thymol levels and relatively higher carvacrol contents (Fig 9c). As thymol increases toward 60–66%, *γ*-terpinene declines to values below 4–5%. This trend supports the notion that *γ*-terpinene, another upstream precursor, is progressively depleted as the biosynthetic flux shifts toward oxygenated monoterpenes. The contours further indicate that thymol exerts a stronger control over γ-terpinene levels than carvacrol, highlighting thymol's central role in regulating precursor–product relationships. Finally, the contour map of EO yield as a function of fresh yield and EO percent (Fig. 9d) demonstrated that maximum EO production (180–200 kg ha^{-1}) is achieved only when high fresh biomass yield (13,000–15,000 kg ha^{-1}) coincides with elevated EO concentration (>5.0%). High fresh yield alone does not guarantee high EO yield if EO percentage remains low (<3.5–4.0%). These results emphasize that EO yield is jointly determined by biomass production and oil concentration, and that agronomic nutrietinal conditions optimizing both parameters simultaneously are critical for maximizing overall EO output.

Table 4 The contribution of variables in components and eigenvalues and variance portion in PC1 and PC2

Trait	PC1	PC2	Trait	PC1	PC2
Thymol	-0.72	0.52	*Cis*-sabinene hydrate	0.39	0.47
Carvacrol	0.64	-0.23	Linalool	-0.09	-0.74
p-Cymene	0.61	-0.52	Myrcene	-0.38	0.72
Borneol	0.16	0.63	Terpinen-4-ol	-0.33	-0.68
α-Terpineol	0.03	-0.56	Linalyl acetate	-0.32	-0.42
γ-Terpinene	0.10	-0.55	*α*-Terpinene	0.66	0.16
α-Pinene	0.08	0.42	Methyl carvacrol	-0.26	0.28
E-Caryophyllene	0.77	0.08	Essential oil	-0.23	0.57
Camphene	0.36	-0.51	Fresh yield	0.87	0.23
1,8-Cineole	0.83	-0.01	Dry yield	0.88	0.01
α-Thujene	0.70	0.23	EO yield	0.67	0.41
Eigenvalue	6.30	4.67	Eigenvalue	6.30	4.67
Percentage of variance	28.63	21.24	Percentage of variance	28.63	21.24
Cumulative variance	28.63	49.86	Cumulative variance	28.63	49.86

DISCUSSION

The essential oil (EO) composition were interactively influenced by genotype, plant density, and organic fertilization under rainfed conditions. The intraction effect of genotype × plant density × fertilizer levels was not significant for yield traits, however plant yield and EO yiled were significantly affected in response plant density and organic fertilization. Optimizing plant density and row spacing is critical to improving the yield and nutritional quality of crops [49]. The superiority of medium planting density (6 plants m^{-2}) combined with 50 t ha^{-1} farmyard manure for maximizing fresh biomass, dry biomass, and EO yield corroborates broader findings that appropriate plant spacing optimizes light interception, nutrient capture, and water use efficiency in medicinal plants [46, 50]. Specifically, optimum densities reduce excessive competition while maintaining high canopy cover, which together enhance total photosynthetic capacity and assimilate partitioning into EO-secreting structures. Similar density effects have been documented in basil, where low density enhanced plant-level biomass accumulation and high– density increased field-level yields [51].

Organic fertilization enhanced yield traits and modulated EO composition, likely through improved soil structure, increased nutrient availability, and enhanced water retention factors known to enhance nutrient uptake and photosynthetic efficiency [50, 52-57]. Organic amendments enhance soil organic carbon (OC), gradually release nitrogen, phosphorus, and potassium integral to chlorophyll synthesis, ATP/NADPH generation, and carbon skeleton formation, thus bolstering both primary and secondary metabolism, including terpenoid biosynthesis [58-61]. Organic fertilizers can enhance nutrient uptake, resulting increase plant growth and EO yield without the synthetic fertilizers, consistent with sustainable agriculture principles [62, 63].

Organic fertilization has been shown to improve soil physical and chemical properties and can increase nutrient uptake and water retention. The key drivers of biomass and EO yield under semi-arid conditions [47, 64]. These outcomes support sustainable cultivation strategies that align with findings from other essential oil-bearing species, where organic matter enhanced vegetative growth, phytochemicals, and EO yield and components [50, 65-68].

The essential oil of *T. kotschyanus* contains many important phenolic compounds with antibacterial and antioxidant activity. In the present study, the compounds thymol, carvacrol, *p*-cymene, *α*-terpineol, *γ*–terpinene, and borneol were identified as main components. Comparably, in a research, the thirty-eight compounds were identified from EO of different populations of *T. kotschyanus* which thymol (6.8-66.15%), carvacrol (2.38-20.06%), linalool (1.39-22.23%), *α*-terpineol (0.16-11.64%), and geraniol (0.36-11.37%) were the major compounds [19]. Also, in another study, ten chemicals were identified in the essential oil of wild-growing *T. kotschyanus* in Iran, and the main constituents were thymol (89.08%) and *γ*-Terpinene (4.62%) [20].

The distinct shifts in EO composition between treatments highlight how secondary metabolism responds to physiological cues. In comparing EO composition, the predominance of thymol and carvacrol in *T. kotschyanus* corroborates recent profiling studies of wild *T. kotschyanus* populations reporting high thymol variability (up to ~81%) and diverse chemotypes across regions [21]. Thymol and carvacrol predominance across treatments parallels reports in Lamiaceae– related species, where chemotypic profiles remain stable yet responsive to management practices [2, 46, 47]. However, the treatment-dependent shifts in other monoterpenes (e.g., *α*-terpineol, linalool, borneol) underscore the plasticity of terpenoid biosynthesis in response to nutrient and density cues. This echoes findings in *Thymus vulgaris* and allied species, where management factors such as water status or bio-fertilizers significantly alter both quantitative EO yield and specific compound abundances (e.g., thymol, *p*-cymene, *γ*-terpinene) under stress conditions [69].

The variations in oxygenated monoterpenes (e.g., linalool, linalyl acetate) and hydrocarbons (e.g., *α*-pinene, *γ*-terpinene) reflect altered carbon allocation and enzymatic flux within the methylerythritol phosphate (MEP) and mevalonic acid pathways under different density/fertilizer regimes. For example, high density may increase abiotic stress signals (e.g., shading, water limitation), which typically upregulate stress-responsive terpene synthases and reactive oxygen species (ROS) signaling that shifts metabolic flux toward defensive or volatile compounds [64, 70].

Conversely, medium density and nutrient sufficiency favor robust primary metabolism, enabling greater partitioning of carbon and ATP toward secondary metabolites such as *E*-caryophyllene and *1,8*-cineole, compounds positively correlated with yield in this study. Similar variability has been documented across Thymus taxa, indicating that biosynthesis of specific terpenoids is plastic and responsive to cultivation practices [70].

Multivariate analyses revealed clear groupings of treatments that integrate productivity with specific EO profiles, underscoring the potential to define and select functional chemotypes. Genotype 2 consistently exhibited high yields and favorable terpene profiles across medium density × manure treatments, shaping phenotypic expression in aromatic crops [70, 71]. These multivariate patterns mirror recent work in organic Thymus cultivation, where chemotypes with distinct main constituents responded differently to growing conditions, suggesting that both agronomy and genetic selection are necessary to optimize cultivar performance for specific industrial goals.

Physiologically, increased biomass and EO yield under manure application likely reflect enhanced leaf area and oil gland development, offering greater sites for terpenoid synthesis and storage. Organic matter also influences stomatal behavior and water use efficiency, which can indirectly affect carbon assimilation and secondary metabolite formation [72, 73]. Biochemically, nutrient availability influences enzyme activities involved in isoprenoid precursor synthesis (e.g., deoxy-D-xylulose 5-phosphate synthase (DXS) is the first and main rate-determining enzyme of the MEP pathway), affecting downstream terpenoid accumulation[74, 75]. Additionally, stress modulation by planting density may shift source–sink relations, with denser stands favoring protective volatiles, while moderate stands allocate more resources to growth and economically important terpenoids [76, 77].

The present findings are consistent with previous studies on other *Lamiaceae* species, where cow manure application significantly enhanced essential oil content and yield components in *Satureja mutica* and *S. bachtiarica* [78-80]. Similarly, Esmaielpour et al. (2019) reported that cow manure supplementation increased the essential oil percentage in summer savory (*Satureja hortensis*) [81].

Overall, the integration of genotype selection, optimal planting density, and organic fertilization promotes sustainable *T. kotschyanus* cultivation. Our findings provide a mechanistic basis for tailoring agronomic practices to maximize both biomass and essential oil yield, offering a viable strategy to reduce wild thymus harvesting pressure while maintaining high commercial thymus quality.

The present study highlighted the critical interplay between thymol, carvacrol, and essential oil (EO) accumulation in *Thymus kotschyanus*, showing that EO content is strongly structured by their relative proportions rather than the concentration of any single compound. Contour analysis demonstrated that high EO percentages (5.0–5.5%) occurred with intermediate carvacrol (6–8%) and elevated thymol (62–66%), while higher carvacrol coupled with lower thymol corresponded to reduced EO (<4.0%). This synergistic pattern aligns with observations in related Lamiaceae species, where chemotype balance influences overall oil yield [47].

The inverse relationship between *p*-cymene and thymol confirmed the biochemical role of *p*-cymene as a precursor in the monoterpene pathway, where *p*-cymene and *γ*-terpinene are sequentially converted into thymol and carvacrol via monoterpene synthases and cytochrome P450 enzymes [19, 82, 83]. As thymol increases, residual *p*-cymene and *γ*-terpinene decline, reflecting intensified flux through oxygenation reactions. The stronger influence of thymol over *γ*-terpinene suggests that downstream enzymatic control exerts priority in steering the pathway toward phenolic.

Achieving synergistic balances between major phenolics and maximizing biomass formation through optimized density and organic nutrition is essential for commercial EO production under rain-fed conditions. Present findings have practical implications for the cultivation of *T. kotschyanus* as a high-value aromatic crop. However, this study's scope was restricted to a single environment and short-season observation, limiting direct extrapolation to other climates or multi-season dynamics. Future research should include multi-location and temporal replicates to assess stability of metabolite responses. Additionally, evaluating diverse organic and biological amendments (e.g., compost, biochar, plant growth-promoting rhizobacteria) may offer further.

The reduction in *γ*-terpinene and *p*-cymene content in response to manure application is likely associated with enhanced enzymatic activity in the isoprenoid/monoterpene pathway as a result of improved nutrient availability, particularly micronutrients such as Fe, Zn, and B. These nutrients stimulate key enzymes (e.g., terpene synthases and cytochrome P450 monooxygenases) involved in converting *γ*-terpinene and *p*-cymene into downstream oxygenated monoterpenes such as thymol and carvacrol. Consequently, greater enzyme activity promotes the metabolic flux toward these final phenolic monoterpenes, reducing the concentration of their precursor compounds. Similar trends have been reported in *Satureja mutica* and *Satureja spicigera* [2, 47]. Pearson's correlation analysis revealed clear and biologically meaningful association patterns among chemical constituents and yield-related traits. Thymol showed strong negative correlations with major monoterpenes, particularly carvacrol, *p*-cymene, and *γ*-terpinene, indicating a trade-off in their biosynthetic accumulation, while its positive association with myrcene and essential oil content highlights thymol-rich profiles as contributors to higher oil concentration. The PCA clearly demonstrated that manure application and plant density exert a strong influence on both quantitative (yield and EO yield) and qualitative (essential oil composition) traits. The close alignment between specific treatments and key metabolites confirmed that manure fertilizer and plant density interact with genotype to regulate terpene biosynthesis pathways, thereby determining the final essential oil profile and agronomic performance. This multivariate separation highlights the potential for targeted management strategies to optimize both yield and essential oil quality simultaneously.

CONCLUSION

Our findings confirm that both agronomic performance and essential oil characteristics of *Thymus kotschyanus* are significantly shaped by genotype, planting density, and organic fertilization. Although essential oil percentage varied within a relatively narrow range, large differences in biomass meant that essential oil yield was mainly determined by dry matter production. Among the evaluated treatments, medium planting density combined with 50 t ha^{-1} farmyard manure provided the most favorable balance between growth and oil productivity, particularly for genotype 2, which consistently outperformed the others in fresh biomass, dry biomass, and total essential oil yield. Thymol and carvacrol remained the main constituents of the oil across treatments, while compositional shifts in other monoterpenes reflected changes in plant density and nutrient availability. Multivariate analyses reinforced these findings by separating genotypes according to both oil profile and yield-related traits, clearly distinguishing genotype 2 as the most promising candidate for cultivation under rainfed conditions. The strong positive correlations among fresh biomass, dry biomass, and essential oil yield further highlight the role of agronomic management in determining final oil output. Overall, the results demonstrate that optimizing planting density and adopting organic fertilizer strategies can substantially improve the domestication potential and sustainable production of *T. kotschyanus*. Medium-density planting with organic manure, especially when applied to genotype 2, represents an effective approach for maximizing yield while preserving desirable essential oil quality in dryland systems.

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Data availability

Data is provided within the manuscript.

Ethics approval and consent to participate

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Competing interests

The authors declare no competing interests.

Seed preparation statement

The seeds of different *Thymus danensis* genotypes were prepared from the Research Institute of Forests and Rangelands (RIFR), Tehran, Iran.

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