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# Interactive Effects of Organic Manure and Planting Density on Growth, Essential Oil Production, and Chemotype Expression in Iranian *Thymus daenensis* Genotypes Under Dry Farming

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## ABSTRACT
The cultivation of *Thymus daenensis*, an endemic Iranian medicinal species rich in phenolic monoterpenes, requires optimization under rainfed conditions to ensure sustainability and productivity. This study investigated the interactive effects of organic manure application (0 and 50 t $ha^{-1}$), planting density (4, 6, and 8 plants $m^{-2}$), and genotype (four distinct genotypes) on biomass production, essential oil (EO) yield, and EO composition in a dry farming system in western Iran. A split-plot factorial experiment (CRBD, three replications) was conducted over the 2021–2022 growing seasons. Results indicated that plant density, genotype, and manure application significantly influenced fresh and dry biomass yields as well as EO yield. The combination of medium planting density (6 plants $m^{-2}$) with manure application generally maximized productivity, yielding up to 16,398 g $m^{-2}$ fresh biomass and 7,122 g $m^{-2}$ dry biomass. EO concentration was less variable but ranged from 4.30% to 6.07%, while EO yield increased markedly with manure and optimal density, reaching 435.3 Kg $ha^{-1}$. GC and GC–MS analyses identified thymol, carvacrol, γ-terpinene, p-cymene, and α-terpineol as major compounds. Thymol content varied widely (59.66–72.76%) and was highest under low-density, unfertilized conditions. In contrast, monoterpene hydrocarbons such as p-cymene and γ-terpinene increased under manure application and higher densities. Multivariate analyses (PCA, correlation, clustering) revealed a consistent trade-off: agronomic practices that enhanced biomass and EO yield promoted monoterpene-rich oils, whereas resource-limited conditions (low density, no manure) favored the accumulation of high-value phenolic monoterpenes like thymol and carvacrol. Significant genotype × fertilization × density interactions underscored the need for genotype-specific management strategies. We conclude that medium planting density combined with organic manure optimizes biomass and EO yield under rainfed conditions, while lower inputs enhance EO quality by increasing phenolic content. These findings provide a basis for tailoring cultivation practices according to production goals—whether maximizing yield or obtaining phenolic-rich essential oils for pharmaceutical and industrial uses.
**Keywords:** Rainfed agriculture, Phenolic monoterpenes, Biomass allocation, Organic fertilization, Chemotype plasticity

## INTRODUCTION
The plants of mint family including Mentha, Thymus, Satureja, Nepeta, Salvia, etc. are the major source of bioactive compounds that are widely used in medicinal, sanitary, and food industries [1, 2]. Their biologically active constituents including flavonoids, terpenoids, phenolics and alkaloids, are characterized by antioxidant, anti-inflammatory, and antibacterial assets [3-6].

Thymus, one of the most well-known Lamiaceae plants, besides applying in traditional medicine and cooking as spice, but also tremendously in medicine, food and hygienic industries [7]. The essential oil of thyme is an antifungal, antibacterial, and anti-inflammatory agent and its sedative, antiseptic, antioxidative, expectorant, and antispasmodic properties have been proven [8-10]. The EO of thyme contains a wide range of phytochemicals such as thymol, *p*-cymene, *γ*-terpinene, linalool, carvacrol, geraniol, nerol, nerolidol, caryophyllene oxide, myrcene, germacrene, etc. The main compounds of EO are diverse, depending on genetic variation, feeding, and environmental conditions. However, the major compounds generally belong to phenolic monoterpenes or sesquiterpenes [11-15].

Intraspecific competition for environmental growth factors leads to a reduction in plant yield and economic productivity [16-18]. Planting density is a key factor influencing competition among plants for the absorption of nutrients, water, and sunlight [19, 20]. Row spacing narrower than the optimum intensifies interplant competition, thereby limiting access to essential resources and restricting growth [19]. Conversely, row spacing wider than the optimum may enhance the growth of individual plants but can ultimately reduce total yield per unit area [21-23]. Studies on several Lamiaceae species have shown that increasing plant density up to an optimal threshold results in a linear increase in total biomass and essential oil (EO) production. However, densities beyond this optimum cause a decline in both plant yield and EO yield [1, 24]. Thus, deviations from the optimal planting density—either higher or lower—lead to reduced productivity. Therefore, determining the optimal planting density for each plant species under specific environmental conditions is crucial for achieving maximum yield [20].

Achieving maximum on-farm performance requires optimal and readily available levels of essential nutrients for crop growth. Although chemical fertilization is a common strategy for improving crop productivity, excessive use of chemical fertilizers can have detrimental effects on soil structure, microbial activity, water-holding capacity, nutrient balance, and overall soil fertility, as well as causing environmental contamination [25-28]. Consequently, there is a growing need to adopt sustainable and environmentally friendly agricultural practices, such as the use of organic fertilizers, which support optimal plant nutrition while mitigating the negative impacts of synthetic fertilizers [29-32]. Organic fertilizers, derived from plant or animal residues, enhance soil fertility by improving chemical and biochemical soil properties, positively affecting plant nutrition, biomass production, and overall soil health [33-38].The application of animal manure increases soil organic matter content, improves the uptake of micronutrients, and gradually converts N, P, and K into plant-available forms, thereby enhancing nutrient use efficiency [39-41]. Moreover, increased soil organic matter improves water infiltration and soil moisture retention, which is particularly beneficial under dryland and drought-prone conditions [40].

*Thymus daenensis* Celak., an Iranian endemic plant, grows exclusively in western and southwestern Iran [42, 43]. This widely used medicinal and culinary herb is characterized by a diverse array of bioactive constituents and aromatic compounds [44-46]. This species is particularly rich in secondary metabolites, including phenolic compounds, flavonoids, and substantial levels of terpenoids [47, 48]. The essential oil of *T. daenensis* is dominated by monoterpenes, notably thymol and carvacrol, as well as sesquiterpenes such as $\beta$-caryophyllene and $\beta$-caryophyllene oxide [47].

Due to the excessive harvesting of *T. daenensis* from natural vegetation, this species is currently under serious threat. Moreover, wild populations of *T. daenensis* growing under semiarid conditions generally exhibit low essential oil yield and reduced thymol content [49]. Consequently, the domestication of this species has attracted considerable attention from Iranian agricultural researchers, with particular emphasis on its cultivation under dryland farming systems. In addition, given the high industrial and economic importance of *Thymus daenensis*, along with the growing demand for sustainable cropping practices in arid and semiarid regions and the need to minimize reliance on chemical fertilizers, the present study aimed to evaluate the domestication potential of this species under rainfed conditions while simultaneously assessing biomass production and essential oil yield in response to organic manure application.

## MATERIALS AND METHODS

### Experimental Farm

This field experiment was carried out at the Mahidasht Research Station of the Kermanshah Agricultural and Natural Resources Research and Education Center (34°16′ N, 46°50′ E; 1,380 m a.s.l.). According to comprehensive soil survey analyses, the experimental site is characterized as Fine, Mixed, Thermic Vertic Calcixerepts based on the USDA Soil Taxonomy classification. The soil exhibits a heavy to very heavy texture, ranging from silty clay to silty clay loam. The site receives a long-term (10-year) mean annual precipitation of approximately 335 mm. Climatic conditions during the 2020-2022 growing season are illustrated by the ombrothermic diagram presented in Fig. 1.

### Plant Materials

Four genotypes of *Thymus daenensis* were investigated in the present study. Seeds of the selected populations were supplied by the Gene Bank of the Research Institute of Forests and Rangelands, Tehran, Iran. The climatic specification of experimental farm is presented in Fig 1. The plant materials were evaluated over the 2021–2022 growing seasons with respect to yield performance and phytochemical characteristics. Detailed descriptions of the studied populations are summarized in Table 1.

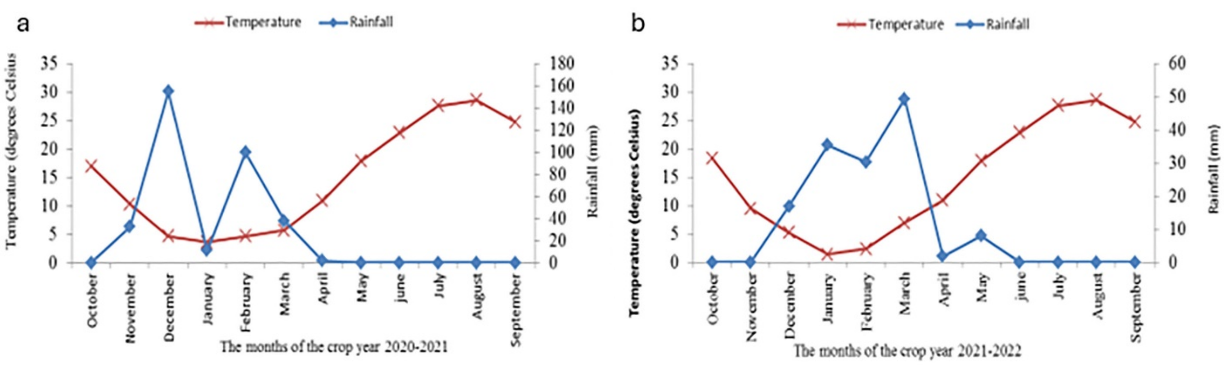



**Fig. 1** The monthly ombrothermic diagram of the crop years 2020-2021 and 2021-2022

**Table 1** The origin and specifications of *Thymus daenensis* seeds prepared from Research Institute of Forests and Rangelands, Iran

| Species | Origin of seeds preparation | Gen bank number | Genotype Code | Primary origin of seeds collection |
|---|---|---|---|---|
| *Thymus daenensis* | RIFR gen bank, Iran | 18209 | G1 | Boien, Daran, Isfahan,Iran |
| *Thymus daenensis* | RIFR gen bank, Iran | 27221 | G2 | Chadegan, Isfahan, Iran |
| *Thymus daenensis* | RIFR gen bank, Iran | 14245 | G3 | Khoram–Abad, Lorestan, Iran |
| *Thymus daenensis* | RIFR gen bank, Iran | 10126 | G4 | Faraidon–Shahr, Isfahan, Iran |

### Experimental Design

The study was implemented as 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 tratments inclues the organic fertilization in tow regimes: no manure application (control) and the application of farmyard manure at a rate of 50 t ha⁻¹, three planting densities (4, 6, and 8 plants m⁻²) and four genotypes of *Thymus daenensis*, a wild thyme species indigenous to Iran. Overall, 72 experimental units were established (2 fertilizer levels × 3 planting densities × 4 genotypes × 3 replications).

### Land Preparation

Primary tillage was performed by plowing in early autumn, followed by two perpendicular diskings to ensure adequate soil pulverization. Prior to transplanting, the field surface was leveled, and ridges and furrows were constructed at 50 cm intervals. In plots receiving organic fertilization, well-decomposed farmyard manure was manually incorporated into the soil at a rate of 7.5 kg plot⁻¹ (equivalent to 50 t ha⁻¹), applied uniformly within the furrows according to the experimental design.

### Seed Cultivation

Seeds of the four *T. daenensis* genotypes were sown in a 1:1 (v/v) peat–coco peat growth medium under controlled greenhouse conditions (day/night temperature of 24 ± 2 °C / 18 ± 2 °C and 60% relative humidity) in late September. Following successful seedling establishment, uniform and healthy transplants were transplanted to the field in mid-November. The physicochemical properties of the soil at the experimental site are reported in Table 2. Each subplot consisted of four rows, each 3 m in length, giving a total plot area of 3.0 × 1.5 m (4.5 m²). Inter-row spacing was fixed at 50 cm, while intra-row spacing was adjusted to achieve the designated planting densities: 25 cm for 8 plants m⁻², 35 cm for 6 plants m⁻², and 50 cm for 4 plants m⁻².

**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

Weed control was carried out manually at three stages during the growing season to ensure weed-free conditions and facilitate uniform crop establishment. No chemical fertilizers were applied during the course of the experiment. Given the rainfed nature of the study, crops relied exclusively on natural precipitation, and no supplemental irrigation was provided.

### Harvesting

In the second growing season, plants were harvested manually in a single harvest at the 50% flowering stage, which is widely recognized as the optimum phenological stage for maximum essential oil accumulation.

### Fresh and Dry Biomass Determination

At 50% flowering, all plants within each plot were cut at a height of 5 cm above the soil surface. To minimize border effects, 0.5 m from both ends of each plot was excluded from sampling. Fresh biomass was immediately determined using a digital balance with a precision of 0.5 g and expressed as fresh weight (g m⁻²).

The harvested plant material was then shade-dried at ambient temperature for seven days until a constant weight was attained. Dry biomass was measured using the same balance and recorded as dry weight (g m⁻²).

### Essential Oil Content

Shade-dried aerial parts were finely ground, and an 80 g subsample was collected from each experimental unit, resulting in a total of 72 samples. For all smaples (without replicate), essential oil extraction was carried out by hydrodistillation using a Clevenger-type apparatus for 2 h. Essential oil content was calculated on a dry matter basis and expressed as a percentage (w/w).

### Identification of Essential Oil Constituents

The qualitative and quantitative composition of the essential oils was determined using gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS).

GC analysis was performed using a Shimadzu GC-9A gas chromatograph equipped with a flame ionization detector (FID) and a Chromatopac C-R3A data processor. Separation was achieved on a DB-5 capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness). The oven temperature was programmed from 60 °C to 180 °C at a heating rate of 3 °C min⁻¹. Injector and detector temperatures were maintained at 300 °C and 280 °C, respectively. Helium was used as the carrier gas at a constant pressure of 1.5 kg cm⁻².

GC–MS analysis was conducted using a Varian 3400 gas chromatograph coupled to an ion-trap mass spectrometer and equipped with a DB-5 capillary column of identical specifications. The oven temperature program was the same as that used for GC analysis. The injector temperature was set at 10 °C above the final oven temperature. Helium served as the carrier gas at a pressure of 1.5 kg cm⁻². Mass spectra were acquired with a scan time of 1 s under electron ionization at 70 eV.

Identification of individual compounds was based on the calculation of Kovats retention indices (RI) using a homologous series of n-alkanes analyzed under identical chromatographic conditions. The calculated retention indices and mass spectra were compared with published reference data and confirmed by comparison with authentic standards and computerized spectral libraries [50].

### Statistical Analysis

All data were subjected to statistical analysis using R software (version 4.3.1). Univariate and multivariate analyses included analysis of variance (ANOVA) appropriate for the split-plot experimental design, mean separation using Tukey's honestly significant difference (HSD) test, GGE biplot analysis, Pearson's correlation analysis, and hierarchical cluster analysis.

## RESULTS

The combined analysis of variance revealed that most agronomic and phytochemical traits of *Thymus daenensis* were significantly influenced by genetic and management factors, with numerous notable interaction effects.Total essential oil (EO) percentage was significantly affected by density and replication effects, whereas fertilizer, genotype, and all interaction terms were not significant, indicating relatively stable EO concentration across genotypes and fertilizer treatments. Fresh yield, dry yield, and EO yield were strongly influenced by density and genotype ($P < 0.001$), with fertilizer exerting a major effect on biomass production and EO yield. Notably, EO yield was significantly affected by fertilizer, density, and genotype as main effects, but none of the interaction terms were significant, suggesting additive rather than interactive control of EO productivity. Overall, these findings demonstrate that while oil composition is largely genotype- and interaction-driven, EO yield is primarily governed by biomass responses to agronomic factors, especially plant density.

### EO Percent

The highest content of EO was observed in genotype 2 under low density cultivation without manure application (6.07 ± 0.23, group a), while the lowest arose in genotype 4 in response to manure application and high planting density × (4.30 ± 0.34) (Fig. 2a). Low-density plantings generally led to slightly higher EO percentages, while high-density plantings slightly reduced EO content.

### Essential Oil Yield

The maximum value was recorded in genotype 1 at medium density plantation + manure application (435.3 ± 55.8, kg ha$^{-1}$, group a), and the minimum in genotype 3 under manure application and low density (103.1 ± 0.63, kg ha$^{-1}$, group f) (Fig. 2b). EO yield increased substantially in medium-density planting and the application of manure, while low-density plantings generally resulted in lower EO yield. EO yield under several medium-density + manure supplementation treatments was significantly higher than its values under low-density without manure application, highlighting the importance of optimizing both fertilizer application and plant density to maximize essential oil production.

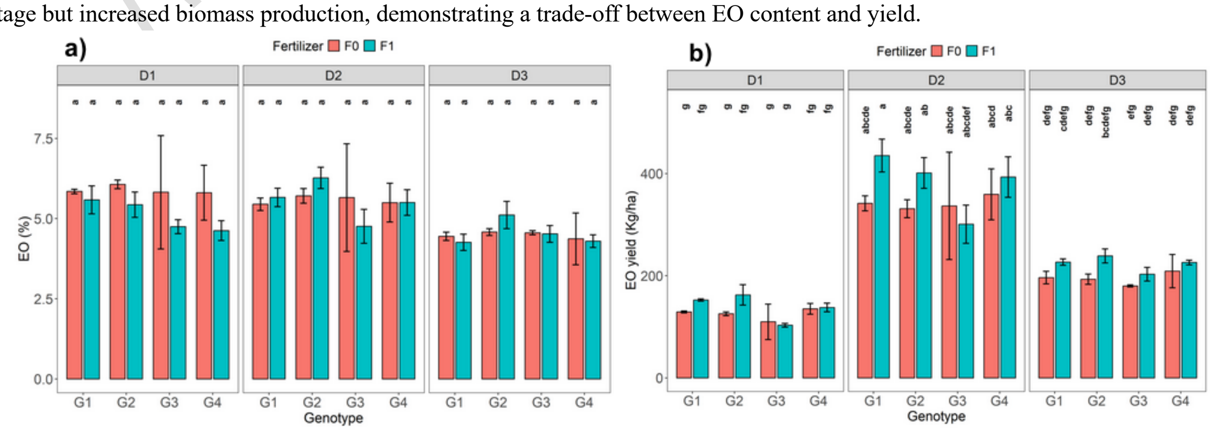
### Plant Fresh Yield

The maximum value of fresh yield was recorded in genotype 4 in response to manure application under medium density planting (16,398.02 ± 133.72, group a), and the minimum in genotype 3 under low density without manure (6,104.45 ± 182.58, group i) (Fig. 2c). High-density plantings typically increased fresh yield relative to low-density treatments within the same genotype and fertilizer, although medium density often produced the highest yields. Manure supplementation generally enhanced fresh yield compared to control across all genotypes and densities, and Tukey post-hoc comparisons highlighted significant differences among several treatments.

### Dry Yield

The uppermost dry yield (7,121.93 ± 336.48, group ab) were obtained in genotype 4 under manure application and medium density, while the lowest was observed in Fertilizer 1 × Low density × Genotype 3 (1,869.22 ± 54.21, group j) (Fig. 2d). Dry yield followed a similar pattern to fresh yield, with medium-density plantings generally producing the highest biomass and low-density plantings the lowest. Manure supplementation enhanced dry yield across most genotypes.

In summary, manure application and medium-density plantings consistently enhanced fresh yield, dry yield, and essential oil yield, whereas low-density plantings tended to maximize EO concentration as a percentage of biomass. High-density planting generally reduced EO percentage but increased biomass production, demonstrating a trade-off between EO content and yield.



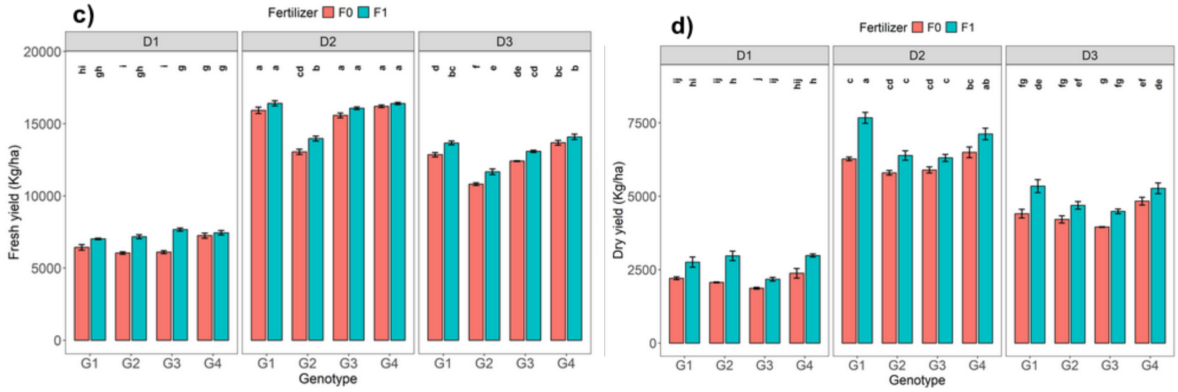



**Fig. 2** The effect of different organic fertilizers × plant density on essential oil content (a), essential oil yield (b), plant fresh yield (c), and dry yield (d) in four genotypes of *Thymus daenensis* (Tukey's Test, α = 0.05, r = 3, n = 10). D1, D2, and D3: respectively, low density (4 plant $m^{-2}$), medium density (6 plant $m^{-2}$), and high density (8 plant $m^{-2}$), G1 – G4: genotypes 1 – 4; Fertilizer 0: non-manure application and 1: manure application.

### Essential oil Phytochemical Compounds

The ANOVA revealed that thymol content was strongly influenced by plant density and genotype, with both factors showing highly significant effects ($P < 0.001$), whereas fertilizer alone had no significant effect. Importantly, all two-way interactions (fertilizer × density, fertilizer × genotype, and density × genotype) and the three-way interaction were significant, indicating that thymol accumulation depends on complex interactions among management practices and genetic background. Similarly, carvacrol concentration was significantly affected by density and genotype ($P < 0.001$), while fertilizer had no independent effect. However, several interaction terms involving fertilizer were significant, suggesting that fertilizer indirectly modulates carvacrol levels through its interaction with density and genotype rather than through a direct main effect.

The monoterpene hydrocarbons and oxygenated monoterpenes including *γ*-terpinene, *α*-terpineol, *p*-cymene, myrcene, methyl carvacrol, *α*-pinene, *α*-thujene, camphene, *1,8*-cineole, *cis*-sabinene hydrate, linalool, and terpinen-4-ol, consistently emerged as a dominant source of variation, often explaining highly significant proportions of the total variance ($P < 0.001$). Plant density also exerted a strong and significant influence on most of these compounds, while fertilizer effects were compound-specific: highly significant for *p*-cymene, myrcene, linalool, terpinen-4-ol, and *E*-caryophyllene, but non-significant for others such as camphene and some terpinene isomers. Numerous significant interaction effects, particularly density × genotype and fertilizer × genotype, indicate that secondary metabolite profiles are highly plastic and strongly conditioned by genotype-specific responses to agronomic factors.

For sesquiterpenes and minor constituents including *E*-caryophyllene and borneol, distinct response patterns were observed compared with monoterpenes. *E*-caryophyllene was strongly affected by fertilizer and genotype, while density alone was not significant, suggesting a greater sensitivity of sesquiterpene biosynthesis to nutrient availability. Borneol content, in contrast, was significantly affected by all main factors (fertilizer, density, and genotype) as well as by all interactions, highlighting its strong responsiveness to both genetic and nutrishments modulation. These results underscore that even minor compounds are subject to complex regulatory controls.

### Major Phytochemical Compounds

### Thymol

Thymol content was affected significantly by fertilizer × density × genotype interaction ($P \leq 0.05$). The highest thymol concentration was observed in genotype 1 under non– application of manure and high density (72.76 ± 1.91%) (Fig. 3a). In contrast, the lowest thymol content was detected in genotype 4 under manure application and high planting density (59.66 ± 1.16%), which was significantly lower than all other treatments. Overall, genotypes 1 and 3 consistently produced higher thymol, whereas genotype 4 predominantly had lower thymol, demonstrating strong genotypic control over thymol accumulation modulated by density and fertilizer.

### Carvacrol

Carvacrol concentration also showed clear variations among treatments. The maximum carvacrol level was recorded in genotype 4 at low cultivation density without fertilizer supplementation (6.58 ± 0.63%), which was significantly higher than carvacrol content in other genotypes and in response to all other treatments (Fig. 3b). Conversely, the minimum carvacrol concentration was found in genotype 2 under high density (3.03 ± 0.83%). Most treatments exhibited substantial overlap among genotypes 1–3 across densities. These results confirm that carvacrol accumulation is maximized under low-density conditions in genotype 4, whereas higher densities generally suppress carvacrol content regardless of manure application.

### *γ*-Terpinene

The highest *γ*-terpinene content (6.06 ± 0.05%) was observed in genotype 2 under medium density without manure application (group a). Similarly high values were recorded for genotype 4 in response to manure supplementation at high planting density (5.86 ± 0.64%), although these belonged to slightly lower but overlapping groups (ab) (Fig. 3c). In contrast, the lowest *γ*-terpinene concentration detected in genotype 1 under low density cultivation plus manure application (3.49 ± 0.23%), which differed significantly from high-performing treatments. The widespread variations in terpinene content across treatments indicate a strong and treatment-specific redirection of monoterpenes biosynthesis, particularly influenced by manure regime.

### *a*-terpineol

The *a*-terpineol content showed highly significant variations in response to treatments. The highest *a*-terpineol value was recorded in genotype 4 under manure application and high plant density (7.98 ± 0.15%), which was significantly greater than all other values. In contrast, the lowest *a*-terpineol concentration was observed in genotype 1 cultivated with low plant density plus manure supplementation (3.22 ± 0.10%) (Fig. 3d). Most remaining treatments produced intermediate values, reflecting moderate but statistically significant differences among management combinations. These findings indicated that *a*-terpineol accumulation is strongly promoted by manure supplementation under high-density conditions, particularly in genotype 4.

### *P*-cymene

*P*-cymene exhibited substantial variation in response to treatments. The highest *p*-cymene concentration was observed in genotype 4 under manure fertilization and medium density plantation (9.23 ± 0.64%), followed closely by the same genotype under manure fertilization and high density (8.26 ± 0.27%). In contrast, the lowest *p*-cymene value was recorded in genotype 1 in response to high planting density (3.52 ± 0.77%), which was significantly lower than most other values (Fig. 3e). The predominance of genotype 4 supports the inverse relationship between *p*-cymene and phenolic monoterpenes, particularly thymol, under intensified manure fertilization.

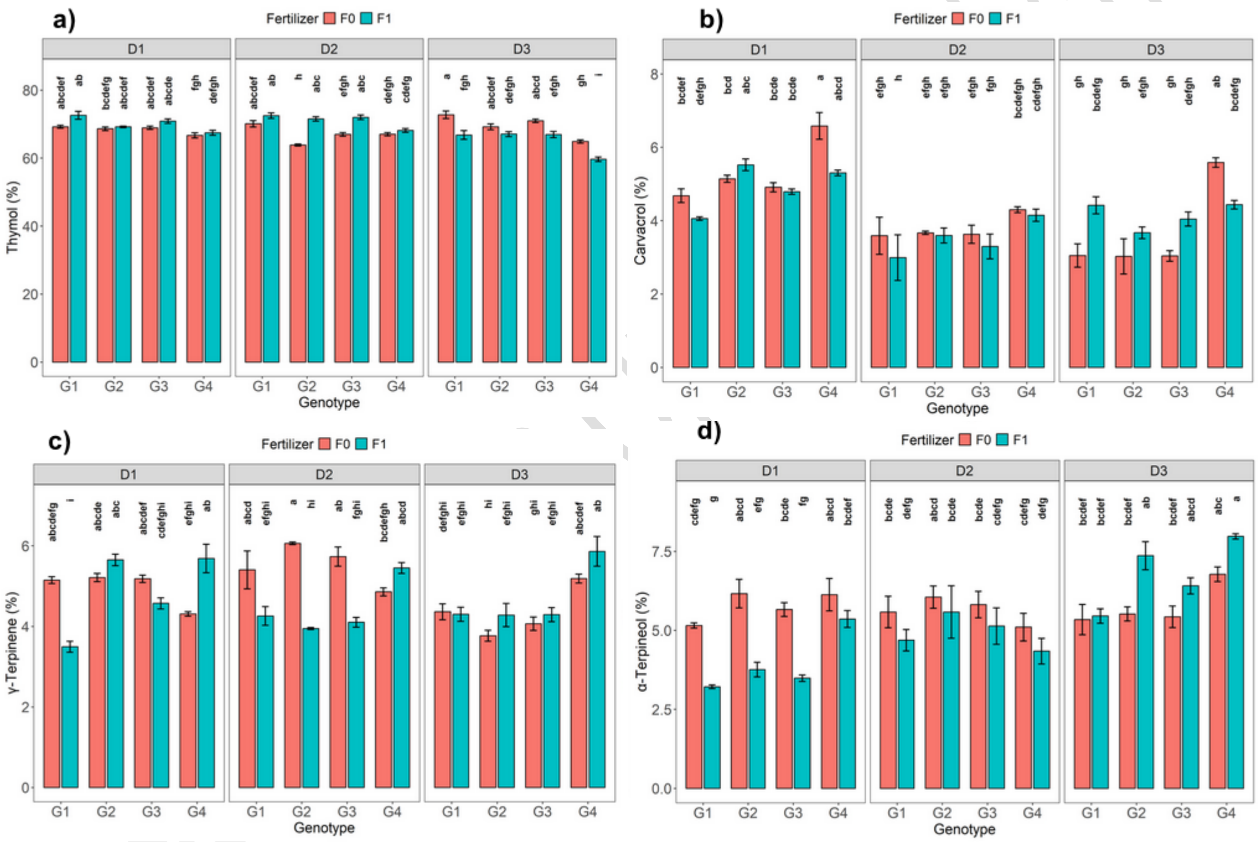



**Fig. 3** The effect of different manure levels × plant density on essential oil compounds in four genotypes of *Thymus daenensis*: thymol (a), carvacrol (b), gamma – terpinene (c), *α* –terpineol (d), and *ρ* – cymene (e) (Tukey's Test, α = 0.05, r = 3, n= 10). D1, D2, and D3: respectively, low density (4 plant $m^{-2}$), medium density (6 plant $m^{-2}$), and high density (8 plant $m^{-2}$), G1 – G4: genotypes 1 – 4; Fertilizer 0: non -application of manure and 1: manure application.

## Minor Phytochemical Compounds

### *E*-caryophyllene

The significant difference was observed among treatment combinations for *E*-caryophyllene. The highest mean value (4.29 ± 0.03) was observed in genotype 2 at medium density cultivation without manure application, indicating significantly greater accumulation than most other treatments. Comparably high values were also recorded under high-density plantation for genotype 2 (3.76 ± 0.71) and under medium density for genotype 3 (3.66 ± 0.05) (Fig. 4a). In contrast, the lowest *E*-caryophyllene content was observed in genotype 4 in response to manure application under medium density (1.21 ± 0.09). Overall, manure application consistently limited *E*-caryophyllene accumulation across genotypes and densities.

### Borneol

Borneol concentration varied significantly among treatments according to Tukey's test. The maximum borneol content (2.68 ± 0.12) was recorded for genotype 2 under medium density without manure application, which constituted the highest statistical group (a), followed closely by genotype 4 (2.67 ± 0.74) under low density (Fig. 4b). Conversely, the lowest mean value (0.90 ± 0.01) was identified for genotype 4 under manure application and medium density (group h). These results demonstrate that borneol biosynthesis was maximized at medium density, while manure application markedly reduced borneol levels in several genotypes.

### Myrcene

Significant treatment effects were also detected for myrcene. The highest myrcene concentration (1.87 ± 0.03) was observed under manure application and high planting density in genotype 2 (1.91 ± 0.07), which was not significantly different from its value in genotype 3 in response to manure application × high planting density (Fig. 4c). However, the minimum myrcene values were consistently associated with genotype 4, with the minimum recorded under manure application × medium density (0.73 ± 0.15), classified in the lowest group (j).

### Methyl carvacrol

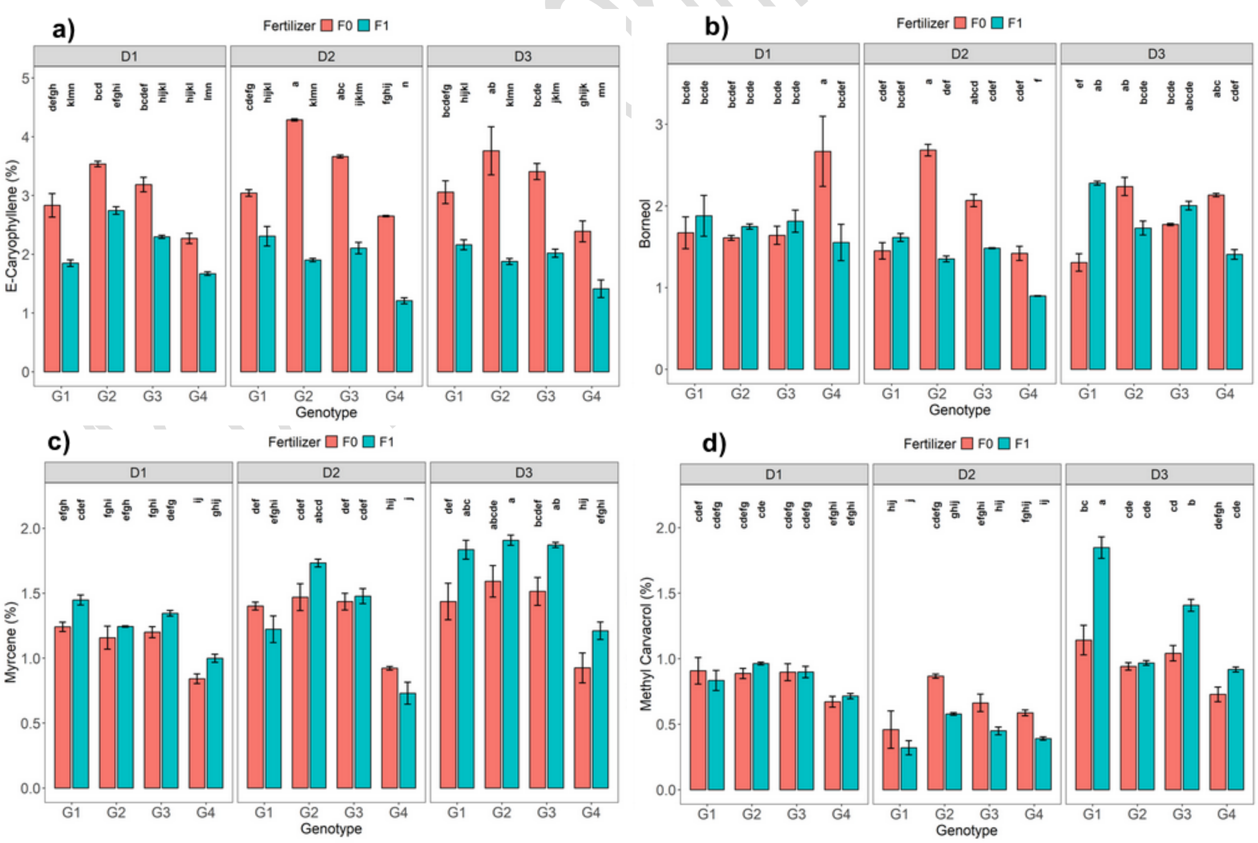
The utmost methyl carvacrol (1.85 ± 0.14) was found in genotype 1 in response to manure fertilization plus high plantation density, indicating significantly greater accumulation than all other values. High values were also observed in genotype 3 under manure supplementation × high density cultivation (1.41 ± 0.08). The lowest methyl carvacrol concentration was gained from genotype 1 under manure application × medium density planting (0.32 ± 0.09) (Fig. 4d). These results suggest that high plant density combined with manure fertilization strongly enhances methyl carvacrol formation in specific thyme genotypes.

### *α*-Pinene

*α*-Pinene content exhibited wide variation among treatments. The peak value by 4.10 ± 0.58 was recorded for genotype 4 at medium plant density, followed by genotype 4 in response to manure application × high density (3.87 ± 0.05) (Fig. 4e). In contrast, the minimum *α*-pinene level was detected under non–fertilization × medium density in genotype 2 (0.56 ± 0.04). This indicates a strong genotype-specific response, with genotype 4 showing exceptional *α*-pinene accumulation under specific density plantation.

### *a-* thujene

Also, the maximum thujene concentration was obtained from genotype 4 under high plant density plus manure fertilization (1.84 ± 0.06), significantly higher than all other values (Fig. 4f). Conversely, the lowest values were detected under low density plantation from genotype 2 (0.56 ± 0.02) and under high density for genotype 1 (0.57 ± 0.18), both assigned to the lowest statistical groups (d). These results demonstrate that manure fertilization**,** particularly at high density, strongly enhances thujene accumulation in genotype 4.



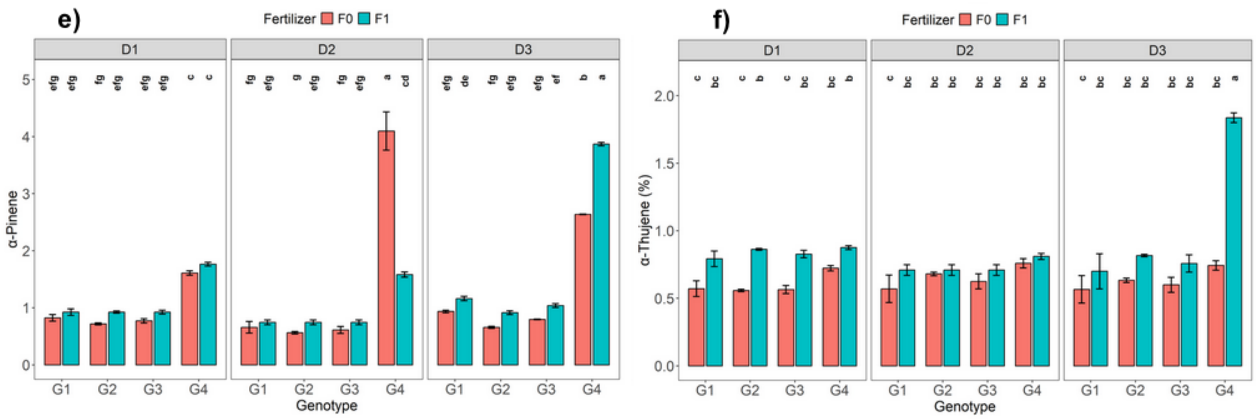



**Fig. 4** The effect of different organic fertilizers × plant density on essential oil compounds in four genotypes of *Thymus daenensis*: *E*–caryophyllene (a), borneol (b), myrcene (c), methyl carvacrol (d), *α*-pinene (e), and *α*-thujene (f (Tukey's Test, α = 0.05, r = 3, n= 10). D1, D2, and D3: respectively, low density (4 plant $m^{-2}$), medium density (6 plant $m^{-2}$), and high density (8 plant $m^{-2}$), G1 – G4: genotypes 1 – 4; Fertilizer 0: non -application of manure and 1: manure application.

In general, organic fertilization with manure produced higher camphene levels across all densities and genotypes, while genotype 4 consistently exhibited lower concentrations. As well as low-density planting produced higher camphene content than medium and high densities for the same genotype. The highest concentration was observed in genotype 1 under manure application and low-density cultivation (0.6263 ± 0.0741, group a), whereas the lowest was gained in genotype 4 cultivated under high-density planting without manure application (0.2058 ± 0.0623, group g) (Fig. 5a).

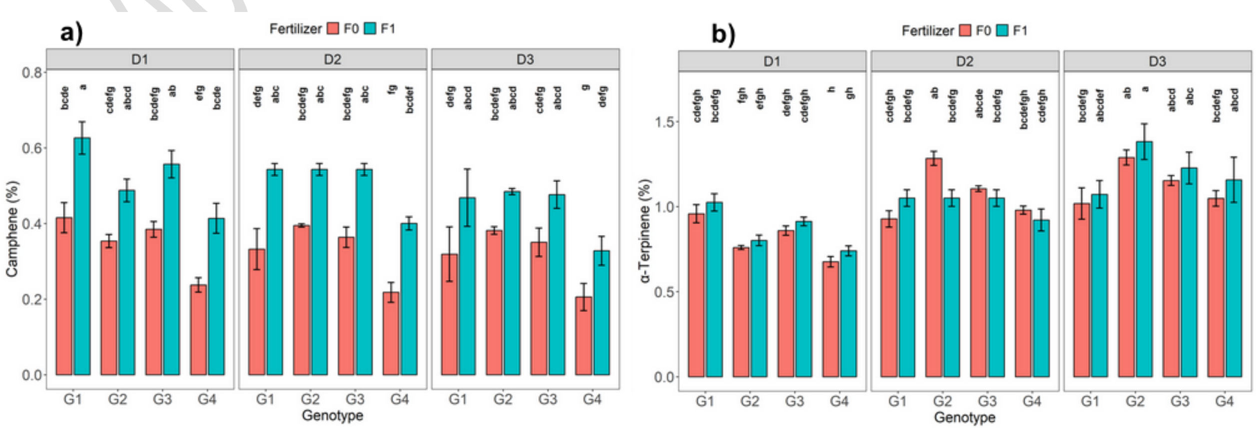
The *α*-terpinene concentrations were highest in genotype 2 under manure application and high density (1.3821 ± 0.1814, group a) and lowest in genotype 4 under low density cultivation (0.6761 ± 0.0533, group h) (Fig. 5b). Manure supplementation consistently outperformed, and high-density plantings often resulted in elevated *α*-terpinene, especially for genotypes 2 and 3.

The concentration of 1*,8*-cineol generally increased with manure application across densities, with high-density showing variable responses depending on genotype. Genotype 4 consistently yields the lowest concentrations. The maximum value of *1,8*-cineol (0.7143 ± 0.1218, group a) was found in genotype 1 under manure application and medium density, while the minimum (0.1677 ± 0.0306, group i) was obtained in genotype 4 under low density without manure supplementation (Fig. 5c).

Across all genotypes, manure supplementation increased the *Cis*-sabinene hydrate content, and low-density planting generally promoted higher accumulation. The highest *Cis*-sabinene hydrate value was observed in genotype 1 with manure application under low density planting (0.4198 ± 0.2453, group a), while the lowest 1 (0.0683 ± 0.0189, group cd) was gained in genotype 3 under high density without manure application (Fig. 5d).

Manure application consistently enhanced linalool levels across all genotypes and densities, with medium-density treatments producing higher concentrations than low or high density for most genotypes. Linalool concentration was maximized in genotype 2 in response to manure fertilization under medium plant density (0.9678 ± 0.0094, group a) and minimized in genotype 2 under high density without fertilization (0.6235 ± 0.0266, group j) (Fig. 5e).

Finally, terpinene-4-ol exhibited the highest mean in genotype 4 at medium density condition plus manure application (0.4065 ± 0.0087, group a), and the lowest in genotype 1 under high density plantation without manure supplementation (0.0433 ± 0.0208, group g) (fig. 5f). Manure fertilization consistently produced higher concentrations of terpinene-4-ol compared to control, and medium-density plantation tended to yield greater accumulation than high-density plantings.



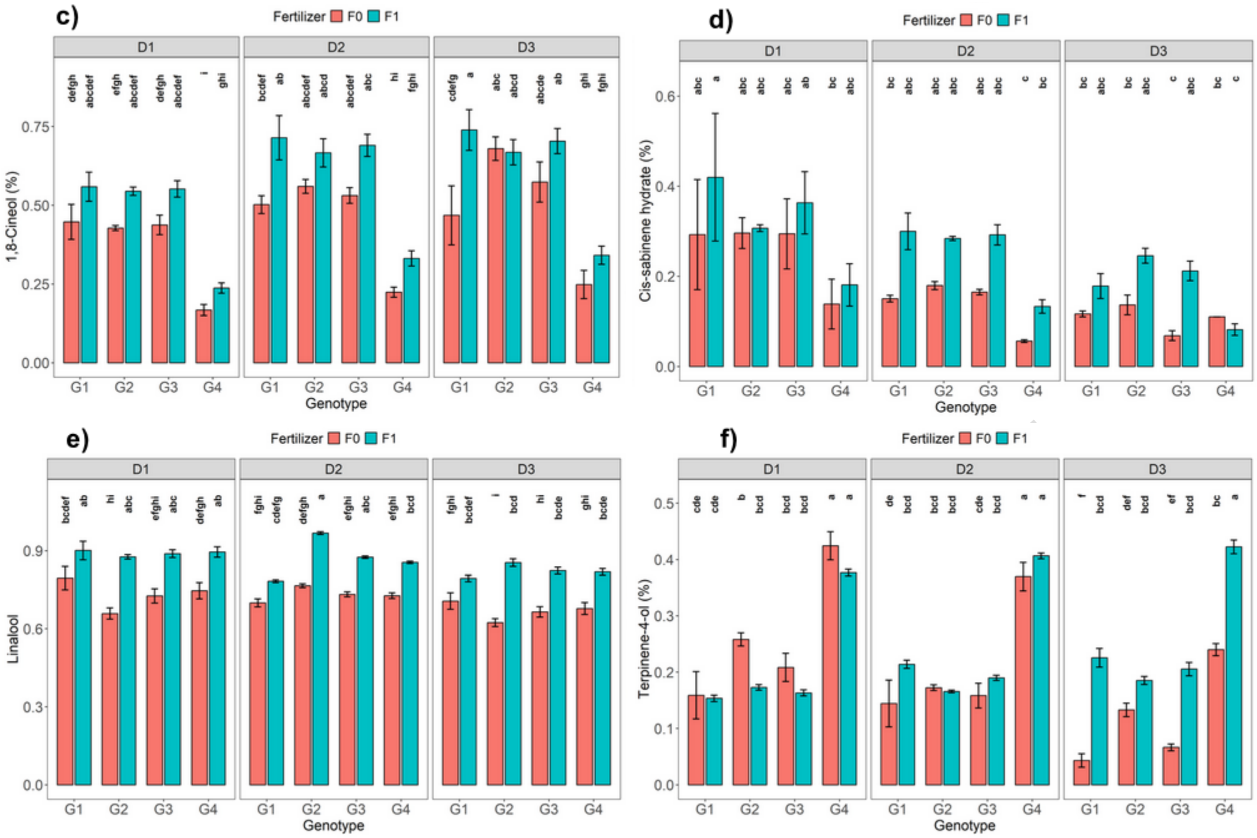



**Fig. 5** The effect of different organic fertilizers × plant density on essential oil compounds in four genotypes of *Thymus daenensis*: camphene (a), *α*-terpinene (b), 1,8- cineol (c), *Cis*-sabinene hydrate (d), linalol (e), and terpinen-4-ol (f) (Tukey's Test, α = 0.05, r = 3, n= 10). D1, D2, and D3: respectively, low density (4 plant $m^{-2}$), medium density (6 plant $m^{-2}$), and high density (8 plant $m^{-2}$), G1 – G4: genotypes 1 – 4; Fertilizer 0: non -application of manure and 1: manure application.

## Principal Component Analysis (PCA)

The principal component analysis (PCA) (Fig. 6) discovered clear differentiation among treatments based on genotype, organic fertilization, and planting density (G × F × D). The first two principal components (PCs) explained 44.3% of the total variation, with PC1 accounting for 25.0% and PC2 for 19.3% of the variance (The eigenvalue values of first and second PC was 5.3 and 4.1, respectively). PC1 primarily separated treatments based on biomass production and essential oil yield, as indicated by the strong positive loadings of fresh yield, dry yield, and EO yield along the positive direction of this component. The treatment associated with higher planting density (D3) and manure application (F1) was positively correlated with increased productivity. Several monoterpene hydrocarbons, including *α*-pinene, γ-terpinene, terpinene-4-ol, *p*-cymene, and thujene, also loaded positively on PC1, suggesting that enhanced growth conditions favored the accumulation of these compounds. In particular, genotypes 3 and 4 (G3 and G4) under manure application at medium to high plant densities (F1 × D2–D3) clustered closely with yield-related vectors, indicating superior agronomic performance and EO productivity under these conditions. PC2 mainly discriminated treatments based on phenolic and oxygenated monoterpene composition, especially carvacrol, thymol, borneol, methyl carvacrol, and *E*-caryophyllene. Notably, several treatments without manure application (F0), particularly at low to medium planting densities (D1–D2), were located closer to the carvacrol and thymol vectors and strongly associated with carvacrol and thymol, compounds of high pharmaceutical and industrial value. This pattern suggests that moderate resource limitation may stimulate the biosynthesis of phenolic compounds, likely as a stress-related metabolic response.

## Genotype-Specific Responses

The PCA clearly highlighted genotype-dependent responses to fertilization and planting density: G4 showed strong association with high biomass and EO yield, especially under manure application and higher plant densities, indicating superior adaptability to rainfed condition cultivation. G3 demonstrated a balanced response, combining relatively high yield with favorable EO composition under manure application and medium planting density (F1 × D2) conditions. G1 and G2 tended to cluster closer to phenolic-rich compounds such as thymol and carvacrol, particularly under non-fertilized and lower-density treatments, suggesting their suitability for quality-oriented rather than yield-oriented production.

Overall, the PCA indicates a clear trade-off between biomass/EO yield and phenolic compound concentration. High planting density combined with organic fertilization favored biomass accumulation and EO yield, whereas lower densities and absence of manure promoted phenolic-rich essential oil profiles. These findings underscore the importance of optimizing genotype × fertilization × planting density interactions depending on whether the production goal prioritizes yield or essential oil quality.

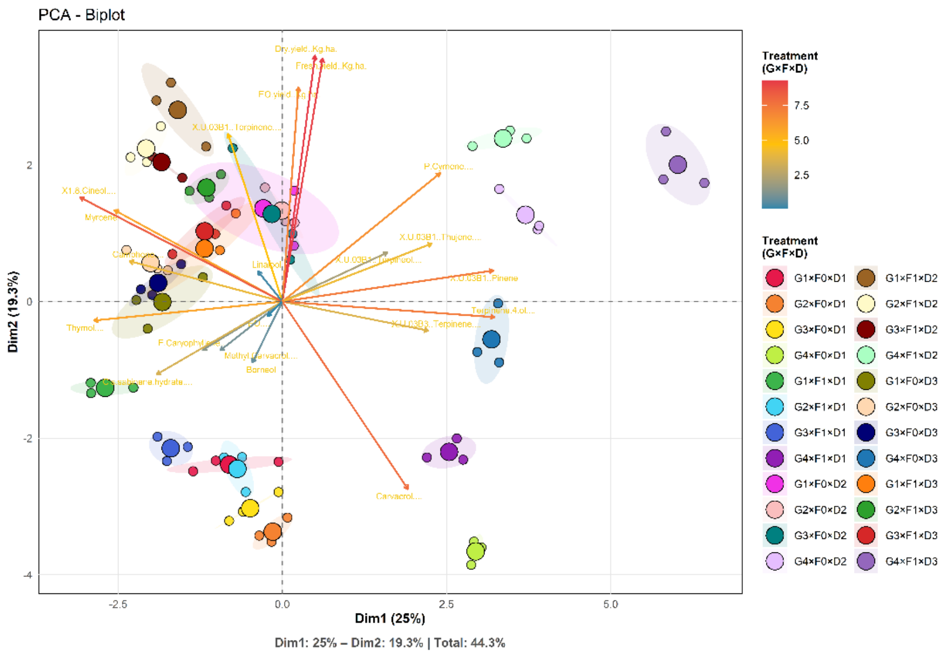



**Fig. 6** The principal component graph for PC1 and 2 of thyme genotypes cultivated under manure application and different plant densities

## Pearson's Correlation Estimation

The Pearson correlation analysis (Fig. 7) revealed strong and biologically meaningful relationships among yield components, essential oil content, and individual EO constituents, which were fully consistent with the patterns observed in the PCA. Fresh yield, dry yield, and EO yield were strongly and positively correlated ($r = 0.95^{**} – 0.89^{**}$), indicating that increased biomass production directly enhanced EO yield. EO percentage showed a moderate positive correlation with EO yield ($r = 0.41^{**}$) but only weak associations with fresh and dry yield, suggesting that EO yield was driven primarily by biomass accumulation rather than changes in oil percent. Yield traits were positively associated with monoterpene hydrocarbons such as *p*-cymene, *α*-terpinene, *α*-pinene, and terpinene-4-ol, whereas they were negatively correlated with phenolic compounds, particularly carvacrol and methyl carvacrol. These relationships indicate a trade-off between high productivity and phenolic-rich EO profiles. Strong correlations were also observed among EO constituents, reflecting shared biosynthetic pathways; for example, *α*-pinene was positively correlated with *α*-thujene, and myrcene showed a strong positive association with *1,8*-cineole (Fig. 7). In contrast, thymol exhibited strong negative correlations with *γ*-terpinene, *p*-cymene, and *α*-thujene, highlighting the metabolic divergence between phenolic and monoterpene pathways. Overall, the correlation structure confirms that agronomic practices promoting higher biomass and EO yield favor monoterpene-dominated profiles, whereas reduced yield conditions tend to enhance phenolic compounds such as thymol and carvacrol.

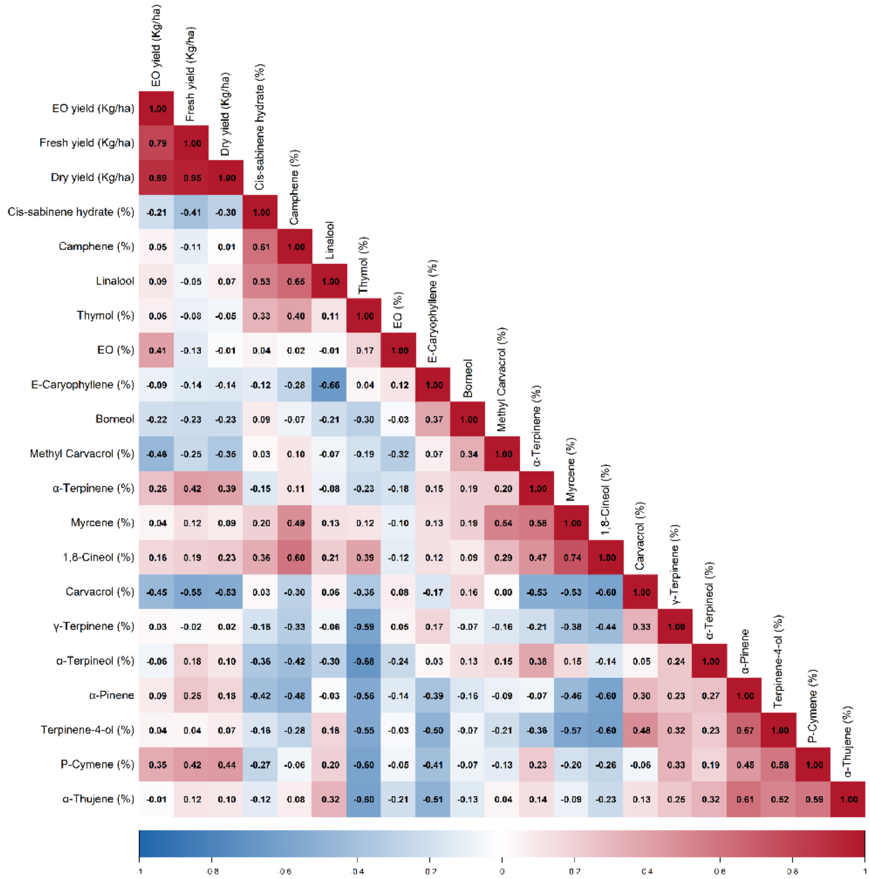



**Fig. 7** The correlation graph for studied variables in thyme genotypes cultivated under manure application and different planting densities.

The hierarchical clustering of treatments and variables (Fig. 8) revealed clear structure in the joint agronomic–chemical response of *Thymus daenensis* to genotype, planting density, and organic manure. On the treatment axis, two major groups were evident. Treatments receiving manure—particularly at medium planting density—clustered together and were characterized by consistently higher fresh and dry biomass and elevated EO yield per hectare, indicating that resource enrichment primarily enhances productivity-driven traits. In contrast, unfertilized treatments and those at lower density tended to cluster separately and were associated with greater relative abundance of phenolic monoterpenes, especially thymol and carvacrol, suggesting a metabolic shift toward stress- or defense-related compounds under reduced competition and nutrient availability. The clustering pattern also showed that genotypes differed in their plasticity: some genotypes grouped across a wider range of densities and fertilizer conditions with relatively stable EO composition, whereas others formed distinct clusters that reflected strong shifts in chemical phenotype under different management regimes. On the variable axis, biomass-related traits (fresh yield, dry yield, EO yield) formed a coherent cluster that was negatively associated with phenolic monoterpenes, whereas monoterpene hydrocarbons such as p-cymene, γ-terpinene, and α-terpineol tended to co-vary positively with increased biomass and EO yield. This configuration indicates a trade-off between biomass accumulation and phenolic monoterpene dominance, consistent with reallocation of metabolic resources between growth and secondary metabolite biosynthesis. Overall, the cluster analysis supports the interpretation that EO yield, biomass, and EO composition are coordinated but differentially sensitive to genotype and management intensity, reinforcing the importance of aligning agronomic strategy with specific production objectives.

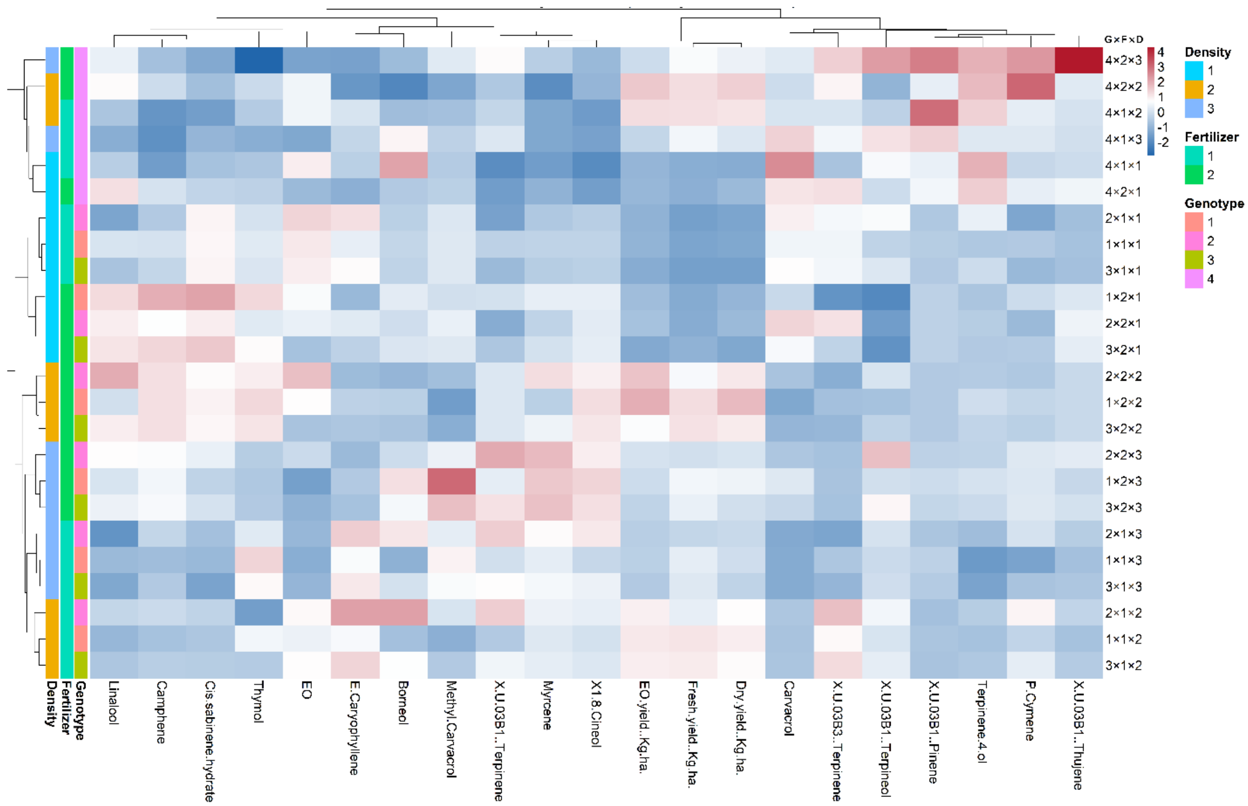



**Fig. 8** The hierarchical clustering heatmap for studied variables and treatments in thyme genotypes cultivated under manure application and different planting densities

## DISCUSSION

Present findings demonstrated that *Thymus daenensis* exhibits pronounced physiological, biochemical, and agronomic plasticity in response to organic manure application, planting density, and genotype under rainfed dryland conditions. The most productive treatments characterized by medium planting density with organic manure consistently yielded the highest fresh biomass (up to 16,398 g $m^{-2}$), dry biomass (up to 7,122 g $m^{-2}$), and EO yield (up to 435.3 kg $ha^{-1}$). In contrast, unfertilized and low-density treatments produced lower biomass and EO yield but tended to accumulate relatively higher proportions of phenolic monoterpenes such as thymol and carvacrol, indicating a critical metabolic trade-off between growth and specialized metabolism. In line with these results, the highest fresh yield (3149.90 kg ha-1) and dry yield (1611.70 kg ha-1) of white savory has gained with supplementation of 50 t/ha manure under 8 plant $m^{-2}$ plantation, while the highest EO percentage (2.19%) resulted under application of 50 t/ha manure and 2.66 plant $m^{-2}$ plantation [23].

Organic manure application enhanced biomass and EO yield primarily by improving soil nutrient availability and structure. Manure increases soil organic matter and enhances nitrogen mineralization and micronutrient availability, thereby promoting root growth and photosynthetic efficiency [41, 51, 52]. Improved soil water retention is especially critical under rainfed conditions, where limited precipitation constrains plant growth. Previous studies have similarly reported that organic amendments augment biomass in aromatic and medicinal plants by improving soil fertility and microbial activity [33, 35, 36]. Also, Manure supplementation has increased essential oil percentage of *Satureja macrantha* from 1.06 to 1.86%, as well as essential oil yield compared with control and NPK-treated plants [53].

Planting density influenced biomass by modulating resource capture and intraspecific competition. [19, 54]. Higher densities can intensify competition for light and water, whereas very low densities may underutilize site resources, thereby limiting overall yield per unit area [19, 21, 22, 55]. This aligns with findings from other rainfed field crops where intermediate densities maximize yield by balancing competition and resource utilization [19, 20].

Although EO percentage remained relatively stable across treatments, its composition shifted according to management practices. Organic manure and medium-to-high density conditions favored an increased prevalence of monoterpene hydrocarbons such as *p*-cymene and *γ*-terpinene. Conversely, treatments with lower resource availability, unfertilized plots, tended to accumulate higher proportions of thymol and carvacrol. This pattern reflects a growth–defense allocation trade-off: under resource-rich conditions, plants invest more in primary metabolic pathways that support biomass accumulation and general terpenoid biosynthesis [56-59]. Under resource-limited conditions, metabolic flux is often redirected toward secondary phenolic compounds that function in stress mitigation and antioxidant defense [47, 60-62]. Thymol and carvacrol are derived through hydroxylation of precursor monoterpene hydrocarbons, a process that may be upregulated under stress to confer enhanced protection against abiotic and biotic factors [58, 63-66]. These findings are consistent with studies showing increased phenolic monoterpenes under nutrient limitation or environmental stress in Thymus and other Lamiaceae species [42, 49].

Genotype significantly mediated responses to density and manure, affecting both yield and EO composition. Some genotypes maintained relatively stable EO profiles across treatments, while others exhibited dramatic shifts in phenolic content depending on the treatment. These differences likely reflect genetic variation in key biosynthetic and regulatory enzymes in the methylerythritol phosphate (MEP) pathway and downstream terpene synthases. Gene expression studies in thyme and related taxa have documented genotype-specific expression patterns of terpene synthase genes that control the relative flux toward thymol, carvacrol, and other monoterpenes [46, 67-70]. The observed interaction effects highlight the importance of considering genotype × farm management interactions when optimizing both yield and EO quality.

Our findings align with earlier research in aromatic medicinal crops, where organic amendments improve yield without necessarily elevating EO concentration [23]. Similar patterns of increased biomass and EO yield under organic inputs and moderate densities have been documented in Satureja species and other Lamiaceae members [1, 24]. Additionally, studies on thyme chemotypes indicate that environmental factors such as nutrient availability and plant competition can shift EO profiles, particularly the balance between phenolic monoterpenes and other volatiles [71-73]. However, present study extends the existing literature by quantifying trade-offs not only in major compounds like thymol and carvacrol but also in a broader suite of monoterpenes and sesquiterpenes. The cluster analysis, for example, revealed coherent grouping of biomass-related traits with monoterpene hydrocarbons, whereas phenolic monoterpenes clustered separately, underscoring coordinated metabolic regulation.

Although our study was conducted over tow year, our data only covers a single harvesting during the second growing season and at one location, which may limit generalizability across different environmental or climatic conditions. Essential oil biosynthesis is known to exhibit seasonal variation and can be strongly influenced by temperature, soil moisture, and other abiotic factors, suggesting that multi-year trials are necessary to determine trait stability. Additionally, the study tested only a single manure application rate. Graded nutrient treatments could provide more precise recommendations for nutrient management.

Thus, we recommend that future work should integrate multi-season field trials to assess the stability of the observed yield and compositional trends under variable precipitation regimes. Studies should also evaluate the impacts of varying manure application rates. Furthermore, assessing biological activity and functional quality of EO under different management regimes would strengthen links between compositional shifts and pharmacological or industrial utility.

In summary, present study illustrates that biomass production and EO yield in *T. daenensis* are most responsive to organic manure and medium planting density, while EO composition, especially the relative abundance of phenolic monoterpenes, is modulated by plant density and genotype. The trade-off between growth and secondary metabolism observed here is consistent with physiological and biochemical theory and has direct implications for optimizing medicinal plant production based on targeted outcomes, whether yield maximization or high phenolic compound content.

In addition, the current study revealed significant genotype- and management-dependent changes in the essential oil composition of *Thymus daenensis*, with major phenolic monoterpenes (thymol, carvacrol) and monoterpene hydrocarbons (*p*-cymene, *γ*-terpinene) showing contrasting responses to planting density and manure application. These patterns can be explained through known physiological and biochemical mechanisms regulating secondary metabolite biosynthesis.

Secondary metabolites such as phenolic monoterpenes are synthesized via the *methyl-erythritol phosphate (MEP)* pathway, which depends on primary metabolic intermediates (e.g., pyruvate, glyceraldehyde-3-phosphate) and reducing power (ATP, NADPH) generated by photosynthesis [58, 74-76]. Under unfavored conditions, the growth differentiation balance hypothesis (GDBH) provides a framework that predicts a trade-off between costs of secondary metabolites (SMs) relative to the demand for photosynthate by growth [77, 78]. Stressful or resource-limited environments favor carbon partitioning into secondary metabolites like phenolics to improve stress tolerance [79-82]. In our study, low density and absence of manure, which limited vegetative growth, correlated with higher *thymol* and *carvacrol* percentages, consistent with GDBH-linked metabolic shifts, where plants channel resources to biosynthesis of phenolic compounds for ecological adaptation and antioxidant capacity. This aligns with broader findings in *Thymus* species showing natural variation in major EO compounds like thymol and *p*-cymene among populations due to genetic and environmental influences on metabolism [83]. Organic manure enhances soil nitrogen (N), phosphorus (P), and potassium (K) availability, which improves amino acid production, photosynthetic capacity, and overall biomass formation [84]. Elevated nutrient status supports increased primary metabolism and carbon assimilation, thereby providing more precursors for both growth and secondary pathways. However, enhanced nutrient availability also tends to favor biosynthesis of *monoterpene hydrocarbons* such as γ-terpinene and p-cymene, as observed with manure application and higher planting densities in this study. This pattern is physiologically explained by enhanced enzyme activity in the MEP pathway and terpene synthase genes under nutrient-replete conditions, shifting metabolic flux towards hydrocarbons that may be less costly to synthesize than highly oxygenated phenolics like thymol and carvacrol [25]. Differential responses among genotypes in major compound accumulation indicate genetic control of key enzymes like *thymol synthase* and *terpene cyclases*, which are sensitive to nutrient and competition stress and inherent genetic regulation [85, 86].

## CONCLUSION

The present study shows that *Thymus daenensis* exhibits a high degree of agronomic and biochemical plasticity under rainfed cultivation, with yield formation and essential oil (EO) biosynthesis being tightly linked to both resource availability and genetic background. Farmyard manure primarily acted through improving biomass accumulation, while planting density modified the balance between vegetative growth and secondary metabolite allocation. As a result, EO yield was largely driven by biomass productivity, whereas EO composition reflected shifts in carbon flow between monoterpene hydrocarbons and phenolic monoterpenes. A key insight from this work is the trade-off between maximizing EO yield and enhancing thymol-rich chemotypes. Environments favoring vigorous growth (medium density with manure) promoted higher

EO yield but diluted the proportion of phenolic monoterpenes, whereas resource-limited conditions enhanced thymol dominance despite lower yield. This pattern suggests differential regulation of biosynthetic pathways in response to plant competition and nutrient status, likely mediated through stress-linked metabolic signaling, and underscores the importance of aligning agronomic strategies with end-use priorities. Equally important is the strong genotype × management interaction, indicating that no single management regime is optimal across populations. Some genotypes responded more strongly to inputs with respect to biomass accumulation, while others maintained more stable EO profiles. This confirms that genotype choice is not only a biological decision but also an agronomic one, and cultivar development for dryland production should explicitly integrate management response traits. In the present study, some limitations, such as the application of a single level of manure without standard control of chemical fertilizer, reduced the accuracy of the research, so it is recommended that different levels of manure and chemical fertilizer control be applied in future studies.

## Ethics Approval and Consent to Participate

Not applicable. This study is not a clinical trial and no human participants are involved in this research.

## Consent for Publication

Not Applicable.

## Data Availability

All data generated during this study are included in this article.

## Competing Interests

The authors declared no potential conflicts of interest regarding the research, authorship and publication of this article.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## Seed Preparation Statement

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

## Authors' contributions

The authors conducted the experiments in collaboration and wrote the manuscript.


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Not Applicable.




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