# INFLUENCE OF HUMUS FERTILIZATION ON YIELD AND ESSENTIAL OIL ACCUMULATION OF *THYMUS × CITRIODORUS*

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**Abstract**

*Thymus × citriodorus* is medicinal, aromatic, essential oil bearing plant, this hybrid can accumulate commercial important chemical compound – geraniol in it's essential oil. The aim of the present study was to evaluate the effect of fertilization with humus on the yield, for parameters of some leaf epidermal structures,as well as on the quantitative and qualitative composition of essential oils of *T. × citriodorus* plants that were grown in an open ground for three years. The experiment was done in eight experimental plots: four experimental plots were used as control and other four– for fertilization with humus. The essential oil was isolated by hydrodistillation and analysed by GC–FID and GC–MS. The results showed that fertilization with humus significantly positive affected the height of plant, the area covered by plant and the length of inflorescences in the first experimental year, the density of stomata in both epidermis of leaf in the first and second experimental years, but significantly negative affected the density of glandular trichomes in lower epidermis of leaf in the second experimental year. Fertilization with humus did not affect the percentage and composition of essential oil.

**Keywords:** *Thymus × citriodorus;* essential oil; geraniol; humus; fertilization.

## 1. Introduction

Perennial interspecific hybrid *Thymus × citriodorus* (parental species *Thymus vulgaris* and geraniol chemotype of *Thymus pulegiodes*) is medicinal, aromatic, essential oil bearing plant (Ložienė et al., 2021). The main compound of essential oil of this hybrid is monoterpenic alcohol geraniol with pleasant rose – like aroma and used in cosmetics and in household products (Vaičiulytė et al., 2022). This monoterpenic alcohol also has antioxidant, anti–inflammatory, antimicrobial, antihelmintic and anticancer properties (Maczka et al., 2020). The raw material of *Thymus × citriodorus* is used for enrichment of the aroma and taste of teas and for fish dishes, ice creams and chewing gum (Toncer et al., 2017; Paslawska et al., 2020).

Humus is a major component of soil organic matter and can improves the soil fertility, physical, chemical and biological features of soil. Humus also improves uptake process of macro– and micro elements as well as water regime and reduces abiotic stress and uptake of toxic ions for plants (Aşik et al., 2009; Khaled et al., 2009; Celik et al., 2010; Muhamedyarova et al., 2020). Humus performs one of main function in creating necessary conditions for the growth and development the plants (Zymyatin et al., 2020). It can help to increase the yield of plants and improves the quality of plants production (Muhamedyarova et al., 2020).

Published data indicate that of humus can differently affect the yield of raw material, morphological, anatomical parameters and quantitative and qualitative composition of essential oils for medicinal and aromatic plants. Spraying with liquid humus significantly positively affected the number of inflorescences and the total number of leaves in *Coriandrum sativum* (Apiaceae) (Hamza et al., 2020). The humus in soil negatively correlated with the number of flowers producing nectar in *Allium ursinum* (Bodo et al., 2021). After humic acid application amounts of oxygenated terpenoids increased in *Artemisia sieberi*, but the amount of essential oil decreased. The amount of essential oil and amounts of oxygenated terpenoids increased *in Semenovia suffruticosa* after humic acid application (Sardashti et al., 2014). Humus in soil positively correlated with amount of essential oil and negatively with amount of geraniol and geranial in essential oil of *Thymus pulegioides* (Vaičiulytė et al., 2022). Therefore, the aim of the present study was to evaluate the effect of fertilization with humus on the yield, for parameters of some leaf epidermal structures as well as on the quantitative and qualitative composition of essential oils of *Thymus × citriodorus* plants that were grown in an open ground for three years.

## 2. Materials and Methods

### 2.1. Plant cultivation and fertilization

Vegetatively propagated plants of *T. × citriodorus* were grown in eight separate experimental square plots (nine plants per each separate square plot of 1.44 $m^2$) in the open ground at the field collection of the Nature Research Centre (Mažieji Gulbinai, near Vilnius, Lithuania) from 2018.

The fertilization experiment was started in spring of 2019 and continued three years (2019–2021). Fertilization was carried out in four separate experimental plots and four separate experimental plots were appointed as control. Plants were fertilized with humus only.

Humic acids concentrate was bought from the shop and contained humic acids (85%), potassium (12%), iron (1%), nitrogen (1.3%) and other minerals (0.7%). Fertilization experiment started at the beginning of vegetation period (from the end of April to early May) and finished two weeks before blooming. Fertilization was performed three times – once per two weeks (5 g humus was dissolved in 5 L water for one experimental plot). Fertilization was carried out only through soil on cloudy but not on rainy days.

Temperature and rainfall data were obtained from the meteorological newsletters of Vilnius meteorology station of Lithuanian Meteorological Service under the Ministry of Environment. These meteorological data of April–June period in three experimental years (2019–2021) are presented in Table 1.

**Table 1**. The meteorological data of April–June period in different experimental years.

| Month | Average temperature, °C | | | Average rainfall, mm | | |
|---|---|---|---|---|---|---|
| | 2019 | 2020 | 2021 | 2019 | 2020 | 2021 |
| April | 9.0 | 6.6 | 6.0 | 0.6 | 6.2 | 24.7 |
| May | 13.3 | 12.4 | 11.2 | 28.6 | 77.8 | 147 |
| June | 21.1 | 19.7 | 19.5 | 27.5 | 68.5 | 55 |

### 2.2. Collection and analysis of soil material

For chemical analysis soil samples were taken before every fertilization and before harvesting plant materials. Three sub-samples of soil in each of the four experimental plots were taken from the depth 10–15 cm, mixed and dried at room temperature. Soil analysis was performed at the Agrochemical Research Laboratory of the Lithuania Research Centre for Agriculture and Forestry. Soil pH was determined potentiometrically in 1 M KCL extracts. Organic carbon in soil was established by a dry combustion method (in molecular form), total nitrogen by the modified Kjeldahl method (in ionic form); mobile potassium, mobile phosphorus by Egner–Rim–Doming method (in ionic forms).

### 2.3. Analysis of plants' yield

*T.* × *citriodorus* samples for morphometrical analysis were collected every year on full flowering stage. The following morphometrical parameters were estimated at the middle of blooming: height of plant, area covered by plant and the number of inflorescences per plant. Height of plant and area covered by plant were evaluated for each plant per each experimental plot. The area covered by plant estimation was examine in following manner: the diameter of every plant was measured at the widest and the narrowest locations; the mean value of plant diameter was estimated from these two parameters; every plant was considered in terms of circular shape and the area covered by plant was calculated by the formula calculating the area of the circle ($S = \pi R^2$ , there S denotes the area covered by plant, and R denotes radius of plant). The number of inflorescences was estimated for three plants per each experimental plot: for the largest, medium and the smallest plant. After measuring of morphometrical parameters, the above grounded parts of plants were harvested and weighted per each experimental plot. Data about the weight of fresh plant raw material per each experimental were recorded. The length of fifty inflorescences per every experimental plot was measured. Plant raw material of each experimental plot was dried at room temperature separately.

### 2.4. Analysis of leaf epidermal structures parameters

Density of glandular trichomes and stomata in mm$^2$ and size (diameter) of glandular trichomes were estimated on the lower and upper epidermis of leaves. An imprint method (Dagys et al., 1965) was used for anatomical investigation: a thin layer of colourless nail polish was spread on the fresh leaf from the middle of flowering stem. The formed skin of nail polish was ripped off from leaf and observed with light microscope "Leica". Two leaves from each of the 12 stems (3 stems from every experimental plot) were used for evaluation of anatomical parameters. Apex, base, margins and midrib regions of leaves were not used in this analysis to avoid the effect of different parts of the same leaf causing variations in the investigated anatomical parameters.

### 2.5. Essential oil analysis

Cutting plant materials of each experimental plot were dried at room temperature. The essential oils from each sample (from leaves and flowers, stems were not used) were isolated separately by hydrodistillation in Clevenger apparatus for two hours. The distillation of essential oils was carried out in 2–4 replications per each sample. Essential oil of each sample was kept in 2 mL bottles separately. The amount of essential oils was expressed by percentage (quantitative analysis of essential oils). For further investigation (qualitative analysis), essential oil solutions of 1 % were prepared in a mixture of diethyl ether and n-pentane (1:1). Analysis of essential oils was carried out using a FOCUS GC (Thermo Scientific) gas chromatograph with a flame ionisation detector (FID) and a GC-2010 Plus instrument equipped with a GC-QP 2010 Plus (Shimadzu) series mass selective detector according to methods, described by V. Vaičiulytė et al. (2022). Only those compounds that made up at least 10 % of the essential oils were analysed. The identification of compounds was based on comparison of retention indexes (Ris) (Adams, 2007), computer mass spectra library (NBS75K) and the analytical standards. Retention indexes has been determined relative to retention times of a series of alkanes (C7–C30) with linear interpolation. The percentage amounts of investigated compounds were recalculated according to the areas of FID chromatographic peaks, assuming that all constituents of essential oils comprised 100 %.

### 2.6. Statistical analysis

Calculation of means and standard deviations (SD) were carried out for soil elements, leaf epidermal structures and chemical parameters of *T.* × *citriodorus*, calculation of means, standard deviations (SD) and coefficients of variation (CV) were carried out for yield. The Mann-Whitney U test was used to estimate differences between the effect of fertilization with humus and control on soil, biomass and chemical parameters of *T.* × *citriodorus*. Student's t test was used to estimate differences between the effect of fertilization with humus and the control on the yield and anatomical parameters of *T.* × *citriodorus*. Statistical data processing was carried out with the STATISTICA®12 and MS Excel software.

## 3. Results

### 3.1. Influence of fertilization with humus on soil pH and main elements of soil

An analysis of soil parameters in the first experimental year showed that fertilization with humus increased the amount of potassium in soil, this amount was about 8 mg/kg higher in comparison with the control, but this result was not statistically significant. Fertilization with humus also increased the amount of potassium in soil in the second and third experimental years. The amount of potassium was 29.5 mg/kg higher in comparison with the control in the second experimental year and 20 mg/kg higher in the third experimental year and this difference was statistically significant ($p<0.05$). Fertilization with humus slightly increased the amount of sum nitrogen in the second and third experimental years. It is interesting that after fertilization with humus the amount of organic carbon in soil was 0.11 p.p lower in comparison with the control in the second experimental year. After fertilization with humus the amount of phosphorus was slightly higher in comparison with the control in the third experimental year but this result was not statistically significant (Table 2).

**Table 2.** Changes in soil pH and main chemical elements in soil during fertilization with humus through three experimental years. SD–standard deviation.

| Fertilization | | pH | Organic carbon, % | Sum nitrogen, % | K, mg/kg | P, mg/kg |
|---|---|---|---|---|---|---|
| First year | | | | | | |
| Humus | Mean ± SD | 5.70 ± 0.08 | 0.86 ± 0.12 | 0.083 ±0.012 | 115.75 ± 8.92 | 126.25 ± 9.21 |
| Control | | 5.60 ± 0.14 | 0.85 ± 0.08 | 0.086 ± 0.018 | 107.75 ±1031 | 127.25 ± 11.34 |
| Second year | | | | | | |
| Humus | Mean ± SD | 5.78 ± 0.13 | 0.81 ± 0.07 | 0.083 ± 0.013 | 130.75 ± 25.51 | 118.25 ± 6.40 |
| Control | | 5.67 ± 0.10 | 0.92 ± 0.08 | 0.076 ± 0.007 | 101.25 ± 9.46 | 127.25 ± 12.42 |
| Third year | | | | | | |
| Humus | Mean ± SD | 5.68 ± 0.05 | 0.83 ± 0.12 | 0.083 ± 0.012 | 102.50 ± 10.25* | 127.75 ± 14.94 |
| Control | | 5.70 ± 0.27 | 0.84 ± 0.16 | 0.072 ± 0.005 | 82.50 ± 1.73 | 116.00 ± 6.06 |

* – statistically significant differences.

### 3.2. Influence of fertilization on the yield

The results of the first experimental year showed that fertilization with humus significantly ($p<0.05$) increased the height of plant, the area covered by plant and the length of inflorescences: these morphometrical parameters were about 1.2 times higher in comparison with the control. The biomass and number of inflorescences in plant were also higher than in control plants but these differences were not statistically significant. In the first experimental year the least variable morphometrical parameter was the height of plant and the most variable – number of inflorescences (Table 3).

In the second and the third experimental years humus did not give statistically significant differences. After fertilization the height of plant, the area covered by plant were slightly higher, but biomass– slightly lower in comparison with the control in the second experimental year (Table 3). In the second experimental year as in the first experimental year the least variable morphometrical parameter was the height of plant and the most variable – number of inflorescences. In the third experimental year the area covered by plant slightly increased but the number of inflorescences slightly decreased in comparison with the control. The variation coefficient of biomass in control plants was low and the height of plant had the same mean in the control plants and plants after fertilization in the third experimental year. In the third experimental year the most variable morphometrical parameter was the number of inflorescences (Table 3).

**Table 3**. Descriptive statistics of yield parameters *of Thymus × citriodorus* depending on fertilization with humus in three experimental years. SD–standard deviation. CV–coefficient of variation*.*

| **Yield parameter** | | **First year** | | **Second year** | | **Third year** | |
|---|---|---|---|---|---|---|---|
| | | **Control** | **Humus** | **Control** | **Humus** | **Control** | **Humus** |
| Biomass, g | Mean ± SD | 110.3 ± 35.9 | 149.3 ± 86.5 | 282.5 ± 81.2 | 254.8 ± 97.9 | 200.0 ± 11.7 | 195.3 ± 124.0 |
| | Min | 72 | 50 | 216 | 165 | 188 | 97 |
| | Max | 158 | 253 | 396 | 369 | 213 | 370 |
| | CV, % | 33 | 58 | 29 | 38 | 6 | 63 |
| Height of plant, cm | Mean ± SD | 5.8 ± 1.3 | 6.9 ± 1.3* | 6.0 ± 1.8 | 6.6 ± 1.4 | 6.1 ± 2.0 | 6.1 ± 2.1 |
| | Min | 3.1 | 4.6 | 3.5 | 3.8 | 3.0 | 3.0 |
| | Max | 8.5 | 9.1 | 11.2 | 9.5 | 12.0 | 10.5 |
| | CV, % | 22 | 19 | 30 | 21 | 33 | 34 |
| Area covered by plant, $cm^2$ | Mean ± SD | 550.7 ± 164.1 | 641.0 ± 231.3* | 1053.9 ± 397.4 | 1161.8 ± 335.6 | 1238.3 ± 461.9 | 1430.3 ± 890.6 |
| | Min | 143.1 | 248.7 | 397.4 | 706.5 | 547 | 730.2 |
| | Max | 865.3 | 1194 | 2061.9 | 2021.8 | 3316 | 5941 |
| | CV, % | 30 | 36 | 38 | 29 | 37 | 62 |
| Number of inflorescences | Mean ± SD | 133.0 ± 88.7 | 160.7 ± 113.9 | 243.8 ± 193.6 | 255.8 ± 150.1 | 141.0 ± 131.0 | 126.0 ± 105.6 |
| | Min | 26 | 6 | 45 | 94 | 10 | 2 |
| | Max | 350 | 350 | 700 | 490 | 400 | 300 |
| | CV, % | 67 | 71 | 79 | 59 | 93 | 84 |
| Length of inflorescences, cm | Mean ± SD | 1.17 ± 0.30 | 1.32 ± 0.32* | 1.47 ± 0.65 | 1.40 ± 0.51 | 1.14 ± 0.37 | 1.19 ± 0.41 |
| | Min | 0.6 | 0.6 | 0.7 | 0.7 | 0.6 | 0.5 |
| | Max | 2.6 | 2.4 | 3.9 | 3.5 | 2.2 | 3.1 |
| | CV | 26 | 24 | 44 | 36 | 32 | 34 |

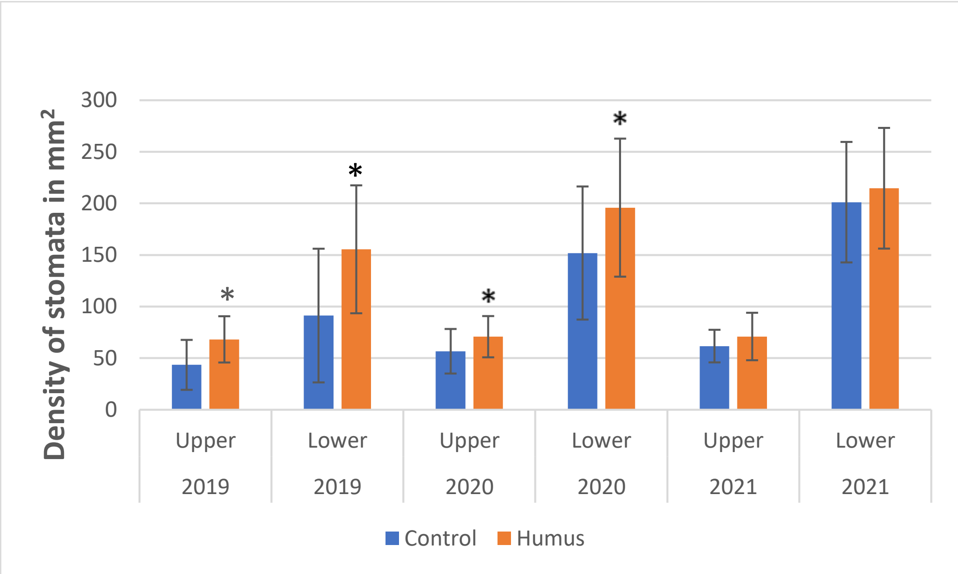
* – statistically significant differences.

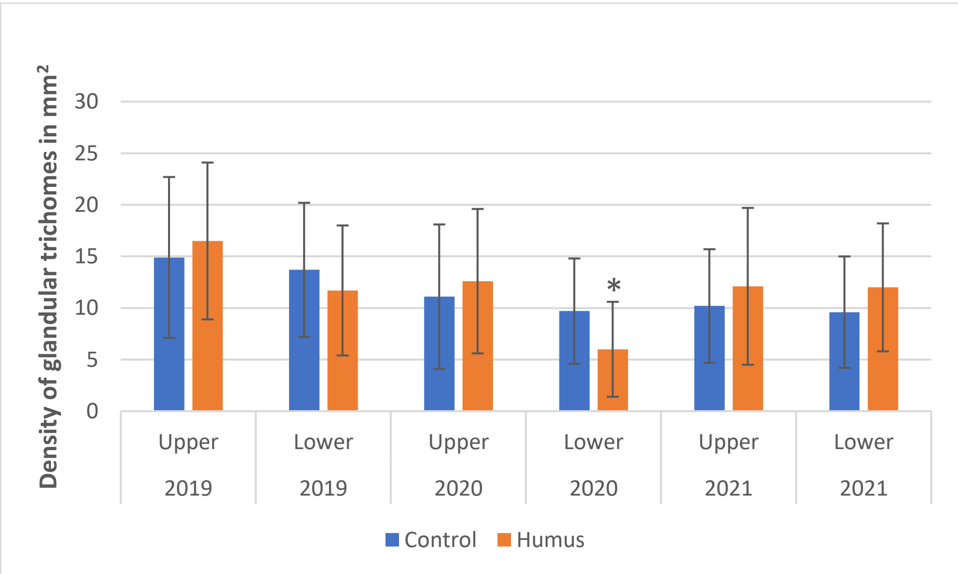
### 3.3. Influence of fertilization on leaf epidermal structures parameters

The fertilization with humus in the first experimental year showed significant ($p<0.05$) positive effects on stomata densities in the upper and lower epidermis of leaves: stomata density was about 1.6 times higher in the upper and about 1.7 times higher in the lower epidermis of leaves. Humus also increased the size of glandular trichomes in the lower epidermis but this result did not significantly different from the control (Figure 1).

Humus also significantly ($p<0.05$) positively affected densities of stomata in the upper and lower epidermis of leaves in the second experimental year: stomata density in 1 $mm^2$ was about 1.2 times higher in the upper and about 1.3 times higher in the lower epidermis. Meanwhile the density of glandular trichomes reacted negatively: after fertilization the density of glandular trichomes in the lower epidermis was significantly ($p<0.05$) 1.6 times lower than in control (Figure 1).

In the third experimental year the fertilization with humus had not significant effect for leaf epidermal structures parameters (Figure 1).



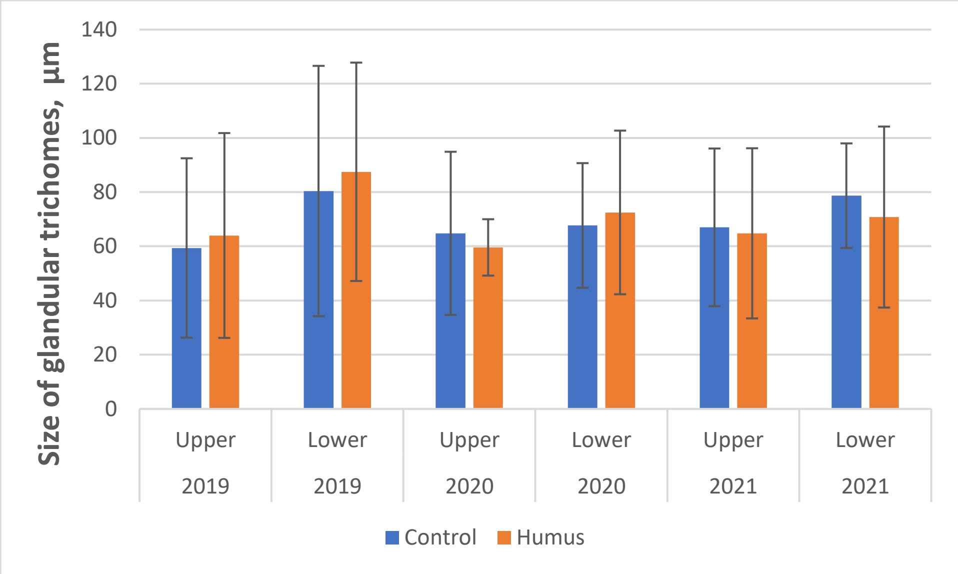



**Figure 1**. Variations in leaf epidermal structures parameters (a – density of stomata, b – density of glandular trichomes, c – size of glandular trichomes) of *Thymus* × *citriodorus* after fertilization with humus. * – denotes statistically significant differences, error bars denote standard deviations.

**3.4. Influence of fertilization on composition of essential oil**

Results of all three experimental years showed that the effect of fertilization with humus on percentage of essential oil in *T.* × *citriodorus* was negative but not statistically significant (Figure 2).

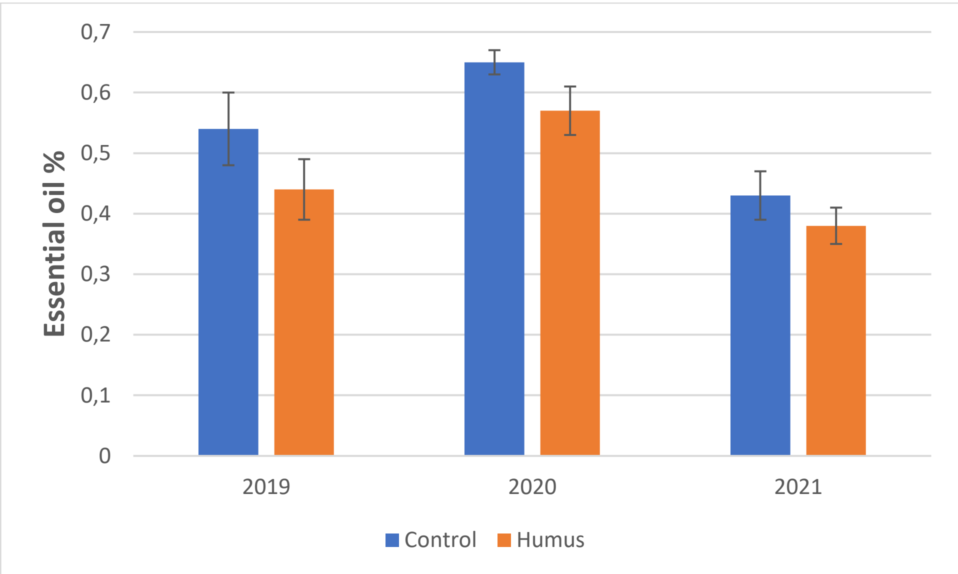



**Figure 2.** Variation in percentage of *Thymus* × *citriodorus* essential oil depending on fertilization with humus in different experimental years. Error bars denote the standard error of mean.

Geraniol was the main compound in the essential oil of *T.* × *citriodorus*. It composed about 22 % of essential oil in control plants and in plants after fertilization with humus. The second compound by plenty was nerol, which composed about 16 % of essential oil in the first and second experimental years, but in the third experimental year the amount of this chemical compound was lower than 15 %. The amount of geranial was 12–14 %, the highest amount of geranial was in the third experimental year. The lowest amount of neral was in the third experimental year, humus application slightly increased the amount of neral in the third experimental year. The fertilization with humus did not significantly affect the percentages of these four chemical compounds of *T.* × *citriodorus* essential oil (Figure 3).

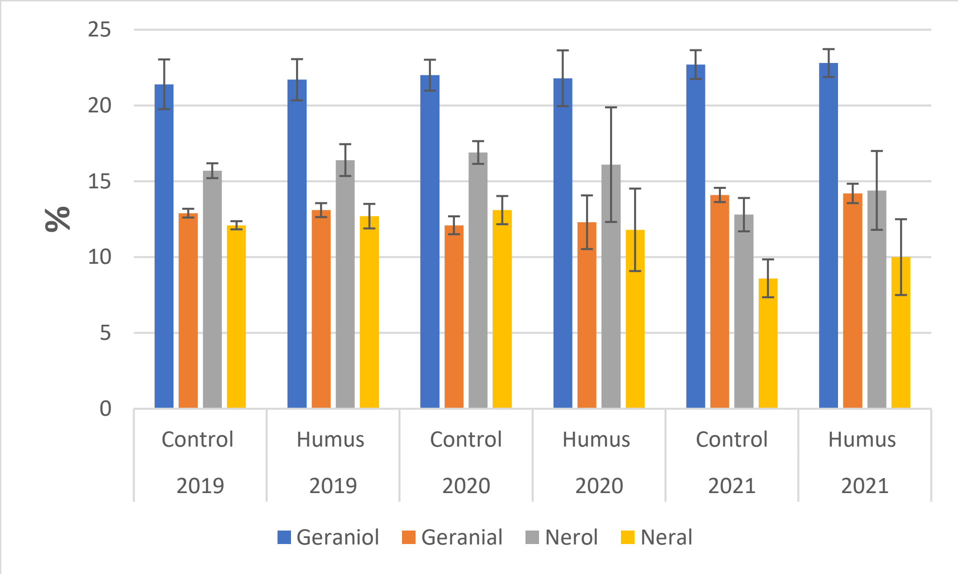



**Figure 3.** Variation in the percentage of main compounds in the essential oil of *Thymus × citriodorus* depending on fertilization with humus in different experimental years. Error bars denote standard deviations.

### 4. Discussion

Humus containing fertilizers can improve fertility of the soil, saturate it with minerals (Merennych et al., 2019). Present study showed that fertilization with humus significantly ($p<0.05$) increased the amount of potassium in soil: it was about 1.2 times higher in comparison with the control (Table 2). Potassium is important macronutrient element for plants: it permits the activation about 60 enzymes in lipid and protein synthesis, controls the opening and closing of stomata, maintains the balance of electrical charges in photosynthesis, promotes the translocation of sugars after photosynthesis, improves a disease resistance (Silva & Uchida, 2000; Dar et al., 2021).

Fertilization with humus also can stimulate growth of plants, increases yield of plants, intensifies biosynthesis of proteins, carbohydrates and vitamins, increases resistance to adverse environmental factors (Nardi et al., 2004; Merennych et al., 2019). Previous study with *T. × citriodorus* showed that humus fertilization with dose 7 g to 1 $m^2$ significantly negatively affected the height of plant and the amount of essential oil of this hybrid (Vaičiulyte et al., 2022). According literature data humus have to increase the yield of plants too (Muhamedyarova et al., 2020). By literature data doses of fertilisers can influenced growth and yield of plants, as well as essential oil yield and composition (Anwar et al., 2010; Garcia et al., 2017; Handayati & Sihombing, 2019). Present study demonstrated that the humus fertilization with 3.5 g to 1 $m^2$ significantly ($p<0.05$) increased the height of plant, area covered by plant and length of inflorescences in the first experimental year (Table 3). In the second and the third experimental years humus application did not significantly affect the yield of *T. × citriodorus* (Table 3). It can be related with colder springs in 2020 and 2021 years (the second and the third experimental years) and very rainy May in 2021 (Table 1). According literature data low temperatures can reduce humus accumulation (Grigal & Vance, 2000). In the third experimental year humus also could wash out from the soil due to very large rainfall in May; large moisture of soil also can reduce the aeration of soil. Humus application can to increase weight of plant, plant height and leaf area (Muhamedyarova et al., 2020). The plants of *Ocimum basilicum var. basilicum*, sprayed with humic acid, grew higher, with more leaves and side branches (Abdul et al., 2012). Fertilization with compost increased the number of branches and the weight in *Thymus vulgaris* in the first and the second seasons (Hendawy et al., 2007). Sometimes the fertilization with humus have no effect for plants yield. For example, the fertilization with humic acid did not increase the growth of lettuce (Hartz, 2007).

The main function of stomata is $CO_2$ uptake for photosynthesis and water loss for transpiration (Sakoda et al., 2020). Higher stomata density can improve biomass production of plants, but it can depend also from plant species and environmental conditions (Kardiman & Rœbild, 2007; Sakoda et al., 2020). Present study showed that fertilization with humus significantly ($p< 0.05$) increased density of stomata in both epidermis of leaf in the first and the second experimental years (Figure 1 a). Humus application significantly increased the height of plant, the area covered by plant and the length of inflorescences in the first experimental year. Meanwhile colder spring in the second experimental year could limit assimilation of humus (Table 1). Fertilization with compost significantly increased the density of stomata in the adaxial side, but significantly decreased in the abaxial side of leaves in *Pogestemon cablin* Benth (Lamiaceae) (Zahara et al., 2021). The main functions of glandular trichomes are secretion and storage of secondary metabolites (Huchhellmannet al., 2017). Present study showed that humus application significantly decreased the density of glandular trichomes in lower epidermis of leaves in the second experimental year (Figure 1 b). The effect of humus fertilization on size of glandular trichomes was not significant (Figure 1 c). Fertilization with organic nitrogen (foliar fertilization) increased the density of glandular trichomes of *Humulus lupulus* L. (Rodolfi et al., 2021).

According literature data fertilization also can influence yield and composition of essential oils for medicinal and aromatic plants (Sardashti et al., 2014; Rehman et al., 2016). Humus application did not significantly affect the yield of essential oil of *T. × citriodorus*, but the percentage of essential oil after humus application was lower than in control plants (Figure 2). Plants use nutrient elements first for increasing the biomass and then for synthesis of secondary metabolites (Caceres et al., 2017). As was mentioned above, humus application increased the height of plant, the area covered by plant and the length of inflorescences (Table 2). Fertilization with compost increased the yield of *Thymus vulgaris* essential oil (Hendawy et al., 2010). Humic acid application increased amount of essential oil in *Semenovia suffruticosa* but decreased in *Artemisia sieberi* (Sardashti et al., 2014). The humic and fulvic acids application had no effect for the yield of essential oil in *Rosmarinus officinalis* L. (Caceres et al., 2017). Fertilization with humus did not significantly influence percentage of main compounds of *T. × citriodorus* essential oil – geraniol, nerol, geranial, and neral (Figure 3). Fertilization with compost increased amount of oxygenated monoterpenes in essential oil of *Thymus vulgaris* (Hendawy et al., 2010). Humic acids application increased amount of oxygenated terpenoids in *Artemisia sieberi* and *Semenovia suffruticosa* (Sardashti et al., 2014).

**Conclusions**

Fertilization with humus had significant effect for *T. × citriodorus* yield and epidermal structures parameters: fertilization with humus increased the yield of *T. × citriodorus* in the first experimental year, increased the density of stomata in both epidermis of leaves in the first and second experimental years, but decreased the density of glandular trichomes in the lower epidermis in the second experimental year. However, humus application did not significantly affect on the percentage and composition of essential oil.

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**Received**: 7 December 2024.
**Accepted**: 27 January 2025.