SCIENTIA PLENA
www.scientiaplena.org.br
VOL. 20, NUM. 04 2024
doi: 10.14808/sci.plena.2024.040202

Sources of fertilizers on the phytotechnical characteristics, oxidative stress and thymol production of *Thymus vulgaris* L.

Fontes de fertilizantes nas características fitotécnicas, estresse oxidativo e produção de timol em *Thymus vulgaris* L.

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(Recebido em 20 de abril de 2023; aceito em 18 de abril de 2024)

The objective of this study was to assess the effects of different fertilizer sources on the phytotechnical characteristics, oxidative stress, and thymol production of *Thymus vulgaris*. The experiment was conducted in 3-dm³ pots containing soil:sand (2:1) + treatment (no fertilizer, organic fertilizer, or chemical fertilizer). The organic fertilizer was 8 kg m^{-2} of an equal mixture of bovine, goat, and quail manures. The variables assessed were leaf, stem, root, and total dry weights; chlorophyll and carotenoid contents; leaf nutrients; total polyphenols; total antioxidant capacity; oxygen radical absorption capacity; DPPH free radical scavenging activity; essential oil content; and thymol production, content, and yield. Organic fertilization significantly increased the variables assessed and reduced oxidative stress indicators. It resulted in gains in the dry weight of the plants and the accumulation of nutrients in dry leaves of *T. vulgaris*. For the quantitative parameters of the essential oil and thymol, organic fertilization was significantly higher than the other treatments, especially for thymol content and yield. *T. vulgaris* plants fertilized with organic fertilizer maximize their plant production and their yield of compounds of interest, such as thymol, possibly because these outputs indicate a balance between improved vegetative production and the elimination of reactive oxygen species.
Keywords: growth, essential oils, antioxidant capacity.

O objetivo deste estudo foi avaliar os efeitos de diferentes fontes de fertilizantes nas características fitotécnicas, estresse oxidativo e produção de timol de *Thymus vulgaris*. O experimento foi realizado em vasos de 3 dm³ contendo solo:areia (2:1) + tratamento (sem adubo, adubo orgânico ou adubo químico). A adubação orgânica foi de 8 kg m^{-2} de uma mistura igual de esterco bovino, caprino e de codorna. As variáveis avaliadas foram massa seca total de folha, caule, raiz, conteúdo de clorofila e carotenoides; nutrientes foliares; polifenóis totais; capacidade antioxidante total; capacidade de absorção de radicais de oxigênio; atividade de eliminação de radicais livres DPPH; teor de óleo essencial; e produção, conteúdo e rendimento de timol. A adubação orgânica aumentou significativamente as variáveis avaliadas e reduziu os indicadores de estresse oxidativo. Resultou em ganhos de massa seca das plantas e acúmulo de nutrientes nas folhas secas de *T. vulgaris*. Para os parâmetros quantitativos do óleo essencial e timol, a adubação orgânica foi significativamente maior que os demais tratamentos, principalmente para teor de timol e produtividade. Plantas de *T. vulgaris* adubadas com fertilizante orgânico maximizam sua produção vegetal e seu rendimento de compostos de interesse, como o timol, possivelmente porque essas saídas indicam um equilíbrio entre a produção vegetativa melhorada e a eliminação de espécies reativas de oxigênio.
Palavras-chave: crescimento, óleos essenciais, capacidade antioxidante.

1. INTRODUCTION

Thyme (*Thymus vulgaris* L., Lamiaceae) is cultivated for commercial purposes in many countries with the purpose of producing dry leaves, essential oil, extracts, and oil resins [1]. In addition to its use as a condiment, thyme has potential utility against infections and infestations of the intestinal system by hookworms, ascarids, gram-positive and gram-negative bacteria, fungi, and yeasts [2]. Thyme essential oil is responsible for the herb's typical pungent aroma and is one

of the 10 main food preservatives in the world. Its oil also found applications in the production of cosmetics, such as deodorants, toothpastes, and mouthwashes [1].

Of the known compounds of *T. vulgaris*, thymol is the most important and attracts considerable attention due to its pharmaceutical and food preservative applications [2, 3]. Thymol is biosynthesized in the glandular secretory trichome plastids of thyme plants and is found on the surfaces of aerial tissues and stored in the subcuticular space of the trichomes [3]. Interest in thymol has grown due to its various functionalities, such as antimicrobial, antioxidant, anti-inflammatory, antibacterial, antifungal, antidiarrheal, anthelminthic, analgesic, digestive, antihypertensive, depigmentation, and insecticidal activities [4].

Despite the wide applicability and economic importance of the species, there is still a need to improve its cultivation to improve its production [5]. Mineral nutrition directly influences the growth, development, and metabolism of plants and thereby changes the yield and chemical composition of secondary metabolites [6]. Organic fertilizers have provided the best mineral nutrition for the cultivation of medicinal and condiment species. The application of organic fertilizers positively affects the growth of medicinal and condiment plants, as well as their production and composition of essential oil and even oxidative stress [7, 8]. In general, environmental stresses can damage the normal pattern of plant development, resulting in reduced production yield [9]. Several abiotic stresses lead to overproduction of reactive oxygen species (ROS) in plants, which are highly reactive and toxic, causing damage to proteins, lipids, carbohydrates, and DNA, ultimately resulting in oxidative stress [10]. The ROS, including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$), are byproducts of physiological metabolisms and are precisely controlled by enzymatic and nonenzymatic antioxidant defense systems that work together.

Several studies have shown positive responses of thyme cultivation to organic fertilization with composting, biofertilizers, phosphate rock powder, and cattle, sheep, and chicken manure [11]. Complete analyses that encompass oxidative stress and phytotechnical, phytochemical, and nutritional data are practically nonexistent for this species. On this background, the objective of this study was to assess the effects of the type of fertilization on oxidative stress and phytotechnical characteristics and on the production of essential oil and thymol by *T. vulgaris* L.

2. MATERIAL AND METHODS

2.1 Design and conduction of the experiment

The experiment was accomplished in a completely randomized design with three treatments, control (without fertilization), chemical fertilizer, and organic fertilizer (manure), with seven replicates, two plants per pot and six pots per replicate, totaling 126 plants. The seedlings were obtained by cutting plants of *T. vulgaris*, whose exsiccate was deposited in the herbarium of the Agricultural Research Company of Minas Gerais (Empresa de Pesquisa Agropecuária de Minas Gerais - EPAMIG) under registration number 58576.

Cuttings of ± 5 cm were placed in 128-cell Styrofoam trays using the commercial substrate Tropstrato HA™ and kept in an oven with automated irrigation. Seedlings 10 to 15 cm in height (45 days old) were transplanted into 3-dm³ pots to receive the treatments. For the preparation of the substrate, soil and coarse sand were mixed at a ratio of 2:1. The soil used in the substrate was collected from the 0-20-cm-depth layer of a Latossolo Vermelho distroférrico (Dystroferric Red Latosol). The chemical characteristics of the substrate were as follows: pH in water = 5.4; K (mg dm^{-3}) = 25.89; P-Rem (mg L^{-1}) = 15.22; Ca^{2+}, Mg^{2+}, Al^{3+}, H + Al ($cmol_c$ dm^{-3}) = 0.68; 0.05; 0.07; 2.93; base saturation index (V%) = 21.35; organic matter (dag kg^{-1}) = 1.03; Zn, Fe, Mn, Cu, B, and S (mg dm^{-3}) = 0.46, 43.3, 15.34, 1.38, 0.02, and 1.6, respectively.

The group fertilized with chemical fertilizer followed the recommendation of Baranauskiene et al. (2003) [12]. The chemical fertilizer dose was equivalent to 135 kg ha^{-1} of N, 120 kg ha^{-1} of phosphorus (P_2O_5), and 150 kg ha^{-1} of potassium (K_2O). The fertilizers used as sources of these nutrients were urea (45% N), simple superphosphate (18% P_2O_5; 18% Ca; 11% S), and potassium chloride (58% K_2O). This corresponded to an application per pot of 45 mg of urea, 100 mg of

simple superphosphate, and 39 mg of potassium chloride, with the N and K_2O divided into three applications.

The dose used for organic fertilization was 8 kg m^{-2}. It was a mixture of bovine manure, goats, and quail in equal proportions. This dose corresponded to the application of 120 g of manure per pot at the time of substrate preparation. The analysis of the organic fertilizer indicated the following values: C/N ratio = 23; moisture (65 °C) and organic carbon = 88 and 308 (g kg^{-1}); N, P_2O_5, K_2O, Ca, Mg, and S (g kg^{-1}) = 16, 17, 25, 47, 4, and 3; Cu, Fe, Zn, Mn, and B (mg kg^{-1}) = 42, 2787, 169, 466, and 105.

The growth conditions of the plants were conducted under sunlight in the experimental field with maximum (29°C) and minimum (19°C) average temperature and relative humidity (73%) located at the geographical coordinates 21° 14′ S and 45° 00′ W at 918 m altitude. To conduct this experiment, each irrigation time was 2 days, with a water depth of 13 mm for the first 60 days and 26 mm after that, to keep the substrate at a moisture between 70 and 90% of field capacity. During the experiment, weeds were removed manually and there was no need to control diseases or pests. The variables assessed 120 days after transplanting the seedlings were leaf, stem, root, and total dry weights; chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid contents; leaf nutrients; total polyphenols; total antioxidant capacity (TAC); oxygen radical absorption capacity (ORAC); DPPH free radical scavenging activity; essential oil content; and thymol production, content, and yield

2.2 Dry weight of the vegetative organs

To obtain the dry weight, the organs of each plant were separated and dehydrated in a forced-air oven at 37 °C to constant weight. The variables measured were leaf dry weight, stem dry weight, root dry weight, and total dry weight, expressed in g $plant^{-1}$.

2.3 Leaf analysis of macronutrients and micronutrients

Samples of dry leaves from each group were sent to the Laboratory of Agricultural Analysis 3rlab, Lavras, MG, Brazil to determine the content of N, P, K, Ca, Mg, S, B, Cu, Mn, Zn and Fe. The macronutrients are expressed in g kg^{-1} of leaf dry weight and the micronutrients in mg kg^{-1} of leaf dry weight. With the mean leaf weight per plant data, the accumulations of macronutrients and micronutrients were calculated.

2.3 Tests indicative of oxidative stress

2.3.1 Sample preparation and reading equipment

Thyme leaves were sprayed into a knife mill, and a mass of 50 mg was directly weighed in microtubes. Then, extraction was performed with 1 mL of 50% hydroalcoholic solution by sonication at 50 Hz using two cycles of 15 min. At the end of each cycle, the samples were centrifuged at 6000 rpm for 10 min. The supernatants were combined to obtain an extract at 25 mg mL^{-1}. The extracts were stored in a freezer at -4 °C until analysis. Spectrophotometric data of the assays were measured in a TECAN INFINITY M200 PRO spectrophotometer operated with the I-Control® data processing system (version 3.37).

2.3.2 Total phenols

The content of total phenolic compounds was determined by the Folin-Ciocalteu method according to Singleton et al. (1999) [13]. The concentration shown by the calibration curve ($y = 14.314x + 0.157$; $R^2 = 0.9986$) ranged from 0.1250 to 0.0078 mg mL^{-1} in an ethanolic solution of

gallic acid (Sigma-Aldrich, USA). The results are expressed in milligrams of gallic acid equivalents per gram of dry leaf weight (mg GAE g^{-1}).

2.3.3 DPPH free radical scavenging

The extracts were also tested for their ability to eliminate the free radical (2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA). The results are expressed by the antioxidant activity index (AAI) and were calculated according to the formula:

$$AAI = \frac{\text{Final concentration of DPPH } (\mu g\ mL^{-1})}{IC_{50}\ (\mu g\ mL^{-1})}$$

The plant extract was considered to have weak antioxidant activity (AAI ≤ 0.5), moderate antioxidant activity (0.5 ≤ AAI ≤ 1.0), strong antioxidant activity (1.0 ≤ AAI ≤ 2.0), and very strong antioxidant activity (AAI ≥ 2.0).

2.3.4 TAC/phosphomolybdenum assay

TAC was assessed according to Prieto et al. (1999) [14]. In this assay, where the reduction of molybdenum (VI) to molybdenum (V) in the presence of a reducing agent (antioxidant) forms a green phosphomolybdate (V) complex. The calibration curve ($y = 0.6418x + 0.1227$, $R^2 = 0.9961$) constructed with aqueous solution of ascorbic acid (Sigma-Aldrich, USA) in the working range of 5.0000 to 0.0390 mg mL^{-1} was used in the calculation of the results. The assessment was performed in quintuplicate, and the results are expressed in milligrams of ascorbic acid equivalents per gram of dry leaf (mg EAA/g).

2.3.5 ORAC/peroxyl radical scavenging

The ORAC assay is based on the inhibition of oxidation induced by the oxyradical 2,2′-azobis-(2-methylpropionamidine) dihydrochloride (AAPH) by substances with antioxidant properties. Over time, the ROS generated from the thermal decomposition of AAPH extinguish the fluorescent probe signal (fluorescein), as described by Cao et al. (1993) [15]. To assess the fluorescence over time (λ_{ex} = 485 nm and λ_{em} = 520 nm), readings were taken every minute for 150 min in a microplate reader. The area under the fluorescence–time curve (AUC) of each sample was subtracted from the area under the curve of the target to find the net area: $AUC_{net} = AUC_{sample} - AUC_{target}$. The analysis was performed in quintuplicate. The concentration of the calibration curve ($y = 1E^{+11}\ x + 296396$; $R^2 = 0.9983$) ranged from 125 to 7.8125 µM Trolox. The results are expressed in micromoles of Trolox equivalent (TE) per milligram of dry leaf weight (µmol TE/mg).

2.4 Analysis of photosynthetic pigments

The extraction and dosing of photosynthetic pigments followed the method developed by Hiscox and Israelstam (1979) [16]. In summary, fresh leaves (50 mg) were weighed directly in Falcon tubes covered with aluminum foil, and 10 mL of dimethyl sulfoxide (DMSO) saturated with calcium carbonate ($CaCO_3$) was added, and they were incubated in an oven at 65 °C for 48 h. The samples were prepared in quadruplicate. Three 3-mL aliquots of each replicate were transferred to a quartz cuvette, and the optical density values at 480, 649, and 665 nm were measured against pure DMSO (target). The specific optical density value of each sample was calculated by the average of the readings of the three aliquots.

The wavelengths and the equations used for the calculations were based on the method by Wellburn (1994) [17], where:

$$\text{Chlorophyll } a_{649} = (12.47 \times A_{665}) - (3.62 \times A_{649});$$
$$\text{Chlorophyll } b_{665} = (25.06 \times A_{649}) - (6.5 \times A_{665});$$
$$\text{Carotenoids}_{480}: (1000 \times A_{480} - 1.29 \times C_a - 53.78 \times C_b)/220.$$

Total chlorophyll (*a* + *b*) was calculated by summing the results of the equations for chlorophyll *a* and *b*. All results are expressed in mg g^{-1} fresh matter.

2.5 Quantitative analyses of essential oil and agricultural yield of thymol

2.5.1 Essential oil content

The essential oil was distilled by hydrodistillation in a modified Clevenger apparatus for 90 min. A sample of 15 g of dry leaves ($n = 5$) was placed in a 2-L distillation flask containing 1 L of distilled water. Liquid–liquid partitioning with dichloromethane (3 × 5 mL) was performed to purify the essential oil. The organic phase was combined and treated with anhydrous magnesium sulfate. After simple filtration, the solvent was evaporated at room temperature under a gas exhaust hood. The essential oil was placed in an amber bottle under refrigeration at 4 °C. The essential oil content (%) represents the average weight of the oil (mg) per 100 mg of dry weight of the leaves.

2.5.2 Thymol measurement

The essential oil was analyzed by gas chromatography (GC) in an Agilent 5890A system using an HP-5 MS column (30 cm long × 250 µm internal diameter × 0.25 µm thick). The essential oil was diluted in ethyl acetate (1%, v/v), and 1 µL was injected in split mode at a ratio of 30:1. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. The injector and detector temperatures were 220 and 250 °C, respectively. The initial oven temperature was 60 °C. It was kept at 60 °C for 1.5 min, followed by a temperature ramp of 3 °C min^{-1} to 100 °C, a temperature ramp of 4 °C min^{-1} to 200 °C, and a last ramp of 10 °C min^{-1} to 240 °C. To identify the presence of thymol, an authentic sample (Sigma-Aldrich®, declared purity ≥ 99.5%) was analyzed under the same conditions. The retention indices relative to the series of *n*-alkanes C8 to C20 were calculated according to van Den Dool and Dec. Kratz (1963) [18] for the corresponding peaks and compared to retention indices in the literature [19, 20]. For the unequivocal identification of the peak identity supposedly corresponding to thymol, the essential oil was also analyzed in an Agilent® 7890A gas chromatograph coupled to an Agilent® MSD 5975C mass selective detector (Agilent Technologies, California, USA), operated by impact ionization electron at 70 eV, in scan mode, at a speed of 1.0 scan/s, with a mass acquisition interval of 40-400 *m*/*z*. The operating conditions were the same as those used for GC. The identity of the thymol peak in the samples was confirmed by comparison with the mass spectra of the library database of the National Institute of Standards and Technology (2008) [21] and coinjection with the standard.

For the quantitative analyses of thymol, the external standard method was used [22]. The linearity range (2.0 to 10.0 µg) was estimated in the known area of a thymol reference solution and the area corresponding to the thymol peak in the samples. Linearity was determined by injecting, in triplicate, different volumes of reference solutions (2.5 and 5.0 µg mL^{-1}). The calibration curves were drawn in relation to the injected thymol mass on two consecutive days. The data obtained for each thymol analytical curve were subjected to linear regression analysis by the least squares method, and the coefficients of determination (r^2) were calculated. The curves obtained on the two consecutive days were statistically compared by analysis of covariance ($p < 0.05$). From the mean analytical curve ($y = 43350186.76x + 10093465.13$, $R^2 = 0.9979$), the thymol production (mg g^{-1} of essential oil), the content (g100 g^{-1} of dry leaf), and yield (µg $plant^{-1}$) were determined. The yield equaled the production of thymol times the mass of oil produced per plant.

2.6 Statistical analysis

The data obtained were first assessed for homogeneity and normality and then subjected to Tukey's test at 5% probability. Statistical analyses were performed using the SISVAR 5.3 statistical software. Statistica® software, version 13.3 (StatSoft, Tulsa, OK, USA), was used for principal component analysis (PCA).

3. RESULTS

3.1 Analysis of plant growth

The vegetative growth of thyme increased significantly with either chemical or organic fertilization compared to the control treatment (Figures 1 and 2). The organic fertilizer most significantly influenced the dry matter accumulation of the organs and total dry matter accumulation. Leaf dry weight was 148.5% higher under organic fertilization than control and 66.6% higher than with the chemical fertilizer. The increase in stem dry weight with organic fertilizer was approximately 158% and 56% when compared to the control and chemical fertilizer, respectively. The increase in root dry weight was even greater, reaching 270% and 52%, respectively. Consequently, the total dry weight was higher under organic fertilization, reaching a weight almost 3 times greater than that under the control and 1.6 times greater than that under chemical fertilization. These results corroborate the observations of Sharaf EL-Din et al. (2019) [11], in which thyme responded positively to growth variables when subjected to organic or chemical fertilization.

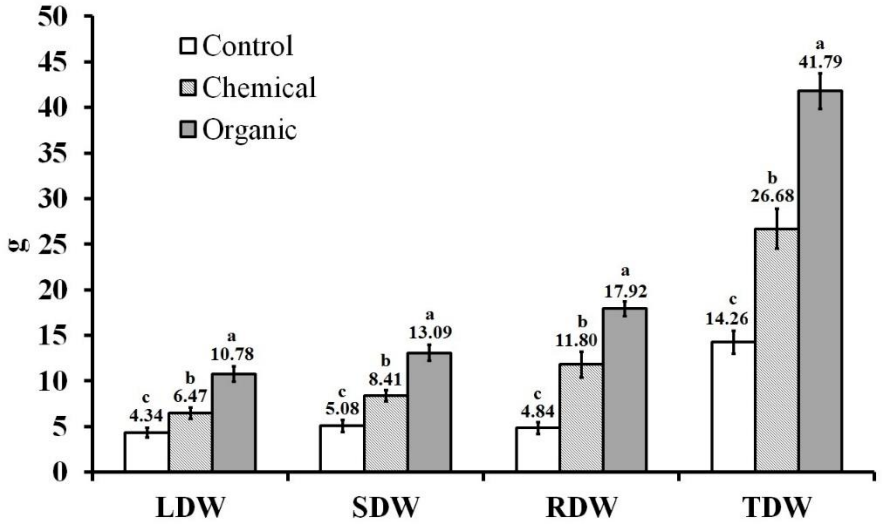

*Figure 1. Leaf (LDW), stem (SDW), root (RDW) and total (TDW) dry weight of Thymus vulgaris L. cultivated with different types of fertilization (Control – Substrate; Chemical – Substrate fertilized with chemical fertilizer; Organic – Substrate fertilized with organic fertilizer). *Means followed by the same letter within the column do not differ significantly by Tukey's test (5%).*

Figure 2. Plants of Thymus vulgaris L. cultivated with different types of fertilization (Control – Substrate; Organic – Substrate fertilized with organic fertilizer; Chemical – Substrate fertilized with chemical fertilizer;).

In general, the contents of macronutrients and micronutrients were significantly impacted by fertilization, being lower in the groups that were fertilized with manure and chemical fertilizer (Table 1). The values observed in the present study are very close to those described in the literature for macronutrients and micronutrients in thyme: N: 15.3 to 16.1 g kg^{-1}; P: 2.5 to 3.5 g kg^{-1}; K: 7.5 to 9.3 g kg^{-1}; Ca: 11.2 to 11.8 g kg^{-1}; Mg: 1.47 to 1.53 g kg^{-1} and S: 2.4 to 2.9 g kg^{-1}; B: 31 to 34.3 mg kg^{-1}; Cu: 7.4 to 9 mg kg^{-1}; Fe: 200 to 242 mg kg^{-1}; Mn: 45 to 55 mg kg^{-1}; and Zn: 25.6 to 30.5 mg kg^{-1} [23].

Table 1: Macronutrients and micronutrients content in dry leaves of Thymus vulgaris L. cultivated with different types of fertilization (Control – Substrate; Chemical – Substrate fertilized with chemical fertilizer; Organic – Substrate fertilized with organic fertilizer).

	Macronutrients					
	N	**P**	**Ca**	**K**	**Mg**	**S**
	---------- g kg^{-1} ----------					
Control	11.2 a	2.0 a	11.9 b	12.6 a	2.4 a	2.3 a
Chemical	11.4 a	1.8 b	14.4 a	9.8 ab	2.3 a	2.0 ab
Organic	12.7 a	1.6 c	10.8 b	7.1 b	2.3 a	1.9 b
C.V. (%)	5.64	3.02	7.45	10.51	8.67	6.83
	Micronutrients					
	B	**Cu**	**Fe**	**Mn**	**Zn**	
	---------- mg kg^{-1} ----------					
Control	33.0 b	15.1 a	241.6 a	145.3 a	60.4 a	
Chemical	40.6 a	12.6 b	187.7 b	68.9 b	25.9 b	
Organic	31.7 b	10.3 c	129.0 c	39.7 b	24.5 b	
C.V. (%)	5.70	2.21	6.84	8.20	5.73	

*Means followed by the same letter within the column do not differ significantly by Tukey's test (5%), CV - coefficient of variation.

The highest levels of P, K, and S (2.0, 12.6 and 2.3 g kg^{-1}, respectively) were observed in the control, as well as those of Cu, Fe, Mn and Zn (15.1, 241.6, 145.3 and 60.4 mg kg^{-1}, respectively) (Table 1). The lowest P, Cu, and Fe contents (1.6 g kg^{-1}, 10.3 mg kg^{-1}, and 129 mg kg^{-1}, respectively) were observed under organic fertilization, lower even than those under chemical fertilization. On the other hand, the accumulation of nutrients in thyme plants was completely significant for the treatment with organic fertilization (Table 2). The significant weight gain of the fertilized plants, especially with organic fertilizer, caused a "dilution" effect on many of the nutrients, especially those with high and moderate mobility in the plant. Thus, we can say that organic fertilization promoted greater absorption and accumulation of nutrients in thyme.

On the other hand, the highest Ca and B contents (14.4 g kg^{-1} and 40.6 g kg^{-1}, respectively) were observed under chemical fertilization. The increase in Ca content is explained by the addition of this element to chemical fertilizer with simple superphosphate, which has 18% Ca.

Table 2: Accumulation of macronutrients and micronutrients in dry leaves of Thymus vulgaris L. cultivated with different types of fertilization (Control – Substrate; Chemical – Substrate fertilized with chemical fertilizer; Organic – Substrate fertilized with organic fertilizer).

	Macronutrients					
	N	**P**	**Ca**	**K**	**Mg**	**S**
	---------- mg $plant^{-1}$ ----------					
Control	24.4 c	4.3 c	25.9 c	27.3 b	5.2 c	5.0 c
Chemical	77.4 b	11.9 b	98.2 b	66.8 a	15.3 b	13.6 b
Organic	136.9 a	16.7 a	115.8 a	76.5 a	24.8 a	19.9 a
C.V. (%)	5.64	3.02	7.45	10.17	7.59	6.83
	Micronutrients					
	B	**Cu**	**Fe**	**Mn**	**Zn**	
	---------- mg $plant^{-1}$ ----------					
Control	0.072 c	0.033 c	0.525 b	0.316 b	0.131 c	
Chemical	0.277 b	0.086 b	1.279 a	0.469 a	0.177 b	
Organic	0.341 a	0.111 a	1.391 a	0.427 a	0.263 a	
C.V. (%)	5.70	1.95	6.84	8.20	5.73	

*Means followed by the same letter within the column do not differ significantly by Tukey's test (5%), CV - coefficient of variation.

3.2 Tests indicative of oxidative stress

Fertilized thyme plants had less oxidative stress than nonfertilized plants (Table 3). The total polyphenol content varied between all treatments, the lowest value (51.72 mg GAE/g) being under chemical fertilization and the highest (65.58 mg GAE/g) in the control group. TAC followed the same trend as the phenols, where the control showed a TAC almost 33% higher than the chemical fertilizer group and 23% higher than the organic fertilizer group. ORAC and DPPH scavenging activity showed the same trend, where the control was significantly more active ($p < 0.05$) than the chemical and organic fertilizer groups. The AAI of DPPH classified the control as having high antioxidant activity and the chemical and organic fertilizer groups moderate activity, which well reveals the benefits of the treatments.

Table 3. Effect of the type of fertilization (Control - Substrate; Chemical - Substrate fertilized with chemical fertilizer; Organic - Substrate fertilized with organic fertilizer) on the content of Total Polyphenols (Phenols) expressed in mg of gallic acid equivalents / g of dry leaf (mg GAE / g); total antioxidant capacity (TAC) in mg of ascorbic acid equivalents / g of dry leaf (mg AAE / g); Oxygen radical absorbance capacity (ORAC) expressed in µmol Trolox equivalent/g dry leaf (µmol TE / mg); and DPPH, Antioxidant Activity Index (AAI), in dry leaves of Thymus vulgaris L.

	Phenols	**TAC**	**ORAC**	**DPPH**
	mg GAE/g	mg AAE/g	µmol TE / mg	AAI
Control	65.58 a	76.63 a	1.84 a	1.30 a
Chemical	51.72 c	57.71 c	1.58 b	0.82 b
Organic	62.14 b	62.17 b	1.60 b	0.77 b
C.V (%)	1.14	1.33	5.37	4.87

*Means followed by the same letter within the column do not differ significantly by Tukey's test (5%), CV - coefficient of variation.

The lower values of phenols and TAC in the chemical fertilizer group (Table 3) may be explained mainly by the high values of Ca (Table 1).

3.3 Photosynthetic pigments

In general, the concentrations of chlorophyll and carotenoids were affected by the treatments to which the plants were subjected. The chlorophyll *a* and total chlorophyll were significantly higher in the control and organic fertilization groups; their levels were 0.90 and 1.92 mg g^{-1} fresh weight in the control and 0.89 and 1.82 mg g^{-1} fresh weight in the organic fertilizer group, respectively, whereas they were only 0.75 and 1.65 mg g^{-1} fresh weight in the chemical group (Figure 2). The chlorophyll *b* and carotenoid contents tended to decrease under any type of fertilization, so the control plants had the highest chlorophyll *b* and carotenoid contents (Figure 3).

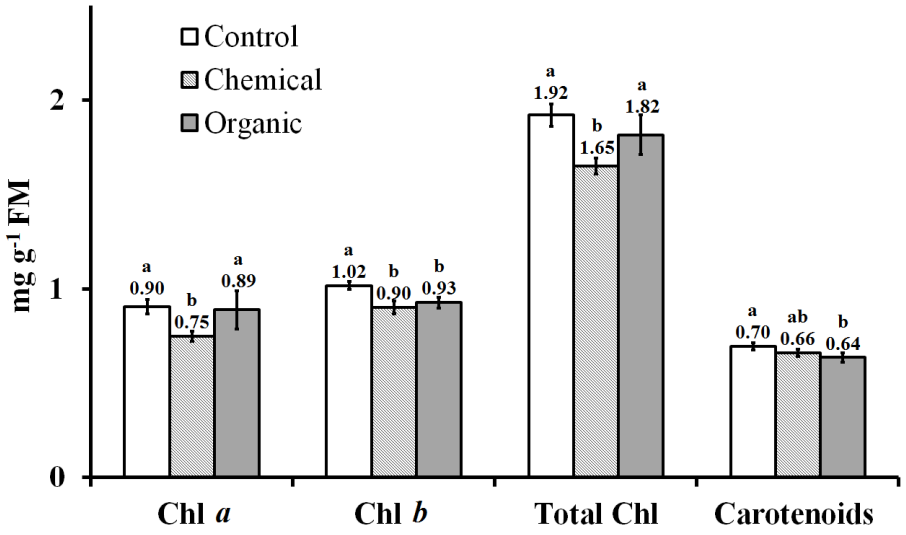

*Figure 3. Chlorophyll a, chlorophyll b, total chlorophyll and carotenoids (mg g-1 of fresh matter) from leaves of Thymus vulgaris L. cultivated with different types of fertilization (Control - Substrate; Chemical - Substrate fertilized with chemical fertilizer; Organic - Substrate fertilized with organic fertilizer). *Means followed by the same letter within the column do not differ significantly by Tukey's test (5%).*

3.4 Quantitative analyses of essential oil and thymol

The essential oil content and thymol production (mg g EO^{-1}) were considerably affected by the treatment (Table 4). These variables were significantly higher under organic fertilization, with a mean increase of 16.3 and 7.8%, respectively, over their values in the control and chemical treatments. The thymol content and yield under organic fertilization were also significantly higher than those under the other treatments. The significant increase in thymol content with organic fertilization provided a gain of 16.4% over the control and even more over the chemical fertilizer (33.3%). For the thymol yield, the organic fertilizer brought a gain of almost 6-fold per plant when compared to the control and more than twice as much when compared to the chemical fertilizer.

Table 4. Essential oil content, Thymol production (mg / g of essential oil (EO), Thymol content (g / 100 g of dry leaf = %) and Thymol yield (µg $plant^{-1}$) extracted from the dry leaves of Thymus vulgaris L. cultivated with different types of fertilization (Control – Substrate; Chemical – Substrate fertilized with chemical fertilizer; Organic – Substrate fertilized with organic fertilizer).

	Oil content	Thymol	Thymol content	Thymol yield
	%	mg g EO^{-1}	%	µg $planta^{-1}$
Control	0.88 b	625.21 b	0.55 b	5.95 c
Chemical	0.84 b	604.31 b	0.48 c	16.53 b
Organic	1.00 a	662.57 a	0.64 a	34.51 a
C.V. (%)	8.46	1.60	1.65	2.98

*Means followed by the same letter within the column do not differ significantly by Tukey's test (5%), CV - coefficient of variation.

3.5 Principal component analysis

The objective of the PCA was to assess the interactions between the variables and the type of fertilization so that we could better explain our results. The PCA explained 100% of the total variation in the variables considered. PC1 contributed 63.96% and PC2 contributed 36.04% of the total variation (Figure 4).

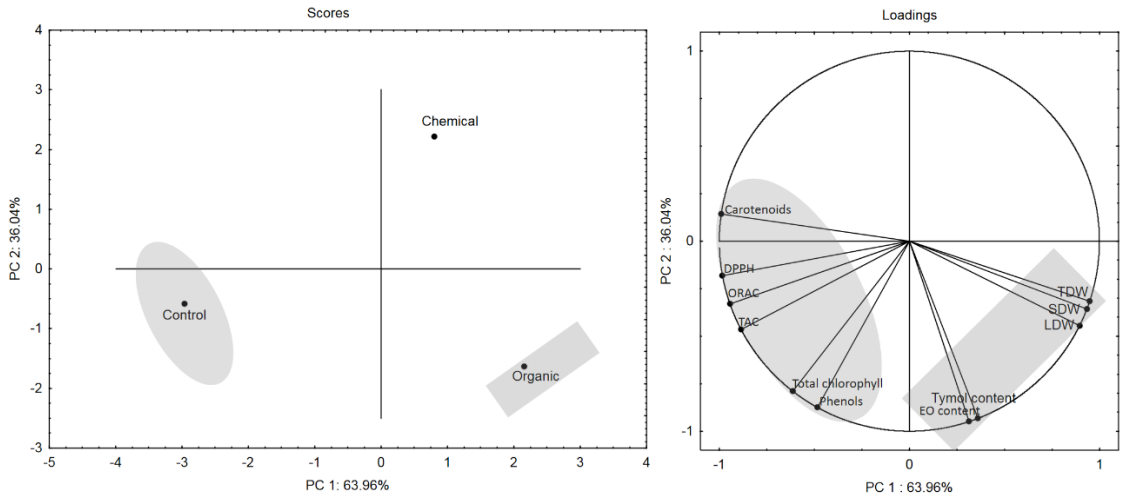

Figure 4. Principal component analysis (PCA) of the matrix correlation constructed using data from Leaf Dry Weight (LDW), Stem Dry Weight (SDW), Total Dry Weight (TDW), Total Chlorophyll, Carotenoids, Total Polyphenols (Phenols), Antioxidant Capacity total (TAC), Oxygen radical absorbance capacity (ORAC), DPPH, essential oil content and Thymol production of Thymus vulgaris L. cultivated with different types of fertilization (Control – Substrate; Chemical – Substrate fertilized with chemical fertilizer; Organic – Substrate fertilized with organic fertilizer).

The graph of scores in Figure 4 shows the separation of PC1 into two groups: chemical or organic fertilization (positive scores) and control treatment (negative score). In this same graph, PC2 was separated into control or organic fertilization (negative scores) and chemical fertilization (positive score). These separations left each treatment in different quadrants of the graph, showing that there was a marked difference between treatments. The control treatment (without fertilization) positively influenced the levels of total chlorophyll, carotenoids, phenols and oxidative stress indicators. In general, organic fertilization provided the plant with favorable conditions for vegetative growth (LDW, SDW and TDW) and production of essential oil and thymol. There was a positive correlation between phenols and total chlorophyll and between the essential oil and thymol contents. Furthermore, the analysis of the vectors showed that the oxidative stress and carotenoid indicators were negatively correlated with the vegetative growth variables of the plant, corroborating the hypothesis of an environmental stress caused by the unavailability of nutrients.

The PCA explained 100% of the total variation in the variables considered. PC1 contributed 73.77% and PC2 contributed 26.23% of the total variation (Figure 5). Corroborating the results of the previous PCA, the analysis of the scores in Figure 5 also indicated marked separation between the treatments. PC1 separated the chemical and organic fertilization treatments from the control treatment, and PC2 separated the control and organic fertilization treatments from the chemical fertilization treatment. This indicates that the contents of macronutrients and micronutrients in thyme leaves were also highly influenced by the chemical composition of the substrates. Organic fertilization favored a high N content, and chemical fertilization favored high Ca and B contents. All other nutrients (P, K, Mg, S, Zn, Mn, Cu, and Fe) had a positive correlation with the control treatment.

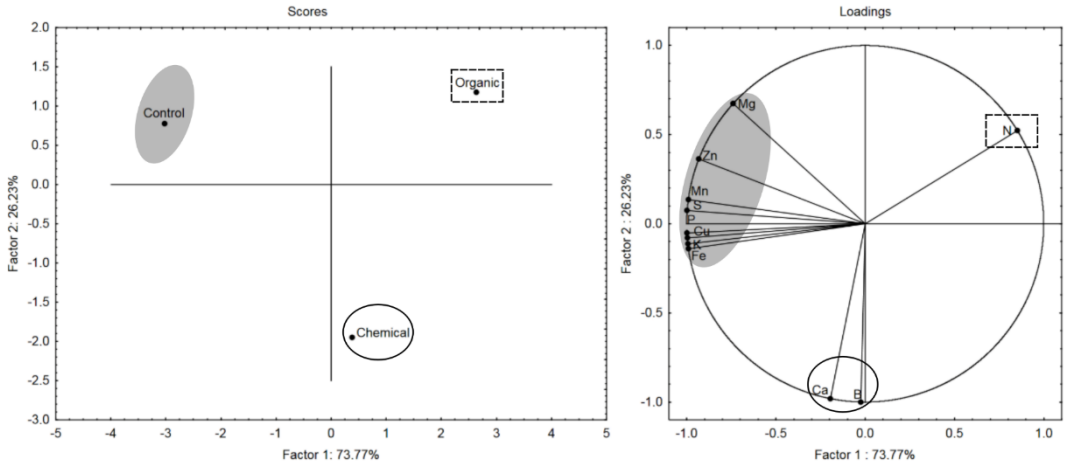

Figure 5. Principal component analysis (PCA) of the matrix correlation constructed using data on the amounts of macronutrients and micronutrients from dry leaves of Thymus vulgaris L. cultivated with different types of fertilization (Control - Substrate; Chemical - Substrate fertilized with chemical fertilizer; Organic - Substrate fertilized with organic fertilizer).

4. DISCUSSION

4.1 Analysis of plant growth

According to da Cunha Honorato et al. (2024) [8] and Alinejad et al. (2020) [24], organic fertilizers increase the vegetative growth of species grown under their fertilization compared to chemical fertilizers by providing all the essential nutrients at the same time and gradually making them available in the soil solution. Fallah et al. (2020) [25] observed an increase in the weight gain provided by poultry manure in *Dracocephalum kotschyi* Boiss.

Organic fertilizers can bring other benefits, such as improved structure, aeration, water storage, and internal soil drainage; improved nutrient adsorption; a gradual increase in the soil cation exchange capacity; and an increase in the biodiversity of useful microorganisms that act in the solubilization of various fertilizers to release nutrients to the plants [26].

Considering the participation of Ca and B in the same functions or processes, Mills and Benton (1996) [27] concluded that the two nutrients should be balanced for adequate plant growth, and Ca can be characterized by synergistic increases. One possibility is that the functions of B in the stabilization of cell wall components depend on joint action with Ca, probably aiding in the metabolism or incorporation of Ca into the cell wall [28].

4.2 Tests indicative of oxidative stress

The exposure of plants to environmental conditions, such as nutritional status, can increase the production of ROS. The accumulation of ROS as a result of various environmental stresses is one of the main causes of crop yield loss worldwide, as ROS hinder many cellular functions. The balance between ROS production and elimination at the appropriate time and place plays a crucial role in the survival of plants under environmental stress [10].

Bistgani et al. (2019) [3] observed that the highest values of free radical scavenging activity in thyme extracts were those with the highest contents of phenolic components, which corroborates the results of the present study (Table 3). The antioxidant activity of phenolic compounds in plants is mainly due to their redox properties and chemical structures, which may play an important role in ROS neutralization [29]. We expected the controls to have the most oxidative stress because these plants were under nutritional stress. Under some types of stress (light, water deficit, nutritional deficiencies, etc.), plant metabolism generates high amounts of

ROS, which are highly harmful to plants, promoting the peroxidation of lipids, membranes, chlorophylls, thylakoids, proteins, DNA, and RNA [10]. ROS are produced constantly in plant cells as a result of aerobic metabolism in most intracellular organelles, such as mitochondria, peroxisomes, and chloroplasts [30]. In each aerobic cell, a dynamic balance is struck between ROS generation and the antioxidant defense system. Abiotic stress increases ROS generation and disrupts the balance in favor of the oxidative reactions, thereby creating oxidative stress [10, 31]. In plant cells, the main source of ROS generation is chloroplast, which occurs because under most tensions, there is an adequate energy indulgence in photosynthesis, and a successive reduction in molecular oxygen produces ROS, including hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$) [30, 31].

Ca plays a significant role in stress tolerance, triggering the activity of antioxidant enzymes and decreasing the lipid peroxidation of cell membranes under abiotic stress conditions [32]. In addition, Ca is essential to stabilize the permeability of the cell membrane, which is due to selective ion uptake, and increases the rigidity of the cell wall, inducing crosslinking of the wall pectins [28].

4.3 Photosynthetic pigments

The photosynthetic pigments (chlorophylls *a* and *b*, carotenoids) are essential to estimate the photosynthetic potential of plants due to their direct connection with the absorption and transfer of light energy and thus their roles in the growth, development, and adaptation of plants to biotic and abiotic conditions. Plants with more chlorophyll can undergo photosynthesis at higher rates [33].

Working with the key gene expression patterns of pigment biosynthesis, Liu et al. (2010) [34], Shi et al. (2012) [35], and Huang et al. (2018) [36] observed that the application of organic fertilizer can markedly promote the formation of chloroplasts (chlorophylls) and the development and accumulation of plastid pigments (β-carotene, violaxanthin, lutea, and neoxanthin). According to Alinejad et al. (2020) [24], a decrease in chlorophyll may be due to reduced synthesis of the main photosynthetic pigment complexes encoded by the chloroplast precursor gene family (light-harvesting complex (LHC) II type I: chlorophyll *ab* binding protein) but also to the destruction of pigment–protein complexes that protect the photosynthetic apparatus, or even to oxidative damage to lipids and chlorophyll proteins [37]. This is probably a mechanism of protection of the photosynthetic apparatus against damage, which is considered essential for the short-term adaptation of plants to stress factors. In the photosynthetic process, the light absorbed by carotenoids and/or chlorophyll *b* in the proteins of the LHC is quickly transferred to chlorophyll *a* and then to other antenna pigments closely associated with the reaction center [38]. The LHC is involved in regulatory processes of the photosynthetic apparatus of the plant, so the increase in chlorophyll (*a*, *b*, and total) and carotenoids observed in the control treatment can be a regulatory mechanism by which the plant absorbs more energy and compensate for the reduced photosynthetic area (Leaves – Figure 1). On the other hand, the higher values of chlorophyll *a* and total chlorophyll observed in the group with organic fertilization may have been stimulated by the high levels of N (Table 1), since the Mg^{2+} ions share electrons with the N atoms of chlorophyll *a*.

In addition to their function as accessory pigments, carotenoids play an essential role in the photoprotection of plants. Carotenoids are responsible for the extinction of singlet oxygen; therefore, the increase in their content under stress may also have been related to this function in the control group of the present study. These results corroborate the tests indicative of oxidative stress in that they identify greater stress in plants given the control treatment.

4.4 Quantitative analyses of essential oil and thymol

Thyme is one of the main sources of thymol, containing between 10 and 64% of this compound in its essential oil. Interest in thymol has been growing due to its wide range of functional possibilities in the pharmaceutical, food and cosmetic industries [39].

Data on the content of essential oil, as well as the biosynthesis of thymol and its accumulation, vary between tissues and in response to different environmental factors and stimuli (temperature, precipitation, soil, mineral nutrition, etc.) in plant species of Lamiaceae, such as *T. vulgaris* [11, 40]. Thymol is a phenolic monoterpene biosynthesized from isopentenyl diphosphate and dimethylallyl diphosphate, which are derived from the methyl erythritol phosphate pathway located in plastids [40]. Some authors have correlated the development and accumulation of plastid pigments with the application of organic fertilizer [33-35]. It may be suggested, therefore, that in the present study, the increase in thymol in the organic fertilization group was linked to the increase in plastid number. According to da Cunha Honorato et al. (2022) [41] the highest percentage of thymol (65.42%) was obtained in the group with 9 kg m^{-2} cattle manure and 3 kg m^{-2} green manure. Also, organic sources such as vermicompost and animal manures improve the quantity and quality of the damask rose (*Rosa damascena*) volatile oil [42].

4.5 Principal component analysis

The PCA data confirm the results discussed above and point to others not observed. The higher N content, for example, explains the increase in pigment content in organic fertilization due to the structural functions of this nutrient in chlorophyll. This also explains the significant biomass gain in this treatment, since approximately 90% of the total N of the plant is in the organic form, so it performs its functions as a structural component of macromolecules and constituents of enzymes. This result suggests that organic fertilization favors the optimization of N by the plant. Alinejad et al. (2020) [24] also observed that the pigment content and biomass were higher when using organic fertilizers, especially poultry manure, due to the accessibility of *Datura stramonium* L. plants to the absorption of N and other nutrients involved in the formation of pigments during the growing season.

Mg showed a strong positive correlation with the control treatment (Figure 5). The most well-known role of Mg is as part of the chlorophyll molecule; this element corresponds to 2.7% of its weight and represents approximately 15 to 20% of the total Mg of the leaves of the plants. This information may explain the increase in chlorophyll content of the control group. Mg performs other important functions in plants, such as activating antioxidant enzymes [43].

Thyme plants that do not receive fertilization suffer oxidative stress, negatively impacting their vegetative growth and thymol production. Therefore, their cultivation requires nutritional supplementation with chemical or organic fertilizer. This species responds better in dry weight gain and thymol production to organic fertilization, possibly because these variables indicate a balance between improvement of phytotechnical characteristics and elimination of ROS. Although no cost/benefit analysis has been performed, some relevant considerations can be inferred from the results presented. The yield gains provided by organic fertilization indicate that, depending on local availability, transport costs, and purchase prices, the organic cultivation of *T. vulgaris* should be prioritized.

5. CONCLUSION

Organic fertilization significantly increased the variables evaluated and reduced oxidative stress indicators.

6. ACKNOWLEDGEMENTS

The authors would like to thank the National Council for Scientifc and Technological Development (CNPq - Conselho Nacional de Desenvolvimento Científco e Tecnológico), the Coordination for the Improvement of Higher Education Personnel (CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and the Minas Gerais State Research Foundation (FAPEMIG - Fundação de Pesquisa do Estado de Minas Gerais) for fnancial support (scholarships and research grants).

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