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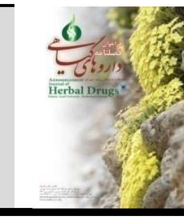

Influence of chitosan concentration on morpho-physiological traits, essential oil and phenolic content under different fertilizers application in *Thymus daenensis*

Zohreh Emami Bistgani[1*], Seyed Ata Siadat[1], Abdolmehdi Bakhshandeh[1], Abdollah Ghasemi Pirbaluti[2], Masoud Hashemi[3]

[1]*Department of Agronomy (Physiology of Crop Plants). Ramin Agriculture and Natural Resources University of Khozestan, Iran;*

***Email:** zohreemami66@gmail.com*

[2]*Department of Medicinal and Aromatic Plants, Research Center for Medicinal Plants and Ethnoveterinary, Shahrekord, Iran;*

[3]*Stockbridge School of Agriculture, University of Massachusetts, Amherst, MA 01003-9294. U.S.A.*

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ABSTRACT

Background & Aim: *Thymus daenensis* is recognized as aromatic herbs, which used in food, flavoring, pharmaceutical, and perfumery industries. This study was conducted to investigate the response of *T.daenensis* to different fertilizers (chemical and organic) along with chitosan.

Experimental: Treatments consisted of different fertilizers including: F1 =C (control without manure and fertilizer); F2 = CF (chemical fertilizers 100-150-100 kg ha^{-1}NPK, respectively), F3= CM (cow manure, 20 tonha^{-1}), F4= VC (vermicompost, 10 tonha^{-1}), F5=CF+CM+VC (chemical fertilizers + cow manure + vermicompost) and three chitosan levels (0, 0.2%, 0.4%) and acetic acid. This experiment conducted in split plot based on randomized complete block design with three replications during the period from 2014 to 2015 in Shahrekord, Iran. Morphological traits, essential oil yield from aerial parts and phenolic content were studied.

Results: Results indicated that canopy diameter, lateral branches, number of flower and leaf area index increased by CF+CM+VC treatment as compared to the control in 2014 and 2015. Chitosan increased biomass yield in both of growing year. Phenolic content increased in CF+CM+VC treatment in 2015 and it was also affected by application of 0.4% chitosan.

Recommended applications/industries: Since essential oil of *T. daenensis*, especially thymol and carvacrol, have a lot of applications in pharmacological and also has antimicrobial and antioxidant activities, the use of integrated fertilizer along with chitosan as a elicitor is recommended to obtain more essential oil yield and phenolic content.

1. Introduction

Thymus daenensis Celak. is a perennial herb distributed in many regions of Iran, (Nickavar *et al*. 2005; Ghasemi Pirbalouti *et al.* 2011). This herb is an endemic species of Iran and grows in the regions in Zagros Mountains chain, western and south western of Iran (Ghasemi Pirbalouti *et al*., 2014; Ghasemi Pirbalouti *et al*., 2013;

Nickavar *et al.*, 2005). It is widely used as a flavoring in meat products, salads, soups, and sauces but is also commonly used in traditional medicine to treat various diseases such as cold, cough, and digestive disorders (Nickavar *et al.* 2005; Ghasemi Pirbalouti, 2009).

*T. daenensis*is known for its powerful antimicrobial and antioxidant activity, which may to some extent explain its use in traditional medicine. The culinary function of this herb is due to its relatively high concentration of essential oils, which normally consist of the monoterpenes, thymol and carvacrol accompanied by their biosynthetic precursor's p-cymene and γ-terpinene as the primary components. These compounds are responsible for the aroma and flavor of this herb but also significantly contribute to its antimicrobial activity (Ghasemi Pirbalouti, 2014).

The major challenge for agriculture is to enhance crop production in a manner that is sustainable for present and future as well. The agricultural production and sustainable intensification goals in commercially important crops over both short and long terms demand proper plant nutrient and soil management. In arid and semi-arid areas, organic matter level of the soil is often very low. Thus, conservation and improvement of the soil organic matter is crucial for maintaining soil health and sustainability of farming in these regions. Moreover, successful crop production requires replacing adequate nutrients that have been removed by crops from the soil or lost to the environment one way or another. It is generally accepted that continuous application of chemical fertilizers may have negative impacts on soil health and environment (Yadav, 2003). Soil organic amendments therefore are considered as sustainable alternative sources of nutrients compared with synthesized fertilizers. Reports indicated that poultry manure and vermicompost are amongst efficient soil organic amendments for crop production (Keshavarzafshar *et al*., 2014).

Elicitors are extensively used to enhance the yield of secondary metabolites in medicinal plants (Yin *et al.*, 2012) The most widely used elicitors are fungal carbohydrates, yeast extracts, and chitosan (Karuppusamy *et al*., 2009) Chitosan is frequently used in cell cultures of medicinal plants as well as plant organs for inducing the accumulation of bioactive secondary metabolites. For example, it has been demonstrated that the total amounts of phenolics and terpenoids in sweet basil (*Ocimumbasilicum* L.) increased after chitosan treatment (Shetty et al., 1997).

The hypothesis of this study is that chitosan may be used as an additional method to the purely agronomic approaches to enhance the content of bioactive secondary metabolites in *T. daenensis*. In this study, we used chitosan levels and fertilizers in different kinds to treat *T. daenensis* plants and to study the effect of chitosan on plant growth, essential oil yield and phenolic content in aerial parts under different fertilizers.

2. Materials and Methods

2.1. Site description

A 2-year (2013 and 2014) experiment was conducted at the Research Farm of Shahrekord (latitude 32° 20´ N, longitude 50° 51´ E, altitude 2061 m above sea level), southwestern Iran. Type of study area climate by Emberger's climatology method is cold and semiarid and semi humid with temperate summer and very cold winter by Karimi's climatology method (IRIMO, 2012).

2.2. Experimental details

The experiment was laid out with a split plot pattern in randomized complete block design with three replicates. Five kinds of fertilizers treatment including F_1 =C (control without manure and fertilizer); F_2 = CF (chemical fertilizers 100-150-100 kg ha^{-1}NPK, respectively), F_3= CM (cow manure 20 $tonha^{-1}$), F_4= VC (vermi compost 10 $tonha^{-1}$), F_5=CF+CM+VC (chemical fertilizers + cow manure + vermicompost) and three chitosan levels (0, 0.2%, 0.4%) and acetic acid were studied.

2.3. Soil amendments

Prior to planting, soil samples were taken from 0 to 30 cm depth and were analyzed for selected physical and chemical characteristics The soil was classified as silt clay, pH=7.6, OC=0.42%, EC=0.43 ds m^{-1}, total nitrogen = 200 mg kg $^{-1}$, available phosphorus = 5.1 mg kg $^{-1}$, potassium = 189 mg kg $^{-1}$.

Accordingly, vermicompost was applied at the rate of 10000 kg ha^{-1} and manure fertilizer was used at the rate of 20000 kg ha^{-1}in the experiment. Before planting, organic amendments were evenly spread on the soil surface and incorporated manually into the top 20 cm of the soil.

2.4. Plant Material

T. daenensis Seeds were purchased from the seed company (Esfahan, Iran). In Apil 2014, transplants were produced in a green house in the pot (20 × 35 cm), and in June 2014, the seedlings were transplanted in the farm system (Sandy loam soil). Experiment conducted in split plot based on randomized complete block design with three replications, during the period from 2014 and 2015 in the research farm of Islamic Azad University of Shahrekord. (latitude 32° 20´ N, longitude 50° 51´ E, altitude 2061 m above sea level). The distance between plants in each row was 30 cm, each experimental plot size was 2.5×2 m.

2.5. Measurements

Plant height, canopy diameter, number of lateral branches, number of flowers, leaf area, as well as dry matter were determined at harvesting time (full flowering stage, on July 20, 2014 and June 30, 2015).

2.6. Essential oil isolation

Fifty g of dried plant materials was subjected to hydro-distillation for 3 h using a Clevenger-type apparatus. Anhydrous sodium sulphate was used to dry essential oil and oil samples were saved in sealed vials at 4 ºC prior to analyses.

2.7. Leaf phenol content

Leaf total phenol content was measured according to the Folin–Ciocalteu method (Spanos and wrostand, 1990). Sample extract (10 µ lit) was mixed with distillation water (490 µ lit) and Folin–Ciocalteu reagent, (500 µl) of saturated sodium carbonate (1%) was added, and then tubes were vortexed for 1 min.After incubation at room temperature for 2 h, absorbance of the solution was measured at 765 nm, UV spectrometry. Gallic acid (0.015 and 0.5 mg ml−1) was used to create a calibration curve (r2= 0.998) and the concentration of total phenolic compounds was calculated as mg gallic acid equivalents (GAE) per g dry weight (DW).

2.8. Leaf flavonoid content

Total flavonoid content of leaf in the extracts was determined by the method of (Quettier-Deleu *et al.*, 2000). Fresh leaf sample (0.1 g) were mixed with 10 ml of ethanol (96%). Two ml of aluminum chloride and 6ml of acetate potassium were added to the extract which were subsequently incubated in the dark for 40 min. Absorbance was measured at 450 nm using UV-1800-Shimatzo (UV-spectrometry. Rutin (0.011and 0.370 mg ml−1) was used for calibration (r2= 0.999). All tests were done in triplicate and total flavonoid content was expressed as mg quercetin equivalent (QE) per g dry weight (DW).

2.9. Statistical analysis

For statistical analysis an ANOVA method for factorial design was conducted using SAS software (version9.1). Mean comparisons were applied using least significant difference (LSD) tests at 5% level of probability.

3. Results and discussion

3.1. Effect of different fertilizers and chitosan on plant morphology

Plant height, increased under different fertilizers in both years and this effect was more pronounced with the chemical fertilizer (Table 1). Except plant height, other traits were higher in second year. That was expected since plants were produced more biomass and the plant roots were deeper in the second year. The highest values for canopy diameter, lateral branches, number of flower, Leaf area index were observed under chemical and integrated fertilizer in 2014 and 2015 respectively (Table 1). The most of morphological traits were unaffected by the application of chitosan (Table 1).

Fertilization is the main factor affecting plant growth of active substances per unit area in herbaceous plants (Kozera *et al.*, 2013). According to Koeduka *et al.* (2006) nitrogen plays a key role in the biosynthesis of many organic compounds such as amino acids, proteins, enzymes, and nucleic acid. This phenomenon boosts nutrient availability resulting in higher nutrient content leading to higher plant growth. Nitrogen is the essential constituents of the chlorophyll molecule. The higher levels of N resulted in enhanced chlorophyll concentration leading to higher photosynthesis activity and plant growth. Further, organic amendments increased the availability of nutrients for plant growth and simultaneously decreased the nitrogen leaching. The combined phenomenon boosted nutrient availability resulting in higher nutrient content and plant growth (Pandry *et al.*, 2015).

Table 1. The effect of different fertilizers and application of chitosan on morpho-physiological, essential oil, ssential oil yield and phenolic content in *T. daenensis*.

Year	Treatments	PH	CD	NF	NS	LAI	DW	OC	OY	Phenol	Flavonoid
2014	**Fertilizers**										
	Control	21.25^{b}	105.58d^{c}	144.25^{d}	253.8^{d}	0.78^{a}	1355.42^{d}	1.50^{d}	20.57^{d}	97.35^{a}	39.69^{a}
	CF	33.58^{a}	119.66a^{b}	267.5^{b}	340.8^{b}	0.94^{b}	2635.16^{a}	2.00^{c}	52.77^{a}	97.07^{a}	37.78^{b}
	CM	23.00^{b}	103.16^{d}	147.4^{d}	263^{cd}	0.81^{dc}	1794.17^{c}	2.28^{b}	41.27^{c}	97.37^{a}	40.1^{a}
	VC	24.75^{b}	113.75^{bc}	163.75^{c}	274.9^{c}	0.87^{c}	835.17^{e}	1.41^{e}	12.07^{e}	97.23^{b}	36.90^{b}
	CF+CM+VC	30.66^{a}	124.58^{a}	284.9^{a}	362.5^{a}	1.43^{a}	2023.33^{b}	2.40^{a}	50.35^{b}	97.1^{c}	39.69^{b}
	LSD	4.08	9.23	10.2	18.36	0.06	153.54	0.05	1.58	0.005	0.69
	ANOVA	P≤0.01	P≤0.01	P≤0.01	P≤0.01	P≤0.01	P ≤0.01.	P≤0.01	P≤0.01	ns	P≤0.01
	Chitosan										
	AA	26.40^{a}	114.2^{a}	200.86^{a}	298.3^{a}	0.94^{b}	1506.33^{c}	1.79^{d}	28.74^{d}	96.62^{b}	38.25^{a}
	0.0	26.46^{a}	113.8^{a}	200.80^{a}	298.5^{a}	0.96^{ab}	1552.67^{c}	1.86^{c}	30.46^{c}	96.62^{b}	38.63^{a}
	0.2	26.86^{a}	112.7^{a}	201.80^{a}	300.4^{a}	0.97^{ab}	1842.8^{b}	1.96^{b}	38.14^{b}	96.74^{b}	38.77^{a}
	0.4	26.93^{a}	112.73^{a}	202.80^{a}	298.8^{a}	0.99^{a}	2012.79^{a}	2.05^{a}	44.26^{a}	98.90^{a}	39.40^{a}
	LSD	1.46	4.85	7.60	10.34	0.03	94.57	0.05	1.27	2.20	1.5
	ANOVA	ns	ns	ns	ns	ns	P ≤ 0.01	P≤0.01	P≤0.01	P≤0.01	ns
2015	**Fertilizers**										
	Control	21.25^{b}	105.58^{dc}	144.25^{d}	253.83^{d}	0.78^{d}	2013.33^{e}	1.72^{e}	34.97^{e}	97.08^{b}	24.16^{c}
	CF	33.58^{a}	119.66^{ab}	267.50^{b}	340.83^{b}	0.94^{b}	2826.67^{c}	2.55^{c}	72.47^{c}	97.87^{b}	21.79^{d}
	CM	23.00^{b}	103.16^{d}	147.41^{b}	263^{cd}	0.81^{dc}	2154.17^{d}	2.43^{d}	52.78^{d}	88.45^{c}	26.58^{b}
	VC	24.75^{b}	113.75^{bc}	163.75^{c}	274.91^{c}	0.87^{c}	2916.67^{b}	3.00^{b}	87.77^{b}	98.79^{b}	26.83^{b}
	CF+CM+VC	30.66^{a}	124.58^{a}	284.91^{a}	362.50^{a}	1.43^{a}	3334.17^{a}	3.33^{a}	111.45^{a}	107.87^{a}	34.83^{a}
	LSD	4.08	9.23	10.20	18.36	0.06	57.67	0.1	4.20	7.78	1.81
	ANOVA	P≤0.01	P≤0.01	P ≤ 0.01	P ≤ 0.01	P ≤ 0.01	P ≤ 0.01	P≤0.01	P≤0.01	P≤0.01	P≤0.01
	Chitosan										
	AA	26.40^{a}	114.30^{a}	200.86^{a}	298.33^{a}	0.94^{b}	2445.33^{d}	2.44^{d}	62.21^{d}	96.53^{c}	25.16^{c}
	0.0	26.46^{a}	113.80^{a}	200.80^{a}	298.53^{a}	0.96^{ab}	2588.67^{c}	2.52^{c}	67.86^{c}	97.36^{bc}	26.20^{bc}
	0.2	26.86^{a}	112.66^{a}	201.80^{a}	300.40^{a}	0.97^{ab}	2708.67^{b}	2.67^{b}	74.96^{b}	97.76^{ab}	27.50^{ab}
	0.4	26.93^{a}	112.73^{a}	202.80^{a}	298.80^{a}	0.99^{a}	2853.33^{a}	2.80^{a}	82.53^{a}	99.40^{a}	28.50^{a}
	LSD	1.46	4.85	7.60	0.06	0.03	31.69	0.05	1.73	2.01	1
	ANOVA	ns	ns	ns	ns	P≤0.01	P≤0.01	P≤0.01	62.21d	P≤0.01	P≤0.01

Similar letter (s) in each column are not significantly different at 5% probability level using LSD test PH: plant height (cm); CD: canopy diameter (mm); NLB: number of lateral branches; NF: number of flowers ; LAI: leaf area index; DW: dry weight (kg ha^{-1}); OC: oil content (%); OY: oil yield (kg ha^{-1}); PHE: phenol (mg g-1);FL: flavonoid (mg g-1): AA: acetic acid.

Table 2. Interactive effect of different fertilizers and application of chitosan on morpho-physiological, essential oil, essential oil yield and phenolic content of *T. daenensis* in 2014.

Year	Treatments	PH	CD	NF	NS	LAI	BY	OC	OY	Phenol	Flavonoid
2014	C×AA	27.33^{f}	78.66^{g}	99.33^{efg}	158.66^{m}	0.55^{h}	1174.66^{d-g}	1.40^{jk}	16.41^{m}	39.73^{abc}	96.80^{c}
	C×0.0	30.30^{bcd}	79.00^{g}	99.00^{fgh}	159.33l^{m}	0.73^{def}	1333.33^{c-g}	1.40^{jk}	19.44^{l}	39.00^{a-d}	96.90^{c}
	C×0.2	28.00^{ef}	80.00^{fg}	98.33^{efg}	161.33^{kl}	0.74^{c-f}	1463.33^{c-g}	1.50^{hij}	22.39^{k}	39.70^{a-c}	98.10^{ab}
	C×0.4	29.00^{c-f}	80.00^{fg}	100.3^{ef}	163.00^{jk}	0.53^{h}	1450.33^{c-g}	1.60^{h}	24.03^{k}	40.31^{a}	96.76^{c}
	CF×AA	36.00^{a}	123.33^{b}	132.66^{a}	259.66^{c}	0.77^{cd}	2450.3^{abc}	1.90^{g}	46.20^{ef}	37.60^{def}	96.73^{c}
	CF×0.0	36.33^{a}	124.3^{ab}	133.33^{a}	260.66^{c}	0.82^{ab}	2490^{abc}	1.90^{g}	48.74^{de}	36.81^{f}	96.83^{c}
	CF×0.2	36.66^{a}	126.00^{a}	133.66^{a}	262.66^{a}	0.84^{a}	2700^{ab}	2.00^{fg}	54.40^{cd}	38.74^{a-e}	98.00^{a}
	CF×0.4	37.66^{a}	126.00^{a}	134^{a}	265.00^{b}	0.75^{c-f}	2900.29^{a}	2.13^{ef}	61.73^{a}	37.96^{c-f}	96.73^{c}
	CM×AA	31.00^{bc}	102.33^{c}	97.33^{h}	197.66^{f}	0.75^{c-f}	1553.3^{b-g}	2.18^{e}	33.89^{i}	40.12^{ab}	96.63^{c}
	CM×0.0	30.30^{bcd}	102.66^{c}	98.00^{gh}	198.33^{f}	0.78^{bc}	1390^{c-g}	2.20^{de}	30.99^{j}	40.16^{a}	96.73^{c}
	CM×0.2	29.30^{c-f}	103^{c}	98.6^{fgh}	201.00^{e}	0.83^{a}	1800^{a-d}	2.30^{cd}	41.54^{gh}	40.00^{ab}	99.50^{a}
	CM×0.4	30.00 $^{b-e}$	104.33^{c}	101.00^{e}	204.00^{d}	0.74^{c-f}	2433.33^{abc}	2.40^{bc}	58.55^{bc}	40.00^{ab}	96.63^{c}
	VC×AA	28.33^{d-f}	81.33^{f}	99.33^{efg}	160.00^{lm}	0.67^{g}	536.66^{g}	1.26^{l}	7.01^{n}	36.70^{f}	96.53^{c}
	VC×0.0	27.66^{f}	81.33^{f}	99.66^{efg}	161.00^{klm}	0.73^{d-f}	690.0f^{g}	1.36^{kl}	8.84^{n}	37.00^{ef}	96.70^{c}
	VC×0.2	30.66^{bc}	81.66^{f}	99.66^{efg}	164.00^{ij}	0.76$_{cde}$	1050.6^{efg}	1.50^{ijk}	15.62^{m}	37.00$_{ef}$	99.13^{a}
	VC×0.4	28.50^{d-f}	82.0^{f}	100.00^{ef}	166.00^{i}	0.72$_{ef}$	1063.33^{defg}	1.57^{hi}	16.82^{lm}	37.20$_{ef}$	96.56^{c}
	CF+CM+VC ×AA	31.00^{b-e}	85.66^{e}	110.33^{d}	178.00^{h}	0.71$_{fg}$	1816.7^{a-d}	2.20^{e}	40.19^{n}	37.14$_{ef}$	96.43^{c}
	CF+CM+VC ×0.0	32.00^{b}	87.66^{de}	111.6^{cd}	178.00^{h}	0.75$_{c-f}$	1860.00$_{a-d}$	2.40^{bc}	44.27^{fg}	40.00$_{ab}$	96.60^{c}
	CF+CM+VC ×0.2	32.00^{b}	87.66^{de}	112.3^{b}	181.00^{g}	0.72^{e-f}	2200^{a-e}	2.50^{ab}	56.77^{c}	38.34$_{b-f}$	98.93^{ab}
	CF+CM+VC ×0.4	31.00^{b-e}	88.66^{d}	113.6^{b}	182.66^{g}	0.72^{ef}	2216.66^{a-d}	2.57^{a}	60.16^{ab}	36.68$_{f}$	96.46^{c}
	LSD	2.08	2.01	1.90	2.35	0.04	211.5	0.12	2.84	1.78	0.97
	ANOVA	ns	ns	ns	ns	ns	P≤ 0.01	ns	P ≤0.01	ns	ns

Similar letter (s) in each column are not significantly different at 5% probability level using LSD test

PH: plant height (cm); CD: canopy diameter (mm); NLB: number of lateral branches; NF: number of flowers ; LAI: leaf area index; DW: dry weight (kg ha^{-1}); OC: oil content (%); OY: oil yield (kg ha^{-1}); PHE: phenol (mg g-1);FL: flavonoid (mg g-1): AA: acetic acid.

Table 3. Interactive effect of different fertilizers and application of chitosan on morpho-physiological, essential oil, essential oil yield and phenolic content in *T. daenensis* in 201

Year	Treatments	PH	CD	NF	NS	LAI	BY	OC	OY	Phenol	Flavonoid
2015											
	C×AA	21.33^{e}	108.66^{def}	144.00^{d}	252.33^{g}	0.75^{b}	1753.33^{i}	1.58^{l}	27.82^{n}	96.66^{b}	22.33^{ghi}
	C×0.0	21.00^{e}	104.00^{ef}	144.00^{d}	250.66^{g}	0.79^{b}	2006.66^{j}	1.69^{kl}	33.91^{m}	96.33^{b}	22.00^{hi}
	C×0.2	21.33^{e}	104.66^{ef}	144.00^{d}	260.00^{f}	0.79^{b}	2076.66^{j}	1.79^{jk}	37.16^{lm}	97.66^{b}	26.00^{c-h}
	C×0.4	21.33^{e}	105.00^{ef}	144.00^{d}	252.33^{g}	0.81^{b}	2216.66^{i}	1.85^{j}	40.99^{kl}	97.66^{b}	26.33^{c-h}
	CF×AA	32.70^{abc}	122.33^{abc}	266.66^{b}	340.00^{b}	1.41^{a}	2690.00^{g}	2.36^{hi}	63.68^{h}	96.66^{b}	32.50^{ab}
	CF×0.0	33.70^{ab}	122.66^{a-d}	266.00^{b}	341.66^{b}	1.41^{a}	2750.00^{g}	2.41^{hi}	66.43^{h}	96.83^{b}	34.50^{a}
	CF×0.2	34.00^{a}	117.00^{a-d}	267.33^{b}	341.66^{b}	1.44^{a}	2866.66^{f}	2.69^{fg}	77.10^{g}	98.66^{b}	36.50^{a}
	CF×0.4	34.0^{a}	116.66^{a-d}	270.00^{b}	340.00^{b}	1.46^{a}	3000.00^{e}	2.75^{ef}	82.71^{f}	99.33^{b}	35.83^{a}
	CM×AA	23.0^{de}	102.00^{f}	146.33^{d}	261.66^{ef}	0.82^{b}	1883.33^{k}	2.31^{i}	43.57^{k}	86.66^{c}	23.16^{fghi}
	CM×0.0	22.33d^{e}	102.00^{f}	146.33^{d}	263.00^{de}	0.81^{b}	2033.33^{j}	2.36^{hi}	48.02^{j}	87.16^{c}	20.66^{e-h}
	CM×0.2	23.33d^{e}	104.00^{ef}	148.33^{d}	264.33^{d}	0.80^{b}	2266.66^{i}	2.46^{h}	55.92^{i}	89.83^{c}	21.00^{i}
	CM×0.4	23.66^{de}	104.00^{ef}	148.66^{c}	263.00^{de}	0.81^{b}	2433.33^{h}	2.61^{g}	63.61^{h}	90.16^{c}	22.33^{i}
	VC×AA	24.66^{d}	114.33^{b-e}	163.66^{c}	275.00^{c}	0.83^{b}	2733.33^{g}	2.76^{ef}	75.50^{g}	97.00^{b}	23.66^{e-i}
	VC×0.0	24.66^{d}	113.66^{c-e}	163.33^{c}	275.00^{c}	0.85^{b}	2883.33^{f}	2.87^{e}	82.84^{f}	99.00^{b}	28.66^{b-d}
	VC×0.2	24.66^{d}	113.00^{c-e}	163.33^{c}	274.33^{c}	0.88^{b}	2933.3^{ef}	3.08^{d}	90.51^{c}	98.83^{b}	26.83^{c-g}
	VC×0.4	25.00^{d}	114.00^{b-e}	164.66^{c}	275.33^{c}	0.90^{b}	3116.66^{d}	3.28^{bc}	102.24^{d}	100.33^{b}	28.16^{b-e}
	CF+CM+VC ×AA	30.33^{c}	123.66^{a-c}	283.66^{a}	362.66^{a}	0.92^{b}	3166.66^{d}	3.17^{cd}	100.48^{d}	105.66^{a}	24.16^{d-i}
	CF+CM+VC ×0.0	30.66^{bc}	126.66^{a}	284.33^{a}	362.33^{a}	0.94^{b}	3270.0^{c}	3.30^{b}	108.12^{c}	107.50^{a}	25.16^{d-i}
	CF+CM+VC ×0.2	31.0^{abc}	124.66a^{b}	285.66^{a}	361.66^{a}	0.94^{b}	3400^{b}	3.35^{b}	114.12$^{.b}$	108.83^{a}	27.16^{c-f}
	CF+CM+VC ×0.4	30.66^{bc}	123.33a^{b}c	286.00^{a}	363.33^{a}	0.96^{b}	3500^{a}	3.51^{a}	12309.16^{a}	109.50^{a}	29.83^{bc}
	LSD	3.28	10.85	5.32	2.35	0.23	70.88	0.12	3.88	4.51	4.33
	ANOVA	ns	ns	ns	ns	ns	$P \leq 0.01$.	ns	$P \leq 0.01$.	ns	ns

Similar letter (s) in each column are not significantly different at 5% probability level using LSD test
PH: plant height (cm); CD: canopy diameter (mm); NLB: number of lateral branches; NF: number of flowers; LAI: leaf area index; DW: dry weight (kg ha^{-1}); OC: oil content (%); OY: oil yield (kg ha^{-1}); PHE: phenol (mg g-1); FL: flavonoid (mg g-1): AA: acetic acid.

3.2. Effect of different fertilizers and chitosan on biomass of thyme

Biomass of thyme plants were higher in 2015 compared to 2014. This was not a surprise since plants sowed in 2014, extended their growth in 2015 in all treatments (Table 1). The results presented in Table 1 showed that the amount of dry matter yield was enhanced progressively by chemical fertilizers in 2014, but in 2015, combination of organic manures and chemical fertilizer significantly increased the crop biomass. The biomass yield increased by 64.5 % and 46.2% in CF and CF+CM+VC treatments, in 2014 and 2015 respectively. Increasing in biomass yield in CF in 2014 can be attributed to applying nitrogen in different growths stage has stimulating effects in organic compounds of the plant system. Moreover, the increasing effect of N fertilization on plant biomass may be due to the positive effects of nitrogen on activation of photosynthesis and metabolic processes of organic compounds in plants. According to Baranauskienne *et al.* (2003) found that N fertilizer increased biomass yield of *Thymus vulgaris.*

In 2015, when thyme plants were exposed to integrated fertilizers, an increase in biomass yield was observed which may be due to integration of organic manures and chemical fertilizers that can increase the organic matter level of metabolic processes in plant. This event increases nutrient availability resulting in more nutrient content leading to higher biomass yield. Only use of organic manures or chemical fertilizer indicated lower nutrient uptake leading to lower biomass yield. Organic manure and chemical fertilizer alone need to more complex compound in the soil for development and growth in each plant cell for its proper functioning (Pandey *et al.*, 2015).

Application of 0.4% chitosan improved biomass yield by approximately 25.8% and 25.3% in 2014 and 2015, respectively. (Table 1). Results also showed that suitable chitosan oligomer concentration for the growth of thyme was 0.4%.

3.4 Effect of different fertilizers and chitosan on essential oil and oil yield of thyme

Data presented in Table 1 indicated, essential oil percentage and oil yield were higher in 2015 than in 2014. The highest essential oil percentage was found in plants under integrated fertilizer sprayed with 0.4% in both of growing year (Table 3), However, in 2014 due to substantial enhancement in dry matter yield of plants that under application of chemical fertilizer, the highest oil yield (5958.24 kg ha^{-1} in 2014) was obtained from plants grown in cow manure, sprayed with 0.2% chitosan (Table 2). CF+CM+VC treatment resulted in maximum essential oil yield sprayed with 0.4% with (12309.16 kg ha^{-1}in 2015) (Table 3). Other fertilizers treatments also indicated significant difference as compared to the control (Table 1).

The enhance in essential oil yield by using of integrated fertilizer (CF+CM+VC) may be due the higher supply of N from cow manure, vermicompost and chemical fertilizer, which could lead to higher essential oil yield and dry matter (Pandey and Patra, 2015). The synthesis of essential oils is dependent on photosynthetic activity. Providing of photosynthetic nutrient boost and metabolic processes correlated to cell division and elongation (Hatwar *et al.*, 2003). According to Pandey *et al.* (2015) nitrogen plays a key role in the division, growth and development of cells that stimulate essential oil accumulation via higher density of oil glands due to the improvement in biomass yield. Thus, it is useful to combine organic fertilizer with chemical fertilizer for optimum thyme productivity and essential oil yield.

Spraying chitosan increased essential oil content, possibly it acted as a potent inducer for increasing the biosynthesis of secondary metabolites. Chitosan is also considering as an elicitor of signaling molecule which increase essential oil. Yin *et al*. (2014) indicated that 0.4% chitosan significantly increased the biomass and essential oil yield of *Origanum vulgare*.

3.5. Effect of different fertilizers and chitosan on phenol and flavonoid content

It was found that the phenolic contents of aerial parts of *T. daenensis* increased under integrated fertilizer in 2015, but the effect of fertilizers wan not significant on this trait. The effect of foliar application of chitosan on phenol content of *T. daenensis* was affected by different fertilizers (Table 2, 3). The highest phenol content was obtained by foliar application of 0.4% chitosan in 2014 and 2015, respectively. (Table 1).

According to table 2 and table 3, phenol content was not affected by fertilizers and application of chitosan in both of growing years (2014 and 2015). The highest flavonoid content was obtained from plants which received manure fertilizer in 2014, however the highest flavonoid content was obtained from integrated fertilizer in 2015 (Table 1). Among different levels of chitosan, concentration of 0.4% improved flavonoid content (Table 1).

In our results, phenol and flavonoid content increased when thyme plants exposed to organic manure and integrated fertilizer. Thus, application of organic amendments remarkably improved phenolic content. The improvement in phenolic content in case of organic manure treatments may be due to the presence of easily available water-soluble carbon, which acts as a source of energy for soil microorganisms (Pandry and Patra, 2015). Organic fertilizers enhance plant growth by providing nutrients and improving the effectiveness of the soil by favorably modifying its water retention and aeration. The results support the previous findings of beneficial effects of combined application of organic and inorganic nutrients on soil fertility and productivity in menthol mint (Patra *et al.*, 2000).

Foliar application of chitosan resulted in the highest phenolic content, in both years (Table 1). Yin *et al* (2011) indicated that chitosan is a significant secondary signal messenger, which can play a key role in many plants activities such as plant growth and secondary metabolite production. Also they indicated that chitosan has the potential to induce the accumulation of polyphenol in *Origanum vulgare*. Kim (2005) reported that application of chitosan solution improved the total phenolic and terpenoids content in sweet basil.

4. Conclusion

The results recommended that combination of organic manure and chemical fertilizer gave higher canopy diameter, lateral branches, number of flower, leaf area index, essential oil and also increased phenolic content in *T. daenensis*. Combination of organic manure and chemical fertilizers was the most suited combination for improving the crop productivity, oil yield and overall profitability and economics of cultivation of the *T. daenensis*. Moreover, cow manure or vermicompost had potential to reduce the application of chemical fertilizers with improvement in the essential oil yield. Foliar application of chitosan increased essential oil content and oil yield in *T. daenensis* by up-regulating the biomass. Thus, foliar application of chitosan for improving oil yield was of great practical value and could be an effective method to reduce the costs associated with essential oil production.

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