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Effect of Nitrogen and Cytokinin on Quantitative and Qualitative Yield of Thyme (*Thymus vulgaris* L.)

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ABSTRACT

In order to investigate the effect of nitrogen and cytokinin on the quantitative and qualitative characteristics of thyme, a three-year factorial experiment was conducted at Razi University based on a randomized complete block design with 3 replications. Factors included nitrogen levels (0, 50 and 100 kg N/ha) and cytokinin concentrations (0, 100, 200 and 400 µM). The results of analysis of variance showed that the interaction effect of nitrogen × cytokinin on plant height, number of branches, leaf, stem and whole plant dry weights, essential oil percentage and yield was significant ($p \leq 0.01$). With increasing the amount of nitrogen and cytokinin, all the studied traits (except harvest index) increased significantly compared to the control. The highest plant height and number of branches were obtained in the treatment of 100 kg N/ha × 400 µM cytokinin. The treatment of 100 kg N/ha × 100 µM cytokinin resulted the highest production of dry weights of leaf (3287 kg/ha), stem (3110 kg/ha) and whole plant (6398 kg/ha); aproximately 60.5, 59.8 and 60.9% more than the control treatment, respectively. Also, the highest percentage of essential oil (2.45 %) was obtained by applying 50 kg N/ha × 400 µM cytokinin. Application of 100 kg N/ha and foliar application of 200 µM resulted in the highest essential oil yield (77.60 kg/ha). In general, the results of this experiment showed that the quantitative and qualitative yield of thyme can be increased by consuming the appropriate amount of nitrogen and cytokinin.

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1. Introduction

Thyme (*Thymus vulgaris* L.) is a perennial plant, belongs to lamiaceae family. Due to adaptation of thyme to a wide range of environmental conditions, it grows in Southern and Central Europe, Asia, North America, and Africa. Thyme is widely used in various industries, such as in the medicinal, cosmetic, health, and food industries (Stahl-Biskup and Sáez, 2002; Naghdi and Maki, 2003; Guine and Goncalves, 2016).Thyme yield can be affected by environmental factors and agricultural operations (Stahl-Biskup and Sáez, 2002). Plant growth depends on sufficient amount of nutrients in the root environment. Nutrients play an important role in regulating the metabolism, production, and development of new tissues and structural components of plants (Salas *et al*., 1997; Adelusi and Aileme, 2006). Nitrogen and phosphorus are the most important essential elements for plant growth (Kirkby 1981; Guo *et al*,. 2012). Among the nutrients, nitrogen has particular importance, because it plays an essential role in the formation of nucleic acids, amino acids, proteins, and other cellular components (Salas *et al*., 1997; Adelusi and Aileme, 2006). Nitrogen in the form of nitrate and ammonia in the soil is absorbed by plants through the roots (Bahara and Shanks, 1997; Morgan *et al*., 2000). Nitrate uptake depends on various factors such as plant species, root pH, temperature, *etc*. (Marschner, 1995).

Plant growth regulators are very important in plant growth (Nishiyama *et al*., 2011; Kang *et al*., 2012). Cytokinin affects on all stages of plant growth from

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ovule fertility to aging and death. It plays important roles in plant processes such as cell division, stem growth and differentiation, delayed aging, photomorphogenic development, chloroplast division and growth, and also control of metabolism in response to environmental stimuli (Chernyad'ev, 2000; Del Pozo *et al.*, 2005; Hirose *et al.*, 2008). There are reports on the effect of cytokinin on the morphological characteristics of plants. For example, it has been reported that foliar application of cytokinin increased leaf area, plant height, root and shoot weight of maize (Ali *et al*., 2011).

The leaves as well as the essential oil are the economic yield of the thyme plant. Previous researches shows that the nitrogen consumption has been effective in increasing the plant vegetative growth. Also, foliar application of cytokinin has induced leaf growth under laboratory conditions. However, the combined use of nitrogen and cytokinin on thyme plant has not been evaluated under farm conditions. Therefore, the purpose of this experiment was to investigate the effect of different amounts of nitrogen fertilizer and cytokinin on some quantitative and qualitative characteristics of thyme.

2. Materials and methods

This experiment was performed during three consecutive years 2018-20 at Campus of Agriculture and Natural Resources, Razi University, Kermanshah, Iran (longitude 45°9´E, latitude 34°21´N and 1319 m above sea level). After initial soil preparation, to obtain the soil physicochemical properties, sampling was done (Table 1). Also, the meteorological information of the region was summarized in Table 2 The experiment was performed as a factorial based on a randomized complete block design with 3 replications. The first factor included different amounts of nitrogen (0, 50, and 100 kg N/ha) and the second factor included cytokinin concentrations (0, 100, 200, and 400 µM). To supply nitrogen, urea fertilizer (46% N) was used. Cytokinin was used as benzylaminopurine (BAP) (Duchefa company, Netherlands). Each plot consisted of six planting rows, 0.4 m apart and 3 m length. Distance between the two plants on the planting row was 0.25 m. Seedlings were planted at early May 2018. Nitrogen treatments were added as soil application in two stages (three weeks after planting and before flowering). The N fertilizer was placed about 2.5 cm distance from the plants. Cytokinin treatments were applied before flowering. To prevent hormone evaporation, the foliar application was performed near sunset. Tween 20 (0.1%) was added to the hormone solution as surfactant. Foliar spraying was performed with water for the control treatment.

Plants irrigated as sprinkler irrigation according to environmental conditions and plant growth stage. Weed control during the growing season was done manually. To study the traits, sampling was performed at the middle of flowering. Plant height was measured by a ruler from the soil surface until the end of the tallest stem. Main stem diameter was measured by a digital caliper 10 cm above the soil surface. The number of lateral branches was randomly selected from five plants. The plants located in 3 m^2/plot were harvested to measure leaf, stem and whole plant dry weight and harvest index. The samples were shade dried and their dry weight was measured.

Two cuttings were harvested per each year, and both summations were considered as plant dry weight of that year. Essential oil was extracted from dried leaves by water distillation method with Clevenger. After the essential oil extraction, sodium sulfate was used as dehumidifier in the essential oil (Omidbeigi, 2000). Essential oil yield was calculated with multiplying the essential oil percentage by the leaf dry matter yield per hectare. Combined analysis of variance in three years was performed using SAS version 9 software. In combined analysis, the year was considered as random effect and the nitrogen and cytokinin as fixed effects. Mean comparisons was done using LSD test at 5% probability level.

Table 1. Physical and chemical analysis of the soil

Soil texture	Calcium carbonate (%)	pH	Organic carbon (%)	Nitrogen (%)	Phosphorus (mg/kg)	Potassium (mg/kg)	Manganese (mg/kg)	Iron (mg/kg)	Zinc (mg/kg)	Copper (mg/kg)
Clay-silt	28	7.8	0.99	0.09	18	360	14	4.5	0.48	1.8

Table 2. Meteorological information of the region during 2018-20

Year	Parameter	Apr	May	June	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar
2018	Temperature (°C)	14.90	15.90	23.90	30.60	31.30	27.10	21.10	12.30	7.40	4.50	5.40	6.50
	Humidity (%)	47.00	64.00	33.00	16.00	17.00	17.00	32.00	62.00	71.00	61.00	60.00	60.00
	Precipitation (mm)	63.40	169.00	5.20	0.00	0.00	0.00	29.10	125.30	104.00	41.50	96.30	79.10
2019	Temperature (°C)	10.40	16.70	26.00	29.90	31.10	26.30	21.60	10.20	5.90	4.40	3.70	9.70
	Humidity (%)	65.00	51.00	29.00	17.00	18.00	18.00	25.00	53.00	67.00	58.00	55.00	56.00
	Precipitation (mm)	194.8	17.50	0.00	0.00	0.00	0.00	15.40	56.10	114.80	25.60	43.50	148.20
2020	Temperature (°C)	11.50	17.77	25.27	28.89	29.64	26.09	19.10	13.16	6.33	-	-	-
	Humidity (%)	55.23	39.97	13.52	13.48	13.29	12.06	17.20	36.27	73.60	-	-	-
	Precipitation (mm)	88.13	38.86	0.00	0.00	0.00	0.00	0.76	44.20	132.00	-	-	-

3. Results and discussion

The results (Table 3) showed that the simple effects of year, nitrogen, and cytokinin on the studied traits except harvest index were significant ($p \leq 0.01$). Also, the interaction effect of year×nitrogen on plant height and the interaction effect of year×cytokinin on plant height and stem dry weight were significant ($p \leq 0.01$). The interaction effect of nitrogen×cytokinin on the measured traits (except for stem diameter and harvest index) was significant ($p \leq 0.01$).

Mean comparisons showed that the plant height increased with increasing the amount of nitrogen and cytokinin. The highest plant height (26.8 cm) was obtained by the interaction of 100 kg N/ha × foliar application of 400 µM cytokinin (Table 4). The highest plant height was related to the amount of 100 kg of nitrogen during 2020 (Fig. 1). The plant height increased significantly with increasing cytokinin concentration. The highest plant height (26.26 cm) was obtained at 400 µM cytokinin during 2020 year (Fig. 2). The number of sub-branches showed a significant increase with increasing cytokinin concentration and nitrogen amount. Treatment of 100 kg N/ha × 400 µM cytokinin produced the highest number of sub-branches (26 sub-branches) (Table 4).The number of sub-branches per plant in 2020 was more than other years (Fig. 3). Increasing the nitrogen amount increased the leaf dry weight (Table 4). Consumption of different cytokinin concentrations increased leaf dry weight compared to the control (Table 4). The highest leaf dry weight (3287 kg/ha) was obtained in 100 kg N/ha × 100 µM cytokinin treatment (Table 4). In 2020, the highest leaf dry weight was produced (Fig. 4). Compared to the control, all cytokinin concentrations increased the stem dry weight. The highest stem dry weight was obtained in 100 kg N/ha × 100 µM cytokinin treatment. This treatment increased stem dry weight 59.8% compared to the control (without nitrogen and cytokinin application) (Table 4). Increasing the nitrogen amount and cytokinin concentration increased the total dry weight of thyme.

The highest total dry weight (6398 kg/ha) was obtained in 100 kg N/ha × 100 µM cytokinin treatment (Table 4). The maximum total dry weight of thyme was produced in 2020 year (Fig. 5). Mean comparison showed that the highest stem diameter was obtained in 2020 year (Fig. 6). Consumption 100 kg N/ha increased stem diameter compared to the control (8.8%) (Fig. 7). Increasing the cytokinin concentration in comparison with the control caused a significant increase in thyme stem diameter. The highest stem diameter (2.04 cm) was obtained in the treatment of 400 µM cytokinin (Fig. 8). The results showed that the application of nitrogen and cytokinin increased the thyme essential oil content in comparison with the control. The highest essential oil content (2.45%) was obtained in the treatment of 50 kg N/ha × 400 µM cytokinin (Table 4). This treatment increased the plant essential oil content about 0.5%. Essential oil yield of thyme increased significantly with increasing cytokinin and nitrogen compared to the control. The highest essential oil yield (77.6 kg/ha) was related to 100 kg N/ha × 200 µM cytokinin treatment, which increased the essential oil yield almost twice compared to the control (Table 4).

Table 3. Combined analysis of variance (mean squares) for year, nitrogen, cytokinin and their interaction effects in terms of studied traits in thyme plant

Sources	df	Plant height	Number of branches	Leaf dry weight	Stem dry weight	Total dry weight	Stem diameter	Harvest index	Essential oils	Essential oil yield
Year (Y)	2	88.82**	52.62**	1208446*	1040510**	4321382*	0.760**	0.00005^{ns}	0.810**	1976.96**
Block (Year)	6	4.36	2.51	149031	121978	515158	0.040	0.00010	0.000	78.97
Nitrogen (N)	2	207.10**	50.67**	1648744**	1636177**	6396676**	0.250**	0.00003^{ns}	0.090**	948.21**
Cytokinin (CK)	3	50.02**	215.04**	3042517**	2744949**	11281184**	0.700**	0.00004^{ns}	0.180**	1726.59**
Y × N	4	4.19**	0.93^{ns}	113^{ns}	429^{ns}	4552^{ns}	0.010^{ns}	0.00010^{ns}	0.000^{ns}	2.05^{ns}
Y × CK	6	2.72**	0.18^{ns}	3313^{ns}	4173**	17186^{ns}	0.010^{ns}	0.00007^{ns}	0.000^{ns}	4.40^{ns}
N × CK	6	2.65**	2.91**	145897**	163618**	562949**	0.009^{ns}	0.00005^{ns}	0.220**	380.45**
Y × N × CK	12	0.57^{ns}	0.61^{ns}	3308^{ns}	841^{ns}	4668^{ns}	0.007^{ns}	0.00009^{ns}	0.000^{ns}	2.15^{ns}
Error	66	1.07	0.71	8864	6665	23404	0.004	0.00006^{ns}	0.002	1.17
CV (%)		4.54	3.79	3.29	3.01	2.74	3.67	1.54	2.12	4.16

ns, * and **: not significant, significant at 5 and 1% probability levels, respectively

Table 4. Mean comparisons of nitrogen × cytokinin interaction in terms of the studied traits in thyme

Factors Nitrogen (kg/ha)	Cytokinin (μM)	Plant height (cm)	Number of branches	Leaf dry weight (kg/ha)	Stem dry weight (kg/ha)	Total dry weight (kg/ha)	Essential oils (%)	Essential oil yield (kg/ha)
0	0.00	17.96^{f}	17.00^{g}	1991.21^{i}	1860.58^{k}	3901.80^{i}	1.95^{h}	38.97^{f}
	100 .00	20.23^{ef}	20.00^{ef}	2965.06^{f}	2813.96^{e}	5779.03^{de}	2.25^{de}	66.84^{c}
	200.00	20.90^{e}	22.33^{cde}	2888.22^{h}	2723.31^{g}	5611.53^{f}	2.32^{bc}	67.07^{c}
	400.00	22.19^{cde}	24.11^{bc}	2815.87^{i}	2649.39^{i}	5465.23^{g}	2.22^{ef}	62.60^{d}
50	0.00	21.75^{de}	20.22^{ef}	2321.68^{k}	2171.78^{j}	4493.47^{h}	2.28^{cd}	53.06^{e}
	100.00	22.23^{cde}	21.44^{def}	3085.92^{c}	2899.38^{c}	5985.30^{c}	2.18^{f}	67.41^{c}
	200.00	23.47^{bcd}	23.56^{bcd}	2986.32^{e}	2843.66^{d}	5829.99^{d}	2.25^{de}	67.31^{c}
	400.00	24.45^{abc}	25.33^{ab}	2913.18^{g}	2766.65^{e}	5679.83^{ef}	2.45^{a}	71.49^{b}
100	0.00	24.21^{bcd}	19.44^{fg}	2793.58^{j}	2681.17^{h}	5474.76^{g}	2.35^{b}	65.80^{c}
	100.00	24.21^{bcd}	21.89^{cdef}	3287.61^{a}	3110.69^{a}	6398.30^{a}	2.02^{g}	66.66^{c}
	200.00	25.20^{ab}	24.33^{abc}	3201.56^{b}	3029.58^{d}	6231.14^{b}	2.42^{a}	77.60^{a}
	400.00	26.82^{a}	26.78^{a}	3073.79^{d}	2914.01^{c}	5987.81^{c}	2.18^{f}	67.11^{c}
LSD (5%)		2.48	2.51	7.66	16.09	107.60	0.04	2.52

In each column, the presence of at least one similar letter indicates that there is no significant difference based on the LSD test

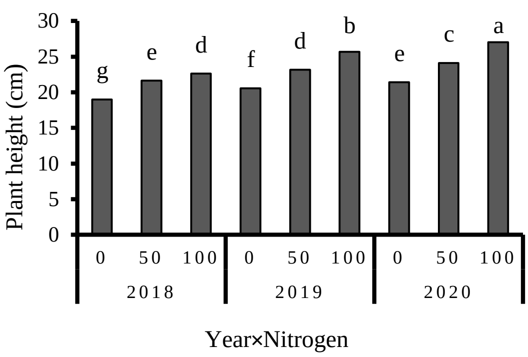

Figure 1. Mean comparison of year×nitroge interaction effect in terms of plant height in thyme

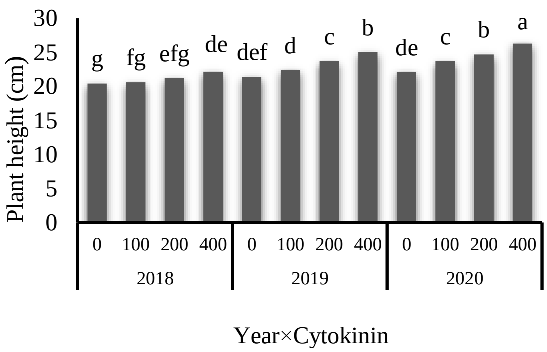

Figure 2. Mean comparison of year×cytokinin interaction effect in terms of plant height in thyme

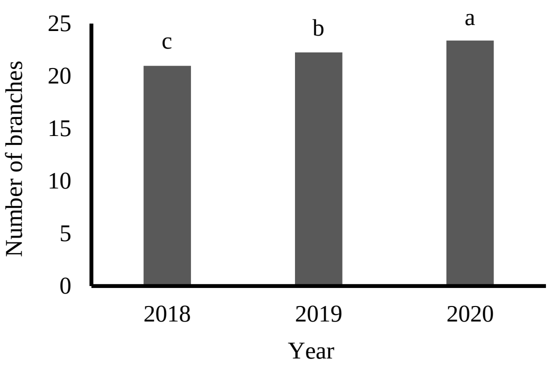

Figure 3. Mean comparison of the year effect in terms of the number of branches in thyme plant

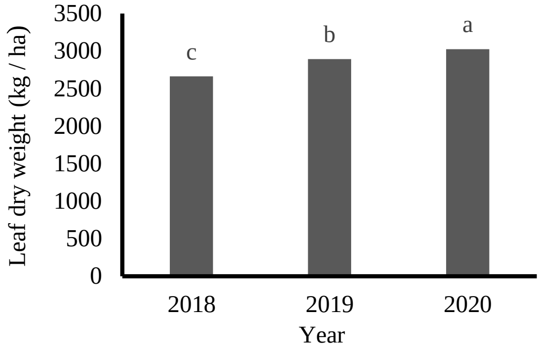

Figure 4. Mean comparison of the year effect in terms of leaf dry weight in thyme

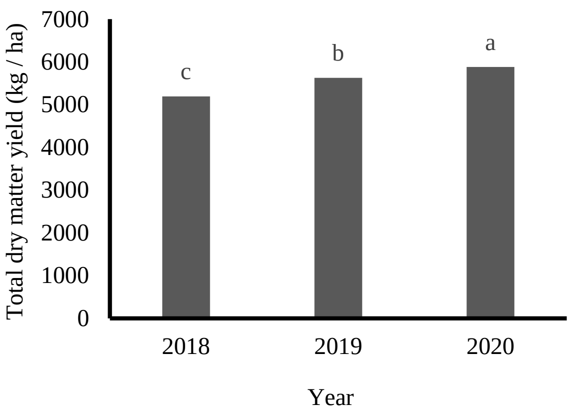

Figure 5. Mean comparison of the year effect in terms of total dry matter yield in thyme

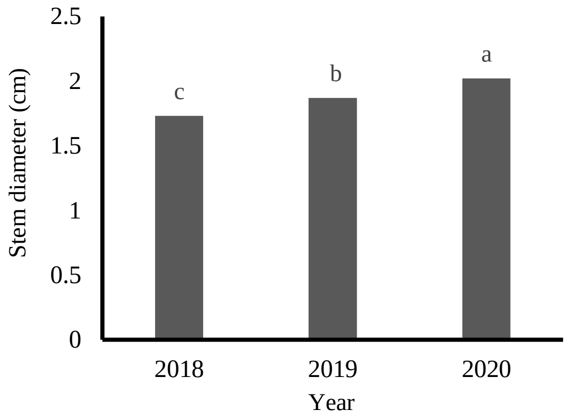

Figure 6. Mean comparison of the year effect in terms of thyme stem diameter

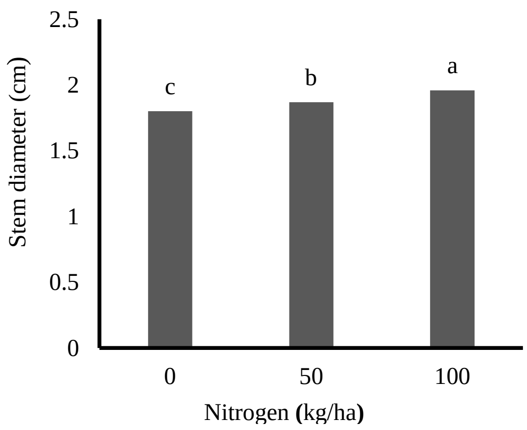

Figure 7. Mean comparison of nitrogen amounts in terms of thyme stem diameter

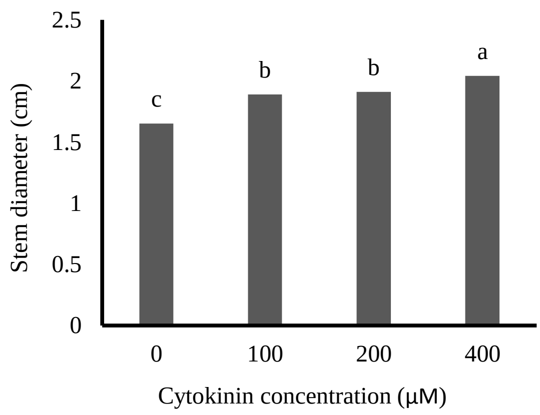

Figure 8. Mean comparison of cytokinin concentrations in terms of thyme stem diameter

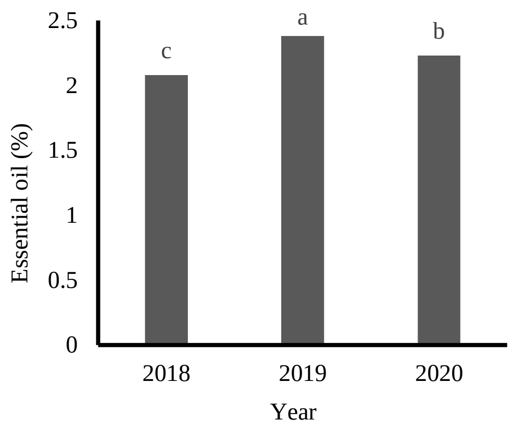

Figure 9. Mean comparison of the year in terms of thyme essential oil percentage

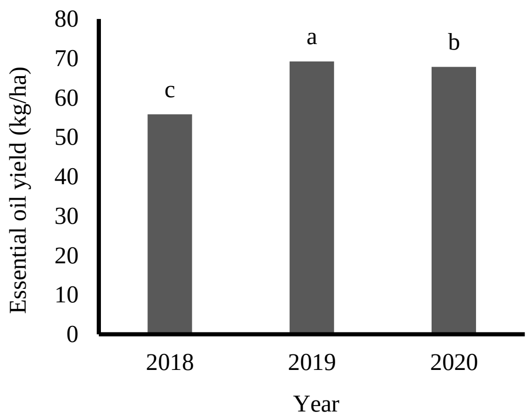

Figure 10. Mean comparison of the year in terms of thyme essential oil yield

Percentage and yield of thyme essential oil in 2019 were obtained more than the other two years (Fig. 9 and 10).

The results of this study showed that the application of nitrogen and cytokinin in appropriate amounts increased the plant dry matter and essential oil. The plant dry matter yield and essential oil were different during three years experiment. This difference among the years may be related to the plant age and the climatic conditions (for example, the higher humidity during summer 2019 than other two years). Also, it is possible that more nitrogen uptake due to more root length during third year (2020) had a positive effect on the studied traits. The results of a study showed that the environmental conditions and plant age affected on thyme yield and the range of essential oil content was from 0.32 to 0.83 (Ozguven and Tansi, 1998).

The results of the present experiment showed that the amount of 100 kg N/ha had more positive effect on the studied traits (except harvest index). Pavela et al. (2018) reported that application of nitrogen (30 kg/ha) as foliar application increased the essential oil content of thyme from 18.76 to 42.47%. Emami Bistagani et al. (2018) in a study on *Thymus daenensis* reported that the combination of organic and chemical fertilizers significantly increased biomass and essential oil yield 39 and 68%, respectively. Abbaszadeh et al. (2006) to study the influence of nitrogen (60 and 90 kg/ha) on *Melissa officinalis* reported that the effect of nitrogen on dry matter yield and plant height was significant. Application of nitrogen (200 kg/ha) increased significantly plant height and dry matter yield of lavender (*Lavandula angustifolia* Mill) but decreased phenols content (Biesiada *et al.*, 2008). Golder and Vangelder (1998) reported that adding nitrogen amount from 50 to 150 kg/ha increased the yield of peppermint. Baranauskiene et al. (2003) reported that nitrogen fertilizer increased the vegetative growth of thyme, but had no effect on the percentage of essential oils and secondary metabolites. The positive effect of nitrogen on vegetative growth is attributed to its participation in the structure of large molecules such as proteins, amino acids and nucleic acids (Zhao, 2006). Saiedi-Gragani et al. (2014) investigated the effect of different nitrogen amounts on parsley (*Petroselinum crispum*) and reported that the highest plant dry weight was obtained in 150 kg N/ha. The results of a study showed that thyme dry matter increased with increasing the nitrogen fertilizer amount and the essential oil percentage increased from 0.78 to 3.1% (Ceylan *et al.*, 1995). The response of thyme to spraying biostimulants (amino acids and methanol) showed that the effect of the treatments on shoot and leaf dry weight, number of leaves, plant height, number of branches, stem diameter, essential oil, thymol and carvacrol was significant (Naghdi Badi *et al.*, 2015).

In the present experiment, the effect of cytokinin concentrations on the studied traits was not the same. In some traits (leaf, stem, and total dry weight) concentration of 100 µM cytokinin and in some traits (plant height and number of lateral branches) concentration of 400 µM cytokinin had the most positive effect. Plant growth regulators can stimulate cell division and elongation by increasing cell wall properties, thereby increasing growth (Rohamare *et al.*, 2013). Our results showed that the concentration of 200 μM cytokinin produced the highest percentage and yield of essential oil. It has reported that this change in essential oil content is due to the number of glandular trichomes on the leaf surface (Erbelgin *et al*., 2006; Prins *et al*., 2010; Gershenzon, 1994). The change in essential oil content after foliar application of growth regulators can be described by changes in leaf area and leaf glandular trichomes density. Palai et al. (1997) reported that the combination of cytokinin and auxin caused more branching. High concentrations of auxin with cytokinin (BAP) prevent the branching induction. Increasing auxin to an optimal concentration increases callus formation, but concentrations above the optimum have inhibitory effects (Dixon and Gonzales, 1996; Bagheri and Safari, 2004). Foliar application of lemon balm with benzylaminopurine (the most common type of cytokinin) increased the number of sub-branches, chlorophyll content, leaf number, plant height, stem diameter, fresh shoot weight, internode length, and the number of nodes (Valiyari and Nourafcan, 2018). It has been reported that foliar application of benzylaminopurine on potato increased plant height and number of stems (Doustipour *et al*., 2016). Foliar application of benzylaminopurine increased leaf number, plant height, leaf area, fresh and dry weight of eggplant (*Solanum macrocarpon* L.) (Opabode and Owojori, 2018). Plant growth regulators can alter some metabolic pathways, photosynthetic efficiency, yield, and morphological characteristics of plants. Plant growth regulators can improve plant growth and development by increasing protein synthesis, nitrate reductase activity, water uptake and mineral nutrition, photosynthesis efficiency, carbohydrate transport and pigment biosynthesis (Muthulakshmi and Pandiyarajan, 2015; Dar *et al*., 2015; Khan *et al*., 2015).

In general, the results of the present experiment showed that the combined use of nitrogen and cytokinin improved the quantitative and qualitative yield of thyme compared to their separate consumption or non-consumption. In this regard, application of appropriate amount of nitrogen and cytokinin was very important. Also, the positive effect of nitrogen and cytokinin consumption on thyme was greater with increasing plant age.

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