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Full Length Research Paper

Biofertilizers effects on quantitative and qualitative yield of Thyme (*Thymus vulgaris*)

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A factorial experiment in randomized complete block design was conducted in order to evaluate the effects of bio and chemical fertilizers on essence, dry and fresh weight production in Thyme (*Thymus vulgaris*) in a field study in Shahrekord, Iran. The applied fertilizers were: (a) phosphate-solubilizing bacterium, (b) frowzy manure from mutton, (c) Nitroxin biofertilizer and (d) phosphate from super phosphate triple at level of 0, 20, 40 and 60 kg/ha. Control experiments were carried out without soil fortification with any fertilizers. Significant differences were observed in dry and fresh weight, essential oil production and number of lateral shoots while soils were fortified with one of the chemical or bio fertilizers or combination of both. Results of Duncan's multiple range test showed the best treatment was simultaneous application of phosphate-solubilizing bacterium, frowzy manure from mutton, nitroxin bio fertilizer and 40 kg/ha of super phosphate triple (essence production = 0.66% of plant dry weight). However, in control experiments without soil fortification with any fertilizers, the minimum production of the essence and other parameters of plants were obtained. The essence production in this treatment was 0.047% of plant dry weight. The application of biofertilizeres (phosphate-solubilizing bacterium, nitroxin and frowzy manure) caused the highest biomass and essence production. It was found that soil fortification with biofertilizers make more plant production comparing to usage of chemical fertilizer.

Key words: Essence production, frowzy manure from mutton, nitroxin, phosphate-solubilizing bacterium, super phosphate triple, Thyme (*Thymus vulgaris* L.).

INTRODUCTION

Thyme (*Thymus vulgaris* L.) belonging to the Lamiaceae or Labiatae family, has many applications in medicinal industries and ethno botany. Propagation is done by stem cutting, stem in division and seed. The best time for harvesting of this plant is the beginning of flowering period. At this time, the highest proportion of dry and fresh weight, as well as the highest essential oil content can be obtained (Naghdi Badi et al., 2004). Thyme has 0.8 to 2.6% essence. The major components are phenols (mainly thymol and carvacrol), monoterpen hydrocarbon and alcohol. Among these, thymol is the main compound. The essence of thyme was used widely in medicinal, hygienic, garnish and drinking industries (Naghdi Badi et al., 2004). Leaves of this plant have also many uses mainly in food industry. It is known that the use of chemicals, particularly nitrogen fertilizers, reduces the essential oil content in vegetation period. In a sense, biofertilizeres (beneficial microbial populations) are unique alternatives for the sustainable agriculture and safe production. Indeed, application of beneficial microbes in agricultural practices started 70 years ago and there is now increasing evidence that they can also enhance plant resistance to adverse environmental

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Table 1. Physical and chemical characteristics of the experimental farm soil.

pH	O.C (%)	E.C (ds/m)	Zn	Fe	Mn	Cu	K	P	Total nitrogen (%)	Texture	Depth (cm)
			(Available ppm)								
8.1	0.79	0.4	1.136	3.453	9.5	1.108	245	2.8	0.065	Loam	0-30

stresses, for example, water and nutrient deficiency and heavy metal contamination (Shen, 1997). Phosphate (P) and potassium (K)-solubilizing bacteria may enhance mineral uptake by plants through solubilizing insoluble P and releasing K from silicate in soil (Goldstein and Liu, 1987). Microbial inoculums not only increased the nutritional assimilation of plant (total N, P and K), but also improved soil properties, such as organic weight content and total N in soil. The nutrient deficiency in soil resulted in a larger population of N-fixing bacteria and higher colonization of arbuscular mycorrhizal fungi (AMF). Biofertilizers have the social community of bacteria and other microorganisms that live in rhizosphere, colonize upon roots and as a consequence of this symbiotic relationship; the host plant achieves a better growth (Dokora et al., 2002; Han et al., 2006). For instance, plant growth-promoting rhizobacteria (PGPR) are beneficial native soil bacteria that colonize on plant roots and result in increased plant growth (Dashti et al., 1998; Remans et al., 2008; Afzal and Bano, 2008). PGPR can positively affect plant growth through different mechanisms such as nitrogen fixation, production of plant growth regulators (Vessey, 2003) and increasing plant water and nutrient uptake (Dey et al., 2004). PGPR can also inhibit soil-borne plant pathogens through antifungal activity and siderophore production (Sindhu et al., 2002).

The objectives of this study were to evaluate the effects of both bio and chemical fertilizers on growth habit of *T. vulgaris* L. and to compare the amount of essence production in bio and chemical treatments.

MATERIALS AND METHODS

Plant material and fertilizers

Shoot segments of Thyme (*T. vulgaris* L.) were obtained from Iranian Seeds and Plant Improvement Institute (ISPII). Plants of thyme were planted after they had 2 to 4 leaves. Table 1 shows physicochemical properties of the soil. Chemical and bio fertilizers which are used for soil fortification were as follow:

1. Phosphate-solubilizing bacterium, *Bacillus licheniformis,* in powder form applied at the level of 100 g/ha (a_1 and a_2 were used as symbols to show soil fortified with biofertilizer and without respectively).
2. Frowzy manure from mutton applied at the level of 700 kg/ha (b_1 and b_2 were used as symbols to show soil fortified with biofertilizer and without respectively).
3. Nitroxin bio-fertilizer which its effective site is the group of nitrogen fixing bacteria belongs to *Azospirillum* and *Azotobacter* genera in liquid form applied at the level of 4 l/ha (c_1 and c_2 were used as symbols to show soil fortified with biofertilizer and without respectively).
4. Phosphate from super phosphate triple (this chemical fertilizer was applied at the levels of 0, 20, 40, 60 kg/ha which were showed by symbols of d_1, d_2, d_3 and d_4 respectively).

Experimental conditions

Field trials were established in 2011 at Shahrekord (50°56′ E 32°18′ N) South Western Iran. Experiments were arranged in a randomized complete block design with a factorial layout. All experiments were carried out in triplicate. 20 plants were used for each trial. At the beginning of the blooming stage, shoots of plants were harvested. Several parameters including dry and fresh weight of plant, number of lateral shoots and the amount of essence production as well as the chemical component of the essence were determined. Essential oil composition was monitored by capillary GC/MS (Shibamoto, 1987).

Data analysis

All data were subjected to ANOVA using the statistical computer package SAS and treatment means separated using Duncan's multiple range test at $P < 0.05$ level.

RESULTS AND DISCUSSION

Shoot dry weight

Table 2 shows different treatments and the combination of soil fortification with different fertilizers which reveals remarkable differences in plants production. It was observed that soil fortification with combination of three biofertilizers and super phosphate triple at the level of 40 kg/ha achieved maximum production of dry weight. The treatment of $a_1b_1c_1d_3$ showed maximum production of dry weight than $a_1b_1c_1d_4$ whose soils are fortified with three biofertilizers and super phosphate triple at the level of 40 and 60 kg/ha respectively (Figure 2b). Figure 2b shows that the treatments of $a_1b_1c_1d_2$ (with super phosphate triple at the level of 20 kg/ha) and $a_1b_1c_1d_1$ (without super phosphate triple fortification) have the most dry weight productions after two previous mentioned treatments. Comparing among dry weight production of treatments of $a_1b_1c_1d_1$, $a_1b_1c_2d_1$, $a_1b_2c_1d_1$ and $a_2b_1c_1d_1$ shows the trend of $a_1b_1c_1d_1 > a_1b_1c_2d_1 > a_2b_1c_1d_1 > a_1b_2c_1d_1$. It was indicated that the influence of frowzy manure from mutton on dry weight production is more than two other biofertilizers as well as phosphate-solubilizing bacterium had more production than Nitroxin biofertilizer (Figure 2b). Also, this finding has been supported by comparing dry weight

Table 2. Analysis of variance of essence, fresh weight, dry weight and number of shoots that affected by several biofertilizeres on Thyme.

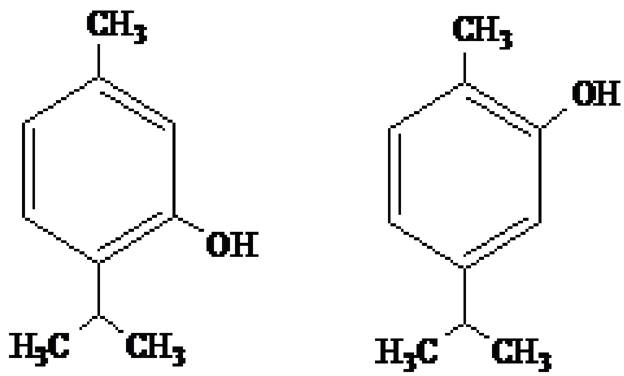
Source of variation	Degree of free	Number of shoot		Dry weight		Fresh weight		Essence	
		MS	P< α	MS	P< α	MS	P< α	MS	P< α
Block	2	2966.3	--	1270.5	--	36951	--	0.0002	--
Treatments	31	3015.4	0.001	9182	0.001	43937.2	0.001	0.13	0.001
A	1	17455.5	0.001	49716.9	0.001	249801.1	0.001	0.54	0.001
B	1	65151	0.001	203032.9	0.001	982293.9	0.001	3.12	0.001
C	1	4688.6	0.001	18106.5	0.001	71819.4	0.001	0.15	0.001
D	3	407.2	0.001	1239.2	0.001	5748.5	0.001	0.02	0.001
a×b	1	3022.9	0.001	5158.2	0.001	27612.5	0.001	0.09	0.001
a×c	1	511.1	0.001	20.4	Ns	91.1	ns	0.00006	ns
a×d	3	34.8	ns	104.8	Ns	32.2	ns	0.0009	0.001
b×c	1	705.7	0.001	2779.9	0.001	10079.2	0.001	0.023	0.001
b×d	3	52.2	ns	177	Ns	533.2	ns	0.001	0.001
c×d	3	4.2	ns	99.7	Ns	47.4	ns	0.0008	0.001
a×b×c	1	337.8	0.001	244	Ns	311.6	ns	0.024	0.001
a×c×d	3	3.9	ns	69.2	Ns	21	ns	0.0003	0.001
a×b×d	3	12.9	ns	35.8	Ns	117.9	ns	0.0006	0.001
b×c×d	3	6.1	ns	75.7	Ns	45.4	ns	0.0004	0.001
a×b×c×d	3	13.7	ns	59.1	Ns	135.1	ns	0.0009	0.001
Error	62	32.6		71.5		224.1		0.0001	
Total	95	1067.7		3069.6		15261.5		0.04	
R-Square			0.98		0.98		0.99		0.99
CV			10.7		6.5		5.4		1.48

ns = not significant.

productions of treatments of $a_1b_2c_2d_1$, $a_2b_1c_2d_1$, $a_2b_2c_1d_1$ and $a_2b_2c_2d_3$ which revealed the trend of $a_2b_1c_2d_1 > a_1b_2c_2d_1 > a_2b_2c_1d_1 > a_2b_2c_2d_3$ (Figure 2b). Figure 2b also shows the range of plants dry weights productions were from 47.2 to 234.3 g per plant after the growth period, whereas very poor growth rate was observed in control plants due to nutrient deficiency. The results of this study have good consistency with previous studies (Akbarinia et al., 2002; Darzi et al., 2009; Preetha et al., 2005; Jeliazkova, 1999; Khan and Azam, 1999; Fallahi et al., 2009; Wu et al., 2005).

Shoot fresh weight

Similar trend was observed in shoot fresh weight. Statistical analysis on fresh weight production showed significant fluctuation among different treatments (Table 2). Table 2 shows simultaneous soil treatment by biophosphate, frowzy manure from mutton and nitroxin biofertilizer was the most effective treatment to achieve maximum productions of dry and fresh weight. Bio and chemical fertilizers treatments of $a_1b_1c_1d_3$ and $a_1b_1c_1d_4$ were the best treatment because of the highest amounts of fresh weight achievement (Figure 2c). Same trends were observed in plant growth and yield production under field conditions in banana and tomato in previous studies (Jeeva et al., 1988; Kohler et al., 2007). The maximum fresh weight of 502.7 g per plant was obtained from the treatment of $a_1b_1c_1d_3$ and $a_1b_1c_1d_4$ thus treatment of $a_1b_1c_1d_3$ is the best because of high productivity and less usage of chemical fertilizer which is more environmental friendly. The combination of all biofertilizers and chemical phosphate in soil treatment seems to be more effective than soil fortification with biofertilizers and chemical fertilizer separately. It was found that the effect of these three biofertilizeres on plant yields were not same, however, the use of biofertilizers individually showed more efficiency than treating with superphosphate triple which is a chemical fertilizer (Table 2). It was deduced that dual inoculation with beneficial bacteria (Phosphate-solubilizing bacterium *B. licheniformis* and *Azospirillum* and *Azotobacter* genera) and frowzy manure are able to compensate for the lack of nutrient in soils. It was reported that wheat yield increases up to 30% with Azotobacter inoculation and up to 43% with Bacillus inoculants (Kloepper et al., 1989). Yield enhancement in field trials was produced by using a combination of *Azotobacter chroococcum* and *Bacillus megaterium* (Brown, 1974). Strains of *Pseudomonas* have increased root and shoot elongation in canola, lettuce and tomato (Hall et al., 1996; Glick et al., 1997). The biofertilizer therefore may have a potential to decrease the input cost of agricultural production, and be applied to the



a b

Figure 1. The chemical structure of thymol (a) and carvacrol (b).

revegetation of low commercial value sites, such as metal tailings ponds (Carlot et al., 2002). The effect of biofertilizers on growth properties, yield and quality property in medicinal plants was studied by other researchers and they showed same beneficial effects of biofertilizeres (Koocheki et al., 2008; Leithy et al., 2006; Youssef et al., 2004; Vital et al., 2002; Ratti et al., 2001).

Number of shoots

Table 2 shows the mean squares of the results of a, b, c, and d treatments which indicates wide range on number of shoots in the plants. The best treatment which was achieved maximum number of shoots was using biofertilizers with superphosphate triple at the level of 40 kg/ha simultaneously. However, The Duncan's multiple range result revealed using biofertilizers with superphosphate triple at the level of 20 and 60 kg/ha had same influence on number of shoots (Figure 2d). Simultaneous usage of Nitroxin biofertilizer and Phosphate-solubilizing bacterium with superphosphate triple into the soil would help bacteria to provide required minerals for plants growth. Indeed microorganisms play a main role on medicinal plants growth. Figure 2d shows the fluctuation of number of shoots versus different soil fortifications which indicate similar trends happen in number of shoots. This data can also support previous discussed results of dry and fresh weight. Comparing between number of lateral shoots of treatments of $a_1b_1c_1d_1$, $a_1b_1c_2d_1$, $a_1b_2c_1d_1$ and $a_2b_1c_1d_1$ shows the trend of $a_1b_1c_1d_1>a_1b_1c_2d_1>a_2b_1c_1d_1>a_1b_2c_1d_1$. It was indicated that the influence of frowzy manure from mutton on dry weight production is more than two other biofertilizers as well as Phosphate-solubilizing bacterium had more production than Nitroxin biofertilizer (Figure 2d). This finding has been also supported by comparing between number of lateral shoots of treatments of $a_1b_2c_2d_1$, $a_2b_1c_2d_1$, $a_2b_2c_1d_1$ and $a_2b_2c_2d_3$ which is revealed the trend of $a_2b_1c_2d_1>a_1b_2c_2d_1>a_2b_2c_1d_1>a_2b_2c_2d_3$ and the lowest number of shoots was observed in the plants without fortification of any fertilizers (Figure 2d). Previously the beneficial usage of mycorrhiza, vermicompost and biophosphate was reported (Darzi et al., 2009). The most biological yield was obtained by biofertilizeres (Arbab et al., 2008). Nitrogen fixers and mycorrhiza resulted in the most plant height; fruit yield (Aseri et al., 2008). Increasing shoots of plant by using biofertilizeres was reported by Kapoor et al. (2004). The enhanced phosphatase activity may help the plant to mobilize P and thereby increase the biomass production (Tarafdar and Gharu, 2006). Significant enhancement in total nitrogen-fixing potential as reflected by increased nitrogenase activity in rhizosphere soils was observed. The measurement of these enzymatic activities can provide an early indication of changes in soil fertility, since they are related to mineralization of such important nutrient elements as N, P and C (Ceccanti et al., 1994).

Plant yield of essence

Two major compounds produced were thymol and carvacrol by GC-MS analysis (Figure 1). The ion fragmentation of thymol was observed at m/z 150 (M^+), 135 (M^+-CH_3), 115 (M^+- CH_3-H_2O-2H), 91 ($C_7H_7^+$) and 65 ($C_5H_5^+$). The ion fragmentation of carvacrol was observed at m/z 150 (M^+), 135 (M^+-CH_3), 115 (M^+- CH_3-H_2O-2H), 91 ($C_7H_7^+$) and 66 ($C_5H_6^+$). Except biophosphate and Nitroxin, all treatments had significant effect on yield of essence (Figure 2a). Figure 2a shows that the most effective treatment were using biofertilizers with superphosphate at the level of triple 40 kg/ha, then 60 kg/ha. As it was expected, the lowest yield of essence belonged to blank samples with no fertilizers treatment (Figure 2a). From Table 2, it was deduced that th

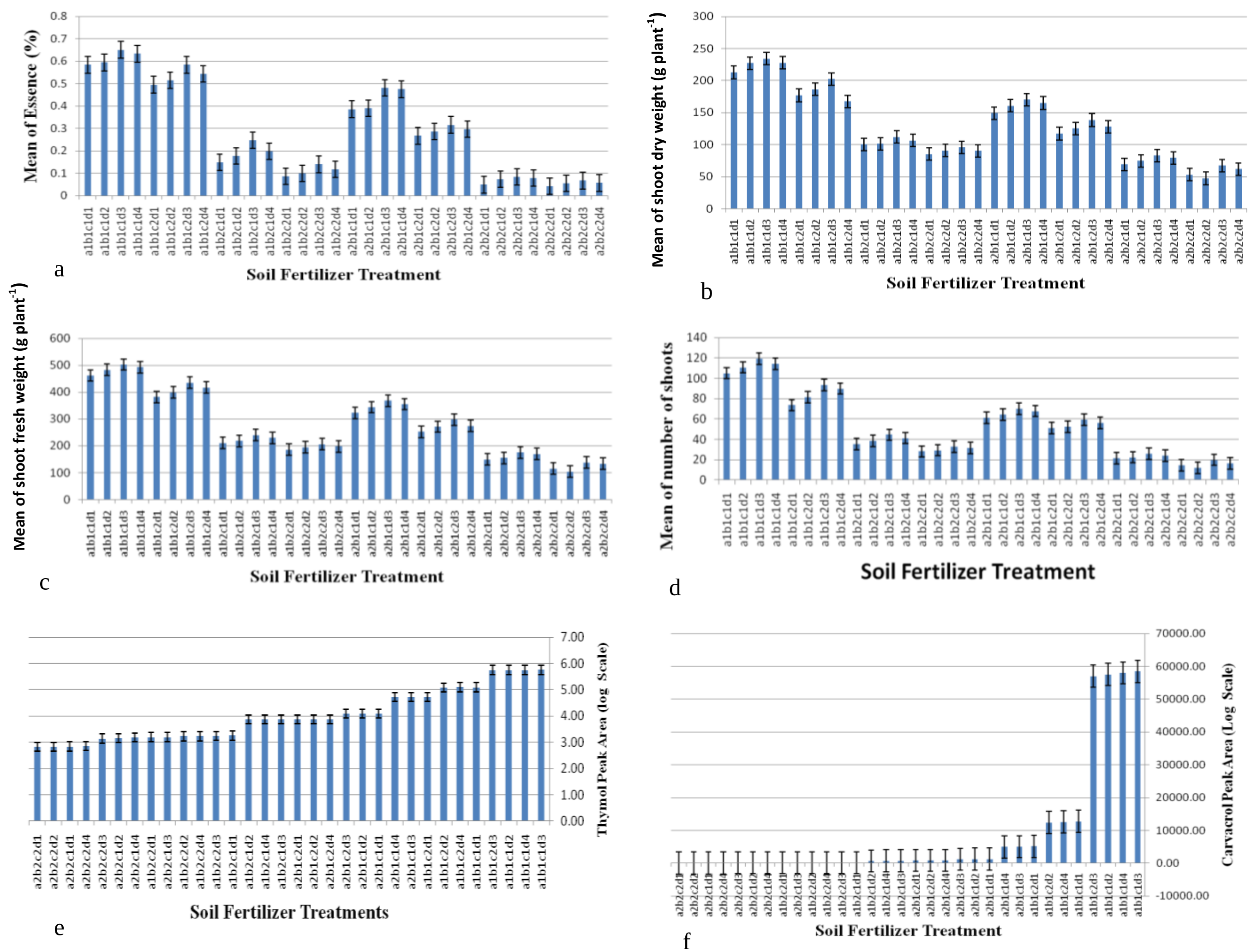


Figure 2. (a) Mean of essence. (b) Mean of shoot dry weight (c) Mean of shoot fresh weight. (d) Mean of number of shoot. (e) Peak area of GC chromatogram of thymol in different treatments. f) Peak area of GC chromatogram of carvacrol in different treatments.

results of Duncan's multiple range test has the best treatment which was simultaneously fortificated with Phosphate-solubilizing bacterium, frowzy manure from mutton, Nitroxin and super phosphate triple at the level of 40 kg/ha (essence production was equal to 0.66% of dry weight of plant). The GC-MS results revealed the amount of thymol and carvacrol produced by the treatment of $a_1b_1c_1d_3$ was higher than other treatments base on their peak area (Figures 2e and 2f). Figure 2e shows that the influence of frowzy manure from mutton fertilizer is more than other treatments. Comparison between these four fertilizers showed, the order of productivity to produce thymol is as follow; frowzy manure from mutton fertilizer > biophosphate > nitroxin > super phosphate triple (Figure 2e). Comparing between $a_1b_1c_1d_1$, $a_1b_1c_2d_1$, $a_2b_1c_1d_1$ and $a_1b_2c_1d_1$ showed that the elimination of nitroxin biofertilizer eventuated to reduction of 10.57 times production of thymol in the plant whereas the elimination of frowzy manure from mutton and biophosphate reduced it to 313.2 and 45.3 times respectively (Figure 2e). Therefore, it was found that the effect of using frowzy manure from mutton fertilizer on thymol production is higher than biophosphate and nitroxin, although biophosphate fertilizer also effected more than nitroxin on plant thymol production. Figure 2e also shows the d_3 treatment (super phosphate triple, 40 kg/ha) is the best treatment of using super phosphate triple. Treatments of d_2 and d_4 which are usage of super phosphate triple at level of 20 and 60 kg/ha respectively showed almost same influence on thymol production in the plant (Figure 2e). It was also observed that the use of super phosphate triple has no remarkable effect on the plant thymol production. On the other hand, the influence of super phosphate triple fertilizer on thymol production is not as much as other three fertilizers. Same trend was observed for carvacrol production in the plant (Figure 2f).

Other researchers showed the effect of nitrogen, phosphorus and potassium on essence production (Mallanagouda, 1995, Bist et al., 2000). Anwar et al. (2005) showed that application of biofertilizers on French basil, the essence production was greater than other treatments. Other researchers showed the beneficial application of biofertilizers on medicinal plants as well as enhancement on quality of plants yield (Copetta et al., 2006; Freitas et al., 2004; Kapoor et al., 2004). Darzi et al. (2009) reported the effects of biofertilizeres on quantity and quality of essential oil in fennel. They showed the highest essential oil content in seed, anethole content in essential oil. They reported that the lowest fenchone content and limonene content in essential oil were obtained with mycorrhiza treatment. Phosphate-solubilizing bacterium also showed significant effects on essential oil content, anethole and limonene contents in essential oil. The maximum essential oil content in seed and anethole content in essential oil were related to the plots with consumption of 60 kg/ha. The lowest limonene content in essential oil was obtained with consumption of 30 kg/ha Phosphate-solubilizing bacterium. The results of this study are in agreement with previous researches, which showed the positive and significant effects of biofertilizeres on essence production of medicinal plants (Vildova et al., 2006; Fatma et al., 2006; Migahed et al., 2004; Sanches et al., 2005; Kalra, 2003).

Conclusion

This study was conducted on application of biofertilizer containing beneficial microbes which showed a promoting effect on the growth of *T. vulgaris*. Optimum nutrition is a key to achieve maximum crop production especially when essential oil is considered as a goal. Nitrogen and phosphorus could act as limiting factors. The results of present study showed that, in thyme, the major ($P \geq 0.05$) difference for herbage biomass was obtained from the combination of bio and chemical fertilizers compared to the other fertilization by single format. The best combination which made the maximum dry and fresh yield as well as essence production was mixture of chemical and biofertilizeres (Table 2). Frowzy manure from mutton and then biophosphate had the maximum influence on desired factors. The maximum effect of biophosphate was achieved as it was used with simultaneous application of enough super phosphate triple. Duncan's multiple range test for fresh weight and essence of plant revealed the best treatment was simultaneous fertilization by Phosphate-solubilizing bacterium, frowzy manure from mutton, Nitroxin and super phosphate triple at the level of 40 kg/ha. The result showed super phosphate triple treatment at the levels of 20 and 60 kg/ha had same effect on dry weight and plant essence production. Duncan's multiple range test showed the best quadruple interaction made by simultaneous fertilization by Phosphate-solubilizing bacterium, frowzy manure from mutton, Nitroxin and super phosphate triple at the level of 40 and 60 kg/ha. The higher uptake of nutrients was due to the synergistic effect of improved biomass and higher nutrient concentration in the inoculated plants. Maximum essence and dry weight was observed in dual inoculation treatment which could be due to the improved symbiotic N_2 fixation, phosphatase activity and finally P mobilization and subsequent P uptake by *B. licheniformis*. The result of this study has a good consistency with previous findings of Singh and Sharma (1993) which reported enhanced N and P concentration in sweet orange which were inoculated with biofertilizeres. The result of this research is also in agreement with the results of Shalby and Razin (1992). The enhancement of dry weight and essence might be due to the production of nutrient-solubilizing enzymes by microorganisms and the ability of *B. licheniformis* for uptake of immobile ions, besides increasing the surface area of roots by tapping larger soil volume. These results clearly show that the biofertilizers have potential to be used practically as a natural nutrition sources in agricultural products. A comprehensive ecological study is required for evaluating the effect of these biofertilizeres and their effective constituents in field condition.

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