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Assessment of Morphological, Physiological, and Biochemical Characteristics of *Thymus kotschyanus* Bioss. and Hohen under Different Bio and Chemical Fertilizers

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ABSTRACT

Biofertilizer has been recognized as an alternative to chemical fertilizer to improve soil fertility and crop production in sustainable agriculture. The objective of this field study was to evaluate the effects of bio and chemical fertilizer on qualitative and quantitative characteristics of *Thymus kotschyanus* Bioss. & Hohen. The experiment was conducted during 2019 at Imam Khomeini International University, Qazvin, Iran, and treatments included control (no fertilizer), chemical fertilizer (NPK), and four types of microorganisms including *Funneliformis mosseae* (AMF), *Azotobacter chroococcum* strain 5, *Pseudomonas stutzeri* strain P-16, and *Pseudomonas putida* strain 41. The results showed that AMF and NPK positively affected plant height, number of branches per plant, and photosynthesis pigments contents compared to other treatments. However, maximum plants' fresh and dry weight, proline, total phenolic and flavonoid contents, DPPH inhibition, essential oil percentage, and carvacrol quantity were obtained from plants that were inoculated with AMF. Also, the activities of Catalase (CAT) and Superoxide Dismutase (SOD) were increased by the application of AMF. According to the obtained results, there were no significant differences in P concentration between plants treated with *P. stutzeri*, *Pseudomonas putida*, AMF, and NPK. Maximum N amount in *T. kotschyanus* leaf was obtained in plants treated with AMF, NPK and *Azotobacter chroccucum*. Plants inoculated with AMF had higher Ca uptake compared to the other treatments, and the maximum total K accumulation in *T. kotschyanus* were obtained in plants inoculated with AMF and NPK. Hence, the use of organic and biological inputs instead of chemical fertilizer for improving crop efficiency and quality with the aim of alleviating pollution and accomplishing sustainable agriculture is highly encouraging.

Keywords: Antioxidant enzymes, Biofertilizer, Essential oil, Mycorrhiza, Thyme.

INTRODUCTION

Industrialization and green revolution have brought an enhancement in productivity but have also resulted in extensive degradation of environment. Large-scale use of synthetic fertilizers to enhance plant productivity and to control pathogens has disturbed the ecological balance of soil and has led to nutrient deficiency. Therefore, there is a necessity to find replacement strategies to improve soil health without inducing damage to the environment. Biofertilizers are, therefore, obtaining importance as they are cost effective, ecofriendly, and non-hazardous. The bio-fertilizers are microbial preparations applied to seed, soil, and seedlings for the enhancement of plant vigor and growth, resulting in a higher yield (Borkar, 2015).

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The bio-fertilizers may broadly be classified into some categories, including: Nitrogen fixing like *Azotobactor*, Phosphorous Solubilizers Microorganisms (PSM), and mycorrhiza fungi (Borkar, 2015). Among various nitrogen-fixing bacteria, *Azotobacter* is one of the most important non-symbiotic nitrogen-fixing bacterium. *Azotobactor* not only provides the nitrogen, but also generates a variety of growth promoting substances like gibberellins, auxin, and vitamins, particularly vitamin-B (Nadeem *et al*., 2014). Fixation of Phosphorus (P) in the soil, which is supplied through chemical fertilizers, causes the problem of availability of this essential element to plants and, therefore, plants suffer from deficiencies. PSMs are prepared from the soil bacteria and fungi, which solubilize fixed phosphorus in soil and mobilize it in plants (Sabzi-Mehrabad *et al.,* 2017).

In natural ecosystems, the majority of plants are associated with mycorrhizae. An increase in roots surface area for nutrient acquisition can be achieved by mycorrhizal colonization. The extrametrical fungal hyphae can expand several centimeters into the soil and absorb large quantity of nutrients for the host root (Oliveira *et al*., 2016). There are strong evidences that *Arbuscular Mycorrhizal Fungi* (AMF) contribute to enhancing uptake and availability of macro and micro nutrients (Tarraf *et al*., 2017; Ortas *et al*., 2019). The mycorrhizal symbiosis can also be regarded as a bridge connecting the root with the surrounding soil microhabitats, by linking the biotic and geochemical portion of the ecosystem. Therefore, AMF have the capacity to promote plant growth by improving the uptake of necessary nutritive minerals (Oliveira *et al*., 2006; Willis *et al*., 2013).

Iran has an amazing ecological diversity and a rich herbal flora, which still needs to be studied in terms of phytochemistry and bioactivity. Lamiaceae, formerly called Labiatae, is one of the most important plant families in which the genus *Thymus*, known as "Avishan" in Persian, is a famous aromatic perennial herb originated in the southern Mediterranean. Among 215 species of thyme genus grown worldwide, about 14 species are distributed in Iranian flora, four of those are endemic (Mozaffarian, 2013). *Thymus* species are well known as medicinal herb due to their biological and pharmacological properties including antispasmodic, antibacterial, antiviral, antifungal and antioxidant activities (Salehi *et al*., 2018; Li *et al*., 2019). In traditional medicine, fresh and dried aerial parts of *Thymus* species are widely used as tonic and herbal tea, spice, disinfectant, antitussive and carminative as well as cold remedy (Zargari, 1990; Mozaffarian, 2013). *Thymus kotschyanus* Bioss. & Hohen. is an aromatic and medicinal plant that grows wild in Iran (Kurdestan, Azerbaijan, Qazvin, Tehran and northern provinces) (Mozaffarian, 2013). This valuable medicinal plant plays an important role in the economy of local people in most regions as its cultivation helps the regional economy (Abbasi Khalaki *et al*., 2016).

The aims of the present study were to determine the effect of bacteria (*Azotobacter chroococcum*, *Pseudomonas stutzeri* and *Pseudomonas putida*) and fungi (*Funneliformis mosseae*) on growth, physiological characteristics, and essential oil content and constituents of *T. kotschyanus*, and assess whether the symbiosis of these microorganisms may serve as a feasible alternative for chemical fertilizer in sustainable production of medicinal plants.

MATERIALS AND METHODS

The Field Information

The field experiment was conducted during 2019 at Imam Khomeini International University, Qazvin, Iran (36° 16′ N latitude and 50 °E longitude). The elevation of the experimental site from the sea level was 1,300 m. The prepared plots were deep

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Table 1. Physical and chemical properties of experimental farm soil.

Texture	Clay %	Silt %	Sand %	Available Phosphorus (PPM)	Available Potassium (PPM)	N%	OM%	pH	EC
Loam	31	35	34	12.7	145	0.08	0.71	7.4	2.31

Table 2. Some climatic data of the experimental city (Qazvin, Iran) for 2019.

Months of the year	Minimum monthly temperature (ºC)	Maximum monthly Temperature (ºC)	Monthly Precipitation (mm)
Jan	-1.8	9.1	73.91
Feb	-1.5	10.6	11.43
Mar	1.3	13.2	62.21
Apr	4.6	18.5	79.51
May	10.4	28.3	15.48
Jun	15.6	35.3	1.78
Jul	18.1	37.9	-
Aug	16.8	35.4	-
Sep	12	31.4	-
Oct	9.5	23.8	51.06
Nov	0.8	13.4	6.09
Dec	-0.1	10.6	37.59

plowed and the topsoil of the plots was disinfected by using 0.1% commercial aqueous formaldehyde. The disinfected soil was covered with a black polyethylene sheet. After 7 days, the plastic cover was removed for decomposition of the fumigant residues. Just before planting, soil samples were taken from 0 to 30 cm soil depth for analysis. Chemical and physical properties of the soil used in the present study are shown in Table 1, and daily values of maximum and minimum air temperature (°C), and precipitation (mm) of 2019 are shown in Table 2.

Treatments and Experimental Design

The experiment was laid out in a Randomized Complete Block Design (RCBD) at 18 plots of 12 m^2 area each, with three replications and six treatments. The microorganisms used for this field experiment were supplied by Soil and Water Research Institute, Karaj, Iran. Three strains including a nitrogen-fixing bacterium (*Azotobacter chroococcum* strain 5) and two phosphate-solubilizing bacteria (*Pseudomonas stutzeri* strain P-16 and *Pseudomonas putida* strain 41) were used. The P solubilizers were cultured with LB (Luria-Bertani) medium, for 48 hours in a shaking incubator under 28±1ºC and 200 rpm. The N-fixing bacteria were cultured under the same conditions with Ashby liquid medium. The density of each bacterial, in a broth culture, was counted using counting chamber (haemocytometer). The bacteria were harvested by centrifugation at 6,000 rpm (Rotofix 32A, Germany). The final population sizes of *A. chroococcum* and P solubilizers bacteria were 2.72×10^8 and 2.05×10^8 CFU g^{-1} inoculum (wet weight), respectively. The inocula were sealed in sterile, airtight plastic bags and stored at refrigerator. The inocula of the AMF (*Funneliformis mosseae*), were purchased from ZPV Company, Iran, with the population of 10^8 CFU g^{-1}. The *Thymus kotschyanus* seeds were collected from the Alborz Mountain Range in northern Iran. Surface sterilized seeds were grown in a controlled environment greenhouse at 24/17ºC day/night temperature. After

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germination and spending the initial growth, the 90-day-old seedlings were transplanted into the field in April 2019. The roots of seedlings were soaked in bacterial suspension for 20 minutes and then transplanted in the field. In addition to the initial inoculation of seedlings, 5 mL of inoculum was injected into the soil of the rhizosphere at a depth of 5 cm (Attarzadeh *et al.*, 2020). AMF treatments received 5 g of inoculum per plant at the time of planting by placing a thin layer of mycorrhizal inoculum around the roots of plantlets (Attarzadeh *et al.*, 2020). Chemical fertilizers (100 kg N ha^{-1} using ammonium nitrate, 50 kg P ha^{-1} using calcium super phosphate, and 50 kg K ha^{-1} using potassium sulfate) were applied before transplanting. At full flowering stage, the aerial part of plants were harvested by cutting 5 cm above the soil surface and plant growth characteristics for three cuts were recorded as plant height, number of branches per plant, fresh and dry weight. Harvested plants were dried in an electric oven at 40°C till constant dry weight.

Determination of Photosynthetic Pigments

Photosynthetic pigments including chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids were determined according to the methods described by Von Wettstein (1957).

Nutrient Analysis

For nutrient analysis, the plant materials were washed in redistilled water twice. The washed samples were then oven dried for 24 hours at 100°C. The dried samples were digested in triacid mixture HNO_3: H_2SO_4: $HClO_4$ (5:1:1 ratio) at 70°C until transparent samples were obtained. Each sample was filtered and diluted with redistilled water up to 30 mL. The samples contents of Ca and K were analyzed by using Atomic Absorption Spectrophotometer. Total nitrogen was estimated by a C/N elemental analyzer. P was measured photometrically as phosphomolybdate blue complex using a microplate spectrophotometer (Khademian *et al*., 2019).

Determination of Proline Content

The proline content of *T. kotschyanus* leaves was measured by the previously reported method (Bates *et al*., 1973). Briefly, 0.5 g of the plant fresh leaves were homogenized in sulphosalycylic acid solution (3% w/v). After filtration, ninhydrin and acetic acid were added to the mixture and heated for 60 minutes at 100°C water bath. Finally, the reaction was stopped by cooling in ice bath and the solution was extracted by toluene. The obtained organic fraction absorbance was read at 520 nm. The proline contents were represented as μmol g^{-1} FW.

Preparation of Plant Extract

The shadow dried leaves of *T. kotschyanus* (5 g) were extracted with methanol at room temperature by maceration method for 2 days. The solvent of the extracts was removed by vacuum evaporation at 40°C to obtain crude extract.

Determination of Total Phenolic Content (TPC)

Folin-Ciocalteu method was used for calculation of the extracts TPC (Bahadori *et al*., 2016). Certain volume (20 μL) of extract methanol solution with 2 mg mL^{-1} concentration were mixed with 100 μL of 1:10 (v/v) Folin-Ciocalteu reagent. After 6 minutes in the dark, 80 μL of sodium carbonate (7.5%) was added to the mixture. Finally, the absorbance of the solution was measured at 740 nm after 2 hours of

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incubation in the dark at room temperature. TPC of the extracts were expressed as mg of Gallic Acid Equivalents per gram of dry weight of extracts (mg GAEs g^{-1} DW). The calibration curve range of gallic acid was 1-1,000 mg L^{-1}. All samples were analyzed in three replications.

Determination of Total Flavonoid Content (TFC)

Total flavonoid content of *T. kotschyanus* extracts was determined by aluminum chloride colorimetric method (Bahadori *et al*., 2015). Twenty µL of extract or standard solution of quercetin (1 to 200 µg mL^{-1}) was mixed with 60 µL of methanol and 10 µL of 5% $AlCl_3$. Then, total volume of the mixture was made up to 200 µL by adding 10 µL of 0.5 M potassium acetate and appropriate amounts of distilled water. The solution was mixed well and, after 30 minutes, the absorbance was read at 415 nm. All tests were carried out in three replications, and mean values of flavonoid contents are expressed as mg of Quercetin Equivalents per gram of dry weight of extracts (mg QEs g^{-1} DW).

Measurement of DPPH Radicals Scavenging Capacity

The ability of *T. kotschyanus* extracts in scavenging DPPH radicals was determined from the bleaching of purple-colored solution of the free radicals (Asghari *et al*., 2018). The appropriate volume (20 µL) of samples dissolved in methanol was mixed with 180 µL of DPPH solution (0.1 mM) and, after 30 minutes incubation in dark, discoloration of the mixtures was measured at 517 nm. Inhibition of DPPH in percent was calculated as given below:

$$I\ (\%) = [(Ac - As)/Ac] \times 100$$

Where, Ac is the Absorbance of the control reaction (containing all reagents except the test sample), and As is the Absorbance of the extracts. All the assays were run in three replications and the results were expressed as average values with Standard Deviation (SD).

Antioxidant Enzymes Assays

A 0.5 g of each leaf sample was chilled in liquid nitrogen, homogenized in phosphate buffer (50 mM, pH 7.8) and centrifuged (10 min, 12,000×g at 4°C). The supernatant was used for the determination of different enzyme activities.

Catalase activity was measured by recording a decrease in absorbance of H_2O_2 reduction reaction mixture for 60 seconds (Asghari *et al.*, 2020). The reaction was started by adding 200 µL of 100 mM H_2O_2 to 100 µL of plant extract and 1.7 mL of phosphate buffer (50 mM) prepared in 0.1 mM EDTA (pH 7.0). The decrease in absorbance was measured at 240 nm. The enzyme activity was calculated using extinction coefficient of 39.6 mM^{-1} cm^{-1} for H_2O_2 and expressed as units mg^{-1} protein. One unit of enzyme was defined as 1 mM of substrate reacted per min per mg protein.

Superoxide Dismutase (SOD) activity in treated and untreated samples was measured according to the photochemical *p*-Nitrobluetetrazolium (NBT) method (Asghari *et al.*, 2020). The reaction mixture was prepared by adding 40 mL of enzyme extract, 1.3 µM riboflavin, 75 µM *p*-Nitrobluetetrazoliumchloride (NBT) and 13 µM methionine to 50 mM phosphate buffer (pH 7.8). The reaction tube was placed under fluorescent light with 80 µmol m^{-2} s^{-1} for 10 minutes. To stop the reaction, test tubes were placed in dark and, finally, the mixture absorption was read at 560 nm. One unit of SOD activity is defined as the amount of enzyme required to cause 50% inhibition in reduction of NBT.

Isolation of Essential Oil

The Essential Oil (EO) of dried aerial parts samples of *T. kotschyanus* (50 g, three times) were extracted with hydro distillation

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method for 3 h using a Clevenger-type apparatus. The essential oil samples were dried over anhydrous sodium sulfate and stored in sealed vials at 4°C until analysis (British Pharmacopoeia, 1988).

GC and GC/MS Analysis

The obtained essential oils were injected to Gas Chromatography (GC) and Gas Chromatography–Mass Spectrometry (GC/MS). GC analyses were performed using an Agilent Technologies 7890A gas chromatograph equipped with a DB-5 fused silica column (30 m×0.25 mm id, film thickness 0.25 μm). Oven temperature was programmed to be held at 50 °C for 5 minutes and then increased to 280 °C at a rate of 10°C min^{-1}. Injector and detector (FID temperatures were 290°C and helium was used as carrier gas with a linear velocity of 32 $cm^3 s^{-1}$, and split ratio 1:60.

GC/MS analyses were carried out using a system equipped with DB-5 fused silica column (30 m× 0.25 mm id) on a Varian 3400 GC/MS system. Oven temperature was 50°C increasing to 280°C at a rate of 10°C, transfer line temperature 260°C. The carrier gas was helium with a linear velocity of 32 $cm^3 s^{-1}$, split ratio 1:60, Ionization energy 70eV, scan time1 s and mass range of 40-300 amu. The percentages of compounds were calculated by the area normalization method, without considering response factors. The constituents of the essential oils were identified by matching their mass spectra with those of a computer library or with authentic compounds, and confirmed by comparison of their retention indices either with those of valid compounds or with data published in the literature (Adams, 2007).

Statistical Analysis

All the data were subjected to Analysis Of Variance (ANOVA) using Statistical Analysis System (SAS) 9.3. Means comparing between the treatments were performed by Duncan's Multiple Range Test (DMRT) at 5% confidence interval.

RESULTS

Plant Growth Parameters

The plant height was significantly different among the applied treatments ($P \leq 0.01$). The results showed that the maximum plant height was related to the plants that received AMF and NPK treatments (13.61 and 13.39 cm, respectively). Inoculation with bio and chemical fertilizers significantly increased the fresh weight of *T. kotschyanus* compared with the control. Fresh shoot weight (24.78 g $plant^{-1}$) was recorded in the plants treated with AFM, which showed almost 68% enhancement in comparison with the control (14.76 g $plant^{-1}$) (Table 3). The results indicated that fertilizers had significant effects on total dry weight per plant as the highest (7.1 g $plant^{-1}$) and lowest (4.24 g $plant^{-1}$) dry matter were shown in the plants treated with AMF and the control, respectively. All the applied treatments, except *P. stutzeri*, had a remarkable effect on the number of branches per plant relative to the control.

Chlorophyll and Carotenoid Content

AMF and NPK fertilizers contributed to an increase in chlorophyll a (0.46 and 0.44 mg g^{-1} fresh weight, respectively), b (0.24 and 0.21 mg g^{-1} fresh weight, respectively) and total chlorophyll (0.7 and 0.65 mg g^{-1} fresh weight, respectively) in the *T. kotschyanus* leaves (Table 2). Also, the results suggested that the use of fertilizer had a positive effect on the production of total carotenoids. The highest amount of carotenoid was obtained from plants treated with AMF (0.4 mg g^{-1} fresh weight) and NPK (0.37 mg g^{-1} fresh weight) representing 100 and 85% increment compared to the control with the value of 0.2 mg g^{-1} fresh weight (Table 3).

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Table 3. Means comparison of morpho-physiological traits of *T. kotschyanus.*[a]

Treatments	Plant height (cm)	Plant Fresh Weight (g)	Plant Dry Weight (g)	Number of branches per plant	Chlorophyll a (mg g^{-1} fresh weight)	Chlorophyll b (mg g^{-1} fresh weight)	Total Chlorophyll (mg g^{-1} fresh weight)	Total carotenoid (mg g^{-1} fresh weight)
A. chroccucum	12.93 b	20.65 b	6.57 c	18.00 ab	0.37 b	0.15 b	0.52 b	0.29 b
P. stutzeri	12.92 b	19.33 c	6.28 d	14.66 c	0.32 cd	0.12 b	0.44 bc	0.22 c
P.putida	12.55 c	17.57 d	5.73 e	17.33 b	0.33 c	0.14 b	0.47 bc	0.27 bc
AMF	13.61 a	24.78 a	7.10 a	18.66 ab	0.46 a	0.24 a	0.70 a	0.40 a
NPK	13.59 a	20.65 b	6.63 b	19.33 a	0.44 ab	0.21 a	0.65 a	0.37 a
Control	12.18 d	14.76 e	4.24 f	14.00 c	028 d	0.12 b	0.40 c	0.20 d

[a] The same letter within each column indicates no significant difference among treatments ($P \leq 0.05$) using Duncan test.

Nutrients Acquisition

The effect of treatments on the mineral concentrations in the *T. kotschyanus* plants is shown in Table 4. All fertilization resulted in increased P concentration relative to the control. According to the obtained results, there were no significant differences in P concentration between plants treated with *P. stutzeri*, *P. putida*, AMF and NPK.

Maximum N amount in *T. kotschyanus* leaf was obtained in plants treated with AMF (14.46 mg g^{-1}), NPK (14.62 mg g^{-1}) and *A. chroccucum* (14.32 mg g^{-1}). Plants inoculated with AMF resulted in higher Ca uptake compared to the other treatments (1.56 mg g^{-1}). Differences in total K accumulation in leaves indicated a positive effect of AMF and NPK application. The best results of total K accumulation in *T. kotschyanus* were obtained in plants inoculated with AMF (7.65 mg g^{-1}) and NPK (7.83 mg g^{-1}).

Proline

The results indicated that fertilization was effective on improving proline content when compared with the control ($P \leq 0.01$). The highest amount of proline was measured in the plants that were treated with AMF (1.99 µmol g FW^{-1}) (Figure 1). There were no significant differences in proline content between NPK, *Azotobacter*, *P. stutzeri* and *P. putida*, and all the mentioned treatments resulted in higher proline relative to the control.

Total Phytochemical Contents and Antioxidant Activity

The effects of fertilizers on total phenolic and flavonoid contents are shown in Figure 2. The TPC increased significantly by using fertilizers. The highest TPC was observed in the plants inoculated with AMF (132.98 mg GAEs g^{-1} DW) compared with control (83.58 mg GAEs g^{-1} DW). Also, TFC increased in

Table 4. Means comparison of nutrients contents of *T. kotschyanu* leaves.[a]

Treatments	P	N	Ca	K
	(mg/g dry weight)			
A. chroccucum	4.13 b	14.32 a	1.13 c	6.65 b
P. stutzeri	5.53 a	12.18 b	0.82 e	5.69 d
P. putida	6.19 a	11.02 bc	0.99 cd	6.09 c
AMF	6.02 a	14.46 a	1.56 a	7.65 a
NPK	6.32 a	14.62 a	1.29 b	7.83 a
Control	3.38 b	9.87 c	0.86 d	5.47 d

[a] The same letter within each column indicates no significant difference among treatments (P≤ 0.05) using Duncan test.

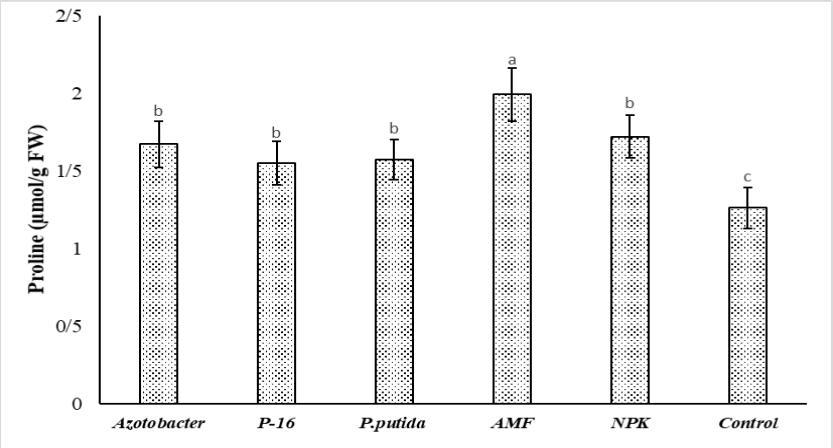

Figure 1. Effect of bio and chemical fertilizers on proline content. The same letter indicates no significant difference among treatments (P≤ 0.05) using Duncan test.

response to AMF treatment (79.7 mg QEs g^{-1} extract) by more than 64% higher than the control treatment. The antioxidant ability of *T. kotschyanus* extracts was evaluated *via* DPPH free radical scavenging assay. AMF led to considerable increase in DPPH scavenging activity compared with the control treatment. The highest DPPH scavenging activity (75.25% inhibition) was related to plants treated with AMF (Figure 3).

Catalase (CAT) and Superoxide Dismutase (SOD) Activities

The activities of CAT and SOD were increased due to the application of mycorrhiza (Figure 4). Maximum CAT (16.19 U mg^{-1} Protein) activity was observed in plants inoculated with AMF compared with other treatments. The SOD activity of mycorrhizal plants was dramatically higher (32.79 U mg^{-1} Protein) compared with other treated plants, but no difference could be found between NPK, *Azotobacter*, *P. stutzeri*, *P. putida* and control (Figure 4).

Essential Oil Percentage and Composition

The highest value of essential oil percentage (2.07%) was observed in the treatment of AMF compared with the control (1.96%). The effect of fertilizers on the main constituents of *T. kotschyanus* essential oil are given in Table 5. In addition, the treatment of NPK recorded the highest percent of thymol (23.36%), borneol (6.27%), eucalyptol (2.98%) and ocymene (10.14%). Moreover, AMF markedly increased the proportion of carvacrol (55.06%) and ocymene (7.41%) (Table 5).

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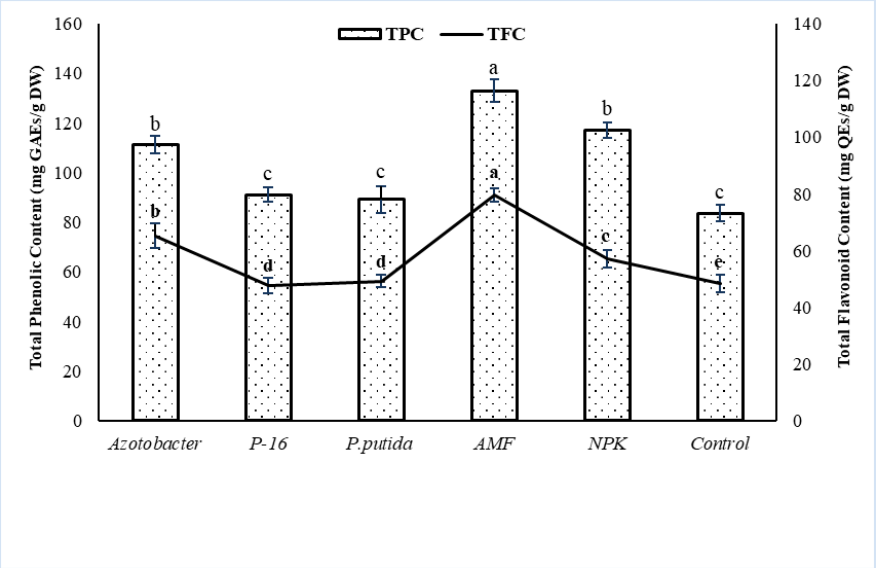

Figure 2. Effect of bio and chemical fertilizers on total phenolic and flavonoid contents. The same letter indicates no significant difference among treatments (P≤ 0.05) using Duncan test.

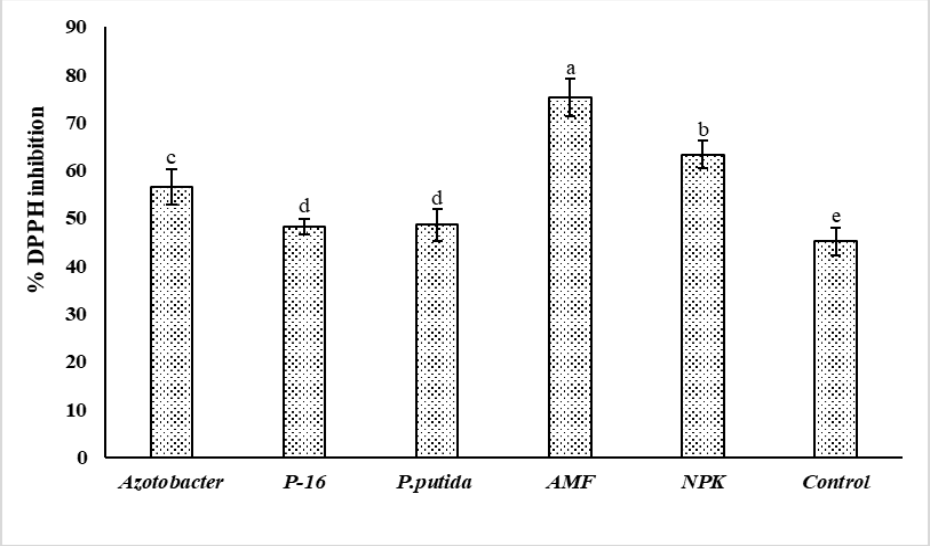

Figure 3. Effect of bio and chemical fertilizers on DPPH inhibition. The same letter indicates no significant difference among treatments (P≤ 0.05) using Duncan test.

Table 5. Means comparison of biochemical traits of *T.kotschyanus.*[a]

Treatments	Essential oil (%)	Thymol	Carvacrol	Borneol	Eucalyptol	Ocymene
Azotobacter	2.01 b	15.15 b	38.88 b	2.48 b	1.66 bc	2.1 d
P. stutzeri	1.98 c	6.29 c	42.06 b	1.23 c	1.85 a	4.34 bc
P. putida	2.01 b	6.330 c	10.53 c	1.63 bc	1.56 bc	5.81 b
AMF	2.07 a	7.75 c	55.06 a	2.486 b	1.62 b	7.41 ab
NPK	2.00 b	23.36 a	12.49 c	6.27 a	2.98 b	10.14 a
Control	1.96 d	5.05 c	11.74 c	1.07 c	0.42 c	2.81 cd

[a] The same letter within each column indicates no significant difference among treatments (P≤ 0.05) using Duncan test.

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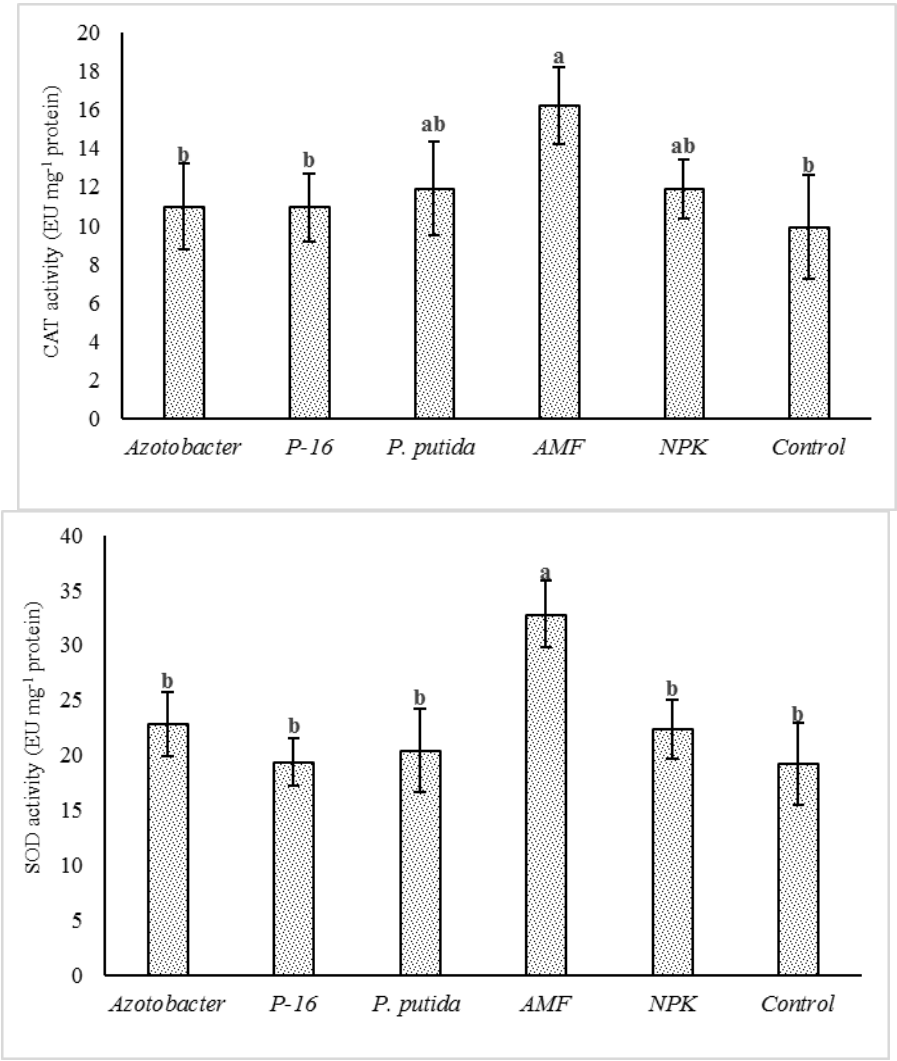

Figure 4. Effect of bio and chemical fertilizers on Catalase (CAT) and Superoxide Dismutase (SOD) activities. The same letter indicates no significant difference among treatments (P≤ 0.05) using Duncan test.

DISCUSSION

The main effect of biological and chemical fertilizers is the enhancement of production, quality and yield of crops (Aggani, 2013). It can be anticipated that the presence of adequate nutrients in chemical fertilizers and quantized release of minerals, during the growing season of plant, as well as useful and positive effects of AMF are important factors that can improve *T. kotschyanus* growth. The positive effects of NPK may be due to the important physiological role of these three macro elements that enhanced growth as a result of increased cell division and growth and maximum biomass conversion into plant growth. The growth promoting role of combined use of N, P and K have been previously reported (Verma *et al*., 2012). Plant nutrition with necessary elements is one of the main factors affecting plant height. Increasing of growth in plants treated with AMF or NPK, could be due to more accessibility of micro and macro elements for plants (Middleton *et al.*, 2015). Improving water and mineral nutrients uptake and enhancing leaf photosynthesis by AMF could be the reasons for increasing growth parameters in AMF inoculated plants (Abdollahi Arpanah *et al.*, 2020). AM are soil-born fungus that can improve plant

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growth and production, including fresh and dry weight and number of branches per plant (Smith and Read, 2008). This productivity stimulation is connected to the fact that AMF support plant nutrition by extending the absorbing network beyond the depletion zones of roots, thereby allowing AMF access to a larger volume of soil to absorb and transport the nutrient more than non-colonized roots (Nakmee *et al*., 2016; Smith and Read, 2008). Generally, the increase in absorption or utilization capacity of nutrients following colonization by AMF depend on the fungal genotype, type of host plant species, and time course for the infection rate (Nakmee *et al*., 2016). The obtained data are in good agreement with those of Azimi *et al.* (2019), Tarraf *et al.* (2017), and Zubek *et al.* (2010).

Fertilization with the AMF and NPK contributed to an increase in chlorophyll content and total carotenoids in *T. kotschyanus* leaf. Our study demonstrated that not only growth was enhanced by mycorrhiza symbiosis, but also AMF improved the synthesis of chlorophylls and carotenoids. By enhancing magnesium and phosphorus uptake, AMF can support a higher chlorophyll concentration and improves the entire performance of mycorrhizal plants (Baslam *et al*., 2013; Zai *et al*., 2012), so, AMF symbiosis improved photosynthetic rate (Haneef *et al*., 2013). In agreement with our results, Ulrichs *et al*. (2008) reported higher fruit pigments such as lycopene and β-carotene content in tomato plants that were inoculated with *Glomus* sp., may be as a result of enhanced photosynthetic performance in host plants. Our finding is in agreement with Baslam *et al*. (2013) and Gheisari *et al*. (2018) in fennel, who found that mycorrhiza can enhance chlorophyll and carotenoid contents in red leaf lettuce and fennel, respectively.

In present research, NPK, AMF and phosphate solubilizing bacteria strains resulted in significant increase in P uptake. Moreover, *Azotobacter*, NPK and AMF enhanced N and K uptake, and maximum Ca in leaf was extracted from plants that received AMF. Microorganisms stimulate the circulation of plant nutrients and reduce the necessity for chemical fertilizers use in agriculture. In a similar study performed by Nell *et al.* (2009), due to the inoculation with AMF, a higher concentration of phosphorus in sage leaf was reported. It has been reported that *Pseudomonas indica* improve N and P assimilation through enhancing the activities of Nitrate Reductase (NR) and phosphate transporters, respectively (Gosal *et al*., 2010). In agreement with our results, Zaidi *et al.* (2003) reported that combined inoculation with Plant Growth Promoting Rhizobacteria (PGPR) and AMF improved the nodulation, nutrient uptake, and grain yield of chickpea. Increase in the absorption of micro and macro elements may be attributed to the high capacity of AMF hyphae to explore more soil volume above the depletion zone, and thus operate P transport from the soil to plant roots (Tarraf *et al*., 2017).

The results illustrated that AMF can play an important role in the synthesis of essential oil by *T. kotschyanus.* Similar results were obtained in *T. vulgaris* (Abdollahi Arpanahi and Feizian, 2019; Habeeb *et al.,* 2020) and some other herbs (Hazzoumi *et al*., 2105; Rydlová *et al*., 2016). As Abdollahi Arpanahi *et al*. (2020), mentioned in their work, improving water and mineral nutrients uptake and enhancing leaf photosynthesis by AMF could be the reasons for increasing essential oil in AMF inoculated plants. Also, they reported more essential oil production in AMF inoculation in *Thymus vulgare* and *Thymus daenensis* plants (Abdollahi Arpanahi *et al.,* 2020).

Nineteen components were identified in the *T. kotschyanus* essential oil. The major components were thymol, carvacrol, borneol, eucalyptol and ocymene. Other components were present at quantities less than 2 %. It has been revealed that AMF inoculation significantly improved the concentration of thymol in *T. daenensis* plants in comparison with the control plants (Bahadori *et al*., 2013; Abdollahi Arpanahi and Feizian, 2019). Other studies have

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shown that inoculation with microorganisms leads to a change in the composition of the EO in different plant species (Sabzi-Mehrabad *et al.*, 2018). It is reported that total EO content, especially concentrations of geranial and geraniol, increased in *Dracocephalum moldavica* following inoculation with AMF (Ghanbarzadeh *et al.*, 2019). Moreover, mycorrhizal association in *Mentha viridis* resulted in relatively high levels of limonene, 1,8 cineolecarvone, eugenol and (e)-methyl cinnamate (Karagiannidis *et al*., 2011). *Salvia officinalis* seedlings inoculated with AMF exhibited changes in the composition of EO with elevated levels of bornyl acetate, 1,8-cineole, α-thujones and βthujones (Geneva *et al*., 2010)

Aerial part of *T. kotschyanus*, is a natural source of antioxidants (Boroomand *et al*., 2018). Evaluation of total phenols, flavonoids and carotenoids is a valuable approach to display the capacity of the antioxidant defense system for scavenging the damaging active oxygen species (Tohidi *et al*., 2017). AMF symbioses promote the accumulation of important secondary metabolites in plants and can raise their antioxidant potential. Our results demonstrated that AMF application resulted in a significant increase in total flavonoids and phenolic compounds in vegetative parts of *T. kotschyanus*. The obtained data are in good agreement with those of Zayova *et al.* (2018), who reported that AM associations with *T. vulgaris* increase the accumulation of antioxidant metabolites such as phenols and flavonoids and enhance the activity of antioxidant enzymes. Higher flavonoids content in *Artemisia annua* L. plants inoculated with Azotobacter under *in vitro* condition was found by Arora *et al*. (2018).

Various enzymes including; Super Oxide Dismutase (SOD) and Catalase (CAT) were the key enzymes in the antioxidative defense system. Different AMF had various effects on antioxidative enzyme activities (Gao *et al.*, 2008). It has been reported that plants treated with bacteria and fungi demonstrated high antioxidant enzymes activity, which contributed to enhance plant protection against stress (Baniaghil *et al.*, 2013). These findings imply that AMF symbiosis could reduce the accumulation of ROS and decrease the damage of oxidative stress by a variety of antioxidant compounds in different ways. It is suggested that increased SOD activity in AMF inoculated plants was directly associated with enhanced plant production and abiotic stress resistance (Gao *et al*., 2008). Several studies have demonstrated that AMF symbiosis can serve to protect the host plants against oxidative damage during environmental stress (Abdel Latef and Chaoxing, 2011; Borde *et al.*, 2011; Estrada *et al.*, 2013; Chang *et al.*, 2018). Higher antioxidant enzyme activity in plants inoculated with mycorrhiza, helps to rapidly and efficiently remove excess ROS (Chang *et al.*, 2018). SOD plays a serious role in protecting membrane consistency in plant cells and catalyzes the reduction of free O_2^- to O_2 and H_2O_2 (Estrada *et al.*, 2013). Our results illustrated that AMF symbiosis significantly influenced the SOD activity in leaves. Similar results were also found in *Elaeagnus angustifolia* L. (Chang *et al.*, 2018) and *Sesbania sesban* (Abd Allah *et al.*, 2015).

CONCLUSIONS

The results indicated that applied biofertilizers, especially AMF, had positive effects on the productivity and biochemical characteristics of *T. kotschyanus*. This effect was probably due to enhanced uptake of sufficient water and soil nutrients mediated by AMF. Avoiding or minimizing the use of chemical fertilizers in the medicinal plants production and their products is of great importance in food, drugs, and cosmetic industries. In general, it seems that inoculation with symbiotic fungi, due to the reduction of the chemicals use and decreases in the possibility of deterioration the environment, could be advantageous to human health. In addition, further researches

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are still needed to reveal the interactions between AMF, chemical fertilizers, and other soil microorganism in larger scales and under more complex environmental conditions.

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ارزیابی خصوصیات مورفولوژیکی، فیزیولوژیکی و بیوشیمیایی آویشن کوهی (*Thymus kotschyanus* Bioss. & Hohen) تحت تاثیر کودهای مختلف زیستی و شیمیایی

ب. اصغری، س. مفاخری، و ف. رجالی

چکیده

کود زیستی به عنوان جایگزینی برای کود شیمیایی جهت بهبود حاصلخیزی خاک و تولید محصولات سالم در کشاورزی پایدار شناخته شده است. هدف از این مطالعه مزرعه‌ای که طی سال زراعی 1397-98 در مزرعه تحقیقاتی دانشگاه بین المللی امام خمینی (ره) ، قزوین، انجام شد، ارزیابی اثرات کودهای زیستی و شیمیایی بر ویژگی های کمی و کیفی آویشن کوهی بود. تیمارهای اعمال شده شامل شاهد (بدون کود) ، کود شیمیایی (NPK) و چهار نوع میکروارگانیسم شامل *Azotobacter chroococcum*, *Funneliformis mosseae* (AMF) *Pseudomonas stutzeri*, *Pseudomonas putida* و بود. نتایج نشان داد که AMF و NPKدر مقایسه با سایر تیمارها بر ارتفاع بوته، تعداد شاخه در بوته و محتوای رنگدانه های فتوسنتز تأثیر مثبت داشتند. با این حال، حداکثر مقادیر وزن تر و خشک گیاه، پرولین، محتوای فنلی کل و فلاونوئید، DPPH، درصد اسانس و مقدار کارواکرول از گیاهان تلقیح شده با AMF بدست آمد. همچنین، فعالیت‌های کاتالاز (CAT) و سوپراکسید دیسموتاز (SOD) به دلیل استفاده از AMF افزایش یافت. با توجه به نتایج بدست آمده اختلاف معنی داری بین گیاهان تیمار شده با *P. stutzeri* ،

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P. putida، AMF و NPK در غلظت P وجود نداشت. بیشترین مقدار N در برگ *T. kotschyanus* در گیاهان تیمار شده با AMF ، NPK و *A. chroccucum* بدست آمد. گیاهان تلقیح شده با AMF جذب بالاتر کلسیم را در مقایسه با سایر تیمارها نشان دادند و حداکثر تجمع کل K در *T. kotschyanus* در گیاهان تلقیح شده با AMF و NPK به دست آمد. از این رو استفاده از نهاده‌های آلی و زیستی به جای کود شیمیایی برای بهبود بهره وری و کیفیت محصول با هدف کاهش آلودگی و انجام کشاورزی پایدار، ممکن و بسیار امیدبخش است.

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