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Original Article

Evaluation of Quantitative Yield of Two Thyme Species Affected as Different Levels of Drought Stress and the Manure Application

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ABSTRACT

The present study aimed to study the effects of drought stress and manure on some physiological characteristics and quantitative yield of two species of the *Thymus*, *Thymus daenensis* Celak and *Thymus vulgaris* L. For this purpose, a factorial layout based on a randomized complete block design with three replications was done at the Agricultural Research Field of University of Birjand, Iran, during two cropping years 2015 and 2016. Experimental factors included water levels (100, 67, and 33% of field capacity), two species of thyme (*T. daenensis* Celak *and T. vulgaris* L.) and manure (Cow manure) (non-application and application of 30 ton/ha). Drought stress reduced plant height, number of lateral branches, number of flowers, relative water content, membrane stability index, and quantitative yield while induced an increase in the amount of ion leakage, proline, phenol, and malondialdehyde compounds. Manure significantly increased the quantitative yield. Further, the amount of ion leakage was lower in the cells of *T. daenensis* indicating the maintenance of the integrity of cell membranes in the face of water stress, which can result in an enhancement in the efficiency of plant production and, ultimately, an increase in yield crop. In addition, *T. daenensis* had higher water use efficiency indicating lower vulnerability of this species. Results differentiated two species and *T. daenensis* can be introduced as superior species in confronting with drought stress.

INTRODUCTION

Environmental stresses are the most important factors limiting the growth and production of crops in the world [1]. Among the environmental stresses, drought or water shortage is considered as the strongest and most sophisticated reducing factor for crop and food security threatening which is solely responsible for 45% of the reduction in crop yield in different parts of the globe [2]. Drought stress also reduces the growth of aromatic and medicinal plants, and plants synthesize secondary metabolites due to the adaptation in response to environmental stresses [3]. The new approach for the growing use of medicinal plants in the world makes these plants more important and currently, the demand for medicinal herbs as products used in the pharmaceutical, health and food industries is on the rise [4]. The Lamiaceae family is the largest plant family in pharmaceutical studies and their medicinal value due to their essences [3]. The genus of *Thymus* belongs to the Lamiaceae family and is a perennial plant [5]. This medicinal species can be considered as a good choice for planting and mass production due to the high yield of the essential oil and valuable phenolic compounds [6]. The quality of medicinal plants is largely determined by the relevant natural compounds (secondary metabolites), and the amount and quality of these compounds are determined by environmental factors such as water availability [7].

In general, water shortage in plant tissues declines the growth of different parts of the plant like the roots and aerial tissues of the plant [8]. In this regard, Sharafzadeh and Zare [3] reported that drought stress reduces dry weight and height of *T. vulgaris*. Drought

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stress influences plant growth by creating morphological changes in plant structure and affecting multiple physiological processes such as cell membrane permeability and stability [2]. Due to the cell membrane damage, its permeability is increased and the cell electrolyte leakage leads to the plant wilting [9]. In general, drought stress increases the amount of membrane lipid peroxidation including malondialdehyde (MDA) which is used as a marker to determine the amount of oxidative damage to lipids and the membrane damage [10]. Further, in conditions of environmental stresses, the production of some essential oil compounds and secondary metabolites like phenolic compounds is increased [11]. Among the secondary metabolites, we it can be refered to the phenolic compounds. Phenolic compounds act as purified reactive oxygen species and consequently, stabilize cell membranes and inhibit lipid peroxidation[12]. Among the organic osmolytes, proline is probably the most abundant and common compatible dissolved material which is accumulated [13]. According to Suriyan and Chalermpol [14], the storage of proline in plant cells is associated with the drought tolerance mechanisms. Misra and Srivastava [15] reported that drought stress increases proline content in *T. daenensis*.

Drought stress is related to the plant nutrition, and a reduction in availability and various nutrients uptake for the plant is considered as one of the most important negative effects of drought stress [16]. The use of natural fertilizers with biological origin has great importance in maintaining the structure, biological activity, exchange and water holding capacity and finally, in modifying the physical and chemical structure of soil [17]. Manure often contains micro and macro nutritional elements for vital activities of the plant. In addition, it can improve soil texture, increase water absorption capacity and provide a suitable environment for the spread of root [18]. Therefore, the assessment of various systems of plant nutrition is one of the important needs of agricultural planning in order to achieve high yields with high quality, especially in medicinal plants. Sanchez-Rodrguez *et al* [19] reported that in drought stress conditions, the moisture needed for the plant is reduced, manure, by keeping moisture in itself, is provided for the plant and reduces the negative effects of drought stress. Based on the experiment of investigating the effect of manure on drought stress, it was reported that manure improves the effects of drought stress and increases the dry matter yield of *Salvia officinalis* plant, compared to the drought stress conditions without using manure and due to the positive effects of manure on improving the physical and chemical properties of soil (increasing water holding capacity) and improving nutritional conditions of the plant, which improves the fresh and dry weight of the plant [20]. Given that the large parts of Iran’s lands are situated in the arid and semi-arid regions and the undeniable role of drought stress on the growth, qualitative and quantitative yield of plants, especially medicinal plants, and the importance of the medicinal plant of thyme, evaluating the effect of drought stress on qualitative and quantitative changes in medicinal plant species of thyme is important. Therefore, the present study aimed to investigate the effect of drought stress and manure on some morphological, physiological characteristics, and quantitative yield of two species of *T. daenensis* and *T. vulgaris*.

MATERIAL AND METHODS

Experimental sites

The experiment was done in two cropping years (2015 and 2016) as a factorial experiment based on a randomized complete block design (RCBD) with three replications at the Agricultural Research Field of University of Birjand with the latitude of 32° and 53 mins North and longitude of 59°and 13 mins East and with the height of 1480 meters above the sea level. Experimental factors were included: three water levels (100% of field capacity as a control, 67 and 33% of field capacity), two manure (Cow manure) levels (no manure (as control) and consuming 30 ton/ha) and two *Thymus* species, *T. daenensis* and *T. vulgaris*. Each block consisted of 12 plots including 4 lines with a length of 4 m and 0.5m distance between rows and 0.4 m line distance. Before preparing the seed bed, sampling was done from 0-30 cm depth based on the soil of testing location for determining the physical and chemical properties (Table 1).

Seedlings with a length of 10-12 cm prepared by Pakan Seed Institute of Isfahan were planted in May 2015. Irrigation was done immediately after transplanting each plot and the second irrigation was done three days later. The replanting of seedlings was done a week after cultivation of seedlings which was dried. Weed management was done manually during the growing season. After the establishment of the plant, the application of drought stress began and for this purpose, the drip irrigation system was established in the farm and a drop-dropper was considered for each plant in each plot. Incoming water to the plots was controlled by embedded taps. In addition, the irrigation water volume for each of the treatments was controlled by a volumetric counter. The times of irrigation was performed with a sampling of 0–30 cm soil depth by auger and determine soil water content as well as using Class A pan evaporation [21].

Method of calculating the volume of water required for stress levels:

The volume of water required for stress levels was measured by measuring the length and width of plot, depth of plant root in the soil, field capacity (FC), and soil specific (Db).

Method of calculating the time of irrigation by soil moisture:

The times of irrigation were determined with a sampling of 0-30 cm soil depth by auger and determine soil moisture as well as using Class A pan evaporation. Table 2 indicates temperature changes during the experiment, In terms of, the average maximum temperature in June and minimum temperature in February.

Table1 Physical and Chemical analysis of the field soil (0-30 cm depth).

Depth	Clay%	Silt%	Sand%	Texture	Db	P (mg/kg)	K (mg/kg)	N%	pH	EC dS/m	Organic matter%	FC%	SP%
0-30	12	38	50	Loam	1.5	12	250	0.03	8.16	5.2	0.29	17	32

Table 2 The maximum and minimum air temperature (°C), evapotranspiration (ET_o) and precipitation (mm) of years of 2015 and 2016.

Months of the year	Minimum temperature °C	Maximum temperature °C	Precipitation (mm)	ET_o (mm)	Time
2015					
Jan	-1.19	14.43	0.73	0	
Feb	-2.03	14.12	0.9	0	
Mar	5.7	20.75	1.04	5.10	
Apr	10.74	23.25	2.12	7.99	
May	14.53	30.1	0.12	10.82	
Jun	16.70	34.64	0.01	14.60	Planting time
Jul	36.04	20.94	0	16.59	
Aug	17.20	33.32	0	15.02	
Sep	13.29	31.58	0	10.87	
Oct	10.37	28.87	0.20	8.16	
Nov	5.83	20.99	0.37	5	
Dec	-1.7	14.39	0.2	3.24	
2016					
Apr	9.22	22.55	0.59	7.026	
May	14.17	31.04	0.15	10.82	
Jun	17.64	35.33	1	14.36	Harvest time
Jul	19.70	35.32	0	16.90	
Aug	17.89	34.05	0	20.9	
Sep	14.8	33.74	0	12.47	

$W\% = \mathrm{W1} - \mathrm{W2}/W2$

W1 = Moisture soil weight (determined by 0-30 cm soil depth)

W2 = Dry soil weight (dried soil in the oven after 24 hours)

Measuring the Studied Characteristics

Vegetative characteristics (plant height, number of lateral branches and number of flowers per plant) and yield

In the sampling stage (the full flowering stage in October month of each year), 3 plants of cultivation lines were harvested with respect to the elimination of marginal effects. After counting the number of lateral shoots and flowers per plant, and measuring plant height, the samples were placed in the individual bags and after drying the samples in an oven at 72 °C for 48 hours, the total dry weight of each sample was measured and finally the average of them selected as Total Dry Matter (TDM).

Relative Water Content

0.1 g from each plant of leaves was measured using a digital scale (FW). Then, each sample was placed inside Petri dish and adding 10 ml of distilled water and was placed in a dark place for 24 hours to be completely saturated. In the following stage, the saturated weight (SW) of the samples was measured after removing from the distilled water and drying. In the next step, samples were dried in an oven at 75 °C for 24 hours and were weighed (DW). Leaf relative water content was calculated from the following equation [22].

$$RWC = \frac{(FW-DW)}{(SW-DW)} \times 100$$

Total Phenol Content

The amount of 2.5 g of plant leaves with 50 ml methanol of 80% was situated on a shaker for 24 hours. The extract was passed through filter paper to obtain a clear solution. The total amount of phenolic compounds was measured by the Folin-Ciocalteu reagent [23]. In this method, 0.5 ml of the extract solution was mixed with 2.5 ml of 10% Folin-Ciocalteu reagent and 2 ml sodium carbonate solution (20%). After shaking, test tubes were put in a water bath at 40 °C for 30 minutes and then, the solution absorbance was read at a wavelength of 760 nm. Gallic acid was used for drawing the standard curve. The total amount of the available phenolic compounds in the extracts was calculated using the equation obtained from the standard curve and results were expressed in mg/g fresh weight of leaf.

The Percentage of Ionic Leakage

The method of Hu *et al* [24] was used for measuring ion leakage. For this purpose, 0.2 g of fresh aerial parts of the plant was put into a test tube with a lid and 10 ml of the distilled water was added to, after washing the potential ions from the surface of the plant. Then, the test tubes were put in a hot water bath at a temperature of 32 °C for 2 hours and the electrical conductivity of the sample (EC_1) was measured using an EC meter device. Then, test tubes were autoclaved for 20 minutes and after cooling pipes to a temperature of 25 °C, the maximum electrical conductivity of samples (EC_2) was measured and the percentage of ion leakage was obtained from the following formula:

$$\frac{\mathrm{Ec1}}{\mathrm{Ec2}} \times 100$$

Membrane Stability Index (MSI)

PEG (polyethylene glycol) was used to measure membrane stability [25]. In this method, leaf samples were separated and washed with distilled water. Then, control samples were placed in 30 ml of distilled water and treated samples in 30 ml of 30% PEG with 6000 molecular weight (MW). The samples were situated at 10 °C for 24 hours and later all samples were washed with distilled water and in pipes of each sample 30 ml of distilled water was poured and they were placed at 10 °C for 24 hours and then, the electrical conductivity of the samples was measured by EC meter device. Then samples were autoclaved for 25 minutes at a temperature of 125 °C and the electrical conductivity was measured again. The percentage of damage to the membrane was calculated using the following formula [26]:

$$\text{Percentage of damage} = \frac{1-T1/T2}{1-C1/C2} \times 100$$

Percentage of damage = stability % - 1

T_1 and T_2 are EC of the treated sample before and after the autoclave respectively and C_1 and C_2 refer to the EC of the control sample before and after the autoclave, respectively.

Malondialdehyde (MDA)

At first, 0.2 g of fresh leaf tissue was weighed and was rubbed in a porcelain mortar containing 5 ml of 0.1% trichloroacetic acid (TCA). The obtained extract was centrifuged at 10000 rpm for 5 minutes and 4 ml of 40% TCA solution containing 0.5% of thiobarbituric acid (TBA) was added to the 1 ml of the supernatant solution obtained by centrifugation and the mixture was heated for 30 minutes at 95 °C water bath. Then, the mixture was immediately cooled in ice and was centrifuged for 10 min at 10000. The absorption intensity of this solution was done by the spectrometer at a wavelength of 532 nm [27]. The measurement results were calculated in micromoles per gram of dry weight.

Proline

0.5 g fresh leaves of the plant were rubbed with 10 ml of 3% solution of sulfosalicylic acid. 2 ml of obtained homogeneous mixture after the filtration was poured in a test tube and then, 2 ml of Ninhydrin acid reagent and 2 ml of pure acetic acid were added to each sample. The resulting solution was placed for one hour in a hot water bath at a temperature of 100 °C and then, the samples were put in an ice water bath to reach the ambient temperature and after that, 4 ml of toluene was added to each sample and was vigorously stirred for 30 seconds and the supernatant solution was used to measure proline. The absorbance value was read at a wavelength of 520 nm and toluene was used as control. Then, the amount of the substance was calculated and announced in milligrams per gram of fresh weight by using the standard curve of proline [28].

Water use Efficiency (kg/m^3)

The efficiency of water use in different experimental treatments was determined by calculating the produced total dry matter (kg/ha) to the volume of the used water (m^3/ha): WUE = DM / WU (4)

In this study, DM is the produced dry matter of plants and WU refers to the amount of water used in control and stress treatments.

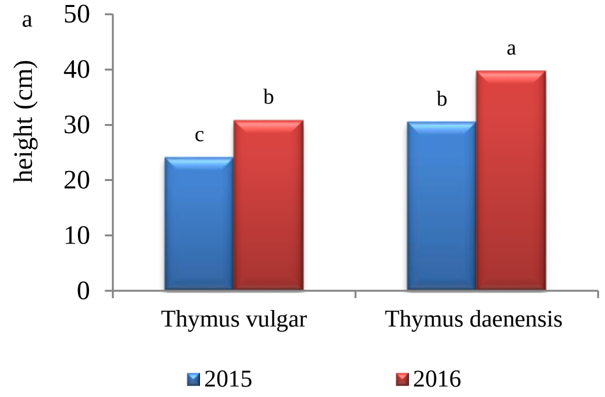
Data Analyses

Finally, the measured characteristics were statistically analyzed and the combined analysis of test years was conducted. The software SAS (V9.1) and Excel were used to analyze the data and draw the figures. Means were compared using the LSD test at 5% probability level.

RESULTS AND DISCUSSION

Plant Height

The combined analysis results of two cropping years indicated that the main effects of year, drought stress, species and manure were significant at 1%, ($P< 0.01$) and among the interaction effects, the triple interaction of drought stress on species with manure and the interaction of year in specie were significant at the 5% probability level (Table 3). The combined mean comparison of year in species showed that the most plant height was obtained in the second year of the experiment from *T. daenensis* and the minimum plant height in the first year from *T. vulgaris* (Fig. 1a). Further, the enhancement of plant height in the second cropping year compared to the first year was 1.3 times (Fig. 1a). Further, the combined mean comparison of the effect of drought stress on the species with manure indicated that the maximum plant height belonged to the *T. daenensis* under irrigation condition at 100% of field capacity and using 30 t/h of manure, though no statistically significant difference was observed between the height of this species in treatments with irrigation at 67 and 33% of field capacity with applying 30 ton/ha of manure and treatments with irrigation at 100% of field capacity and the non-application of manure (Fig. 1b). In contrast, *T. vulgaris* in treatments with irrigation at 33% of field capacity and the non-application of manure had the lowest plant height. In addition, there was no significant difference between the height of this species in treatments with irrigation at 33% of field capacity and using 30 ton/ha manure and the treatment of 67% of field capacity without using manure (Fig. 1b). Drought stress prevents the development of cells, their growth and plant height enhancement. In fact, drought stress disrupts the regulation of genes involved in the development of cell walls and in this way reduces growth [29]. Alavi-Samani *et al* [30] and Sharafzadeh and Zare [3] reported that drought stress reduces dry weight and plant height of *T. vulgaris* and *T. daenensis*. Plant height like other growing processes is affected by water and available adequate water for increasing plant height is essential, manure with increasing soil pores, root growth and improves water and nutrient absorption help to improve the plants height [31]. The positive effects of biofertilizers on the growth of medicinal herbs like *T. vulgaris* and *T. daenensis* [32] and *Ocimum basilicum* [33] have been reported. Further, in this test, there was a positive and significant correlation between plant height and quantitative yield ($r= 0.81^{**}$, N= 24). Therefore, it can be concluded that drought stress with the significant reduction of plant height leads to a decrease in quantitative yield.



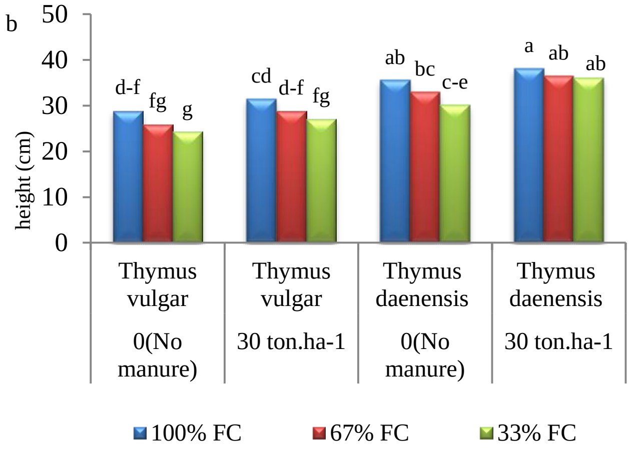


Fig. 1 a- The interaction of water stress levels in the tested species in manure on plant height (cm). b- The interaction of year in the species on plant height (cm). Differences of the columns that have the same alphabets one not statistically significant at 5% (LSD) level of significance.

The number of lateral branches and flowers per plant

The results from the combined analysis indicated the significant effect of year, drought stress, species, manure, and interactions of species in manure and year in manure at a probability level of 1% on mentioned traits (Table 3). The main effect of drought stress showed that by reducing the moisture rate of the soil from 100 to 33% of field capacity, the number of branches and the number of flowers per plant reduced 9.5 and 8.2% respectively (Table 4). Shubhra *et al* [34] in their study on pot *Calendula officinalis* founded that the number of branches and the number of flowers per plant severely reduced under drought stress conditions. In addition, the interaction effect of year on species showed that the largest number of lateral branches and flowers per plant was obtained in the second cropping year from *T. daenensis* (Table 4). The impact of the year on the manure also indicated that the largest number of branches and flowers per plant was in the second cropping year by using 30 tons/ha of animal manure (Cow manure) (Table 4). The number of branches per plant in the second cropping year of the experiment (Mean=70.8) was 1.15 times of the first year (Mean=61.27) and the number of flowers per plant in the second cropping year (Mean=57) was 1.2 times of the first cropping year (Mean=47.47) (Table 4). The results of other studies demonstrated that the application of manure increases the number of lateral branches in the medical herb of *Nigella sativa* [35], *Melissa officinalis* and *Origanum majorana* [18]. The results of the present study showed a significant and positive correlation between quantitative yield and the number of lateral branches ($r=0.92^{**}$, N= 24) and the number of flowers per plant ($r=0.91^{**}$, N= 24). Drought stress led to a reduction in the number of lateral branches and the number of flowers per plant and could be one of the reasons leading to a reduction in the quantitative yield.

Relative Water Content

The results showed that drought stress had a significant effect on the relative water content of the plant (Table 3) as the relative water content had a 99% reduction in the treatment of 33% of field capacity, compared to the control plant (100% of FC) (Table 7). Sanchez-Rodrguez *et al* [19] reported that relative water content of leaf indicates water supply balance for leaves and transpiration rate better than other indicators. Therefore, it can be a good indicator to show the aqueous status of the leaf and the damages caused by the drought stress. The reduction of the relative water content under drought stress in *O. basilicum* is frequently reported [3].

The application of manure increased the relative water content (Table 4). Manure increases soil pores and enhances the growth and further development of plant roots in the soil and improves water and nutrient absorption [31]. Further, the results showed a significant difference between the two species tested and two cropping years and the highest relative water content was obtained from *T. daenensis* in the second cropping year (Table 4). Increased relative water content in the second cropping year (Mean= 76.99) was 1.1 times of the first cropping year (Mean= 67.73). In this experiment, the highest correlation was between relative water content and quantitative yield which was positive and significant ($r = 0.94^{**}$, N = 24). Queensberry and Reitz [36] reported a high correlation between the relative water content of leaves and yield. Thus, the species which can conserve water in their tissues have more stress tolerance and yield. In this experiment, *T. daenensis* had higher relative water content and quantitative yield.

The Content of Phenol (phenolic substances)

The variance analysis results of the content of phenol showed that the main effects of year, moisture stress, species, manure, and interaction effects of a year in species and year in manure at a probability level of 1% ($P < 0.01$) on this feature was significant (Table 3). The mean comparison of year in species showed that the highest content of phenol in both cropping years was obtained from *T. daenensis*. Further, no significant difference was observed between two cropping years in *T. daenensis*. However, the lowest content of phenol was obtained from *T. vulgaris* in the first cropping year that had a significant difference with the second cropping year (Table 4). Further, there was a significant difference between the levels of drought stress as the phenol content increased by reducing the moisture levels (Table 7). Among the secondary metabolites, we can refer to the phenolic compounds. These compounds can prevent oxidative stress and accumulate free radicals due to their antioxidant role directly by engaging in revival reactions and indirectly by chelating iron because they act as a strong electron donor and proton donor group [11]. In this experiment, a significant and negative correlation was observed between the phenol and quantitative yield ($r= -0.74^{**}$, N= 24).

In addition, the mean comparison of the interaction of year in manure showed a significant difference between manure levels and two cropping years and the phenol content decreased in both cropping years by using manure, and the highest content of phenol was obtained from the second cropping year and in the condition of non-application of manure (Table 4) because manure contains the elements necessary for vital activity of the plant [37] which increases water absorption capacity, and provides a suitable environment for the spread of roots [18].

The Percentage of Ionic Leakage

The result showed the significant effects of year, drought stress, specie and manure at a probability level of 1% and the interaction of drought stress in species, drought stress in manure, drought stress in species in manure at a probability level of 5% and year in specie at a probability level of 1% and the triple interaction of year in drought stress in specie at a probability level of 5% (Table 6). The mean comparison of combined analysis of the year in drought stress in species demonstrated that by reducing moisture of control (100% of FC) to drought stress level (33% FC), the ionic leakage of cell membranes increased in both tested species and in both cropping years. However, the enhancement in species of *T. vulgaris* in the first cropping year was more than the *T. daenensis* as the highest amount of ionic leakage from 33% of field capacity was obtained from *T. vulgaris* in the first cropping year and the lowest amount of ionic leakage from the conditions of 100% of field capacity was obtained from *T. daenensis* in the second cropping year which statistically had no significant difference with the treatment of 67% of field capacity of *T. daenensis* in the second cropping year (Fig. 2a). In the second cropping year, a reduction happened in the amount of ionic leakage from the cell membrane while still, *T. vulgaris* had ionic leakage more than *T. daenensis* (Fig. 2a). The ionic leakage reduction in the second year could be due to the full establishment of plant and more development of root system in the second cropping year, compared to the first year which increases plant tolerance and reduces the effects of drought stress and consequently, increases strength and cohesion of cell membrane and reduces the rupture of the membrane and ion leakage. Saneoka *et al* [26] reported that by reducing the amount of irrigation water, water stress exerted on the plant increased and the cells are severely damaged and the cell's ability to control entry and exit of materials from the cells' membrane decreased therefore electrolytic conductivity of solution containing plant tissue increased. Generally, by decreasing the soil moisture rate, the ionic leakage of the leaf increased. Therefore, it can be used as an indicator for measuring the resistance to employed drought stress. In this experiment, the amount of ionic leakage is decreased by using manure (Fig. 2b). Accordingly, the application of manure improves the physical characteristics of soil and develops plant roots largely [18], and thereby, it reduces the damage caused by drought stress on the cytoplasmic membrane. Further, based on the results, an increase in ionic leakage of membrane increased the amount of damage to the cells which will eventually leads to a reduction in plant yield. The results of this experiment showed a significant and negative correlation between ionic leakage and quantitative yield (r= -0.80 **, N= 24). Since the ionic leakage of cell membrane increased under drought stress conditions, it can be concluded that the enhancement of ionic leakage of the membrane increases the amount of damage to the cells, which finally leads to a reduction in plant yield.

Membrane Stability

Although the main effects of year, drought stress, manure, species and the interaction of year in the drought stress were significant on the membrane stability at a probability level of 1%, the interaction of year in drought stress in the species was not significant (Table 6). The mean comparison of year in water stress showed that by reducing the amount of soil moisture from control treatment to 33% of field capacity, the membrane stability decreased in both cropping years so that the highest membrane stability was obtained from the treatment of 100% of field capacity in the second cropping year and the lowest amount was from the treatment of 33% of field capacity in the first year (Fig. 3). The enhancement of membrane stability in the second cropping year was 1.06 times of the first cropping year (Fig. 3). Ahmadizadeh *et al* [38] reported that the cell membrane stability under stress conditions can be considered as the main cause of drought resistance. Gunes *et al* [22] reported that the cell membrane stability is decreased under drought stress.

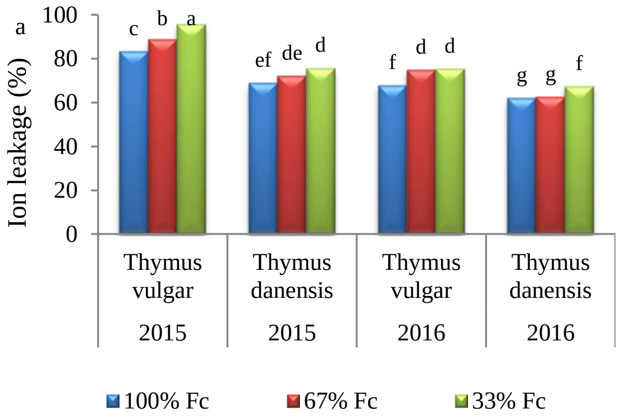
Table 3 values of mean squares in the analysis of variance of the Traits in this study.

Source	df	Height	Branches Number	Flower Number	RWC	Phenol Content
Replication	2	0.3 ns	91.58 **	91.58 **	22.22 ns	57.1*
Year	1	1209.09 **	1632.13 **	1632.13 **	1544.5 **	729.62**
Year × Replication	4	0.0051	0.5	0.5	56.91	49.51
Water stress	2	82.50 **	261.6 **	123.58 **	348.75 **	958.76 **
Species	1	983.53 **	61616.44 **	61616.44 **	10902.81 **	6555.26 **
Manure	1	236.20 **	783 **	783 **	174.46 **	1039.47 **
Water stress × Species	2	4.67 ns	9.98 ns	9.98 ns	1.83 ns	29.34 ns
Water stress × Manure	2	13.7 *	0.67 ns	0.67 ns	20.7 ns	3.7 ns
Species × Manure	1	13.79 ns	178.25 **	178.25 **	23.06 ns	2.57 ns
Water stress × Species × Manure	2	12.61*	0.04 ns	0.04 ns	2.96 ns	9.65 ns
Year × Water stress	2	1.4 ns	0.52 ns	0.52 ns	0.7 ns	16.48 ns
Year × Species	1	16.73 *	712.35 **	712.35 **	487.57 **	727.07 **
Year × Manure	1	4.01 ns	30.37*	30.37*	2.41 ns	124.82 *
Year × Water stress × Species	2	0.079 ns	1.38 ns	1.38 ns	9.5 ns	33.84 ns
Year × Water stress × Manure	2	0.2332 ns	0.13 ns	0.13 ns	2.02 ns	9.78 ns
Year × Species × Manure	1	0.2346 ns	15.04 ns	15.04 ns	23.76 ns	1.74 ns
Year × Water stress × Species × Manure	2	0.21 ns	0.22 ns	0.22 ns	3.58 ns	3.04 ns
Error	44	3.58	6.1	6.1	13.92	17.68
CV%	-	6.02	3.74	4.72	5.15	7.63

*and **: Significant at the 5% and 1% probability levels, respectively., ns: Non- Significant.

Table 4 The interaction of year in the Species and interaction of year in the manure on number of branches, number of flowers per plant, relative water content (RWC%) and the total phenol (Mg/gDw).

Interaction Year × Species					
Year	Species	Number of Branch	Number of Flower	RWC%	Total phenol (Mg.g/Dw)
2015	*T. vulgar*	35.17 c	21.37 c	58.03 c	39.18 c
2015	*T. daenensis*	87.39 b	73.59 b	77.43 b	64.62 a
2016	*T. vulgar*	38.4 c	24.61 c	62.08 c	51.9 b
2016	*T. daenensis*	103.2 a	89.4 a	91.9 a	64.63 a
Interaction Year × Manure					
Year	Manure	Branch number	Flower number	RWC%	Total phenol (Mg.g/Dw)
2015	No manure	57.33 c	43.53 c	66.36 c	54.38 b
2015	30 ton.ha	65.22 b	51.42 b	69.1 b	49.42 c
2016	No manure	68.15 b	54.35 ab	75.25 ab	63.38 a
2016	30 ton/ha	73.45 a	59.65 a	78.73a	53.15 b



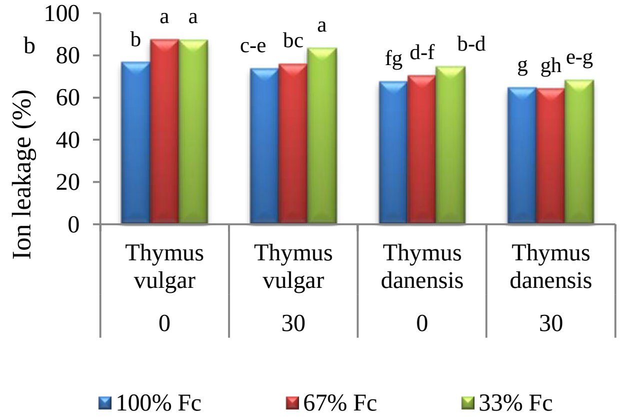


Fig. 2 a- The interaction of year in species in the water stress on the ions leakage (%). b- Effect of interaction water stress in species in manure on the ions leakage (%).Differences of the columns that have the same alphabets one not statistically significant at 5% (LSD) level of significance.

Genotypes with higher tolerance to environmental stresses have less destruction of the cytoplasmic membrane [39]. In general, taking advantage of determining electrolyte leakage and calculating membrane stability index is one of the most widely used markers to estimate the effect of the membrane destruction processes in plant tissues influenced by adverse environmental factors [40]. In addition, the main effect of species showed a statistical difference between the two tested species and the membrane stability of *T. daenensis* was more than *T. vulgaris* (Table 7). The main effect of manure indicated that applying manure increases membrane stability (Table 7). Applying manure in stress conditions provides the required water for plants and prevents materials' accumulation and thereby, prevents cell membrane's destruction. In this experiment, by increasing manure application, the cell's electrolyte leakage decreased and thereby, cell membrane stability increased by applying manure. The results showed a positive and significant correlation between membrane stability and quantitative yield ($r=0.85^{**}$, N=24). Generally, the reaction of cytoplasmic membrane to the different environmental factors and conditions such as drought changed and plant growth is influenced due to its role in controlling the exchange of water and minerals to maintain turgor of cell.

Malondialdehyde (MDA)

The results showed the significant effect of the interaction of year in species at a 1% probability level on Malondialdehyde (Table 6). *T. vulgaris* had higher malondialdehyde than *T. daenensis* in both tested years as well as the amount of this trait in the first cropping year was more than the second cropping year (Fig. 4a).

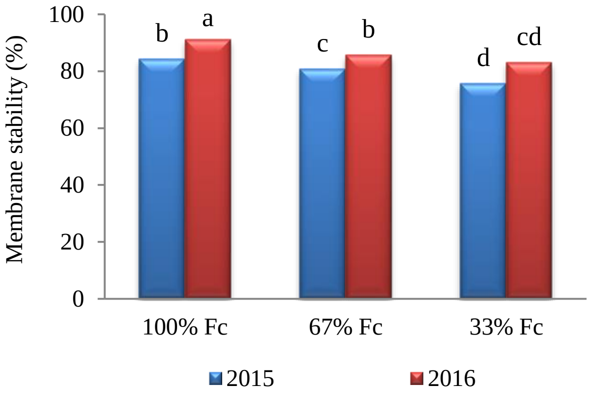


Fig. 3 The interaction of year in the water stress on the membrane stability. Differences of the columns that have the same alphabets one not statistically significant at 5% (LSD) level of significance.

Further, the results of the interaction effect of drought stress on species showed that the amount of malondialdehyde in both tested species increased by decreasing the moisture. However, *T. vulgaris* had more malondialdehyde in such a way that the highest amount of malondialdehyde was obtained from the treatment of 33 % of field capacity and *T. vulgaris* (Fig. 4b). Results showed that the highest and lowest amount of malondialdehyde were obtained from the treatment *T. vulgaris* without applying manure and from *T. daenensis* with applying 30 tons manure per hectare, respectively (Fig. 4c and). The amount of malondialdehyde (MDA) was increased under drought stress conditions, which have been a sign of lipid peroxidation and could be due to decreased super oxidative dismutase and catalase [41]. Overall, lipid peroxidation of the membrane can be considered as a sign of oxidative damage, which is often used as an indicator to determine the extent of damage to under stress membrane [10]. In this experiment, there was a significant and negative correlation between malondialdehyde (MDA) and quantitative yield ($r=-0.59^{**}$, N=24). *T. vulgaris* had the highest amount of malondialdehyde and ionic leakage and in contrast, the lowest amount of membrane stability and quantitative yield, which demonstrated the cell membrane destruction and more vulnerability of the cell membrane and consequently, disruption in growth in this species.

Proline

The results of the combined analysis of two years represent the significance of main effects of year, drought stress, specie and manure, specie in manure and year in specie at a probability level of 1% on the year on the rate of proline (Table 6). The results of the mean comparison of the combined analysis of the year in species showed that the amount of proline in both tested species and in the second year was more than the first year.

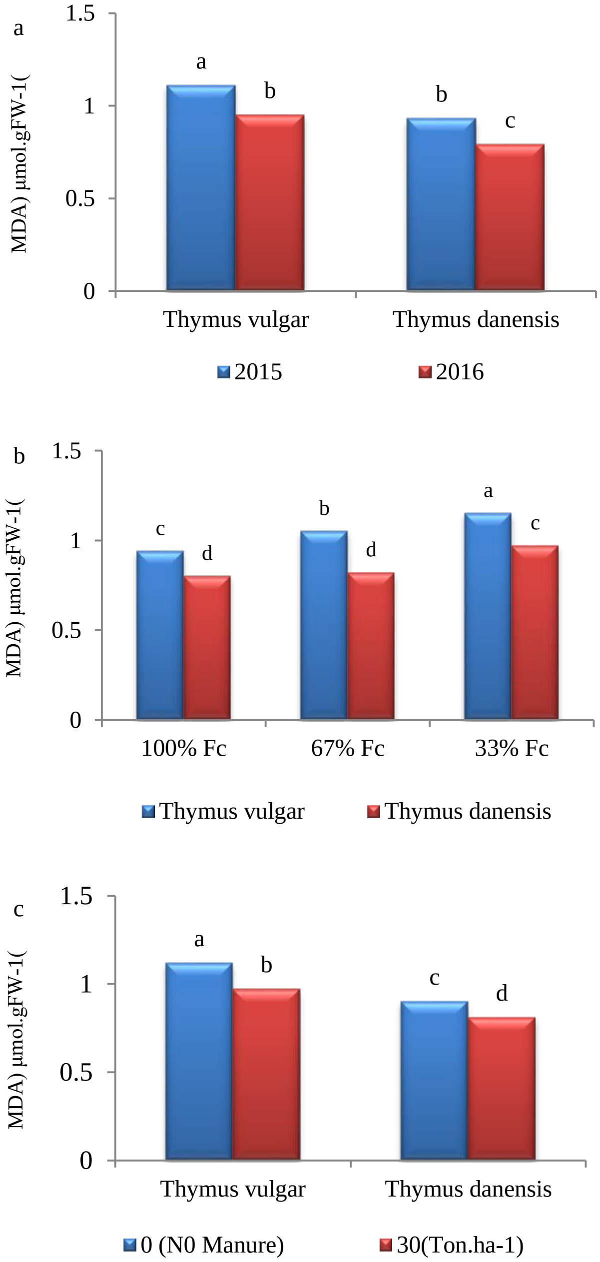
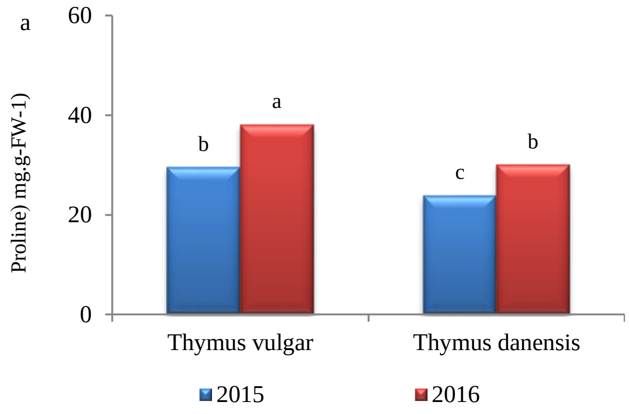


Fig. 4 a- The interaction of year in the species on MDA (µmol/g FW). b- The interaction of water stress in the species on MDA (µmol/g FW) and c- The interaction of the manure in species on MDA (µmol/g FW). Differences of the columns that have the same alphabets one not statistically significant at 5% (LSD) level of significance.

a


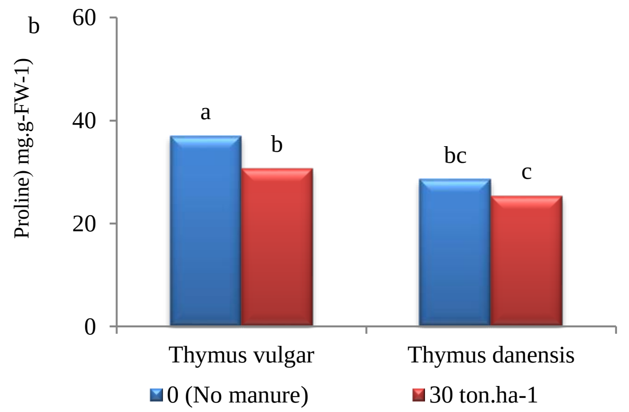
b


Fig. 5 a- The interaction of year in the species on Proline (mg/g/FW). The interaction of manure in the species on Proline mg/g/FW). Differences of the columns that have the same alphabets one not statistically significant at 5% (LSD) level of significance.

However, *T. vulgaris* had more proline, compared to the *T. daenensis* and the highest amount of proline was obtained from *T. vulgaris* and in the second cropping year (Fig. 5a). In addition, the main effect of drought stress showed that by reducing the moisture level from 100 to 33% of field capacity the amount of proline increased (Table 7). Osmotic adjustment is one of the effective mechanisms used by the plant in facing drought stress to maintain cellular turgor and swelling. In this regard, Omidi [42] reported that the amino acid of proline is one of the most important and effective compatible osmolytes which plays an important role in the tolerance of environmental stresses such as drought stress. Further, the amount of proline in plant organs is an indicator of stress and the amount of proline is enhanced by increasing the tension intensity [43]. Proline increases the stress tolerance in plants by osmotic adjustment, preventing enzymatic degradation and removal of hydroxyl radicals [44]. Moein Alishah *et al* [45] reported the enhancement of proline content affected as drought stress conditions in *Ocimum basilicum*. Proline plays a key role in osmotic adjustment in drought stress conditions, it can increase the plant's resistance to the drought and largely decrease the destructive effects of osmotic stress caused by the drought. The result is consistent with the results of Amiri *et al* [46]. In addition, in this experiment, there was a negative and significant correlation between proline content and quantitative yield ($r = -0.53^{**}$, N = 24). This negative correlation can indicate the reduction of the effects of drought stress by increasing the amount of proline and in this experiment. *T. vulgaris* had more proline compared with *T. daenensis* and it can be concluded that *T. vulgaris* spent more energy to endure the stress and survive in these conditions and consequently, had less organogenesis and flowering and quantitative yield, compared to the *T. daenensis*. The mean comparison of species in manure showed that the utilization of manure decreased proline content in both species tested and the highest amount of proline was obtained from the treatment of non-application of manure and *T. vulgaris* (Fig. 5b). It seems that using manure increases the availability of water for plants so that the effect of drought stress is reduced compared with the non-application of manure, due to the reduction of the amount of proline in conditions of using manure. Manure by providing water for the plant and avoiding the occurrence of moisture fluctuations prevents the drought stress in the plants and paves the way for reducing the accumulation of proline in under stress plants. In this experiment, *T. vulgaris* showed better drought resistance, compared to the *T. daenensis*. A further increase of proline in *T. vulgaris* may indicate less need for *T. daenensis* to produce proline or proline in this species is less important than other regulators.

Quantitative Yield

The results indicated the significant effect of treatments of the first year, drought stress, species, manure, drought stress in species, species in manure, year in species and year in drought stress on quantitative yield (Table 6). The mean comparison of the interaction of year in drought stress in species showed that the decrease of moisture stress levels led to the reduction of quantitative yield in both tested species in both cropping years. Further, there was a significant difference between the two cropping years, and between two tested species, *T. daenensis* had higher yield than *T. vulgaris* in both cropping years and the highest quantitative yield was obtained from *T. daenensis* in treatment of 100% of field capacity and the second cropping year and the lowest quantitative yield was obtained from *T. vulgaris* in the treatment of 33% of field capacity and in the first cropping year.

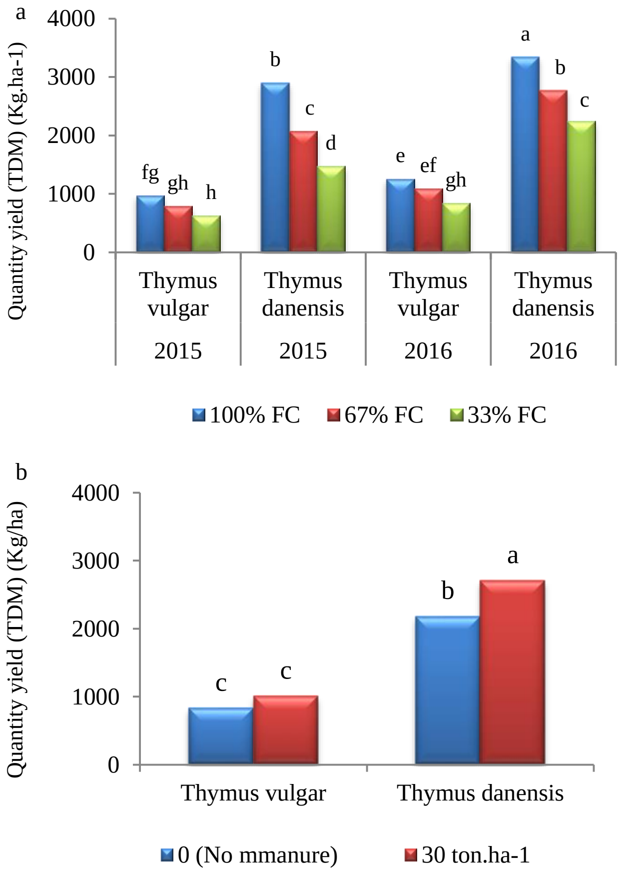
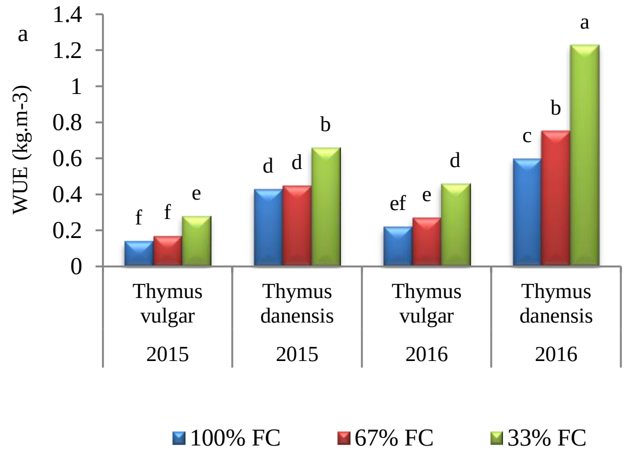


Fig. 6 a- The interaction of year in the water stress in the species on the Quantity yield (TDM, Total dry matter) (Kg/ha). b- The interaction of Manure in the species on the Quantity yield (TDM, Total dry matter) (Kg/ha). Differences of the columns that have the same alphabets one not statistically significant at 5% (LSD) level of significance.

Further, the yield rate in both tested species and at all levels of drought stress in the second cropping year was more than in the first year and in both species tested, the yield enhancement in the second cropping year was 1.3 times of the first cropping year (Fig. 6a). Decreasing yield during the moisture levels' reduction can be related to a decrease in plant height, the number of flowers, the number of lateral branches per plant, and produced leaf area and an increase in allocating photoassimilates to root than the aerial organs of the plant [47]. By reducing cell growth, the size of the aboveground plant parts is limited and consequently, the produced dry matter is decreased in the plant [47]. Drought stress by creating numerous morphological, physiological and biochemical changes in plant and by stopping the spread of cells and reducing the pressure of swelling can influence the dry and fresh weight of the plant and can reduce them [3]. These results are consistent with the study results of Bahreininejad *et al* [48, 49]. In the second year, due to the improved situation of soil and suitable plant nutrition by using manure in which the release of nutrients is gradual, the plant growth and photosynthesis increased and consequently, the yield of the aboveground plant parts increased. Further, the significant effect of species on manure showed that using manure increased the quantitative yield in both tested species, compared to the non-application of manure and the treatment of 30 tons per ha manure and *T. daenensis* had the highest quantitative yield (Fig. 6b) which reflects the positive effects of manure on growth characteristics and the total dry matter. Hendawy *et al* [37] reported the positive effects of manure on the quantitative yield of medical herbs like *T. vulgaris*. The results also showed a 35.56% decline in quantitative yield for *T. vulgaris* in the first cropping year of the control condition of 33% of field capacity and a 33.42% decline in the second cropping year. However, the rate of decline for *T. daenensis* in the first cropping year was 49.09% and 32.97% in the second cropping year which represents more dry matter production and less vulnerability of *T. daenensis* in the second cropping year. Further, *T. daenensis* had a better establishment in the second cropping year and regrowth in the second cropping year in *T. daenensis* was further and faster than *T. vulgaris*. For this reason, *T. daenensis* had more yield stability in the second cropping year.

Water-use Efficiency (WUE)

The result indicated that by reducing the amount of soil moisture, the water use efficiency increased in both cropping years and in both species tested. However, *T. daenensis* in both cropping years had water use efficiency higher than *T. vulgaris* and the highest water use efficiency of *T. daenensis* was obtained at the lowest level of moisture (33% FC) and in the second cropping year (Fig. 7a). WUE in the second cropping year was 1.6 times of the first cropping year, due to the increase of the amount of total dry matter in the second cropping year was 1.3 times of the first cropping year (Table 5). In addition, maximum WUE was obtained from the low level of moisture, due to the consumption of less water in the treatment of high stress. In the present study, due to the proper management of water (having a drip irrigation system and volumetric counter), water loss was reduced and the production of dry matter of plant was higher than water consumption. Therefore, in the present study, the WUE increased by reducing moisture levels by consuming less water. The results of the present study demonstrated that *T. vulgaris* had higher ionic leakage which can led to the cell membranes' damage and consequently, the reduction of the plant production efficiency and ultimately, the reduction of dry matter yield of the plant. Thus, the dry matter production of *T. vulgaris* was less than the identical water used for both species and this reason, *T. vulgaris* in 33% of the field capacity (having the highest ionic leakage) had less water use efficiency. However, *T. daenensis* had less ionic leakage and had higher water use efficiency due to having a dry matter more than the identical water used for both species.



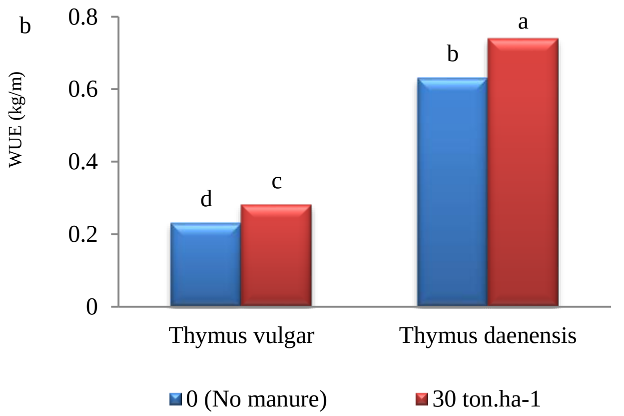


Fig. 7 a- The interaction of year in the water stress in species on the WUE (kg/m). b- The effect of Manure in species on WUE (kg/m). Differences of the columns that have the same alphabets one not statistically significant at 5% (LSD) level of significance.

In this study, the frequency of irrigation in the second cropping year was less than the first year because planting operation was done in transplanting form in the first year and the first three irrigations were done with a tertian interval to prevent the occurrence of stress in seedlings. In addition, irrigation was done once every seven days until the establishment of the plant (the onset of stress). However, in the second cropping year, due to the full establishment of the plant and the development of the root system, irrigation was done under the principles of stress and by reducing the amount of soil moisture leading to less irrigation in the second year. Dhanda *et al* [50] reported that the enhancement of water use efficiency is influenced by drought stress in wheat. In addition, Zhang *et al* [51] concluded that the water deficit stress to 40% of field capacity increases water use efficiency in wheat. The increase in water use efficiency under water deficit stress may be due to the greater effect of water deficit on stomatal conductance compared to carbon fixation [52]. Wakarim *et al* [53] in their studies on beans and Ayas and Korukcu [54] in their studies on potatoes founded that drought stress, due to less transpiration of plant, increases water use efficiency significantly and the results are congruent with the results of the present study. Water use efficiency had a non-significant and positive correlation with quantitative yield (r = 0.34 ns, N = 24). The mean comparison of the effect of manure in species showed that the highest water use efficiency was obtained from applying 30 tons of manure per hectare (Fig. 7b); due to the auxiliary role of manure in the absorption of water and nutrients under tension conditions [55]. Manure may modify the role of drought stress in the reduction of plant photosynthesis by increasing the availability of water and nutrients for the plant and accordingly by preventing the dry weight loss of aerial organs resulting in a significant preference of water use efficiency.

CONCLUSION

The results indicated that plant height, the number of lateral branches and flowers per plant, membrane stability, relative water content and quantitative yield (total dry weight) of thyme were reduced.

Table 5 Water use, Total Dry Matter and water use efficiency (based on total dry matter) of *T. daenensis* and *T. vulgaris* under different drought stress Level in 2015 and 2016

Drought stress Level (Fc%)	Water use (m^3/ha)		TDM (Total Dry Matter) Kg/ha		WUE (kg/m^3)	
	2015	2016	2015	2016	2015	2016
100	6732	5508	1935.85 a	2301.15 a	0.28 c	0.41 c
67	4510.4	3690.36	1428.99 b	1894.58 b	0.31 b	0.51 b
33	2221.56	1817.6	1051.27 c	1539.42 c	0.47 a	0.84 a
Mean	4487.98	3671.98	1472.03	1911.71	0.35	0.58

Values followed by different superscripts (a-c) in the last row are significantly different at $P< 0.05$ (means of the treatments over each year).

Table 6 values of mean squares in the analysis of variance of some Traits in this study

Source	df	Ion leakage	MSI	MDA	Proline	TDM (Total Dry Matter), (Quantity yield)	WUE
Replication	2	1.54 ns	30.79 ns	0.055**	33.82**	367092.92 **	0.03 ns
Year	1	3082.35**	533.19 **	0.52**	980.93**	3479750.08 **	0.98 **
Year × Replication	4	37.98	0.03	0.001	34.61	102082.56	0.007
Water stress	2	434.02 **	629.74 **	0.24 **	331.04 **	4081783.27 **	0.63 **
Species	1	3120.71**	1000.68 **	0.62 **	851.2 **	43237306.53 **	3.32**
Manure	1	788.89 **	184.12 **	0.25**	406.57 **	1372768.92 **	0.12 **
Water stress ×Species	2	21.41*	7.75 ns	0.0093 **	0.52 ns	1166974.92 **	0.10 **
Water stress × Manure	2	22.02*	0.09 ns	0.0006 ns	5.52 ns	2896.46 ns	0.011**
Species × Manure	1	8.81 ns	1.31 ns	0.014 **	44.63**	173154.97 **	0.02 **
Water stress × Species × Manure	2	21.28 *	0.5 ns	0.0028 ns	5.49 ns	10158.31 ns	0.0065 ns
Year × Water stress	2	10.03 ns	44.59 **	0.00095 ns	4.89 ns	25661.43 ns	0.095 **
Year × Species	1	226.94**	1.009 ns	0.015 **	26.54 *	692244.03 **	0.022 **
Year × Manure	1	0.36 ns	0.19 ns	0.0024 ns	4.76 ns	48907.26 ns	0.001 ns
Year ×Water stress × Species	2	27.26 *	0.06 ns	0.000096 ns	2.8 ns	65146.75 *	0.03**
Year ×Water stress × Manure	2	13.67 ns	0.01 ns	0.0000095 ns	2.73 ns	205.57 ns	0.0013 ns
Year × Species × Manure	1	3.38 ns	0.001 ns	0.0001 ns	5.14 ns	409.43 ns	0.00038 ns
Year × Water stress × Species × Manure	2	11.29 ns	0.0006 ns	0.00003 ns	0.27 ns	3333.01 ns	0.00071 ns
Error	44	7.03	4.01	0.0015	5.97	17304.18	0.0027
CV%	-	3.57	2.41	4.16	8.04	7.77	11.07

*and **: Significant at the 5% and 1% probability levels, respectively.,ns: Non- Significant.

Table 7 The mean some of the traits affected by main factors

Drought treatment	Branches number	Flower number	RWC	WUE	MSI	MDA	Proline	Total Phenol
100% Fc	69.32 a	54.32 a	75.92 a	0.13 c	88.38 a	0.86 c	26.99 c	49.03 c
67% Fc	66.06 b	52.56 b	72.83 b	0.15 b	83.17 b	0.94 b	29.83 b	54.59 b
33% Fc	62.72 c	49.82 c	68.34 c	0.24 a	78.14 c	1.06 c	34.35 a	61.64 a
Species treatment								
T. vulgaris	36.78 b	22.98 b	60.06 b	0.096 b	79.50 b	1.05 a	33.83 a	45.54b
T. daenensis	95.29 a	81.49 a	84.67 a	0.25 a	86.96 a	0.86 b	26.95 b	64.62 a
Fertilizer treatment								
No manure	62.74 b	48.94 b	70.80 b	0.16 b	81.63 b	1.01 a	32.77 a	32.77 a
Of manure30 Ton/ha	69.33 a	55.53 a	73.92 a	0.19 a	84.83 a	0.89 b	28.01 b	28.01 b

However, the amount of proline, phenol content, ion leakage from the cell membrane, and malondialdehyde of the medicinal plant were increased. Plants under drought stress by accumulating a series of materials such as proline, phenolic compounds can largely cope with stress, which is regarded as the reason for the enhancement of these features in water stress in this experiment. According to the results of the study, it is recommended that in arid and semi-arid regions where plants are faced with water shortages, proper nutritional management practices should be applied to achieve maximum quantitative yield. Using decayed organic fertilizers with sufficient intervals before planting can exert greater effects on the improvement of plant growth, as nutrients are gradually released. Thus, it can be concluded that using manure had a positive impact on the dry yield of *T. vulgaris* and *T. daenensis*. Modifying the physical properties of the soil and the availability of nutrients for the Thyme plant can be a major reason for the increased yield and growth of the plant.

In addition, the results of this experiment showed a significant difference between the two cropping years, and the quantitative yield of both tested species increased in the second year. In fact, it is logical, because in the first year, seedlings transferred to the main land have not established fully and plants were poor and over time showed better growth in the second cropping year. Further, in the second year, due to the improved situation of soil and proper nutrition of plant by using manure in which releasing nutrient was gradual, plant growth and photosynthesis increased, leading to an increase in yield of aerial organs. The results of the present study could properly distinct two species of *T. vulgaris* and *T. daenensis* in terms of water stress tolerance and introduced *T. daenensis* as superior species in facing drought stress. By maintaining further relative water content, *T. daenensis* decreases the amount of water lost from leaves which indicates the importance of this genotype in maintaining water potential in tension. On the other hand, *T. daenensis* had less ion leakage, which reflects the maintenance of the cell membrane integrity in drought stress. The results distinguished the two thyme species in yield and *T. daenensis* had the highest quantitative yield. In general, *T. daenensis* can grow successfully in arid and semi-arid regions and is recommended for planting in these areas.

Conflict of Interest

The authors report to confirm no conflict of interest and there has been no significant financial support for this work that could have influenced its outcome.

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