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Identification of Drought Tolerant and Sensitive Species of Thyme through Some Physiological Criteria

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Abstract
Drought as the most important abiotic stress has deleterious effects on plants. Developing drought tolerant varieties can help produce plants in a sustainable way. This study was conducted to identify drought tolerant and drought sensitive thyme species including *Thymus vulgaris*, *T. vulgaris* (origin: Spain), *T. carmanicus*, *T. daenensis* and *T. kotschyanus* and to study the mechanism used by them to cope with drought stress. For this purpose, relative water content, water use efficiency, soil water depilation rate, root:shoot ratio, drought resistance index and a new criterion "FC ceased growth" were used. *T. carmanicus* and *T. daenensis* had the lowest and the highest reduction on relative water content, respectively. In terms of water use efficiency and soil water depletion curve, the highest and the lowest values were detected for *T. daenensis* and *T. carmanicus,* respectively. The most and the least root:shoot ratios were recorded for *T. daenensis* and *T. vulgaris* (origin: Spain), respectively. Analyses by drought resistance index and PCA revealed that *T. carmanicus* is drought susceptible, *T. kotschyanus* and *T. vulgaris* are semi-drought susceptible, and *T. daenensis* and *T. vulgaris* (origin: Spain) are semi-drought tolerant species. FC ceased growth analysis showed that *T. carmanicus* stopped its growth at higher FC, while *T. kotschyanus* stopped it at lower FC. Therefore, based on this criterion and considering the sustainability of growth under drought condition, *T. carmanicus* and *T. kotschyanus* are the least and the most drought tolerant *Thymus* species.

Keywords: Thyme, breeding, water deficiency, agriculture, screening, drought tolerance.

Introduction

Environmental stresses (biotic and abiotic stress) are serious threats to agricultural production (Nakabayashi and Saito, 2015). Drought is an important abiotic environmental stressor of plants and water deficit is typically the most limiting factor for plant growth, yield, and productivity (Barchet et al., 2014). In most area of the world, agriculture is more influenced by drought than other competing parts like industry, because of changes in rainfall pattern caused by global climate changes (Sanchez et al., 2012). To increase the agricultural crop yields within the limited land resources, existence of plants that can tolerate undesirable conditions like drought is essential. Understanding the reaction of plants to water-limited conditions is crucial and will paves the way for improving tolerance to drought (Reddy et al., 2004).

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In the evolutionary path, plants developed different ways to cope with drought, which are drought escape, dehydration avoidance and dehydration tolerance (Blum, 2011; Moradi, 2016) and among them the last two mechanisms considered as drought resistance strategy (Levitt, 1980; Norton et al., 2014). Physiological traits, which often used as criteria of drought avoidance are relative water content (RWC), water potential, abscisic acid content, and canopy temperature (Hu and Xiong, 2014). There are different ways to measure dehydration tolerance including: photosynthesis, growth, osmotic adjustment, root:shoot ratio and drought survival (Blum, 2011; Hu and Xiong, 2014). Yield under water-limited environments is another important selection criterion for breeding programs. Stress tolerance (TOL), mean productivity (MP) (Rosielle and Hamblin, 1981), stress tolerance index (STI), geometric mean of productivity (GMP) (Fernandez, 1992), and stress susceptibility index (SSI) (Fischer and Maurer, 1978) are criteria related to yield, which are used for identification of drought resistance cultivar/species.

Genus *Thymus* is one of the largest genera in Lamiaceae family. About 214 species and 36 subspecies are identified in this family (Stahl-Biskup and Sáez, 2002; Moradi et al., 2014). The *Thymus* is the eighth genera of Lamiaceae family with regard to the number of species (Stahl-Biskup and Sáez, 2002). Thyme is a perennial, subshrub or shrub plant, which has been used as a medicinal, aromatic and spicy plant (Stahl-Biskup and Sáez, 2002; Boning, 2010).Demand for Thyme has been increasing, but because of indiscriminate harvesting of thyme as well as insufficient/irregular rainfall in its natural habitats, harvesting from natural habitats is not equal to its vegetation. Therefore, it is necessary to identify/develop stress tolerant thyme plants especially for drought for its sustainable production as well as its biodiversity conservation (Stahl-Biskup and Sáez, 2002)

There is only one study that assessed different thyme species under drought condition done by Moradi et al. (2014). Six species and eleven populations of thyme by root:shoot ratio (length), survivability, water content and water potential were studied, and it was concluded that survivability is more reliable criterion for screening of drought tolerance for that collections of thyme plants, but mechanisms of drought tolerance was not determined in their study.

Some researchers stated that productivity is more important than survival of the crop (Blum, 2011) and rejected the relevance of survivability towards plant production under stress conditions (Passioura et al., 2007).

Plants delays death by using survival mechanisms and can continually and slowly extract soil moisture. Furthermore, plants extend their plant life towards an oncoming rainfall event and subsequent recovery from the imposed drought, but this may not be necessarily related to an economic yield, because survivability might be resulted from a small plant size and very early flowering (Blum, 2011). According to what is mentioned, survivability is more important in natural habitats (ecological system) to reduce the risk of specie/plant extinction and yield is more important for agricultural and economical point of view. We conducted this study to identify drought tolerant and sensitive Thyme species with considering agricultural point of view rather than the ecological one. Furthermore, this study aimed to determine the mechanism(s) involved in drought tolerance in Thyme species.

Materials and methods

Seeds of *Thymus vulgaris*, *T. daenensis*, and *T. kotschyanus* were obtained from Pakan Bazr-e-Esfahan Company (Esfahan, Iran), *T. carmanicus* (accession number: P1006577) was received from Iranian Biological Resource Center (Tehran, Iran) and *T. vulgaris* (origin: Spain) was purchased from Semillas Silvestres Company (Córdoba, Spain).

Two greenhouse experiments were conducted in winter and spring (last one was only performed to assess repeatability of FCG method). Thyme seeds were grown in pots (8.5×12×6 cm) filled with 285 and 215 grams of two different soil mixture of top soil, vermicompost, perlite and sand (for winter 4:2:0.5:1 and for spring 2:1:0.5:1, respectively). Plants were grown in a greenhouse with a day/night temperature of 25°C/20°C ±1, and light/dark cycles of 16/8 h. All pots were daily irrigated with tap water to 95% of field capacity (FC) for 30 days. Next, drought stress was imposed on 30 days old plants by withholding water till last plant died (32 days) and control plants were irrigated same as before.

Soil water content and soil water depletion curve

The soil water field capacity (FC) was estimated by the gravimetric method as grams of water per gram of dried soil and expressed as the percentage of the total water content held in the soil after 2 days of a downward drain under greenhouse conditions, which is the water-holding capacity (Sanchez et al., 2012).

Soil water depletion curve was drawn only for drought treated plants. For this purpose, daily pot's FC was used as Y axes and time, in this case day was used as X axes to draw soil water depletion curve.

RWC and root:shoot dry weight ratio measurement

RWC was measured as described by Barrs and Weatherley (1962).(Barr and Weatherley 1962)

A destructive method was used for calculation of root:shoot dry weight ratio. At beginning of drought treatment as well as 10 and 20 days after that roots were removed from soil and washed with water. Next, the whole plant parts were dried out in 70°C for 48h. Afterward, root and shoot were weighted separately.

Water use efficiency and drought resistance index

Water use efficiency (WUE) was calculated according to Blum (2011) method. TOL, SSI and STI indices were calculated based on produced biomass under normal and drought conditions and were used to categorize the species into different classes of tolerance or susceptibility. TOL, MP (Rosielle and Hamblin, 1981), SSI (Fischer and Maurer, 1978), STI and GMP (Fernandez, 1992) were calculated using equations 1 to 5, respectively, where Y_p, $\bar{Y}_p$, Y_s and $\bar{Y}_s$ are yield under no-stress, yield mean of all genotypes under no-stress, yield under stress and yield mean of all genotypes under stress.

$$TOL = Y_p - Y_s \quad (1)$$

$$SSI = (1-(Y_s/Y_p))/(1-(\bar{Y}_s/\bar{Y}_p)) \quad (2)$$

$$STI = (Y_p \times Y_s)/(\bar{Y}_p)^2 \quad (3)$$

$$GMP = \sqrt{(Y_s \times Y_p)} \quad (4)$$

$$MP = Y_s + Y_p/2 \quad (5)$$

Real-time measurement of relative plant growth and measurement of FC ceased growth

By knowing the daily pot's FC and the day in which relative plant growth was stopped, FC ceased growth (FCG) could be determined (Fig. 1). Relative growth of plant was measured by Chen and colleagues procedure with some modification (Chen et al., 2011). Plants and a 2 cm^2 index were co-photographed every day at 9 am with a 12 megapixels digital camera (Nikon, COOLPIX P500, Tokyo, Japan). Photos were analysed by Adobe Photoshop CS6.13 software to determine plant and index pixels. After determination of the plant and the index pixels, equation (6) was used to convert the pixels to the characters where Pa, Pp, Ia, and Ip are the plant area, the plant pixels, the index area and the index pixels, respectively.

$$Pa = \frac{Ia \times Pp}{Ip} \quad (6)$$

FCG was determined in two distinct soil mixture and season to assess its stability and reproducibility.

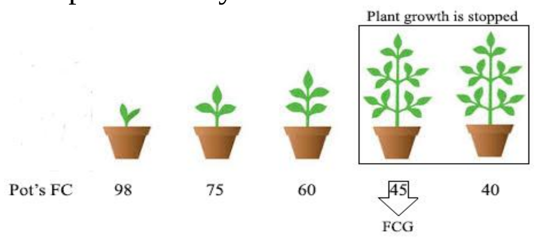

Fig. 1. Schematic view for determining of FCG

Statistical analysis

The factorial experiment in completely randomized design was used as experimental design in which water treatment, sampling time and plant species were the factors. Analyses of variance (ANOVA) were computed for RWC, WUE and root:shoot ratio. Differences between means were tested using Duncan test. ANOVA, mean comparison and Principle component analysis (PCA) were performed using R (R Development Core Team 2009), agricolea (De Mendiburu 2009) and factoextra (https://CRAN.R-project.org/package=factoextra), respectively.

Results

Soil water depletion curve

There were no differences in depletion rate until day fourth of drought treatment. At the fourth day of drought treatment curve of *T. carmanicus* was differentiated from curves of other species until the day 23rd. The *T. vulgaris* (origin: Spain) and *T. vulgaris* differentiate its curve after six and seven days of drought treatment from curves of two remaining species, respectively. The curves of *T. kotschyanus* and *T. daenensis* had almost similar form (Fig. 2).

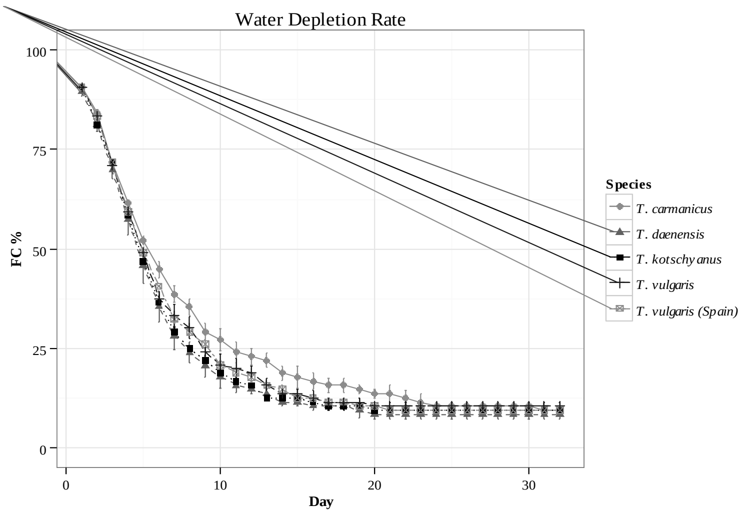

Fig. 2. Water depletion rate of studied species from the day of withholding water till the death of all species.

RWC and Dry weight root:shoot ratio

As shown in Fig. 3 the RWC was significantly affected by species, sampling time and drought treatment. Under control condition, by passing through stage one to four of sampling, RWC increased in *T. carmanicus*, *T. daenensis*, and *T. vulgaris* (origin: Spain), but *T. vulgaris* had an increasing manner until stage three and then a decrease was notified. There were no significant changes in RWC of *T. kotschyanus*. Under drought stress, species had different responses. Five days after beginning of drought treatment (the second sampling) only the RWC of *T. daenensis* significantly decreased. RWC of *T. vulgaris* and *T. vulgaris* (origin: Spain) was significantly declined after ten days of drought treatment (the third sampling). At the third sampling time, a severe drop was observed in RWC of *T. daenensis* and *T. vulgaris* (origin: Spain). Unlike two mentioned species, *T. kotschyanus* did not show any significant reduction at the third sampling time, but its regression line was very close to *T. daenensis* and *T. vulgaris* (origin: Spain). *T. carmanicus* and *T. kotschyanus* had no significant decrease until the tenth day of drought treatment, but RWC was also finally decreased at the fifteenth day of drought treatment (the fourth sampling).

Root:shoot dry weight ratio was affected by plant growth stage and experimental treatments.

In all species, mild drought stress caused a significant increase in root:shoot ratio, while severe drought stress caused significant reductions except for *T. carmanicus* in which this ratio was significantly higher than control.

WUE, Biomass production and drought tolerant indices

Biomass production under control condition ranged from 0.521g/pot (*T. daenensis*) to 0.285g/pot (*T. carmanicus*), and under drought condition ranged from 0.075 g/pot (*T. carmanicus*) to 0.198 g/pot (*T. daenensis*) (Table 1). Results indicated that drought stress significantly reduced biomass production in species. The maximum and minimum reductions in biomass production were observed in *T. daenensis* and *T. vulgaris*, respectively. Figure 5 demonstrates that WUE is affected by either plant species or drought treatment. In control condition, *T. daenensis* (0.541) and *T. carmanicus* (0.26) had the most and the least WUE, respectively. Drought stress significantly decreased WUE of *T. carmanicus*, *T. vulgaris* (origin: Spain), and *T. daenensis*, while, WUE of *T. vulgaris* and *T. kotschyanus* was not significantly reduced by drought. Under drought condition, similar to control, the most and the least WUE were belonged to *T. daenensis* (0.396) and *T. carmanicus* (0.161), respectively. Comparing WUE under control and treatment conditions indicated that maximum and minimum reductions in WUE were in *T. daenensis* (0.145) and *T. vulgaris* (0.029), respectively (Fig. 5).

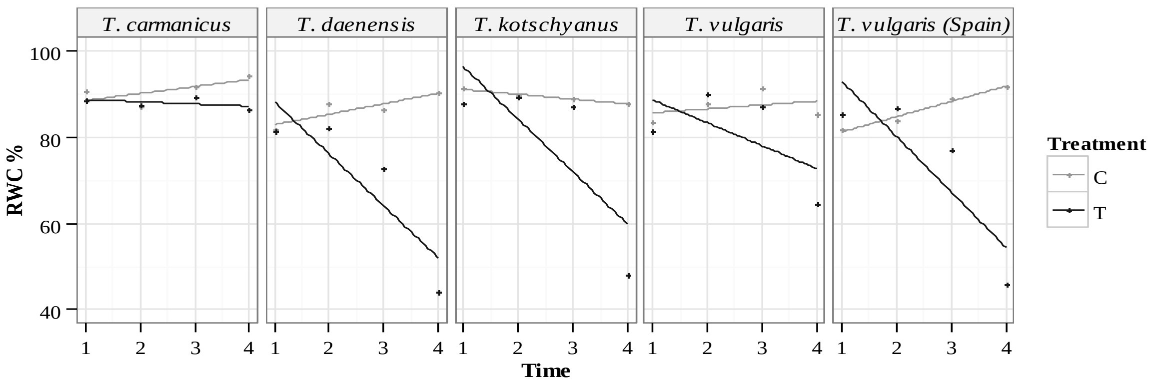

Fig. 3. Relative water content of five thyme species under drought stress at four time points by five days interval. Lines show regression line.

Table 1. Drought tolerant index and biomass production for studied species.

Plant species	STI	SSI	TOL	GMP	MP	Yp	Ys
T. vulgaris	0.478	0.973	0.193	0.235	0.254	0.351	0.158
T. kotschyanus	0.399	0.584	0.087	0.215	0.219	0.395	0.176
T. daenensis	0.891	1.094	0.323	0.321	0.360	0.521	0.198
T. vulgaris (origin: Spain)	0.717	1.085	0.285	0.288	0.322	0.464	0.179
T. carmanicus	0.185	1.301	0.210	0.146	0.180	0.285	0.075

STI: Stress tolerance index, SSI: stress susceptibility index, TOL: stress tolerant, GMP: geometric mean of productivity, MP: mean of productivity, Yp: produced biomass (g) under control condition and Ys: produced biomass (g) under drought condition.

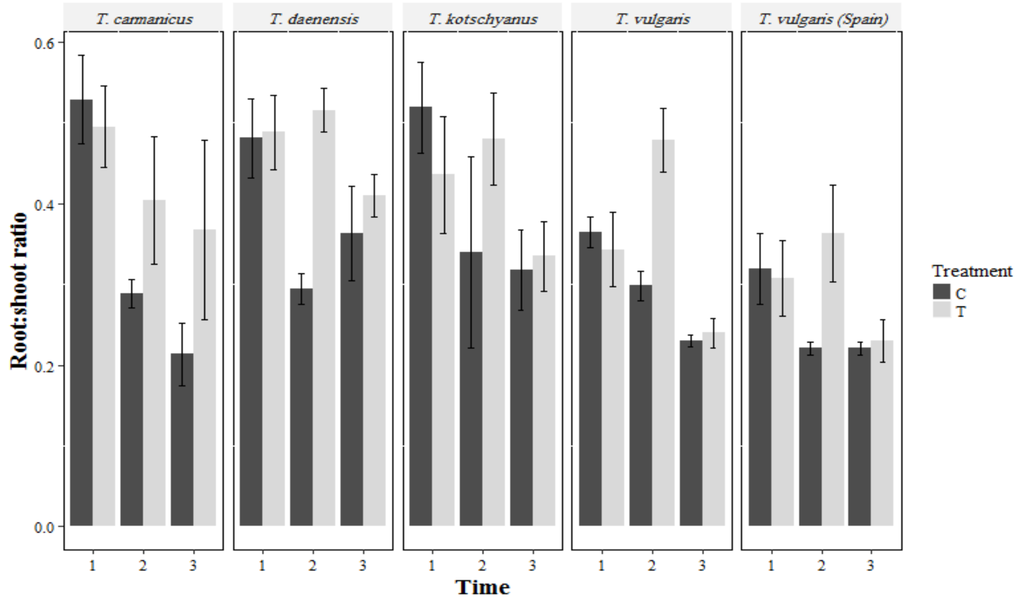

Fig. 4. Dry weight root:shoot ratio of studied species under well-watered and withhold water regimes at three time points by ten days interval. Each bar indicates mean ± standard error. C=Control, T= Treated.

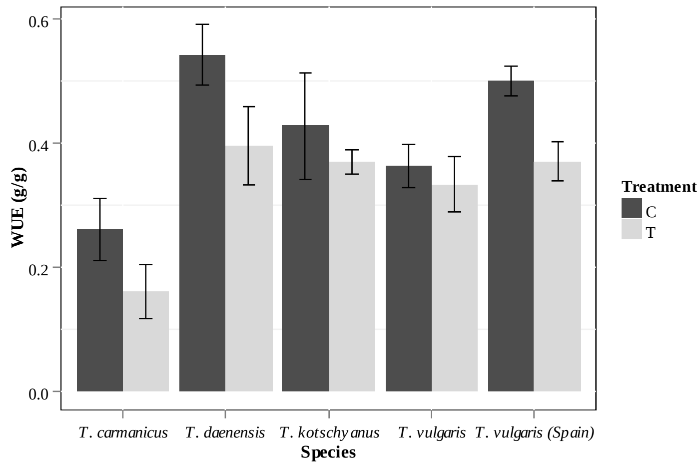

Fig. 5. WUE of studied species under well-watered and drought conditions. Each bar indicates mean ± standard error.

The highest STI, TOL, GMP and MP was observed in *T .daenensis*, while the highest SSI was calculated for *T. carmanicus*. In contrast, the lowest STI, GMP and MP was calculated for *T. carmanicus* and the lowest SSI and TOL was determined for *T. kotschyanus* (Table 1). But, for better visual assessment of the relationships between the species and drought tolerance indices, PCA was performed (Fig. 6).

The first principal component (PC1) had a high and negative correlation with the MP (-0.99), STI (-0.99), GMP (-0.97) and Y_p (-0.97), TOL (-0.78) and Y_s (-0.77) indices as well as it explained 73.22% of the total variation. The second principal component (PC2) had a high and negative correlation with the SSI (-0.99) and TOL (-0.61) and a positive correlation with the Y_s (0.62) indices and explained 26.69% of the total variation. The first principle component constituted 5.12% of the variation in drought indices, and the second principal component constituted 1.86% of such variations. The species *T. daenensis* and *T.*

vulgaris (origin: Spain) ranked strongly in the SSI, TOL and Y_p indices. Alternatively, the STI, GMP, MP and Y_s indices did not ranked in other species as well as the TOL and Y_s indices did not significantly separate the species (Table 2).

FCG

Among all studied species, in both experiments, growth of *T. carmanicus* ceased at higher FC and *T. kotschyanus* stopped growing at lower FC (Fig. 7 A, B). In both experiments growth cessation for the studied species happened as the following: *T. carmanicus* > *T. vulgaris* > *T. vulgaris* (origin: Spain) > *T. daenensis* > *T. kotschyanus*.

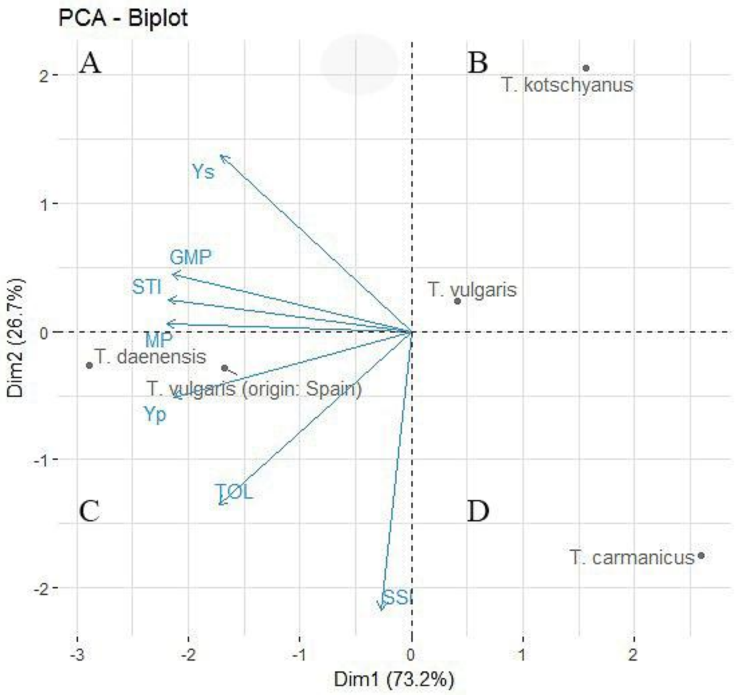

Fig. 6. Principal component analysis (PCA) biplot for drought tolerant indices in studied thyme species. A. drought tolerant region, B. semi drought susceptible region, C. semi drought resistance region, D. drought susceptible region.

Table 2. Principal component loading for drought tolerance indices based on dry mass.

Indices	Dimension 1	Dimension 2
TOL	-0.786	-0.617
SSI	-0.121	-0.991
STI	-0.992	0.115
GMP	-0.978	0.205
MP	-0.999	0.0279
Ys	-0.778	0.627
Yp	-0.972	-0.232
Eigen value	5.12	1.86
Percentage of variation	73.22	26.69
Cumulative percentage	73.22	99.91

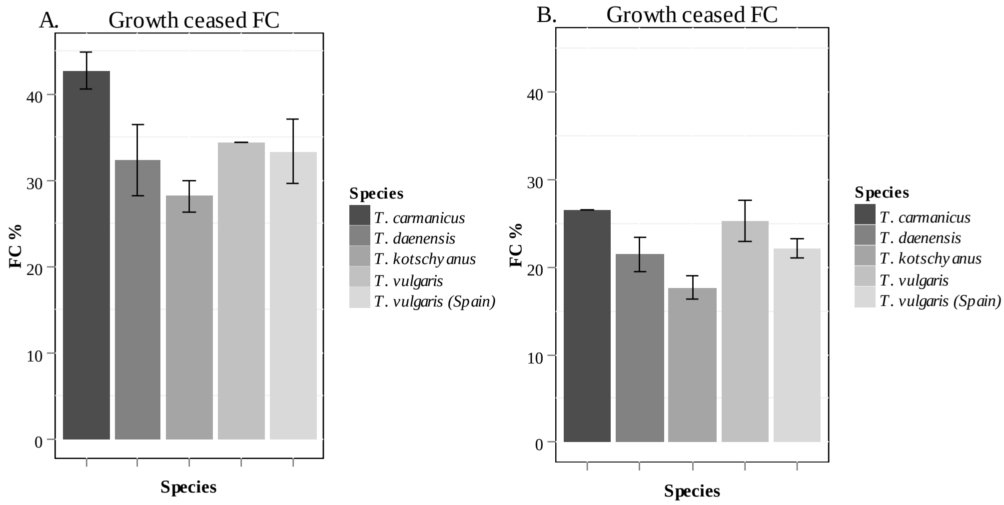

Fig. 7. FCG of studied species in two different soil mixtures. A. Winter experiment, B. Spring experiment. Each bar indicates mean ± standard error.

Discussion

Drought stress is the most limiting factor for plants growth and development. Change of plant water status is the first sign of drought effect that could be monitored through calculation of RWC (Tanentzap et al., 2015). Results indicated that there was a sharp reduction in RWC for all species except for *T. carmanicus*, through sampling time 3 to 4. *T. carmanicus* conserved its own RWC even at the fourth sampling time therefore, this species have good strategies to save water. On the other hands, RWC of *T. daenensis* and *T. vulgaris* (origin: Spain) was declined at the third sampling time revealed that these two species could not have an appropriate strategy for conserving water. According to RWC, the most and the least drought tolerant species were *T. carmanicus* and *T. daenensis*, respectively.

The results indicated that root:shoot ratio decreased as the plant getting older. Alteration in root:shoot ratio over plant's life cycle is part of an intrinsic ontogeny. In general, this ratio decreases by plant aging because of constant accumulation of carbon in aerial parts (Rooty crops would be a notable exception) (Amos and Walters, 2006; Munns et al., 2016), which is in accordance with our observation on control plants (Munns et al., 2016). Amos and Walters (2006) reported that many soil parameters (e.g. soil moisture, soil depth and etc.) can affect the root:shoot ratio. The results of current study revealed that severity of drought also affect root:shoot ratio and the greatest effect was observed in the second sampling time (moderate stress). These observations indicate that carbon allocation is a dynamic process that is affected by drought severity.

Some studies indicated that change in root:shoot ratio under water shortage is mainly an adaptive improvement (drought tolerance), which is genetically inherited (Silva et al., 2012). Generally, drought resistant species or cultivars have greater root:shoot ratio and allocate higher proportion of photosynthetic assimilates towards the roots (De la Barrera and Smith, 2009; Akhzari and GhasemiAghbash, 2013). According to mentioned studies along with this ratio, *T. daenensis* and *T. vulgaris* (origin: Spain) were the most and the least drought tolerant species, respectively.

As shown in Figure 2, slower and faster water depletion rate were observed for *T. carmanicus* and *T. daenensis*, respectively. In contrast to water depletion rate, the least and the most WUE were calculated for *T.*

carmanicus and T. daenensis, whether in control or treated plants. In general, there is a negative relationship between WUE and stomatal conductance, assimilation rate and transpiration (Lawson and Blatt, 2014), but we observed a positive relationship between WUE and water depletion rate for *T. carmanicus* and *T. daenensis*, so further studies should be performed to find the possible reason(s) of this finding.

Drought tolerance index could be assessed by calculation of STI, SSI, TOL, GMP and MP. By use of STI, GMP and MP, higher stress tolerant genotypes could be identified (Rosielle and Hamblin, 1981; Fernandez, 1992) . Among the stress tolerance indicators, the lowest values for SSI and TOL are favorable because the lowest value indicates more tolerance (Fischer and Maurer, 1978; Rosielle and Hamblin, 1981).

Shiri et al. (2010) carried out a study on the drought tolerance of maize plants and showed that PC1 have a high and positive correlation with STI, GMP, MP, Y_p and Y_s and named the first component as yield potential and drought tolerance component. On the other hands, PC2 have a high and positive correlation with TOL and SSI and named this component as drought susceptibility component. So, they concluded that genotypes whit high PC1 and low PC2 will produce high yield under drought and normal conditions (Shiri et al., 2010).

PC1 explained 73.2% of the total variability and had a high and negative correlation with STI, GMP, MP, Y_s and Y_p. PC2 also explant 26.7% of the total variability and had a high and negative correlation with SSI and TOL as well as had a high and positive correlation with Y_s. According to results and Shiri et al. (2010) conclusion, PC1 could be named as drought susceptible and PC2 could be named as drought tolerant (The TOL and Y_s indices were not included in component naming because of inability of them in separation of species), therefore, lower value of PC1 and higher value of PC2 will identify drought tolerant species. Base on this conclusion, *T. carmanicus* was identified as drought susceptible, *T. kotschyanus* and *T. vulgaris* were selected as semi-drought susceptible, but *T. daenensis* and *T. vulgaris* (origin: Spain) were determined as semi-drought tolerance species.

Some studies introduced STI, GMP and MP as the suitable indices for selection of drought tolerant genotypes (Ghaffari et al., 2013). Our results also identified STI, GMP and MP as the appropriate indices for selection of drought tolerant Thyme species.

Plants with ability to maintain a relatively high level of hydration under a specific soil or atmospheric water stress condition either by increasing the capacity for water uptake by roots ("water spender plants") or reducing water loss from leaves ("water saver plants") benefit from dehydration avoidance. On the other hands, plants which continue their function under low plant water status (by use of various mechanisms e.g. osmotic adjustment) benefit from dehydration tolerance (Levitt, 1980; Blum, 2011). It seems that studied species take advantage from dehydration avoidance using water saving and spending mechanisms. Since *T. carmanicus* had the lowest WUE, soil water depletion rate and also the highest RWC, it seems that this species benefited from water saving mechanism and dehydration avoidance, but *T. kotschyanus*, which had relatively high RWC, WUE and soil water depletion rate seems to be a water spender and benefited from dehydration tolerance. Although the highest biomass, WUE and root:shoot ratio was observed in *T. daenensis*, this species could not be a water spender because of its low RWC, so it may benefit from drought escape mechanism. *T. vulgaris* and *T. vulgaris* (origin: Spain) also seem to be a moderate water saver because of medium water depletion rate, RWC and WUE.

Resistance/tolerance mechanisms are complex and despite recent advances in development of drought resistant/tolerant cultivars, a crucial trait is not always obvious for selection of resistant/tolerant cultivars (Blum 2005). By selection of drought tolerant species based on root:shoot ratio, RWC, and stress tolerance indicators different species have been selected as drought tolerant/sensitive. Each of these indicators assesses different parts of the plant (e.g. root, leaf and shoot), which have a complex interaction with other parts of plant as well as environmental conditions. Therefore, selection of drought tolerant/sensitive species using only one of these indicators is not reliable and a more comprehensive, stable and reliable criterion is necessary. Sustaining of growth under a standard managed drought stress specifies integrated ability of the plant, without considering dehydration avoidance or tolerance (Blum, 2011). Evaluation of drought tolerance by considering FC and growth changes is a comprehensive assessment of plant parts and environment interaction. Grouping of species by FCG in two distinct trials revealed that in both trials plant species ceased growth as the following: *T. carmanicus* > *T. vulgaris* > *T. vulgaris* (origin: Spain) > *T. daenensis* > *T. kotschyanus*, which was stable under different environmental conditions (Fig. 5).

Norton et al. (2014) stated that drought-tolerant cultivars ceased growth and senesced its herbage earlier and more completely than the drought sensitive cultivar. Norton et al. (2014) statement and FCG results, *T. carmanicus* is more drought tolerant but today, researchers are attempting to sustain growth under drought condition and believe that sustaining growth under drought will be the main criterion for introducing drought tolerant plants (Ramegowda et al., 2014; Vurukonda et al., 2016) to achieve more yield so, according to these attempts, *T. kotschyanus* is suggested as the tolerant as well as more suitable species of Thyme plant.

Conflict of interest

The authors declare that they have no conflict of interest.

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