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Plant-water responses of different medicinal plant thyme (Thymus spp.) species to drought stress condition

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Abstract

Thymus with more than 200 species is known for its medicinal and culinary uses. There is an increasing demand for thyme and its products, requiring cultivation under different conditions, including drought. To understand the response of thyme to water deficit and its variation across the genus, eleven populations from a range of species (*Thymus daenensis*, *T. kotchyanous*, *T. vulgaris*, *T. serpyllum*, *T. capitata* and *T. zygis*) were evaluated physiologically to identify the response of different populations to water deficit stress. The drought stress was imposed by withholding water 30 days after planting. Root/shoot ratio, survivability, water content and water potential were recorded. The results showed that populations had significantly different ratios of root/shoot under drought conditions. Leaf water potential in watered plants (-3.4 bar) decreased to -10.5 bar in droughted plants. On average, water content for watered plants were 85%± 3.7 and for droughted plants were 73.4%±15.5, after the droughted period before wilting. Analysis of the data found a significant negative association between water content and water potential. This correlation ranged from – 0.075 in *T. kotchyanous* (from Iran) to – 0.91 in *T. serpyllum* (from Spain). *T. serpyllum* (from Spain) survived longer than all other populations (for 22 days) while *T. vulgaris* (Spain) did not survive beyond 12 days. Among the screening indicators tested in this study, survivability was found to be a reliable tool to screen germpalsm for drought stress tolerance. Our experiments identified *T. serpyllum* as being more tolerant than other species, and Spanish *T. vulgaris* population was the most susceptible.

Keywords: Survivability; Physiological; Drought tolerance; Thymus; Water potential.

Abbreviations: DMRT_Duncan's New Multiple Range Test; ANOVA_Analysis of Variance; ROS_Reactive Oxygen Species; ABA_Abscisic acid; WC_Water content; WP_Water Potential

Introduction

The genus *Thymus* is one of the largest genera in the *Labiatae*. At present, there are 214 species and 36 subspecies recognised (Stahl-Biskup and Sàez 2002). *Thymus* products/applications are widespread and include essential oils, oleoresins, fresh and dried herbs. As a medicinal plant, thyme extracts and essential oils can be used as antiseptic, antibacterial and spasmolytical agents (Sagdic et al., 2002; Stahl-Biskup and Sàez, 2002). Demand for thyme products is increasing and cannot be satisfied by collecting from natural populations, particularly given the need to secure their sustained conservation. Improving cultivation of thyme under different conditions, including drought, will facilitate future increased production whilst protecting natural biodiversity.

Drought reduces the availability of water in the soil by evaporation and transpiration (Jaleel et al., 2007). Drought stress in plants is characterized by loss of water, associated stomatal closure and limited gas exchange and decreased leaf water potential followed by failing metabolism and photosynthesis and finally the death of the plant (Smirnoff, 1993; Jaleel et al., 2008). Ability to survive and grow under drought depends on the plant species, growth stage, duration and intensity of water deficit (Chaves et al., 2002; Jaleel et al., 2008). Plants respond to water deficit by a series of physiological and metabolic processes, which depends on the species and developmental stage (Bray 1993). In general, plants have three main approaches to combat drought stress namely escape, avoidance and tolerance. Escape is sidestep of the crisis through fast development (shortening the life cycle) of growth, flowering or seed set. Avoidance involves a balance between water loss and water uptake under mild water reduction in soil. These mechanisms include stomatal closure in the short term and increasing root growth and thickening the cuticle as long term mechanisms. If water stress persists, tolerance mechanisms will apply to maintain the plant metabolic activity while the water potential is low. These mechanisms are used to prevent cellular damage due to water deficit and include metabolic changes, protective solutes and proteins, ROS detoxification (Verslues and Juenger, 2011; Ludlow, 1989).

In medicinal plants, like other plants, environmental factors affect secondary metabolites, and drought stress can be a major factor in increasing concentration of secondary plant products. These secondary metabolites include terpenes, phenols, alkaloids and cyanogenic glucosides (Khalid, 2006; Selmar, 2008). There are limited studies on the effects of stress of *Thymus* species. Jordan et al. (2003) studied the effects of 4 watering levels on *Thymus hyemalis* Lange showing no significant differences in essential oil yields between watering levels. They suggested winter harvesting under low water level for essential oil extraction (Jordan et al., 2003). Another study on *Thymus zygis* subsp. *gracilis* was carried out to understand the effect of drought on phytomass production and essential oil quality. Maximum plant dry matter production, essential oil yield and thymol concentrations were achieved with a moderate watering level (Sotomayor et al., 2004). Babaee et al. (2010) studied the effects of 4 different levels of watering. While water stress decreased plant height, number of secondary branches, dry and fresh weight growth and root mass, dry and fresh weight and length of roots, thymol percentage and proline content increased under severe stress (Babaee et al., 2010). Letchamo et al. (1994) investigated three different soil water levels and two light regimes on two clones of *T. vulgaris*. Maximum plant yield and essential oil was achieved at 70 % soil water level when grown under supplemental lighting (Letchamo et al., 1994).

Understanding the response of plants to water deficit is of great importance, providing information to improve drought tolerance, particularly for screening of useful variation in germplasms (Reddy et al., 2004). Here, we aim to determine the drought tolerance and susceptibility of thyme germplasm (six species and eleven populations) by examining morphological and physiological changes following water stress. This germplasm has been collected because of its economic importance within the genus from different countries including Spain, Germany, UK and Iran. We also aimed to identify most tolerant thyme populations and determine the mechanisms of drought tolerance across a larger range of species/populations than have been previously studied.

Results and Discussion

When studying water stress, three most important traits arguably are the plant growth, survival and plant water status (Verslues et al., 2006), as water stress is described by lower water potential, lower water content and reduced plant growth, and finally wilting (Manivannan et al., 2007; Ekanayake et al., 1985). In the present study, plant shoot and root length and their ratio were recorded as the plant growth index. The water status was measured as both % water content and leaf water potential and to screen for overall drought tolerance. The time of survival (survivability) was also estimated.

Shoot/root ratio as a morphological indicator

Shoot and root ratio calculated based on their length after drought stress period (Fig 1). Species/population responses were placed in three groups. In the first group, namely populations *T. vulgaris* (Iran), *T. daenensis* (Iran) and *T. capitata* (Spain), ratios in droughted plants were greater than plants under watered conditions. The second group consisted of species/populations which had root/shoot ratios under stress lower than control plants. This included *T. serpyllum* (Spain) and *T. kotchyanous* (Iran). The other populations were placed in a third group which had the same ratio under both conditions.

Several factors such as genetics, environment and their interactions affect plant growth rate (Manivannan et al., 2007; Sestak et al., 1971). Both elongation and expansion growth are affected by drought stress (Kusaka et al., 2005; Shao et al., 2008). One of the mechanisms of adaptation to drought is the development of root systems which can enhance water uptake to support plants above ground growth (Passioura, 1981). Increasing root to shoot ratio is one of the avoidance mechanisms that plants employ to maximize the water uptake in water deficit circumstances (Chaves et al., 2003). Regulating root to shoot ratio has been identified as one of the indirect functions of ABA. It has been demonstrated that ABA synthesis is increased in the roots when the soil moisture decreases (Cutler and Krochko, 1999; Thompson et al., 2007; Wilkinson and Davies, 2002). The synthesized ABA is transported to the shoots through the xylem (Davies and Zhang, 1991; Dodd et al., 2008; Wilkinson and Davies, 2002). The higher concentration of ABA in roots limits function of ethylene, which inhibits the growth. In contrast, lower concentration of ABA in the shoots allows ethylene to inhibit the growth. Therefore during water deficit period, root/shoot ratio will increase as a response to drought (Sharp and LeNoble, 2002; Chaves et al., 2003). There are already reports in some other plants regarding this conflict. For instance, increasing root growth in sunflower and *Catharanthus roseus* (Jaleel et al., 2008), decreases the root dry weight in poplar (Wullschleger et al., 2005). No significant difference in maize and wheat (Sacks et al., 1997) has been reported in this case.

In our study, one population showed a different response to drought regarding root/shoot ratio. The highest root/shoot ratio in either control or stressed conditions belonged to *T. serpyllum* species (Fig 1) Showing an absence of such a significant difference. These populations with prolific root systems can efficiently extract water from shallow soil layers and support aerial parts during growth. But populations of *T. capitata* (Spain), *T. vulgaris* (Iran) and *T. daenensis* (Iran) responded to water stress very significantly with increasing root/shoot ratio, compared to watered plants.

Shoot water potential

Application of pressure bomb to measure water potential offers high precision and enables study of a fundamental, not derived, parameter (Clarke and Simpson, 1977). All the populations studied here had a significantly lower leaf water potential in droughted plans, compared to well watered plants (Fig 2). Overall, the average water potential of leaves of well watered plants was -3.4 bar and that decreased to -10.5 bar due to drought stress. Shoot water potential was more negative in droughted plants compared to watered plants across all material ($p \leq 0.01$). The largest difference occurred in *T. vulgaris* (Germany) at around 10 bar (-4 bar in watered decreasing to -14 bar in droughted material). The smallest decline was observed in *T. capitata* (Spain), where it declined from -4 bar to -7 bar. These water potential values are similar to those of Sayar et al. (2007) on wheat (Sayar et al., 2007), although comparison is difficult due to differences in both species and treatment methods.

Water potential quantifies the water availability status (Kramer and Boyer, 1995). When the soil water potential decreases it will be increasingly difficult for plants to take up water from the soil (Verslues et al., 2006). A significant correlation has been reported between high negative water potential in durum wheat and both drought (Benlaribi et al., 1990) and salt stress (Haddad and Coudret 1991) tolerance. To determine significant water potential differences in droughted condition, the one-way ANOVA was used. Within the droughted plants, the group that showed a decline in water potential included *T. vulgaris* (Varico2), *T. vulgaris* (Varico3), *T. vulgaris* (Spain), *T. vulgaris* (Germany), *T. kotchyanous* (Iran) and *T. daenensis* (Iran) evidently more tolerant than the others as they could maintain their internal water potential at a more negative value. Different mechanisms can operate to maintain lower water potential, as explained by Chaves et al. (2003) to allow plants to take up water even under water deficit conditions. But direction of

Table 1. Association of water potential (WP) and water content (WC) in studied populations.

No.	Population/collection region	Pearson Correlation (r)	R^2	Linear Regression model
1	*T. daenensis (Iran)*	-0.48*	0.24	WC=96.19-WP
2	*T. kotchyanous (Iran)*	-0.075n.s.	0.006	WC=81.9-0.33WP
3	*T. vulgaris (Iran)*	-0.51*	0.26	WC=90.2-0.82WP
4	*T. vulgaris (Germany)*	-0.61*	0.37	WC=87.8-0.37WP
5	*T. serpyllum (Europe)*	-0.77**	0.59	WC=90-0.82WP
6	*T. serpyllum (Spain)*	-0.91**	0.83	WC=96-3.3WP
7	*T. capitat (Spain)*	-0.77**	0.59	WC=102.3-3.3WP
8	*T. vulgaris (Spain)*	-0.81**	0.66	WC=95.5-3.18WP
9	*T. zygis (Spain)*	-0.44*	0.17	WC=75.3-1.75WP
10	*Thymus spp.*	-0.27**	0.076	WC=86-0.8WP

* P≤0.05, ** P≤0.01, n.s. non-significant

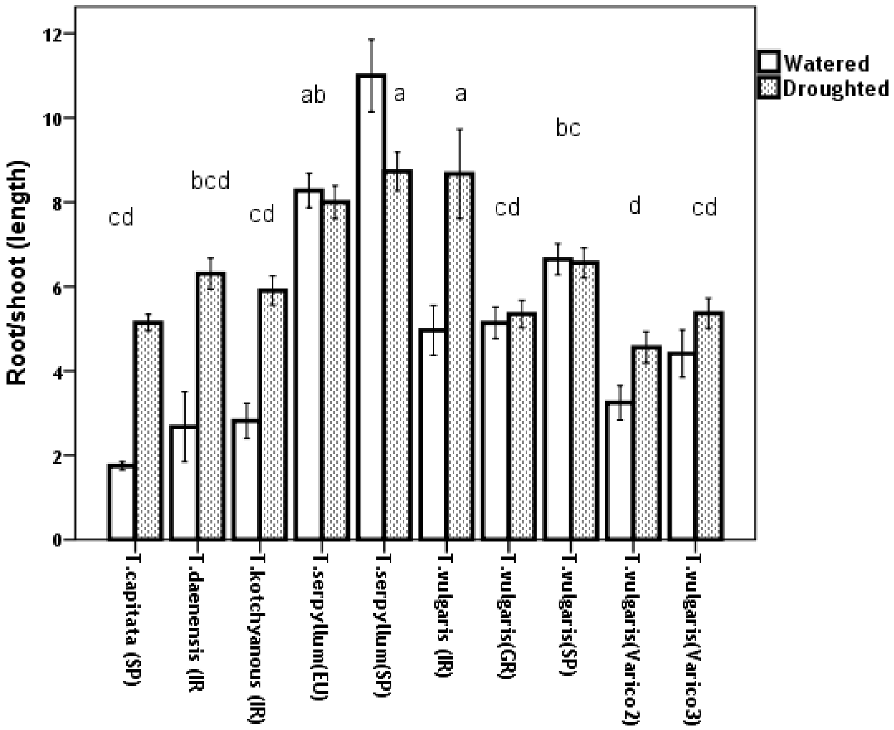

Fig 1. Change in Root/Shoot ratio of 11 thyme populations over a water deficit period. Root and shoot length was recorded in 3 replicates. Studied population responses were placed in four categories, which in the first group [populations *T. vulgaris* (Iran), *T. daenensis* (Iran), *T. kotchyanous* (Iran), *T. vulgaris* (Varico2), *T. vulgaris* (Varico3) and *T. capitata* (Spain)] ratios in droughted were greater than watered plants. Second group consisted of population which had root/shoot ratio in stressed less than control condition. This population was *T. serpyllum* (Spain). The other populations placed in the third group which had the same ratio in both condition. Similar letters on bars show the same mean group based on ANOVA and Duncan mean comparison (p≤0.05). Error bars give ±1 SEM. SP: Spain, GR: Germany, EU: Europe, IR: Iran.

water movement is from higher water potential to lower; thus, in dehydrated soil, the roots will lose water. To uptake more water one of the strategies is to accumulate more solutes as osmolytes or osmoprotectants (Pareek et al., 2010). By increasing osmotic potential, water potential will decrease (Verslues et al., 2006; Zhang et al., 1999). In this way roots can uptake water even in dehydrated soils.

Water content as an alternative indicator of shoot water status

To complement shoot water potential measurements, water loss can be measured by water content as a proportion of fresh weight, using a less technical method (Sunkar 2010). Populations exposed to different external water potentials varied in their water content (% WC) (Fig 3). Under unstressed conditions, populations *T. vulgaris* (Germany), *T. vulgaris* (Spain) and *T. zygis* (Spain) had WC over 80%, which decreased by around 10% at the higher levels of drought. On average, water content for watered plants were 85%± 3.7 and for droughted plants were 73.4%±15.5 after the droughted period before wilting. The larger standard deviation for droughted plants (15.5%) indicates more diverse effects of water stress on different populations.

Table 2. Major volatile compounds (thymol and carvacrol) of studied species.

species	Thymol %	Carvacrol %	Reference
T. vulgaris	49.7	48.8	(Morgan 1989)
T. serpyllum	18.7	-	(Nickavar et al., 2005)
T. daenensis	73.9	3.6	(Nickavar et al., 2005)
T. kotchyanous	38.6	33.9	(Nickavar et al., 2005)
T. zygis	23.8	25	(Salgueiro et al., 2010)
T. capitata	39.3	12.7	(Ravid and Putievsky 1986)

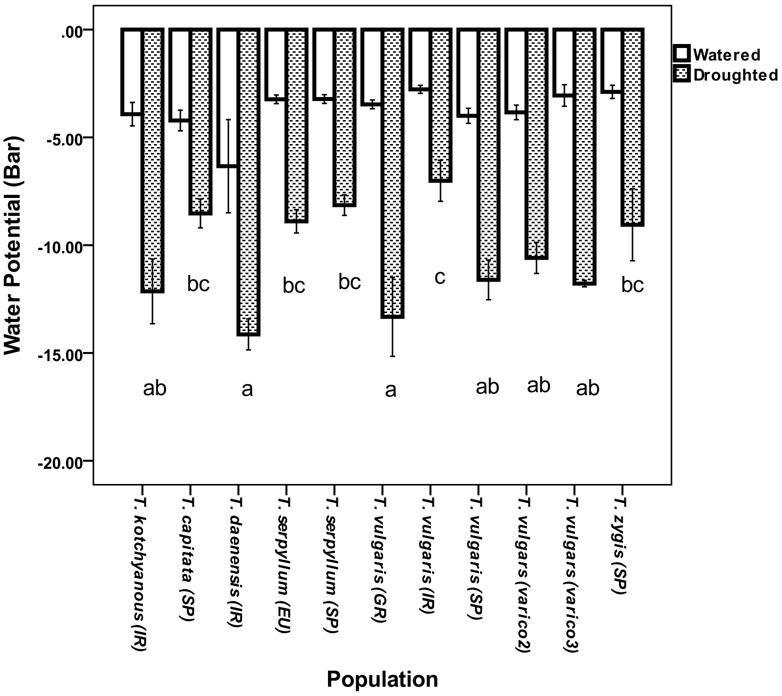

Fig 2. Water potential of shoots from well watered and drought stressed plants. Differences between the well watered plants and the same populations under drought were significant (** $p \leq 0.01$, Student's t test). The Y axis shows water potential in – bar. The largest difference was recorded for *T. vulgaris* (Germany) at around 10 bar (-4 bar decreased to -14 bar) and the smallest for *T.capitata* (Spain) at around 3 bar (-4 declined to -7). Similar letters on bars show the same mean group based on ANOVA and Duncan mean comparison ($p \leq 0.05$). Numbers of replicates=15, Error bars give ±1 SEM. SP: Spain, GR: Germany, EU: Europe, IR: Iran.

The ANOVA followed by Duncan mean separation (DMRT) identified *T. vulgaris* (Spain) and *T. zygis* (Spain) as a lower water content group and *T. serpyllum* (Spain), *T. daenensis* (Iran), *T. kotchyanous* (Iran), *T. serpyllum* (Europe), *T. vulgaris* (Iran), *T. capitata* (Spain) and *T. vulgaris* (Germany) as forming a higher water content group.

The water content (WC) provides a rapid assessment of water in tissues. In some conditions water content is not an adequate measure of water status and water potential should be applied. Tissues with higher lipids have lower water content, and if sampling is done in non-equilibrated (heterogeneous) tissues, in both cases water potential will give a more accurate index of available water (Sunkar 2010). In our study, water potential is more accurate than water content because leaves and stems that used for water content estimation were heterogeneous tissues.

Correlation of shoot water potential and water content

While water potential represents the real water status of the tissue, measuring water potential is technically more difficult. Therefore, correlation between water content and water potential was calculated (Table1).

According to Pearson correlation, there was a significant negative correlation between water potential and water content in all populations except for *T. kotchyanous* (Iran). The highest significant association between WP and WC was observed in *T. serpyllum* (Spain) (-0.91**) and the lowest in *T. zygis* (Spain), which is still significant (-0.44**). Overall analysis of all the data found a significant negative association between water content and water potential.

Since a pressure chamber instrument may not always be available, this correlation can be utilised to predict water potential based on water content using the regression formula in Table 1. This prediction is particularly reliable for *T. serpyllum* (Spain), *T. vulgaris* (Spain), *T. capitata* (Spain), *T. serpyllum* (Europe).

Survivability of thyme populations under drought stress condition

Survival was estimated as the number of days that plants were alive following withholding of water. To follow the progress of soil drying, soil moisture was measured, and plant status was scored against a 5 point index. There was significant variation among populations with *T. serpyllum* (Spain)

Table 3. Plant material obtained from various countries during 2010.

No	Species	Origin	Provided by
1	*Thymus daenensis*	Iran	RIFR*
2	*T. kotchyanous*	Iran	RIFR
3	*T. vulgaris*	Iran	RIFR
4	*T. vulgaris*	Germany	Humber VHB®
5	*T. serpyllum*	Europe	Ball®
6	*T. serpyllum*	Spain	Semillas Silvestres®
7	*Thymus capitatus* (today *Thymbra capitata)*	Spain	Semillas Silvestres®
8	*T. vulgaris*	Spain	Semillas Silvestres®
9	*T. zygis*	Spain	Semillas Silvestres®
10	*T. vulgaris_ cultivar Varico2*	Switzerland	Agroscope ACW
11	*T. vulgaris_cultivar Varico3*	Switzerland	Agroscope ACW

*RIFR(*Research Institute of Forests and Rangelands-Iran)*

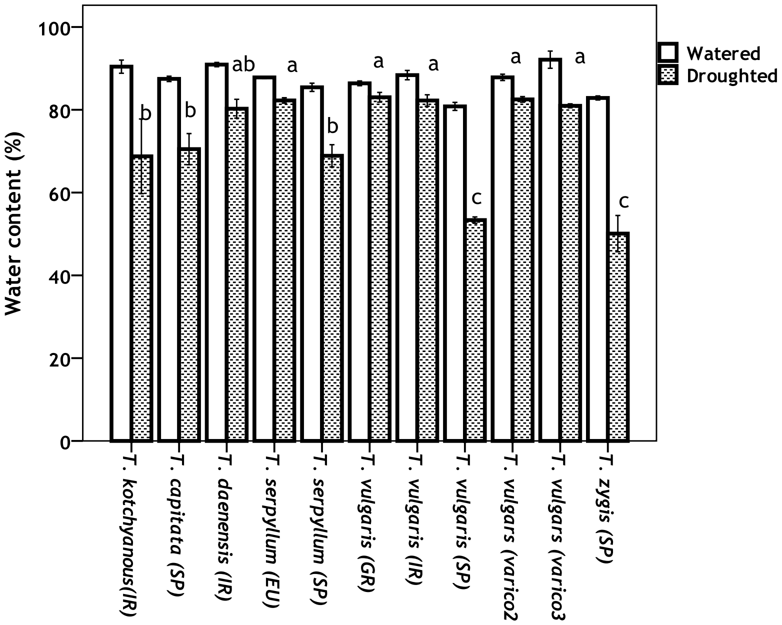

Fig 3. Effect of drought regimes on the water content (WC %) of plant shoots compared to well-watered plants. The WC was calculated as the difference between fresh weight and dry weight divided by fresh weight. The Y axis shows the soil moisture percentage. Similar letters on bars show the same mean group based on ANOVA and Duncan mean comparison ($p \leq 0.05$). Error bars give ±1 SEM. Replicate numbers=4. SP: Spain, GR: Germany, EU: Europe, IR: Iran.

surviving longer than all other populations (22 days), while soil moisture declined to nearly zero percent (Fig 4). The most susceptible population was *T. vulgaris* (Spain), which did not survive beyond 12 days, despite maintaining the soil moisture around 10%. There were both inter- and intra-species differences, in which *T. vulgaris, vulgaris* (Spain) were drought-sensitive, but *T. vulgaris* (VARICO3) was more tolerant. However, both *T. serpyllum* populations were tolerant.

Several criteria have been suggested for screening plants for drought tolerance e.g. water potential (Sinclair and Ludlow 1985), water use efficiency, seedling vigour (Nagarajan and Rane 2000) and survivability (Singh et al., 1999). Survivability is rapid, simple and can be done for large samples of genotypes (Tomar and Kumar, 2004). The approach can accurately be used to distinguish tolerant genotypes from susceptible (Singh et al., 1999). In cowpea, survivability was controlled by one gene, and therefore is free of environmental effects and can be used effectively in plant improvement programs (Mai-Kodomi et al., 1999). Survivability was a central trait in this project, which showed *T. serpyllum* (Spain) as the most tolerant and *T. vulgaris* (Spain) the most susceptible populations evaluated. In contrast, leaf water status traits indicated that the studied populations responded differently to water deficit (Fig. 2 and 4) and that these traits did not necessarily correlate with survival under drought. This variation could be attributed to different underlying physiological mechanisms.

Given the importance of thyme for commercial secondary metabolite production (Stahl-Biskup and Sàez 2002), it is important to consider the effects of drought stress on the populations volatiles studied here. Average volatile content of the species studied is shown in Table 2, based on previous investigations. Some studies have reported a role for secondary metabolites in the adaptability of plants to particular environments (Stahl-Biskup and Sàez, 2002). These studies suggested that plants adapted to dry and hot conditions had enhanced phenolic chemotypes (carvacrol and thymol), but plants with non-phenolic chemotypes (geraniol, a-terpineol, tr-sabinene hydrate or thuyanol-4, linalool) were better adapted to wetter and cooler climates (Stahl-Biskup

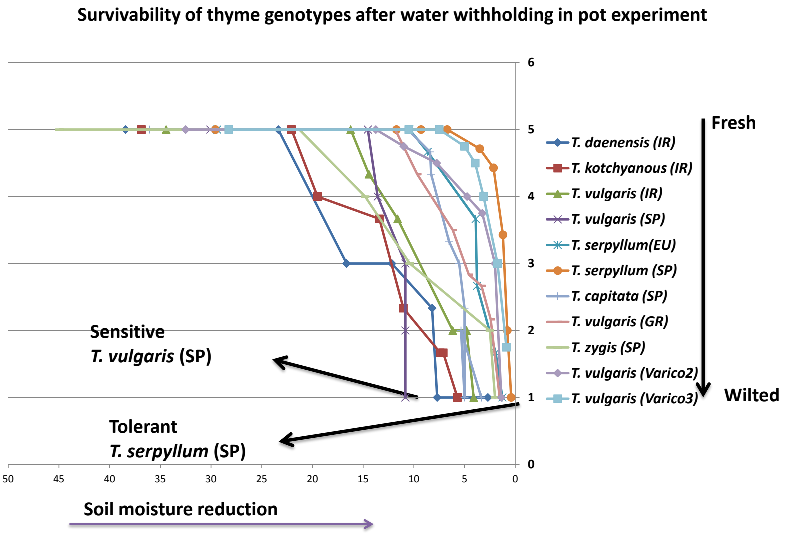

Fig 4. The survivability of 11 thyme populations after withholding the watering. The Y axis shows the drought index from 5- fresh plant to 1- dead plant (as described in materials and methods). *T. serpyllum* (Spain) survived longer than all other populations (22 days) with soil moisture declining to near zero percent. The most susceptible population was *T. vulgaris* (Spain) which did not survive beyond 12 days, despite a soil moisture of around 10%. SP: Spain, GR: Germany, EU: Europe, IR: Iran.

and Sàez, 2002; Echeverrigaray et al., 2001). According to data acquired in this investigation, it appears that no particular trend exists for association of containing phenolic compounds and drought tolerance. The only possible reason for this non-association could be environmental effects on essential oils and these studies reported from different part of the world with different climates. Hence, for accurate correlation, analysis of essential oils for plants of this experiment required.

Materials and Methods

Plant materials, growth conditions and drought treatments

The plant material included eleven populations of 6 different species in the *Thymus* genus (Table 3). Populations were evaluated during the early stage of growth for physiological traits related to drought tolerance, one month after seeds sowing. Seeds were sown in 8cm diameter pots and placed in the growth room with a 16:8 cycle and a temperature of 22^{0}C and watered weekly with tap water. Soil mixture was 4 parts Humax Multipurpose peat based compost mixed in 1 part perlite with Intercept 70wg insecticide added at 0.02 kg/m^3 compost, and about 120 g soil in each pot. Drought stress was applied by withholding water from the day 30th.

Traits measured

The growth, survivability, shoot water potential and water content were recorded before, during and after drought treatment. Root to shoot ratio was used to compare the growth rates of different populations and their response to drought stress. To measure survivability, plant status was recorded daily after withholding water according to the following index: 5 (fresh plants), 4 (some leaves lost due to wilting), 3 (some leaves with dried tips), 2 (more than half the plant dried) and 1 (completely dried). Soil moisture was recorded daily using a sensor model SM300 by Delta-T Devices Ltd. The SM300 measures volumetric soil moisture content with 2.5% accuracy. This sensor with hand-held meter was used to record soil moisture by inserting the sensors in pot soil to a depth of 5 cm.

Shoot water potential was measured using a pressure chamber (Skye Company Model SKPM 1400) and shoots of 10-30 mm length. To record the water potential (before and after drought) stems were cut and sealed into the chamber. Chamber pressure was increased at a rate of bars per minute until sap was observed exuding from the cut point of the stem. This pressure represents the magnitude of the xylem tension present before excision.

Statistical analysis

Data were analysed using an unbalanced one-way analysis of variance with 3 to 15 replicates depending on trait analysed. Trait mean comparisons were performed using DMRT (Duncan's New Multiple Range Test). All analyses of variance, post-hoc and also regression and correlation utilised SPSS 19.0.

Conclusion

Overall, these series of physiological experiments have clearly identified *T. serpyllum* as more tolerant to drought than *T. vulgaris*. Within the *T. vulgaris* population, the population *T. vulgaris* from Spain was the most susceptible and *T. vulgaris* (Germany) the most tolerant. The survivability can offer a powerful tool to screen populations for drought tolerance at early growth stage of thyme even on a large scale. These findings are useful for further identification of germplasms to underpin breeding and selection programmes with aim to improve *Thymus* for drought stress. In addition, future work required to identify the underlying molecular mechanisms responsible for the responses to drought. Unravelling these responses will be possible with novel methods like transcriptomics, proteomics and metabolomics.

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