International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 5, No. 10, p. 134-145, 2014

RESEARCH PAPER OPEN ACCESS

An evaluation of germination efficiency in a range of genotypes of *Thymus* species differing in susceptibility to drought

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Key words: Water Deficit, PEG, Thyme, Germination, drought.

http://dx.doi.org/10.12692/ijb/5.10.134-145 Article published on November 26, 2014

Abstract

The aim of this study was to compare the germination of a range of *Thymus* genotypes subjected to different levels of drought stress, in order to identify differences in germination response to water stress, important to inform plant breeders aiming to produce more drought tolerant crops. Germination is the first, and arguably the most important and vulnerable stage in the life cycle of plants. *Thymus spp* seeds from a range of genotypes were germinated under four external osmotic potentials (0, -2, -4 and -6 Bar) imposed using appropriate concentrations of PEG6000. Following 10 days of exposure to drought, fresh weight, dry weight, radicle and plumule length, water content, percentage germination and germination time were recorded. There was considerable variation between the genotypes in their response to drought. Under mild stress (-2 bar) *T. serpyllum* and *T. vulgaris* (Iran) were more tolerant than other varieties. However, under moderate stress (-4 bar), *T. serpyllum* (Spain) was the most tolerant. Under -6 bar (severe stress), *T. vulgaris* (Spain), *T. zygis* (Spain) and *T. kotchyanous* (Iran) were scored as tolerant.

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Introduction

Drought stress is the main environmental factor that limits plant production worldwide (Boyer, 1982). Water deficit stress like other environmental factors might compromise seedling establishment (Albuquerque and De Carvalho 2003). Plant breeders have improved some varieties to be more drought tolerant by way of improved understanding of the plant response to water deficit stress, but in spite of considerable effort, there are still many unknown areas in the responses of plants to water stress (Lisar, 2011).

Germination and seedling establishment are the first, arguably the most important 'unknown' but this is also the most vulnerable stage in the life cycle of plants (Benech-Arnold and Sanchez, 2004). Low germination rate followed by reduced seedling establishment will result in yield loss for most plants (Hegarty, 1984). Moreover, plant yield is influenced by the timing, pattern and amount of seedling establishment (Finch-savage, 1995), and in this respect seeds take in water in three main stages consisting of rapid-pause-rapid water uptake (Bewley and Black, 1994). The first and second stages of water uptake can occur in both dead and viable seeds, while the last stage occurs only in viable seeds. The first rapid water uptake is known as imbibitions, a physical process. Hydration of cell walls and cell particles generate matric forces to uptake water in the imbibition stage. Too rapid imbibition can cause damage to the seed (Woodstock, 1988; Vertucci, 1989; Finch-Savage, 1995). Several factors influence the amount of water uptake at this stage: soil water potential, soil hydraulic properties and seed composition (Bradford, 1995). In the second stage of water uptake, despite constant seed water content and respiration, various metabolic processes occur (Koller and Hadas, 1982. Water deficit at this stage might result in germination inhibition subsequent to seed drying. The last stage also known as the growth stage occurs alongside the radicle emergence. During this stage, respiration rate increases, cell division begins and finally the radicle emerges (de Miguel and Sanchez, 1992; Ni and Bradford, 1992; Bradford, 1995). The minimum water volume required for germination is referred to as critical water content and the water potential known as critical water potential (Hadas, 1970; Koller and Hadas, 1982). Critical water potential identified for various plants show values for corn, rice, pea and clover seeds as -1.25, -0.79, -0.66 and -0.35 MPa respectively (Benech-Arnold and Sanchez, 2004).

Water stress can be imposed on seeds in a number of ways. One of the frequently used methods to impose low water potential involves use of solutions of high molecuar weight solutes, however it has been demonstrated that mannitol and melibiose are not appropriate, since they are taken up by plants and can damage the roots (Verslues *et al.*, 1998). PEG (polyethylene glycol) is an alternative and an advantage of this method is that water potential can be accurately imposed, with minimal toxic effects (Verslues *et al.*, 2006). PEG has been used successfully to screen different populations and varieties of plants such as wheat (Baalbaki *et al.*, 1999), common bean (Hucl, 1993), barley (Al-Karaki, 1998) and pea (Okcu *et al.*, 2005). For *Thymus* species, Bagheri *et al.* (2011) and Khoshsokhan *et al.* (2012) used solutions of PEG 6000 to compare seed germination under water stress for *T. kotschyanus* and *T. daenensis* species (Khoshsokhan, 2012; Bagheri *et al.*, 2011). They found a significant decline in germination of both species, but their results regarding identification of tolerant material are conflicting, since they reported opposite tolerant population. Hence, our investigation has been conducted with a comprehensive evaluation at the germination stage under the same experimental conditions.

Understanding the response of plants to water deficit is of great importance, providing information to improve drought tolerance, particularly in screening germplasm for useful variation (Reddy, Chaitanya *et al.* 2004). In our study we used PEG 6000 to screen 9 populations of several different species of thyme by measuring early germination and seedling traits to categorise them as tolerant or susceptible.

Materials and methods

Plant material was examined across nine populations of thyme genotypes encompassing 5 different species from the *Thymus* genus (Table1).

Different osmotic potential solutions were made using PEG6000 (polyethylene Glycol 6000 MW) to make four water stress treatments: 0, -2, -4 and -6 bar PEG. Seeds of each of the 9 populations were sown on filter paper in petri dishes moistened (10 ml of solutions) with each of the 4 PEG treatments and replicated three times. Each petri dish contained 20 seeds of each population. The experiment was conducted in a factorial completely randomized design (CRD) with populations as a main factor and PEG levels as a sub-factor (Steel and Torrie, 1980).

Dishes were sealed with lab film and incubated in a controlled environment room at 22° C. From day 2 the number of germinated seeds was counted daily. After 10 days, the traits of fresh weight, dry weight, radicle and plumule length were recorded. Dry weight was measured after drying at 70 degree Celsius for 48 hours. Subsequent data analysis was performed by SPSS 19 and Minitab 15.

Results

Evaluation of seedling growth factors

Imposing drought using PEG had a significant effect on all traits studied (Figure 1). Radicle/plumule ratio increased in the -2 and -4 bar treatments compared to unstressed controls. Analysis of variance (ANOVA) indicated (Table 2) significant differences between the populations for all traits ($p<0.01$). Fresh and dry weights were both significantly increased following incubation at -2 bar but was decreased at -4 and -6 bar. Subsequently, the treatment means were compared with Duncan's New Multiple Range Test, to order the treatment means.

Table 1. Plant materials accessed from different 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. vulgaris*	Spain	Semillas Silvestres®
6	*T. serpyllum*	Europe	Ball®
7	*T. serpyllum*	Spain	Semillas Silvestres®
8	*T. serpyllum*	Spain	Semillas Silvestres®
9	*T . zygis*	Spain	Semillas Silvestres®

* RIFR : Research Institute of Forest and Rangelands, Iran.

Because there was a significant interaction (different behaviour of populations to treatment levels), statistical comparisons were not performed. For instance, in a low water potential level -2 bar, ranking order of germination percent were *T. zygis*, *T. vulgaris* (Germany), *T. vulgaris* (Iran) and *T. vulgaris* (Spain). While at -4 level, population ranking order were different from above; order was *T. vulgaris* (Iran), *T. vulgaris* (Spain), *T. vulgaris* (Germany) and *T. zygis* (Spain). However, for an overall view of the response of the different populations, the mean response of traits to different PEG levels is shown in Figure 2.

Percentage germination decreased with decreasing (more negative) osmotic potential for all species. However some differences were noted between genotypes; all of*T. vulgaris* and *T. zygis* (Spain) species had similar germination as the control at -2 bar and *T. serpyllum* (Spain) has the same reaction. No difference in dry weight was observed between stressed and control plants of *T. kotchyanous* (Iran), *T. zygis* (Spain) and *T. vulgaris* (Spain). The remaining species had similar dry weights at 0, -2 and -4 bar, but all decreased at a stress of -6 bar, except for *T. serpyllum* (Spain) (Figure 2.b). Water content decreased in all species (Figure 2.c). Water stress increased radicle/plumule ratio in all species, apart from *T. serpyllum* (Spain) in which it declined sharply (Figure 2.d). There were no significant differences in mean germination time between the control and -2 bar drought, but as stress was increased to the -4 bar level, *T. zygis* (Spain) had increased Mean Germination Time. Some species such as *T. serpyllum* (EU) had significantly decreased MGT, but others had similar MGT until stress reached –4 bar. Interestingly at -6 bar stress, *T. vulgaris* (Spain) had increased MGT while it decreased in all other species.

Table 2. Analysis of variance in 9 populations treated by 4 levels of PEG6000.

Source	DF	Mean of squares						
		fresh weight	dry weight	radicle	plumule	rad/pl	Germ. percent	Water content
Species	8	0.22**	0.22**	481.38**	28.64**	23.48**	2092**	2169**
Treat	3	1.14**	1.13**	2115.23**	132.03**	79.86**	25555.7**	3371.1*
speciesxtreat	24	0.12**	0.12**	146.52**	7.25*	17.67**	347.1**	334.6**
Error	71	0.04	0.05	18.37	0.77	6.41	144.9	124.9

Effect of drought on percentage germination

There was considerable variation in germination between populations (Figure 3). Under the highest stress (-6 bar osmotic potential), *T. vulgaris* (Spain) and *T. zygis* (Spain) had the highest germination. *T. daenensis (Iran)* and *T. serpyllum* (Spain) showed no germination at -6 bar stress while the other populations had intermediate germination. Treatment means were compared using DMRT. *T. vulgaris* (Spain) was less affected by osmotic stress having the same germination at treatments 0, -2 and -4 bar osmotic potential but lower germination at -6 bar level.

Time course of germination

Percentage germination was evaluated over time for seeds under control, mild, medium and severe water stress. In control conditions most populations showed a similar pattern (Figure 4) with 60-90% of the seeds germinating during the first 3 days. However, *T. serpyllum* (EU) and *T. serpyllum* (UK) germinated more slowly over the nine days of the study. At an osmotic potential of -2 bar, seeds of all populations germinated, but the maximum germination was less than 90%. Some populations, such as *T. vulgaris* (Spain) had almost the same germination behaviour as the controls, and could be defined as tolerant at this level of drought.

At moderate water stress, -4 bar, all populations showed some germination. However, the *T. serpyllum* (UK, Spain, EU) populations were most affected, having the lowest germination. Under more severe drought stress (-6 bar), *T. serpyllum* (Spain) and *T. daenensis* (Iran) showed no germination. In contrast, the populations of *T. vulgaris* (Spain) and *T. zygis* (Spain) had the highest germination among the populations studied at that level of stress (Figure 4).

Classification of populations

To achieve a comprehensive analysis of the action of each osmotic potential, cluster analysis was performed using a Euclidean distance and Ward clustering method (Mohammadi and Prasanna, 2003; Ward, 1963) (Fig5). At an external water potential of -2 bar, 4 populations could be categorized as tolerant: *T. serpyllum* (EU), *T. serpyllum* (UK), *T. serpyllum* (Spain) and also *T. vulgaris* (Iran). *T. zygis* (Spain) and *T. vulgaris* (Spain) were moderately tolerant while all the others were classified as susceptible. A similar analysis was carried out at -4 bar stress but excluding traits radicle/plumule and water content as they are products of linear combinations of other traits. This analysis classified nine populations into 3 groups (sensitive, moderate and drought tolerant). In this analysis, *T. serpyllum* (Spain) was the most susceptible and *T. kotchyanous* (Iran), *T. vulgaris* (Spain), *T. zygis* (Spain), *T. vulgaris* (Germany) and *T. daenensis* (Iran) were the most tolerant species. The other genotypes, (*T. vulgaris* (Iran), *T. serpyllum* (EU) and *T. serpyllum* (UK)) were classified as moderately tolerant. However, populations had different responses at osmotic stress levels. For instance, *T. daenensis* (Iran) was sensitive at all levels, while *T. serpyllum* (Spain) was tolerant at both -2 and -4 bar but appeared sensitive at -6 bar. Likewise, *T. vulgaris* (Spain) was moderately susceptible at -2 and -4 bar but was classified as tolerant at -6 bar.

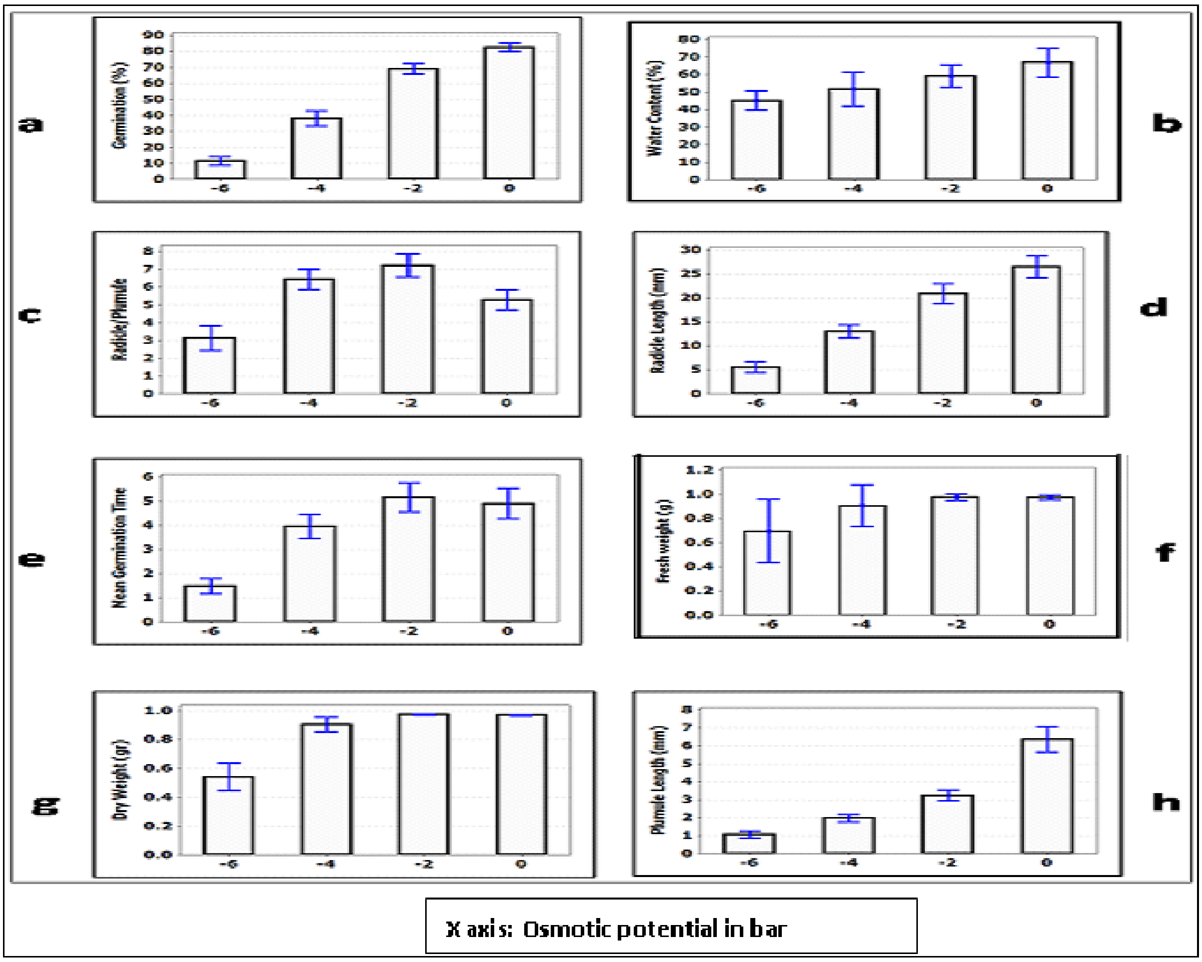

Fig. 1. Four levels of PEG induce changes in all 8 recorded morpho-physiological traits across the nine thyme populations. a) Germination percentage % b) Water content % c) Radicle/Plumule ratio d) Radicle length (mm) e) Mean Germination Time f) Fresh Weight (gr) g) Dry Weight (gr) h) Plumule Length (mm). Treatments were imposed as described in the material and methods. Fresh weight and dry weight were not significantly affected by -2 bars, but were decreased in moderate and severe stress. Mean germination time (MGT) and Radicle/Plumule increased at -2 and -4 bar and decreased under severe osmotic stress. Other traits declined along with stress dose increase. X-axis shows stress levels in bar, where 0 is control and -6 bar is the highest level of osmotic stress. Error bar are means ± SEM.

Germination has different components and hence can be regarded as multivariate. We therefore recorded various traits, and to understand the underlying data structure and/or form, a smaller number of uncorrelated variables (for example, to avoid multicollinearity in regression), principal component analysis was carried out on the -2, -4 and -6 bar data and results summarized as score plots of individuals for the first two principal components (Figure 6). The results of the PCA analysis agreed with the cluster analysis grouping.

The traits involved in this analysis were water content, germination percentage, radicle length, plumule length, radicle/plumule ratio and fresh weight. PCA as an unsupervised method breaks down the large data set to PC1 and PC2. PC1 described the largest variation in the data, which discriminates a drought-tolerant group with a large score for this component and susceptible group with a small score. PC2 had the second largest variation, orthogonal to PC1. PC1, explaining 69% of the total variation, clearly separated population groups according to their tolerance. PC2, describing 16% of existing variation, could further separate populations within the tolerant group (Figure 6).

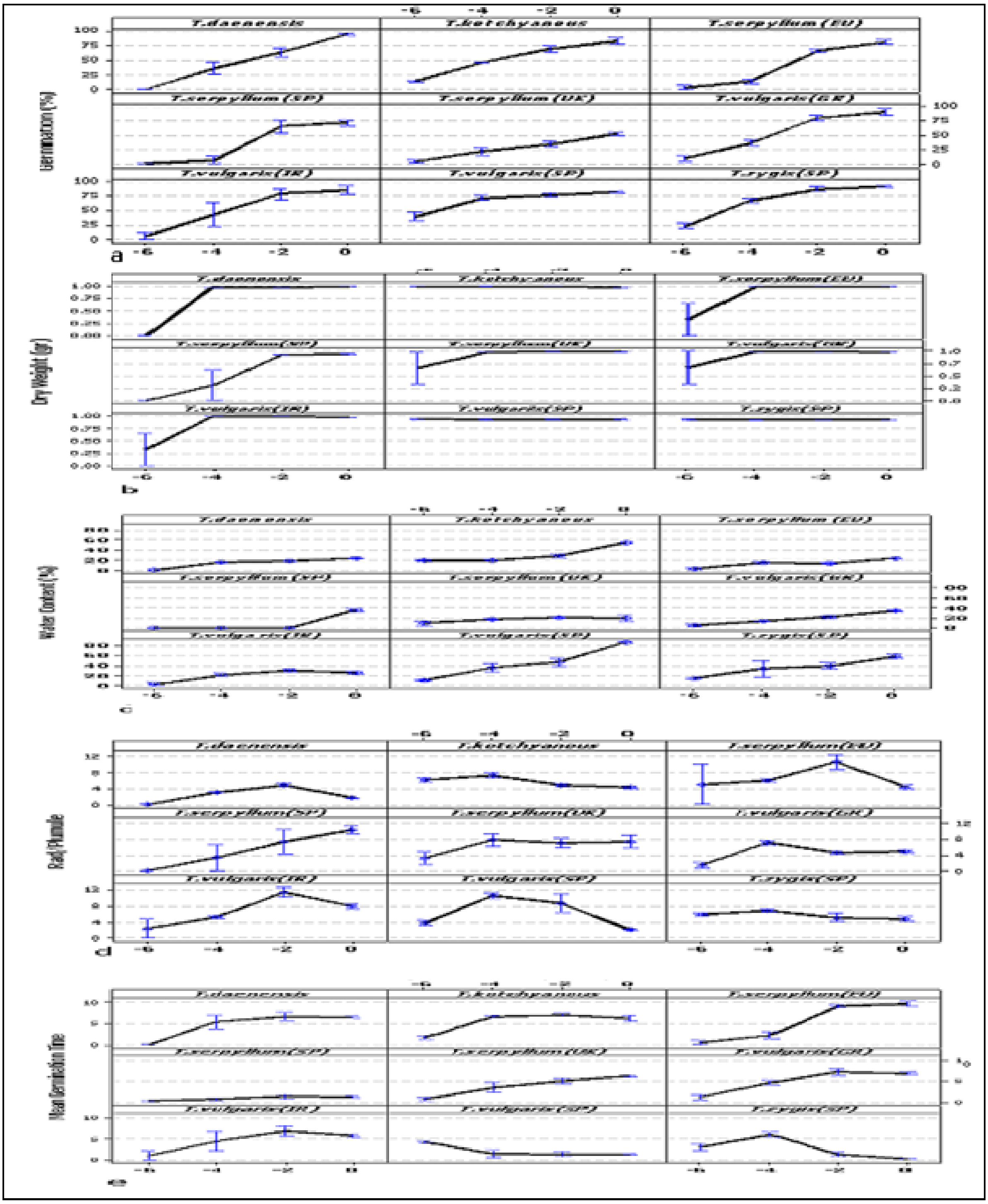

Fig. 2. Effect of water deficit stress on germination and seedling traits in nine populations of thyme. a) Germination percentage (%): Germination percentage decreased with increasing osmotic potential for all the species.. b) Dry weight (gr): There was no difference among stressed and control plants in *T. kotchyanous (Iran)*, *T. zygis* and *T. vulgaris* (Spain). Other species had different trend. c) Water content (%): in all species the water

content declined steadily, except for *T. serpyllum* (EU) and *T. vulgaris* (Iran). d) Radicle/Plumule ratio: water stress increased this ratio in all species, but only in *T. serpyllum* (Spain) it declined sharply. e) Mean Germination Time: there were no significant difference between the control and -2 bar, but with increasing stress to -4 bar, populations exposed diverse behaviour. The X-axis shows osmotic potential in bar and 0 is control condition without any PEG. Error bars are ± 1 SEM. Number of replicates varies from 3-15.

Discussion

Each population showed specific behaviour at different stress levels. For instance, all the *T. serpyllum* populations were tolerant to mild stress, but in severe stress, *T. serpyllum* (Spain) did not germinate. In contrast *T. vulgaris* (Spain) and *T. zygis* (Spain) were tolerant to water stress at all levels. *T. daenensis* (Iran) appeared as the most sensitive at all levels of stress, while at mild and moderate stress, *T. serpyllum* (Spain) was the most tolerant. Finally at severe stress, the most tolerant was *T. vulgaris* (Spain).

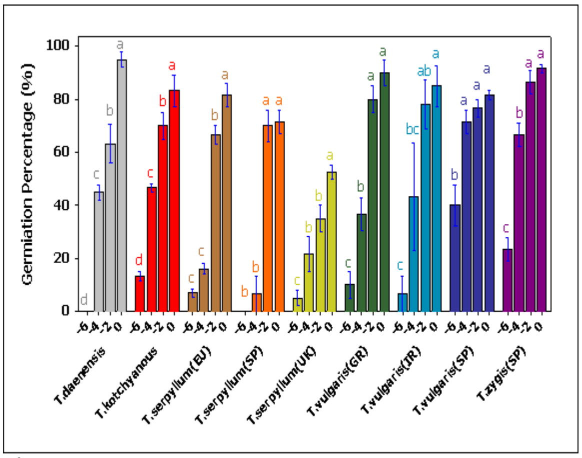

Fig. 3. Responses to PEG levels at germination phase for 9 different populations of thyme.

Ten days after placing the seeds on filter paper, the percentage of germinated seeds (out of 20) was counted in 3 replicates. There was no significant difference for the germination of this population at the applied levels of 0, -2 and -4 bar osmotic stress but a difference was seen at -6 bar. Error bars are Mean± SEM with 3 replicates and letters on the bar show the same group (i.e. means that are not significantly different from one another) within the same population shown with the same colours.

There are several investigations involving the use of PEG to induce controlled drought stress in plant species. Murillo-Amador *et al.* (2002) indicated a significantly decreased germination percentage in cowpea (Murillo-Amador *et al.*, 2002) with similar results demonstrated in pea (Okcu *et al.*, 2005). PEG has advantages compared to other methods of dehydration, as it does not damage plant roots and it imposes low water potential stress more likely reflecting the type of stress caused by water loss from the soil (van der Weele *et al.*, 2000; Verslues *et al.*, 1998). It is also accurate and reproducible (Verslues *et al.*, 2006). One limitation of using PEG can be uptake by the plant, as shoots (with rate of 1 mg/g fresh weight per week) take up PEG slower than the roots (Lawlor, 1970). But in this relatively short-term experiment of 10 days, the problem of uptake is likely to be less than in pot growth experiments. Likewise, we have assessed our plants at the germination phase, which initially depends on cell expansion in the radicle.

Apart from the radicle/plumule ratio (increasing with more stress), the remaining traits declined with decreasing osmotic potential. Thus water stress imposed by PEG inhibits germination and other seedling growth parameters apart from at -2 bar. Similar results have been observed in plants such as pea (Okcu *et al.*, 2005) and rice (Pirdashti *et al.*, 2003) which had similar experimental procedures. In thyme (considering just *T. daenensis* and *T. kotchyanous*) Bagheri *et al.* (2011) reported no change in germination on PEG induced -3 bar water stress, and Mean Germination Time (MGT) was unaffected at -3 bar, increased at -6 bar and decreased at -9 bar. Other traits such as root and shoot length were reduced at more negative osmotic potentials (Bagheri *et al.*, 2011). In our study, germination percentage as well as other traits decreased with greater stress. The variation observed in our study between species may provide valuable germplasm for plant breeders, and also scientists investigating drought mechanisms in this genus (Chaves *et al.*, 2002).

The results of the present study on germination percentage (Figure 3) showed general agreement with previous studies on *Thymus* species germination, in *T. daenensis* (Iran) and *T. kotchyanous* (Iran) (Khoshsokhan, 2012; Bagheri *et al.*, 2011), as those populations had around 90 percent germination under control (well watered) conditions and as *T. kotchyanous* (Iran) was more tolerant than *T. daenensis* (Iran) under severe water deficit condition (-6 bar osmotic potential).

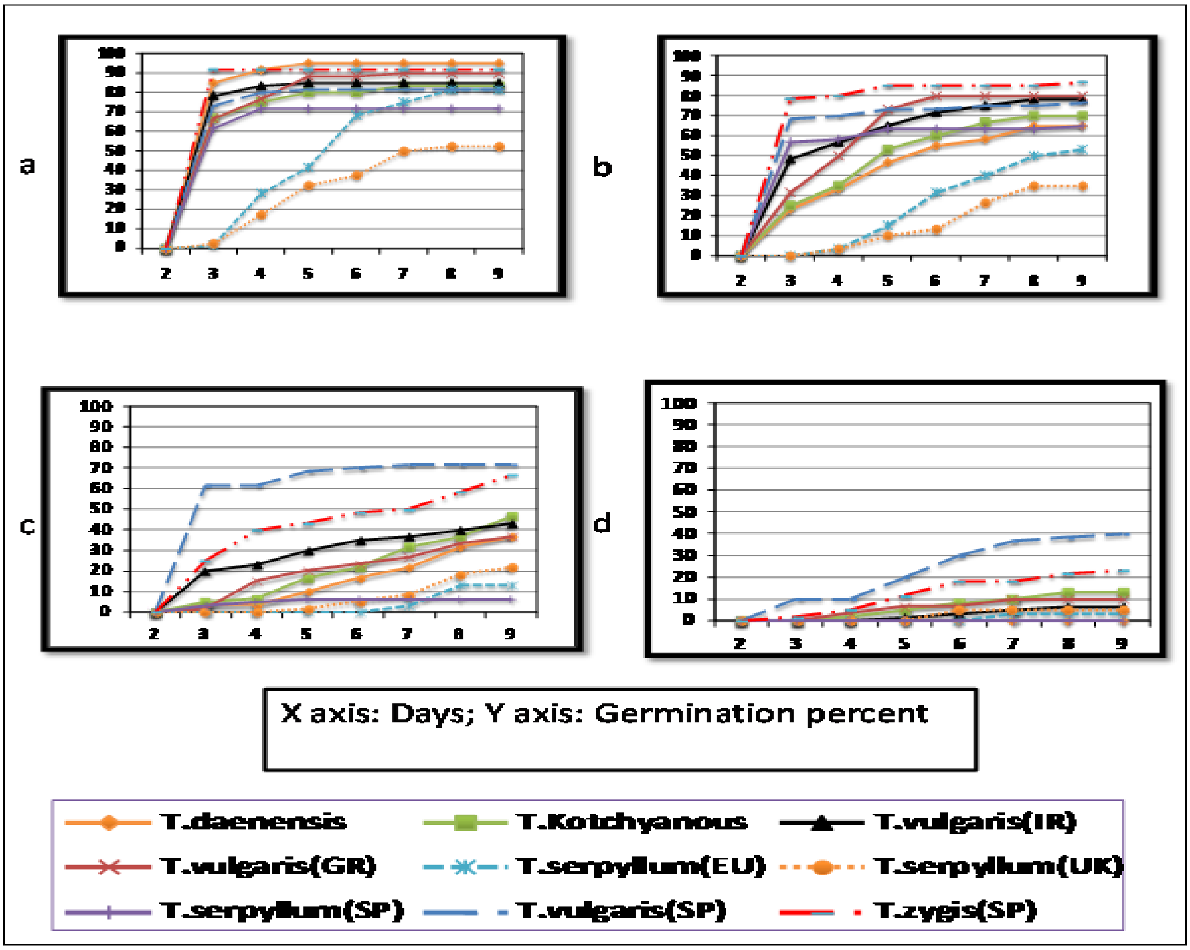

Fig. 4. Monitoring germination percentage in 9 populations of thyme seeds at different levels of water stress. a) Control condition (no PEG added). b) Osmotic potential -2 bar. c) Osmotic potential -4bar. d) Osmotic potential -6 bar. X axis shows the days after sowing the seeds on plate and Y axis indicates the counting number of germinated seeds on that day. In control conditions, most populations germinated about 40% during first 4 days, but they reached 50-95 % germination at the end.

To our knowledge there is currently no study screening different species of thyme under water stress for more than 2 species at germination level. An interesting point is the conflicting results of two published works; Bagheri *et al.* (2011) compared *T. daenensis* and *T. kotchyanous* and concluded that, based on germination, *T. kotchyanous* was more tolerant than *T. daenensis* (Iran), agreeing with our study, (Bagheri *et al.*, 2011), while Khoshsokhan *et al.* (2012) concluded the opposite (Khoshsokhan, 2012). The former used treatment levels from -3 to -18 bar while the latter used just -3 to -9 bar, even though both used PEG 6000 to impose the stress. The contrasting results might be explained by the use of different ecotypes of the species in the two studies, in addition to different treatment levels.

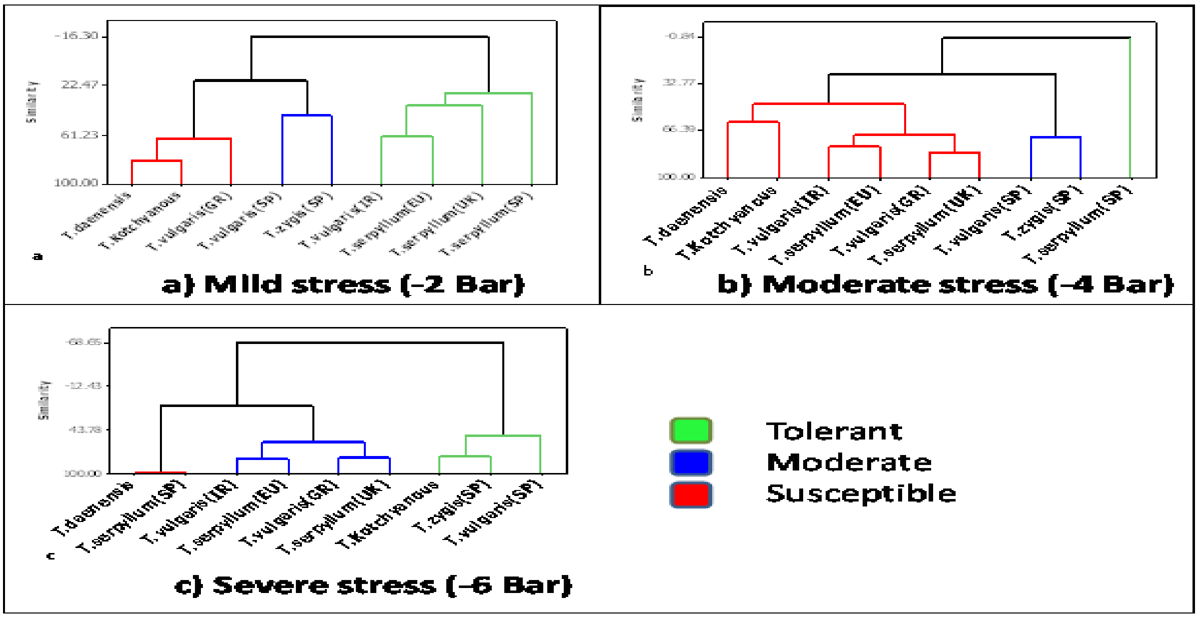

Fig. 5. Classification of nine thyme populations using cluster analysis based on all traits recorded in 3 levels of osmotic potential. X-axis shows the populations and Y-axis indicates the similarity between the individuals. To do this analysis, 6 traits were entered which were the following: fresh weight, germination percentage, radicle length, plumule length, radicle/plumule ratio and water content. Cluster analysis classified all the populations in mild stress conditions (a), moderate stress (b) and severe stress conditions (c).

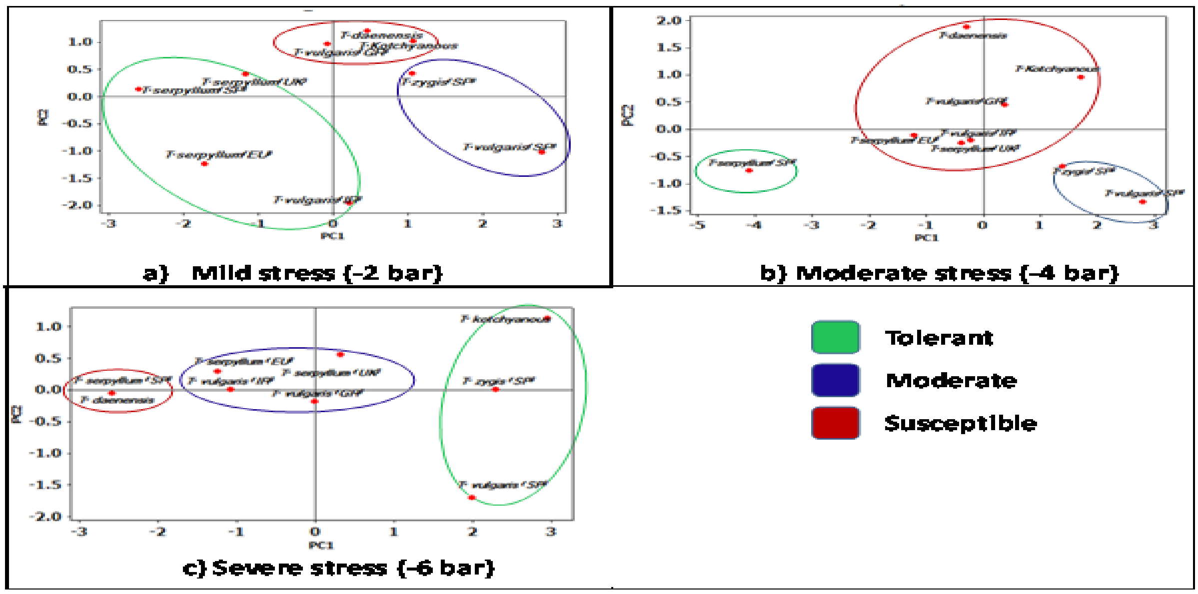

Fig. 61. Principal component analysis of 9 population of thyme based on 6 traits. The traits for PCA analysis were: Water content, Germination percentage, Radicle length, Plumule length, Radicle/Plumule Ratio and Fresh weight.

Conclusion

In conclusion, this experiment demonstrated that different concentrations of PEG effectively imposed different osmotic potential levels in water and results revealed distinct genetic differences between populations respect to their germination.

Further investigations including transcriptomics, proteomics and metabolomics could be undertaken to understand the underlying mechanisms of tolerance to drought as revealed in these genotypes, and in addition, confirmation of these responses requires evaluation under field conditions. Neverthless we are able to propose that under severe stress, *T.vulgaris* (Spain), *T.zygis* (Spain) and *T.kotchyanous* (Iran) can be considered as valuable material for the future development or selection of drought resistant *Thymus* plants.

Acknowledgments

I would like to acknowledge Islamic development bank and RIFR for financial support to do my PhD thesis.I would also like to express my gratitude to my senior colleagues Dr. Laura Vickers, Dr. Teresa Dilks, Dr. Younuss Saidi, Dr. Rashed Hossein and Fay Hughes for their helps to do this project.

Abbreviations

RIFR: Research Institute of Forest and Rangelands; PEG6000 (Poly Ethylene Glycol 6000 MW); CRD: Completely Randomized Design; C: Concentration; T: Temperature; WC: Water content; UK: United Kingdom; EU: Europe; DMRT: Duncan's New Multiple Range Test; MGT: Mean Germination Time; MPa: Mega Pascal.

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