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Determination of cardinal germination temperatures of two ecotypes of *Thymus daenensis* subsp. *daenensis*

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Summary

Thymus daenensis is an important endemic medicinal plant of Iran. In the present study, the germination behaviour of two ecotypes of *T. daenensis* subsp. *daenensis*, Elam and Isfahan, was studied at various temperatures (3 to 40°C) to (1) calculate cardinal temperatures and (2) determine whether germination behaviour varied between ecotypes. To determine the cardinal temperatures, the relationship between germination rate and temperature was considered. Temperature had a significant effect on both final germination percentage and germination rate in both ecotypes. The optimum germination rate occurred within the temperature range 11.2 to 24.3°C for the Elam ecotype and 23 to 34.6°C for the Isfahan ecotype. The calculated base temperatures (T_b) for the Elam and Isfahan ecotypes were between 0.3 and 1.3°C and 1.3 and 4.4°C, respectively. The ceiling temperature (T_c) for the Elam and Isfahan ecotypes was 40.9 and 42.3°C, respectively. The Elam ecotype appears to be adapted for germination at lower temperatures to avoid summer drought and maximise the benefit of water availability in winter. Conversely, the Isfahan ecotype was adapted to germinate at higher temperatures.

Introduction

Germination behaviour is an important part of a species regeneration strategy (Baskin and Baskin, 1998). To minimise the risk of failure to establish, seeds have mechanisms to sense their surroundings and can respond when conditions are favourable (Baskin and Baskin, 1998; Koutecká and Lepš, 2009). Germination is influenced by a range of inherent and environmental factors (Shim *et al.*, 2008; Koutecká and Lepš, 2009). External factors that are important during the germination process include amount and quality of light (red/far-red ratio), temperature, moisture availability, the chemical environment and gaseous exchange (Zalucki and Daws, 2008). Among these factors, temperature is the most important, affecting the maximum germination percentage and rate of germination (Phartyal *et al.*, 2003). Germination may be controlled by a single factor or by a

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combination of factors. If climatic factors such as temperature and precipitation change in a region beyond the tolerance of a species' plasticity, changes in species distribution may be unavoidable. It has been shown that plant species have shifted their altitudinal and latitudinal ranges in response to climate change (Parmesan and Yohe, 2003), but there is also likely to be a rapid increase in extinction risk (Thomas *et al.*, 2004). Furthermore, species might face possible extinction due to habitat degradation. A number of wild medicinal and aromatic plants are facing possible extinction and the mounting demand of these plants necessitates domesticating and propagating them on a large scale (Bannayan *et al.*, 2006).

Thymus daenensis subsp. *daenensis* Celak. is an aromatic and medicinal plant of Iran which is used widely in traditional medicine. The genus *Thymus* belongs to the Lamiaceae and includes about 215 species of herbaceous perennials and sub-shrubs. The Mediterranean area is the centre of origin of this genus. In Iran, this genus is represented by fourteen species/subspecies, five of which (*T. daenensis* subsp. *daenensis*, *T. daenensis* subsp. *lancifolius* (Celak.) Jalas, *T. carmanicus* (Jalas.), *T. persicus* (Roniger ex Reach. F.) and *T. trautvetteri* (Klokov and Desj-Shost)) are endemic. *Thymus* species are commonly used as herbal teas, flavouring agents (condiment and spice) and for medicinal purposes (Stahl-Biskup and Saez, 2002; Baranauskiene *et al.*, 2003).

The ability to predict the germination time has a critical role in determining seedling establishment in both natural and cropping systems (Wang *et al.,* 2004). Thermal time models have considerable potential to characterise and quantify the effects of seedbed environments on seed germination and seedling emergence (Kurtar, 2010). Naylor (2007) used segmented regression to describe genotype-environment interactions for identifying ecological thresholds. Plant growth, crop yields and physiological changes clearly depend on mean temperature and temperature extremes in plant's ecosystem. A thermal time model uses temperature for predicting seed germination and the model can be applied to a wide range of conditions. The goal of the present research was to determine the cardinal temperatures and germination behaviour of two ecotypes of *T. daenensis* subsp. *daenensis* which are found in different climatic conditions.

Materials and methods

Seed material

Experiments were initiated in February 2012. Seeds were collected in 2011 from two natural habitats in different regions of Iran (table 1), Elam ecotype from Mehran region and Isfahan ecotype from Daran region. The seeds were globose with mucilage on the seed coat, brown or black, and the mean thousand-seed weight was 0.5 and 0.1g for the Elam and Isfahan ecotypes, respectively.

After collection, insect-damaged and immature seeds were removed. Seeds were surface-sterilised by soaking in 1% sodium hypochlorite (NaOCl) for three minutes and washed thoroughly with sterilised water. All germination experiments were conducted using four replications of 50 seeds for each treatment. Seeds were placed on double layered Whatman no.1 filter paper moistened with 5 ml distilled water in sterilised Petri

Table 1. Meteorological data of Mehran and Daran regions in Iran where seeds of two ecotypes of *Thymus daenensis* subsp. *daenensis*, Elam and Isfahan, were collected[1].

Meteorology	Ecotype (location)	
	Elam (Mehran)	Isfahan (Daran)
Latitude (N)	33°38'	32°58'
Longitude (E)	46°26'	50°22'
Altitude [m a.s.l.]	370	2290
Mean annual temperature (°C)	24.3	10.8
Mean annual rainfall (mm year^{-1})	195	48
Mean maximum monthly temperature (°C) (month)	49.2 (Aug)	36.3 (Jul)
Mean minimum monthly temperature (°C) (month)	-3.4 (Jan)	-24.6 (Jan)
Mean maximum monthly temperature (°C) (month)	49.2 (Jul)	36.3 (Jul)
Maximum monthly rainfall (mm) (month)	48.6 (Jan)	72.3 (Mar)
Minimum monthly rainfall (mm) (month)	0.0 (May-Jul)	0.9 (Sep)

[1] Source of data: Islamic Republic of Iran Meteorological Organization (IRIMO)

dishes (9 cm-diameter). The filter paper was re-wetted periodically to prevent the seeds from drying out. Petri dishes were placed at 3, 5, 10, 15, 20, 25, 30, 35 and 40°C in dark incubators with 70-75% relative humidity. Seeds began to germinate after 24 hours; germinated seeds were counted and removed every 24 hours until germination finished in all treatments (60 days). A seed was considered germinated when the tip of the radicle had grown free of the seed coat (Auld *et al.*, 1988).

Data analysis and model fitting

In this study, final germination percentage (*FGP*) and germination rate (*GR*) were recorded to evaluate germination performance. Germination rate was calculated by using the following equation:

$$GR = {}^{1}/_{t_{50}}$$

where t_{50} is the time required to reach 50% of the final germination percentage.

The *GR*-temperature relationship was used to determine cardinal temperatures. For this purpose, the triangle (intersected lines) regression model was fitted and the following formulas were used:

$$f(T) = \frac{(T - T_b)}{(T_o - T_b)} \qquad if \quad T_b < T \leq T_o$$

$$f(T) = [1 - \frac{(T - T_c)}{(T_c - T_o)}] \qquad if \quad T_o \leq T < T_c$$

$$f(T) = 0 \qquad if \quad T \leq T_b \quad or \quad T \geq T_c$$

where $f(T)$ is the daily development rate (per day), T is the temperature in °C ($T_b \leq T \leq T_c$); T_b is the base temperature below which $f = 0$; T_c is the ceiling temperature above which $f(T) = 0$; T_o is the optimum temperature. Finally, diagrams and fitted model for the two ecotypes were compared.

The quality of fit was evaluated by comparing the values of coefficients of determination (R^2) and root mean square error (RMSE). RMSE was calculated by the following formula (Roman *et al.*, 2000):

$$RMSE = \sqrt{\Sigma^{(Oi - Pi)^2} / _{n-1}}$$

where O, P, and n represent values predicted by the observed values, fitted model and total number of paired values, respectively. Smaller RMSE values indicate better agreement between predictions and observations than larger RMSE values, and an arbitrarily chosen RMSE value of ≤ 10 was considered to represent an adequate fit between predictions and observations.

The relation between FGP and temperature was described by fitting a segmented linear model. The means comparisons of final germination percentage were separated by Fisher's Least Significant Difference (LSD) at $P \leq 0.01$ levels of probability. Statistical analysis was done using PROC GLM in SAS Statistical Package (SAS 9.2). The software package SigmaPlot Version 11 (Systat Software, Inc.) was used for model fitting.

Results

Germination rate was strongly dependent on temperature and seeds of the two ecotypes responded significantly differently to various temperatures (figure 1). Maximum *GR* was obtained at 20-25°C for Elam ecotype seeds and at 30-35°C for the Isfahan ecotype seeds. In both ecotypes, *GR* at 3-5°C was significantly lower than at other temperatures. Comparing Elam and Isfahan ecotypes at 3-20°C, seeds of the Elam ecotype had a higher *GR* at these temperatures than seeds of the Isfahan ecotype, although after 20°C the *GR* of Elam seeds declined while that of Isfahan seeds increased. At 40°C, the *GR* of Elam ecotype seeds was approximately 0, while there was still germination of Isfahan ecotype seeds. For seeds of the Elam ecotype, T_b = 1.7°C, T_o = 22.9°C and T_c = 37.7°C (table 2). In this model, RMSE was low (0.0118), R^2 was 0.92 and F_S was significant ($P < 0.01$). For Isfahan ecotype seeds, T_b = 5.8°C, T_o = 34.4°C and T_c = 41.0°C. RMSE was low (0.0745), R^2 was 0.90 and F_S was significant ($P < 0.01$).

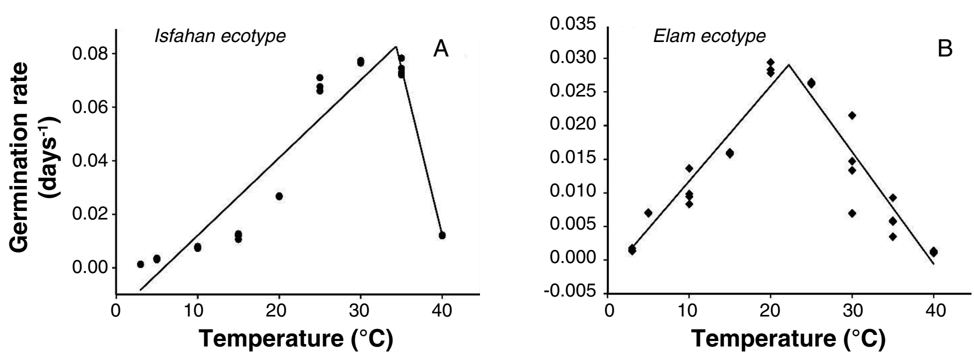

Figure 1. Relationship between germination rate and temperature as fitted using an intersecting model for two ecotypes of *Thymus daenensis* subsp. *daenensis*. See text for an explanation of germination rate.

Table 2. Results of fitting the intersected cardinal temperatures model for two ecotypes of *Thymus daenensis* subsp. *daenensis* (figure 1). T_b, T_o and T_c are cardinal temperatures; RMSE = root mean square error; R^2 = coefficient of determination; SE = standard error; F_S = Fisher test statistic.

Ecotype	$T_b \pm$ SE	$T_o \pm$ SE	$T_c \pm$ SE	RMSE	R^2	SE	F_S
Elam	$1.7 \pm 0.89^{**}$	$22.9 \pm 0.67^{**}$	$37.7 \pm 0.87^{**}$	0.0118	0.92	0.003	128.9^{**}
Isfahan	$5.8 \pm 0.92^{**}$	$34.4 \pm 0.46^{**}$	$41.0 \pm 0.47^{**}$	0.0745	0.90	0.009	104.8^{**}

** Significant difference at level $P < 0.01$.

Maximum FGP was observed at temperatures between 3 and 25°C in Elam ecotype (86-94%) and 10 and 35°C in Isfahan ecotype (94-97%; figure 2). Germination percentage decreased at temperatures higher than 25 or 35°C for Elam and Isfahan seeds, respectively (table 3). Elam ecotype seeds required a temperature of 20°C to reach its maximum germination in minimum time; Isfahan seeds required 30°C (table 3). Seeds of the Elam ecotype required 2-3 days to reach maximum germination at temperatures between 20 and 25°C while seeds of the Isfahan ecotype reached maximum germination in about one day at 25 and 35°C. At 30°C, the *GR* of Isfahan seeds was 3-times higher than Elam seeds.

Isfahan seeds required significantly more days to start germination at 3-10°C than at other temperatures and these conditions resulted in the lowest FGP. The seeds required significantly longer time to start germination at 5 and 35°C and in these conditions FGP decreased (figure 2). FGP of Elam seeds which had a lower T_b was much higher than that of Isfahan seeds at low temperatures (3 to 5°C).

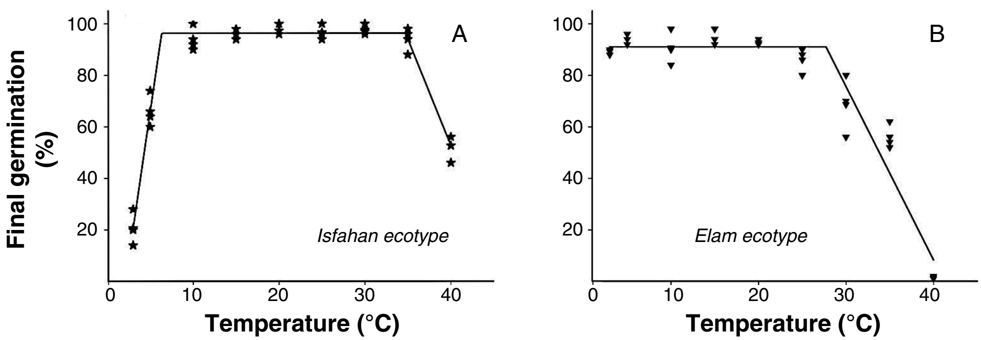

Figure 2. Relationship between final germination percentage and temperature for two ecotypes of *Thymus daenensis* subsp. *daenensis*.

Table 3. Means comparison of final germination percentage (FGP) and germination rate (GR) as affected by temperature for seeds from two ecotypes of *Thymus daenensis* subsp. *daenensis*[1].

	Ecotype	Temperature (°C)								
		3	5	10	15	20	25	30	35	40
FGP (%)	Elam	89^{a}	94^{a}	90.7^{a}	94^{a}	92.7^{a}	86^{a}	68.7^{b}	56^{c}	1.3^{d}
	Isfalhan	20.7^{d}	66^{b}	94^{a}	96^{a}	97^{a}	96.7^{a}	97.7^{a}	94^{a}	52.7^{c}
GR (days^{-1})	Elam	0.001^{e}	0.007^{d}	0.01^{cd}	0.016^{b}	0.028^{a}	0.026^{e}	0.014^{bc}	0.006^{d}	0.001^{e}
	Isfahan	0.001^{f}	0.003^{f}	0.007^{e}	0.01^{d}	0.03^{c}	0.07^{b}	0.08^{a}	0.07^{a}	0.01^{d}

[1] Values with the same letters within each row are not significantly different at $P < 0.01$.

Discussion

In understanding plant thermo-tolerance, information obtained from seed germination studies are of vital importance. Such information is also useful to identify suitable geographical regions for plant establishment and consequently for improvement of yields (Massawe *et al*., 2003). In this study, an intersected lines regression model was used to provide a description of germination behaviour for seeds from two ecotypes of *Thymus daenensis* subsp. *daenensis*, thereby identifying sub-optimal and supra-optimal temperature ranges (Hardegree, 2006). Germination occurred over a wide range of temperatures, from below 5 up to 40°C (figure 2). This indicates the potential for germination and establishment in areas with different thermal conditions.

In both ecotypes, germination rate at 3-5°C was significantly (P < 0.01) lower than other temperatures. This reduced germination could be caused by a decrease in cell metabolism due to low temperature. The results agree with previous findings; Nadjafi (2009) found that lower temperatures, such as constant 5°C, will limit final germination percentage and germination rate of *Thymus daenensis* subsp. *daenensis*. In Isfahan ecotype seeds, germination rate increased with temperature from about 5°C to an optimum temperature of about 34°C and declined sharply thereafter (figure 1A). Seeds of Isfahan ecotype germinated even in high temperatures (35 to 41°C). At temperatures beyond 35°C, germination decreased in Isfahan ecotype and it was noted that death of some seeds occurred, which was resulted from increased respiration and decrease in seeds storage (Wang and Zhou, 2010).

In Elam ecotype seeds, germination rate increased as temperature increased from 5 to 22°C and decreased when temperature went over 22°C (figure 1). In contrast, FGP was relatively fixed (figure 2). This observation is consistent with those of Hardegree (2006), Psallida and Spyropoulos (2006) and Naylor (2007) who found that FGP at 10-30°C was approximately constant for the seeds of the species considered. Increasing temperature from 15 to 35°C, at first increased then decreased the germination rate of *Mimosa foliolosa* (Benth.) seeds, however FGP remained relatively fixed (Silveira and Fernandes, 2006). Sincik (2004) showed that, in *Pisum sativum* L., increasing temperature from 5 to 25°C progressively decreased the time required for 50% germination, and at first caused FGP to increase, then decrease. Nadjafi (2009) found that 20°C was the optimal temperature for *T. daenensis* subsp. *daenensis* seeds. It seems that increasing temperature over the optimum (22°C) is the result of secondary dormancy in seeds of the Elam ecotype. That is, to evade drought and decline of rainfall of its natural environment, Elam ecotype experiences secondary dormancy. Similarly, secondary dormancy in goat grass (*Aegilops cylindrica* Host) delayed germination until autumn when temperatures were low and moisture was available (Fandrich and Mallory-Smith, 2005).

As Nadjafi *et al.* (2009) stated, germination behaviour is highly related to the ecological conditions of a species' natural habitat. The calculated base and optimum temperatures of Elam ecotype confirm its adaptation to low temperatures (table 2) and this ecotype with lower T_b also showed higher germination percentages (figure 2B). In the Elam ecotype, germination rate declined at very high temperatures (30-40°C) and estimation of the ceiling temperature was difficult due to death of seeds at temperatures above 35°C. A similar problem in estimating T_c was mentioned by Massawe *et al.* (2003). In the Elam region, rainfall is concentrated during winter and beginning of spring; during May to August there is no rainfall and temperature is extremely high (about 50°C). Living in a warm and semi-arid climate, it was expected that seeds of Elam ecotype would germinate at high temperatures, but the results showed that the cardinal temperatures of Elam ecotype seeds were low in comparison with seeds of the Isfahan ecotype. This may be a mechanism to evade drought and heat in the natural environment. It seems that Elam ecotype transferred its germination to winter season which has maximum rainfall and ended its life cycle before experiencing maximum temperatures in this region. Conversely, in Isfahan region, the maximum temperature is 36°C and there is summer rainfall. It is possible that to evade cold winters and to be able to make use of spring and summer humidity, Isfahan ecotype prefers to germinate during spring and its cardinal temperatures are higher than those of the Elam ecotype.

Ecological differences in seed characteristics may also be due to genetic variability which results from plant adaptability to environmental conditions. There are evolutionary constraints to the development of species adaptation to environmental conditions. Differences in germination behaviour between closely related species should be related to differences in their habitat preferences (Koutecká and Lepš, 2009). Temperature range response was different for the Elam and Isfahan ecotypes because they come from different ecological regions. This may suggest that environmental adaptation is more important in germination than the genetic characteristics of ecotypes in a species but more studies are needed to confirm this finding.

Measured germination indices in the present study indicate Isfahan ecotype's superiority over the Elam ecotype regarding germination rate. The findings also suggest the Isfahan ecotype having high competitive ability in the natural environment. With climate change, the Isfahan ecotype may be more resistant and would tolerate drought-stress better than the Elam ecotype, at least with respect to seed germination behaviour.

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