

Assessing Effects of Temperature Change on Four *Limonium* Species from Threatened Mediterranean Salt-Affected Habitats

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Abstract

Evaluation of tolerance to temperatures in the germination stage is an important tool to foresee the possible effect of global warming. Moreover, establishing germination protocols for endemic and threatened species is most interesting for restoration and management plans that focus on habitat conservation. Seed germination was analysed in four species of the genus *Limonium* (*L. virgatum*, *L. narbonense*, *L. girardianum* and *L. santapolense*) growing in two saline habitats on the eastern coast of the Iberian Peninsula. The study aim was to compare responses of seed germination to different temperature regimes. Seeds were collected in two protected areas, 'L'Albufera de Valencia' (province of Valencia) and 'Clot de Galvany' (province of Alicante). Responses to temperature were checked within a constant range, 10-30 °C at 5 °C intervals, by considering the environmental conditions at both sampling sites. The final germination percentage and mean germination time (MGT) were calculated after 30 days. In order to compare thermal responses, base temperature (T_b) and thermal time (S) were determined for each species. The possible correlations of soil parameters with the species' germination pattern were also analysed. To identify distinct behavioural groups, a non-linear principal component analysis was performed. Significant differences between species were found in the velocity of germination. A fast response as opportunistic germination was observed in all the species. The base temperature and thermal time showed significant differences in competitiveness between species.

Keywords: endemics, halophytes, salt marshes, seed germination, soil characteristics

Introduction

Coastal salt marshes are ecosystems of much ecological value which, over time, suffer many aggressions and transformations. They have long since been considered unhealthy places given the presence of malaria-carrying mosquitoes. In recent times, they have been profoundly altered by urban development and pressure from tourism. Global warming also poses another threat to such ecosystems, which can influence their structure and functioning. Increased temperatures, heat waves, droughts, floods, storms and forest fires increase the vulnerability of salt marshes and many other ecosystems. Many species, especially endemic ones, face an increased risk of extinction especially when climate change interacts with other stress factors of an anthropic origin.

The vegetation of salt marshes is constituted by halophytes, which are plants adapted to survive and

complete their biological cycle under saline conditions. A more precise definition is that by Flowers and Colmer (2008), who include in this category the species capable of surviving and completing their biological cycle under salinity conditions of at least 200 mM NaCl. Halophytic flora is diverse and includes taxa from different mono- and dicotyledonous families. A genus that typically includes halophytes is *Limonium* Mill. (family Plumbaginaceae), with more than 400 species worldwide. This genus has a diversification centre in the western Mediterranean region and another in the Asian steppes. Mediterranean species usually occupy small isolated areas in salt marshes, interior lagoons, rocky coastlines, or very arid soils. *Limonium* species show extraordinary intraspecific variability due to hybridisation and apomixis phenomena (Erben, 1993). In the region of Valencia, the genus is represented by around 30 species, many of which are endemic and/or threatened (Crespo, 2000). For this reason, the genus aroused the interest of taxonomists and geneticists (Palacios *et al.*, 2000;

Lledó *et al.*, 2005). Given its well-known tolerance to salinity, many anatomic, physiologic and biochemical studies have also been performed on different species of this genus. *Limonium* belongs to the special category of recretohalophyte, which includes plants able to secrete salt from their leaves through saline glands. Such structures, which directly secrete toxic ions outside plants are present only in a few halophyte species and are completely absent in glycophytes (Shabala *et al.*, 2014; Yuan *et al.*, 2016).

Several studies analysed seed germination in *Limonium*, as this process is the most sensitive in plants life cycle, tolerance to stress increasing progressively with age (Vicente *et al.*, 2004). However, the majority of the studies on seed germination in species of this genus focused on responses to salinity, and very few were performed on responses to temperature of germination (Zia and Khan, 2004; Giménez Luque *et al.*, 2012).

Considering that the first effect of global warming is increased temperatures, knowledge of how species respond to different temperature regimes in the germination stage is extremely important, not only from a theoretical point of view, but also to manage endangered or rare taxa in protected areas. The aims of the work were to test germination responses in different temperature regimes and to correlate the germination patterns with local edaphic conditions and the distribution pattern of the analysed taxa.

Materials and Methods

Plant material

Mature fruits were collected from four perennial *Limonium* species, which grow in salt marshes on the East coast of the Iberian Peninsula but have different biogeographical ranges, and were considered for this study.

L. santapolense Erben is a local endemic of the province of Alicante on the Spanish Red List of vascular flora in the IUCN vulnerable category due to its risk of extinction (Moreno, 2008). *L. girardianum* (Guss.) Fourr. is endemic to the north-western Mediterranean coastal salt marshes of France, SE Spain and the Balearic islands (Erben, 1993). This species is also threatened by habitat transformation since it is represented by a few isolated populations, and is protected in France (Baumberger *et al.*, 2012). *L. virgatum* (Willd.) Fourr. and *L. narbonense* Mill. are present throughout the Mediterranean, the former in coastal salt marshes and cliffs, and the latter in salt marshes and littoral prairies.

Sampling sites

Seeds and soil samples were collected from two different areas situated on the coast of the Valencian Community, one of them in the province of Alicante ('Clot de Galvany') and the other in the province of Valencia ('L'Albufera' Natural Park), both located in areas protected according to different protection figures. 'Clot de Galvany' was designed by the local Government Municipal Natural Park (MNP), a Special Protection Area (SPA) and a Site of Community Importance (SCI) by the Nature 2000 network, which also considers the 'L'Albufera' Natural Park a SPA and a SCI.

Soil analysis

Soil was sampled in the seed collection area in summer 2016, when soil salinity is higher. From the area of each species, three soil samples were taken at a depth of 0-15 cm. Once the samples had evenly lost moisture, they were crushed with a roller to break aggregates and then passed through a 2-mm light sieve. Analyses were performed on fine soil (diameter < 2 mm). Soil texture was analysed by the hydrometer method (Bouyoucos, 1962). The following parameters were analysed in a saturation extract: pH, EC, chlorides, Na⁺, K⁺, Ca²⁺, and Mg²⁺. A Crinson pH-meter Basic 20 and a Crinson Conductimeter Basic 30 were used to measure pH and EC, respectively. Sodium and potassium were quantified with a PFP7 flame photometer (Jenway Inc., Burlington, VT, USA), chlorides were measured in a MKII Chloride Analyzer 92 6 (Sherwood, Inc., Cambridge, UK), and divalent cations ions of calcium and magnesium were measured with an atomic absorption spectrometer Spectra 220 (Varian, Inc., CA, USA).

Seed features

Seed dimensions, width and length, were measured by the ImageJ image analysis software (Rasband, 1997-2012). Seed masses were determined from 50 seeds of each species on an Orion Cahn C-33 microbalance. Data were expressed as mean ± standard deviation.

Germination assays

Before running tests, the seeds from both sites were cleaned and stored in open paper bags in a controlled environment, at 20 °C and with 40-50% RH. For each germination test, four experimental replications were performed, each with 25 seeds placed on 0.6% agar in 55 mm-diameter Petri dishes in programmable-environmental chambers with controlled temperature and illumination. Germination responses to temperature were evaluated at five constant temperatures that ranged between 10 °C and 30 °C. Illumination was provided for 12 h daily by fluorescent lights (75-150 μmol m⁻² s⁻¹).

The final germination percentage was determined after 30 days, and the mean germination time (MGT) was calculated according to Ellis and Roberts (1981), to assess the germination rate. All the data in the tables and graphs are expressed as the means of the four replications, with standard deviation (SD).

To compare the thermal responses among the four species, base temperature (T_b) and thermal time (S) (García-Huidobro *et al.*, 1982; Trudgill, 1995) were calculated.

Statistical analysis

An analysis of variance (ANOVA) was applied to assess the significance of the observed effects (p < 0.05) (Khan and Rayner, 2003). Percentages were arcsine-transformed before the statistical analysis to ensure homogeneity of variance. A one way ANOVA and Tukey's HSD test were applied to identify the homogeneous groups when significant differences were found among the studied parameters. A non-linear principal component was applied (NLPCA, de Leeuw, 1982) by the CATPCA programme, included in the SPSS 15.0 software (SPSS Inc.) to compare the species

response and to evaluate the influence of climatic and soil variables on germination behaviour.

Results

Soil analysis

The soil characteristics of each sampling area of the four species are summarised in Table 1. Among the measured parameters, the sand, silt and clay percentages clearly differed in the *L. santapolense* sampling area (located at ‘Clot de Galvany’) from those of the other three species collected from different salt marshes in ‘L’Albufera’ (Valencia). As such, the soil texture for the area of *L. santapolense* was silty-clay-loam, and was sandy for *L. girardianum*, *L. narbonense* and *L. virgatum*. Although there were some differences in soil EC, there was considerable variability in the concentrations of Na⁺ and Cl⁻ ions among the sampling points within one area, and such differences among species were not statistically significant. On the contrary, significant differences among species (ANOVA, p-value ≤ 0.05) were found for bivalent cations Ca²⁺ and Mg²⁺.

Seed features

The morphological analysis detected statistical differences in the mean seed length among the four species (Table 2). The *L. santapolense* seeds gave the highest length values, while *L. girardianum* reached the lowest values for both length and width. This species, along with *L. santapolense*, also had the lower seed mass (Table 2).

Germination assays

Concerning the germination percentage to thermal variation, a similar response, with no remarkable statistical differences, was observed in all the tested *Limonium* species. All the studied species reached high values at the tested temperatures, and at both low and high temperatures, in some cases with values that came close to 100% (Fig. 1). In contrast, significant differences between species were found in the germination velocity at the different tested temperatures. Germination velocity, calculated through

MGT, revealed significant stimulation at the highest temperatures (from 20 °C to 30 °C) in the four *Limonium* species. When considering the germination pattern, the fastest germination times at all the tested temperatures were exhibited by *L. girardianum* and *L. santapolense*, especially at the highest temperatures (Table 3).

The linear regression of the inverse of the germination rates represented in Fig. 2 allowed to calculate the base temperature and thermal time (Table 4). These data provided information about the competitiveness of each species in relation to the range of tested temperatures. From the obtained results, *L. santapolense* was the most competitive species at practically all the tested temperatures, followed by *L. girardianum*, as these two species showed the fastest germination velocity. The other two species proved less competitive at most of the tested temperatures. According to germination competitiveness against increasing temperatures, the following order of species is proposed: *L. santapolense*, *L. girardianum*, *L. narbonense* and *L. virgatum* (Fig. 2).

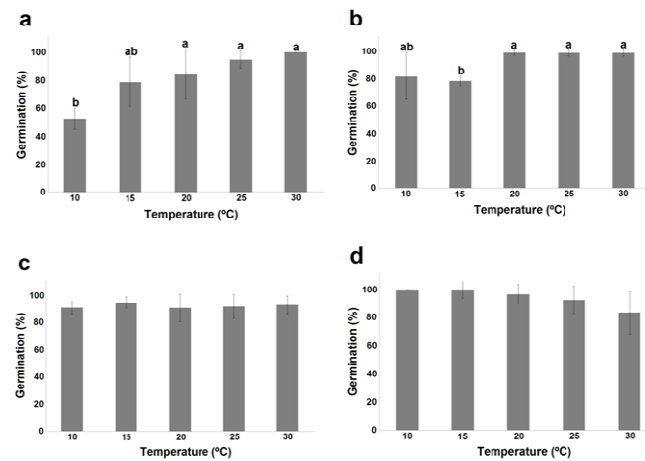


Fig. 1. Germination response to different temperatures for all the studied species. a. *L. virgatum*, b. *L. narbonense*, c. *L. girardianum*, d. *L. santapolense*. Different letters indicate significant differences (Tukey’s test, p < 0.05)

Table 1. Soil variables at the collection sites of the four analysed *Limonium* species

Soil variable	<i>L. virgatum</i>	<i>L. narbonense</i>	<i>L. girardianum</i>	<i>L. santapolense</i>
Silt (%)	2.7 ± 0.6a	2.7 ± 0.6a	6.7 ± 0.6b	55.3 ± 0.5c
Clay (%)	4.3 ± 0.6a	6.3 ± 0.57b	7.0 ± 1.0b	33.7 ± 0.6c
EC (dS m ⁻¹)	59.0 ± 20.2a	50.3 ± 25.5a	33.9 ± 15.1a	63.9 ± 26.8a
pH	8.7 ± 0.2b	8.5 ± 0.2ab	9.0 ± 0.1b	8.2 ± 0.1a
Cl ⁻ (meq L m ⁻¹)	581.1 ± 161.2a	438.2 ± 320.1a	346.0 ± 124.1a	602.2 ± 293.8a
Na ⁺ (meq L m ⁻¹)	367.9 ± 80.9a	291.5 ± 148.1a	239.1 ± 91.2a	373.4 ± 165.5a
K ⁺ (meq L m ⁻¹)	12.9 ± 5.4a	10.4 ± 6.5a	10.2 ± 4.9 a	5.5 ± 3.3a
Ca ²⁺ (meq L m ⁻¹)	67.9 ± 14.6a	62.8 ± 19.2a	23.94 ± 8.7a	147.4 ± 49.2b
Mg ²⁺ (meq L m ⁻¹)	225.9 ± 3.4ab	281.7 ± 47.2b	130.7 ± 3.8a	275.8 ± 76.1b

Note: Different letters within the same line indicate significant differences among species (Tukey’s, p < 0.05). EC: electric conductivity in the saturated extract.

Table 2. Seed features for the studied *Limonium* accessions

Feature	<i>L. virgatum</i>	<i>L. narbonense</i>	<i>L. girardianum</i>	<i>L. santapolense</i>
Length (mm)	1.91 ± 0.8c	1.84 ± 0.1b	1.50 ± 0.1a	2.03 ± 0.1d
Width (mm)	0.47 ± 0.0c	0.47 ± 0.0c	0.41 ± 0.0a	0.44 ± 0.0b
Seed mass (mg)	0.23 ± 0.1b	0.29±0.1c	0.14 ± 0.0a	0.13 ± 0.0a

Note: Different letters within the same line indicate significant differences among species (Tukey’s test, p < 0.05).

Table 3. Mean germination time (MGT) for seeds during the germination tests at different temperatures

Temperature (°C)	<i>L. virgatum</i>	<i>L. narbonense</i>	<i>L. girardianum</i>	<i>L. santapolense</i>
10	11.7 ± 1.7b	11.5 ± 2.6b	6.2 ± 1.0a	4.3 ± 0.3a
15	8.0 ± 1.1c	4.8 ± 0.5b	3.4 ± 0.6a	2.2 ± 0.0a
20	5.6 ± 0.9d	3.8 ± 0.2c	2.4 ± 0.2b	1.2 ± 0.2a
25	4.6 ± 0.4c	2.8 ± 0.3b	1.7 ± 0.3a	1.2 ± 0.1a
30	4.8 ± 0.8c	2.6 ± 0.2b	1.3 ± 0.3a	1.6 ± 0.3a

Note: Distinct letters indicate significant differences in germination velocity among species for each temperature (Tukey's test, $p < 0.05$).

Table 4. Base temperature (Tb) and Thermal time (S) for the four studied species

Species	Tb (°C)	Thermal time (°C day)
<i>L. virgatum</i>	0.7	109.9
<i>L. narbonense</i>	2.5	67.1
<i>L. girardianum</i>	5.8	31.0
<i>L. santapolense</i>	6.5	16.8

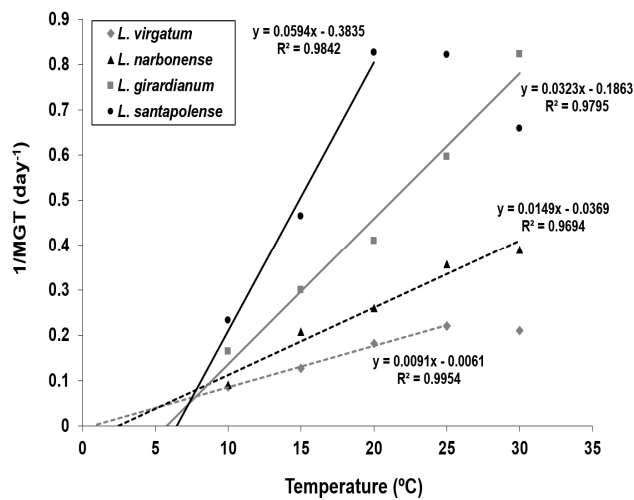


Fig. 2. Germination rate and regression lines for the tested temperatures in the four studied *Limonium*

The variables with the strongest influence on the response to temperature of the *Limonium* species were established by a NLPCA (non-linear principal component analysis; Fig. 3). Morphological features were not included in this analysis as they did not correlate with the four species' germination response to temperature during a preliminary assessment. The two new dimensions explained 88.4% of total variance, with 56.5% corresponding to the first dimension and 31.9% to the second. Cronbach's alpha values indicated high internal consistency, close to 1 for both components, 0.95 and 0.87 respectively, with a total of 0.99. The first axis correlated negatively with the base temperature (Tb), and positively with the increasing values of thermal time (S) and the decreasing ones of germination velocity (increase in MGT), and separates the faster species (*L. girardianum* and *L. santapolense*), especially at the highest temperatures, and also the more competitive ones, which correspond to the species with a more restricted distribution range. The second axis correlated positively with the soil ion concentration, especially Cl^- , Ca^{2+} and Mg^{2+} . The soil properties of the sampled sites are the most important discriminating factor on this axis and the two species with a minor distribution range are separated, *L. santapolense* and *L. girardianum*, depending on soil salinity.

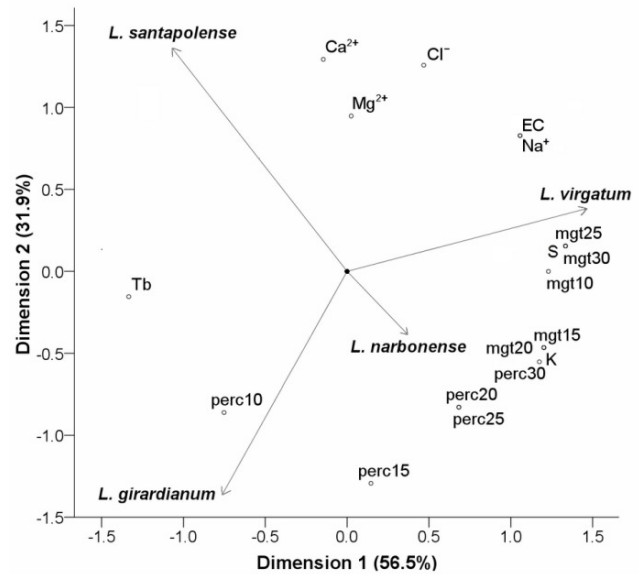


Fig. 3. Ordination plot of the four *Limonium* species with a Non-linear Principal Components Analysis (NLPCA). Species are represented by vectors. The mean germination time at the tested temperature (mgt); Germination percentage at the tested temperature (perc); Base temperature (Tb); Thermal time (S); Electric conductivity (EC)

Discussion

Germination and seedling establishment are the most critical stages in the biological cycle of plants as only the plants that overcome these first stages can survive in a territory (Donohue et al., 2010). The germination stage is a challenge for all plants, especially for those that live in saline habitats as plants must overcome soil limitations to succeed in this stressing environment. Global climate change, with a progressive increase in temperatures and an alteration in rainfall regimes, constitutes a potential threat to these environments and to the species that undergo osmotic stress. The global temperature increase rate in the last 50 years was $0.13 \text{ }^\circ\text{C} \pm 0.03$ per decade (IPCC, 2014). This remarkable increase affects the geographic distribution of species, their physiology and phenology, and also biodiversity as a whole (Thuiller et al., 2005; Menzel et al., 2006).

The response of plants to increasing temperatures depends on several factors, such as magnitude and duration of this increment, and also on plant characteristics. Many plant physiology aspects can be affected by thermal stress, including reserve immobilisation, protein denaturation, and alteration in the fluidity of membranes, enzyme inactivation, protein synthesis inhibition and impaired chlorophyll biosynthesis, among others (Levitt, 1980; Wahid *et al.*, 2007; Hasanuzzaman *et al.*, 2013; Jha *et al.*, 2014). In addition, plant's phenological stage plays a relevant role in the magnitude of adverse effects that result from high temperature episodes. These effects can be especially harmful during the reproductive period. Therefore, studying temperature effect on germination as a critical plant life stage is crucial when the evaluation of the possible effects of future climate change is required.

Limonium is a genus with a cosmopolitan distribution range, which includes species, that are always edaphic specialists, and participate in salt marsh vegetation communities distributed worldwide. The species integrated into this genus have morpho-physiological adaptations that allow them to survive in saline soils. These adaptations are related to salt tolerance and have been well-studied (Liu *et al.*, 2009; Hameed *et al.*, 2014; Fernández *et al.*, 2015). Nevertheless, responses to temperature changes are still poorly understood, although temperature changes are involved in some of the most important life cycle phases of these plants, specifically in the germination phase. From the results of this study, we can state that this group of *Limonium* species is narrowly adapted to germinate at high temperature, where they find their optimum in both percentage and, especially, germination velocity. Thus as a final conclusion, an increase in temperature in the germination phase as a result of climate change is not a problem itself as the species analysed herein show good tolerance to temperature increase, and even a stimulus in germination velocity was observed, at the higher temperatures, especially in *L. santapolense* and *L. girardianum*. Although other species that grow in saline environments show a similar response to temperature, by extending the optimum range even at 35 °C (Al-Ahmadi and Kafi, 2007), the temperature effect on germination is vastly variable in halophytes. Different responses have been reported in the genus *Limonium*, depending on species. *L. stocksii*, *L. iconicum* and *L. lilacinum* showed a similar response to temperature as the four herein studied species, and an optimal response at higher temperatures (Zia and Khan, 2004; Yildiz *et al.*, 2008; Santo *et al.*, 2017), whereas other species like *L. cossonianum* and *L. insigne* were more sensitive to increasing temperature (Giménez Luque *et al.*, 2012; Fernández *et al.*, 2015). Tolerance to a wide range of temperatures could allow these four species to germinate during a longer period of the year. This would depend, on the one hand, on water availability, the real limiting factor in the Mediterranean environments and, on the other hand, on the increasing soil salt concentration.

However, multiple factors influence plants in their natural environment. Thus we cannot rule out that the future temperature increase predicted for the Mediterranean basin (Hertig and Trambly, 2017) will negatively affect the studied species. It also has to be

considered that they inhabit salt marshes, which represent a highly sensitive environment, as a result of complex physical and ecological interactions. This fragile equilibrium can be altered by increasing temperatures and decreasing rainfall, which would lead to a higher soil saline concentration. Our results support the statement that edaphic parameters are relevant factors to be taken into account in such studies.

Previous studies on these same four species have demonstrated that they are not especially tolerant to presence of salt in the substrate in the germination phase (Al Hassan *et al.*, 2017). In addition, endemics and species with a restricted distribution are predictably the most sensitive to climate change despite their broadly tolerant response to high temperatures, which we proved under laboratory conditions.

The real risk of climate change for this species type is not directly higher temperatures, but an increase in the saline concentration in soil, which would derive from both less rainfall and high evaporation (Vicente-Serrano *et al.*, 2014). This is because such a situation narrows the available period for the germination phase, which depends on water availability and the consequent drop in soil salt concentration. This situation could cause these species to displace to less saline soils where they would compete with a larger number of species. Stronger competition could pose heavy pressure for their colonisation capability, and could thus narrow their distribution range and lead to an increasing risk of extinction in endemic species.

Our findings suggest a need to raise the level of protection of some species like *L. santapolense* and *L. girardianum*, or those in a similar situation in their distribution areas, as studies currently indicate greater potential sensitivity in endemic species. Contrarily, germination in *L. virgatum* and *L. narbonense* shows good tolerance to increasing temperature and, according to our previous data, also to increased soil salt concentration. As such, changes in Mediterranean ecosystems induced by global warming would predictably not affect the survival of these two species. Another direct conclusion drawn from this research is that the potential climate change threat cannot be analysed from the single point of view of increasing temperature, but must be approached from a broader perspective that takes into account as many factors as possible.

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