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RESEARCH ARTICLE

Assessing the germinability of coastal *Limonium minutum* (Plumbaginaceae) under different temperature and salinity conditions: implications for its conservation

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Background and aims – *Limonium minutum* is an endemic plant of the eastern Balearic Islands (Majorca and Minorca), where it grows in coastal rocky habitats. The effects of temperature and salinity on seed germination of *Limonium minutum* were evaluated in order to set a protocol for recovery of this species in this habitat.
Material and methods – Experiments to determine the effect of temperature have been carried out at temperature conditions between 10 and 23°C. Tests to determine the effect of salinity have been evaluated at 18°C with concentrations of 0, 100, 200, 300, and 400 mM of $MgCl_2$, $MgSO_4$, NaCl, and Na_2SO_4.
Key results – Maximum germination took place between 16 and 20°C. Based on the salinity tests, the highest germination values were obtained with distilled water. The use of saline solutions resulted in significant decreases in the germination percentage. However, in almost all treatments, seed germination was observed. The T_{50} increased at low temperatures and with increasing salt concentration.
Conclusion – *Limonium minutum* has a wide germination temperature range and a high resistance to salinity. After being exposed to different saline solutions, once washed with distilled water, the seeds recover their full germination capacity; therefore, the effect of salts is an osmotic and non-toxic effect for this species. Sowing seeds in late August ensures that the species has an optimal chance to survive in coastal habitats.

Keywords – Balearic Islands; conservation; germination; *Limonium*; recovery; salinity; seeds; temperature.

INTRODUCTION

In a context of global biodiversity, endemic species have very limited ranges and the knowledge of the reproductive biology of these species is a fundamental prerequisite for establishing effective management plans (Evans et al. 2003). To establish effective management and conservation protocols, it is necessary to know the optimal conditions for seed germination (Andreou et al. 2011). With the prospect of climate change, it is essential to discern how factors such as temperature and salinity affect seed germination, especially for endemic species that are characteristic of coastal habitats.

Temperature is a decisive factor in the germination process and influences the action of catalytic enzymes that regulate the speed of biochemical reactions that occur after the seed's hydration phase (Pita & Pérez García 1998; Fenner & Thompson 2005; Baskin & Baskin 2014). The activity of these enzymes occurs optimally in a specific temperature range for each species, and consequently, germination takes place in a specific temperature range. The optimum germination temperature is defined as "the most appropriate to achieve the highest percentage of germination in the shortest possible time" (Mayer & Poljakoff-Mayber 1975; Eberle et al. 2014).

In addition, salinity is a limiting factor in the germination of almost all species, especially in arid climates (Khan & Gulzar 2003; Rejili et al. 2010). In most cases, high

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salt concentrations in the substrate inhibit or delay seed germination (Ungar 1991). Therefore, salinity seems to be one of the most important selection factors for the establishment of coastal species (Barbour 1970; Keiffer & Ungar 1997; Baskin & Baskin 2014; Estrelles et al. 2015; Murru et al. 2017; Naik & Karadge 2017; Santo et al. 2017a, 2017b, 2019). In arid and semi-arid regions, seed germination usually occurs during the rainy season, during which the concentration of soil salts is reduced (El-Keblawy 2004; Redondo-Gómez et al. 2004; Fenner & Thompson 2005; Delgado et al. 2016). Seeds of halophytes maintain germination capacity after exposure to high soil salinity (Khan & Gul 2006; Orlovsky et al. 2011) and they are adapted to germinate when the saline stress decreases (Pujol et al. 2000; Vicente et al. 2009; Delgado et al. 2016). This ability to germinate after exposure to different levels of salinity is known as recovery (Khan & Ungar 1996; Gul et al. 2013).

Climate projections indicate that many environmental factors will change in the immediate future, especially in the Mediterranean: the average temperature will be higher, cold days will diminish, and droughts will become more common (IPCC 2014). Accordingly, climate change can result in a narrower range of suitable environmental conditions for species to germinate and complete their life cycle (Jiménez-Alfaro et al. 2016; Luna & Chamorro 2016). Coastal halophyte communities are extremely dependent on water supply, and germination adaptation has consequences in the evolution of the species by defining ecological niches and geographic ranges of these species (Estrelles et al. 2015). For this reason, some coastal halophyte species with a highly specialized ecological niche can be displaced by other species (Caperta et al. 2014) or their habitat will disappear below the sea (Flowers & Muscolo 2015). One of these seafront habitats is included in the EU Habitats Directive as “1240 Vegetated sea cliffs of the Mediterranean coasts with endemic *Limonium* spp.”, and this habitat is very well represented on the Balearic Islands. There are 53 species of *Limonium* (Plumbaginaceae) on the Balearic Islands (Llorens et al. 2018), of which 43 are endemic (Rita & Payeras 2006) and most of them live in coastal communities with marine influence (Llorens et al. 1992). *Limonium minutum* is a keystone species characteristic of these habitats on rocky shores.

In ecosystem restoration and species management, it is often more successful to use plants produced in pots in a nursery, and for this, it is necessary to know the biology of the germination. However, planting is expensive and time consuming, which puts limits on the extend of ecosystem restorations with this approach. Sowing seeds makes restoration cheaper and it can also be successful (Lorite et al. 2021) but it requires a high level of knowledge about the germination process and the establishment of protocols to carry out sowings at the time and location that ensure the greatest chance of success.

Our study aims to determine a germination protocol for the successful establishment of *L. minutum*, and for this, we investigate how the species germinates and what time of the year is most optimal for sowing. This information should be useful for species living in similar habitats as well. In order to develop such a protocol, it is necessary to determine, first of all, the effect of temperature on the germination of *L. minutum* seeds. Secondly, we evaluate the effect of concentrations of four salts on the germination of the seeds. In order to assess the effects of temperature and salinity, the percentage of germination, the germination rate (T_{50}), the decreasing germination percentage, and the recovery capacity of the seeds of this endemic species are analysed. Finally, in order to determine the best time to sow seeds in the field, we calculates the germination predictability index.

MATERIAL AND METHODS

Study species and seed collection

Limonium minutum (L.) Chaz. (Plumbaginaceae) (fig. 1) is a perennial cushion-like chamaephyte (3–15 cm), usually glabrous, with a woody and tortuous rhizome. Leaves are imbricated, spatulate, obtuse, and form a basal rosette. Flowering stems are 2–12 cm long, with the tops arranged in small panicle, corymbiform; the spikelets have 1–4 flowers; the calyx lobes are 4–5 mm long; and the chromosome number is 2n = 18 (Erben 1993). Flowering takes place between May and October and the seeds are dispersed between the summer and the end of winter. Sprouted seedlings are observed in the autumn and winter months. Plants grow

Figure 1 – *Limonium minutum* in its coastal rocky shore habitat. Photograph by Carles Cardona.

on substrates rich in carbonates in proportion to sand, but poor in sulphates (Llorens et al. 2018). *Limonium minutum* is a species endemic to the eastern Balearic Islands in the western Mediterranean (Erben 1993). It is characteristic of rocky shores, which according to the EU Habitats Directive (European Commission 1992) corresponds to habitat 1240 ("Vegetated sea cliffs of the Mediterranean coasts with endemic *Limonium* spp.").

Seeds of *L. minutum* were collected in July 2017 (for the temperature tests) and July 2018 (for the salinity tests) in Punta Grossa, Menorca, Balearic Islands (western Mediterranean) (40°01′54.3″N, 4°11′30.7″E). Seeds were dried in silica gel and stored in sealed containers in a refrigerator at 4°C in order to maintain germinability before performing the tests (Bacchetta et al. 2008). Seeds were kept for three months until the germination tests were started.

Germination experiments

In all cases, four replicates of 25 seeds each were used. All tests were performed in germination chambers in dark conditions (MLR-351; SANYO Electric, Osaka, Japan). Seeds were sown in 9 cm diameter Petri dishes on two Whatman No. 1 paper filters and 4 ml of distilled water or 4 ml of saline solution were added. To prevent moisture loss during testing, the Petri dishes were sealed inside zipped plastic bags. Germinated seeds were removed and counted for a period of 30 days (temperature tests) or 40 days (salinity tests). A seed was considered as germinated when the radicle was visible (Song et al. 2006).

Evaluation of the temperature effect was conducted at 10, 14, 16, 18, 20, and 23°C, respectively. This range of temperatures was selected for being the average monthly temperatures with hydric availability for the germination in the study area (Guijarro 1986).

For the evaluation of the salinity effect, salts used were NaCl, $MgCl_2$, $MgSO_4$, and Na_2SO_4 at concentrations of 100, 200, 300, and 400 mM (Pujol et al. 2000; Vicente et al. 2009). These salts correspond to the four most abundant anions and cations in seawater (Margalef 1991). The temperature used in these tests was the optimum temperature obtained in the temperature effect experiment (viz. 18°C).

Recovery experiments

After 40 days, the ungerminated seeds of the different saline treatments were rinsed with distilled water and resown for an additional 30 days to study germination recovery.

Data analysis

Four germination parameters were calculated: final germination percentage (FGP), germination rate (T_{50}), decreasing germination percentage (DGP), and recovery percentage (RP). The final germination percentage (FGP) was calculated as the number of germinated seeds divided by the total number of viable seeds in each petri dish. T_{50} measures the speed of germination following the definition of Thanos & Doussi (1995): it is the time needed for 50% of the final germination percentage and is calculated by linear interpolation from the two germination values closest to median germination (see Bacchetta et al. 2008). Lower T_{50} values mean higher germination speed. The decreasing germination percentage (DGP) was calculated by the following formula, DGP = [(FGP in distilled water - FGP in saline solution)*100]/FGP in distilled water (Zhang et al. 2015). Higher DGP values indicate lower salt tolerance. To investigate the effect of the osmotic potential of salts and concentrations, the values of this osmotic potential for each salt and concentration, described in Zehra et al. (2013) and in Ahmed et al. (2020) have been used. DGP was modelled using generalized linear models with quasibinomial distribution (Zuur et al. 2009). The recovery percentage (RP) is determined by the following formula: RP = [(a-b)/(c-b)]*100; where a is the number of seeds germinated after recovery; b is the total number of seeds germinated in saline solution, and c is the total number of seeds (Pujol et al. 2000; Gulzar et al. 2001). Strictly speaking, these parameters do not obey the assumptions of a normal distribution and arcsine trans-formation used to be the standard advice (Sokal & Rohlf 1981; Scott et al. 1984; Baskin & Baskin 2014). The T_{50} was transformed by applying the logarithm of the value (Zar 1999). The Tukey's HSD test was used for subsequent comparison of the different treatments. If assumptions for ANOVA were not met after data transformations, the non-parametric Wilcoxon signed-rank test was applied (Sall et al. 2012).

Table 1 – Final germination percentage (% ± s.e.) and T_{50} (days ± s.e.) at different temperatures. Different letters indicate significant differences in FGP and T_{50} between the temperatures. Tukey's HSD, $p < 0.05$.

T (°C)	FGP (% ± s.e.)	T_{50} (days ± s.e.)
10	58.1 ± 3.0 [a]	12.1 ± 0.9 [a]
14	63.6 ± 5.1 [a]	6.6 ± 0.2 [b]
16	87.6 ± 0.9 [b]	4.3 ± 0.1 [c]
18	91.0 ± 0.1 [c]	4.3 ± 0.1 [c]
20	89.1 ± 2.2 [bc]	3.4 ± 0.1 [d]
23	80.5 ± 1.1 [d]	4.5 ± 0.1 [c]

Field germination predictability

The germination predictability index (GPI) was calculated with the formula: GPI(x) = [(FGP(Tx)/100) * fa(x) * (FGP(TMx)/100) * (FGP(Tmx)/100)]*100; where x is the month whose germination probability we want to calculate; FGP(Tx) is the final germination percentage at the mean temperature of month x; FGP(TMx) is the final germination percentage at the average maximum temperature of month x; FGP(Tmx) is the final germination percentage at the average minimum temperature of month x; and fa is the aridity factor (fa = 1 if $P/2T \geq 1$; and fa = 0 if $P/2T < 1$; where P is the precipitation of month x and T is the average temperature of month x) (Gil 1994; Llorens et al. 2009). This index allows for the probability of seed germination to be calculated on a monthly basis, making it possible to schedule sowings in the field, in order to achieve greater germination success.

RESULTS

Temperature effect

Optimal germination temperature for *L. minutum* ranged from 16 to 20°C (table 1). The highest FGP was observed at a temperature of 18°C ($\chi^2 = 20.5$; d.f. = 5; p = 0.001). At the lowest temperatures (10 and 14°C), significantly lower germination was observed (table 1). The highest T_{50} value was observed at 10°C, while the lowest T_{50} values were observed between 16 and 23°C ($\chi^2 = 20.4$; d.f. = 5; p = 0.001) (table 1).

Salinity effect

The germination of *L. minutum* seeds were significantly affected by each of the salts ($\chi^2 = 62.8$; d.f. = 16; p < 0.001). FGP reached 100% in the control tests with distilled water (table 2). As saline concentration increased, the percentage of sprouted seeds decreased. Total inhibition of germination was only observed at 400 mM $MgCl_2$. In all salts, the T_{50} increased significantly as salt concentrations increased (F = 4.93; d.f. = 15; p < 0.001) (table 3). Increases in DGP were observed for all salts and concentrations. There was a high positive correlation between DGP and the absolute value of the osmotic potential (fig. 2).

Table 2 – Effect of salts and their concentrations on the final germination percentage (% ± s.e.) at 18°C. Different lowercase letters indicate significant differences in FGP between the four salts. Different capital letters indicate significant differences in FGP between concentrations of the same salt. Non-parametric Wilcoxon signed-rank test, p < 0.05.

Molarity (mM)	Final germination percentage (% ± s.e.)			
	$MgSO_4$	$MgCl_2$	Na_2SO_4	NaCl
0	100.0 ± 0.0 aA	100.0 ± 0.0 aA	100.0 ± 0.0 aA	100.0 ± 0.0 aA
100	86.8 ± 3.1 aB	57.0 ± 5.5 cB	70.6 ± 4.8 bcB	79.0 ± 5.0 abB
200	66.9 ± 8.6 aB	11.3 ± 1.9 cC	11.0 ± 3.4 cC	33.7 ± 3.5 bC
300	38.0 ± 4.8 aC	6.1 ± 3.4 bcCD	2.0 ± 1.2 cD	12.1 ± 0.1 bD
400	16.0 ± 5.2 aD	0.0 ± 0.0 bD	1.0 ± 1.0 bD	9.2 ± 2.6 aD

Table 3 – Effect of salts and their concentrations on T_{50} (days ± s.e.) at 18°C. Different letters indicate significant differences between salts and concentrations. Tukey's HSD, p < 0.05.

Molarity (mM)	T_{50} (days ± s.e.)			
	$MgSO_4$	$MgCl_2$	Na_2SO_4	NaCl
0	6.8 ± 0.1 a	6.8 ± 0.1 a	6.8 ± 0.1 a	6.8 ± 0.1 a
100	8.7 ± 0.6 abc	12.0 ± 1.5 abcde	12.2 ± 0.3 abcde	9.7 ± 0.3 abcd
200	13.9 ± 2.1 bcde	7.8 ± 0.9 ab	17.6 ± 2.9 de	13.7 ± 1.7 bcde
300	14.7 ± 0.5 cde	22.0 ± 4.8 e	11.5 ± 4.0 cd	13.6 ± 2.2 abcde
400	16.1 ± 2.5 cde	–	13.5 ± 0.0 abcde	10.8 ± 1.5 abcde

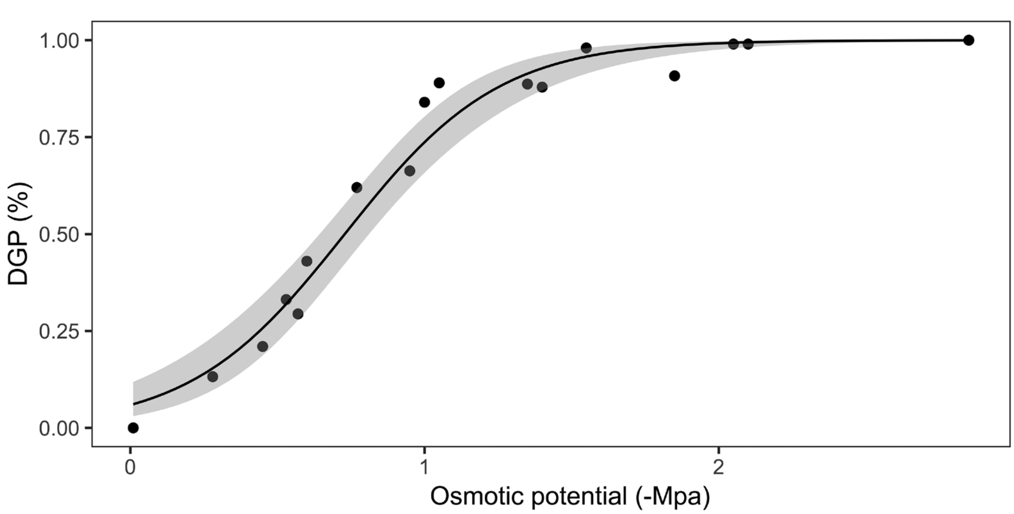

Figure 2 – Correlation between decreasing germination percentage (DGP) and the absolute value of the osmotic potential of the salt germination tests. There is a high positive correlation between DGP and the absolute value of the osmotic potential, which means that germination does not happen when osmotic potential in absolute value is high. R = 0.97; p < 0.01.

Table 4 – Germination recovery percentage (% ± s.e.).

Molarity (mM)	Recovery percentage (% ± s.e.)			
	$MgSO_4$	$MgCl_2$	Na_2SO_4	NaCl
100	93.8 ± 6.3	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
200	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
300	97.1 ± 2.9	100.0 ± 0.0	98.0 ± 1.0	100.0 ± 0.0
400	100.0 ± 0.0	97.9 ± 1.2	98.0 ± 1.0	100.0 ± 0.0

Recovery tests

In all germination recovery tests after the salinity tests, germination took place (table 4). In all cases values were close to 100%; there were no significant differences between all tests ($p = 0.189$).

Germination predictability index

Seed germination only happens between September and April, and October and November are the months with the highest GPI (fig. 3). This coincides with the time of greatest precipitation in the study area. In the field, seedlings are mainly observed between late October and early December (Gil 1994).

DISCUSSION

For most plant species in the Mediterranean region, the most suitable temperature for germination ranges between 15 and 20°C (Fenner & Thompson 2005; Galmés et al. 2006; Narbona et al. 2007; Bacchetta et al. 2008; Chamorro et al. 2013, 2017; Nedjimi et al. 2014; Murru et al. 2017). Among them are some species typical of the coastal rocky shores of the Western Mediterranean (Llorens et al. 2009; Fernández-Pascual et al. 2017; Picciau et al. 2019; Santo et al. 2019).

Most Mediterranean *Limonium* species have the highest germination percentage between 10 and 25°C (Giménez Luque et al. 2013; Delgado et al. 2015, 2016; Melendo & Giménez 2019). Other non-Mediterranean *Limonium* species show similar responses, for example, *L. stocksii* germinates at a day/night temperature of 20/30°C (Zia & Khan 2004). In the present study, it was shown that the temperature range of *L. minutum* was narrower, achieving optimal germination between 16 and 23°C (table 1). In this interval, it presents the highest germination percentage and the lowest T_{50}.

Germination percentage decreases, while T_{50} increases, as the absolute value of osmotic potential of the saline solution increases (tables 2, 3). Other species from coastal habitats show similar responses (Estrelles et al. 2015; Murru et al. 2017; Naik & Karadge 2017; Santo et al. 2017b). For all species, the highest germination percentages occur with distilled water, and salinity causes a decrease in the percentage and rate of germination (Zia & Khan 2004;

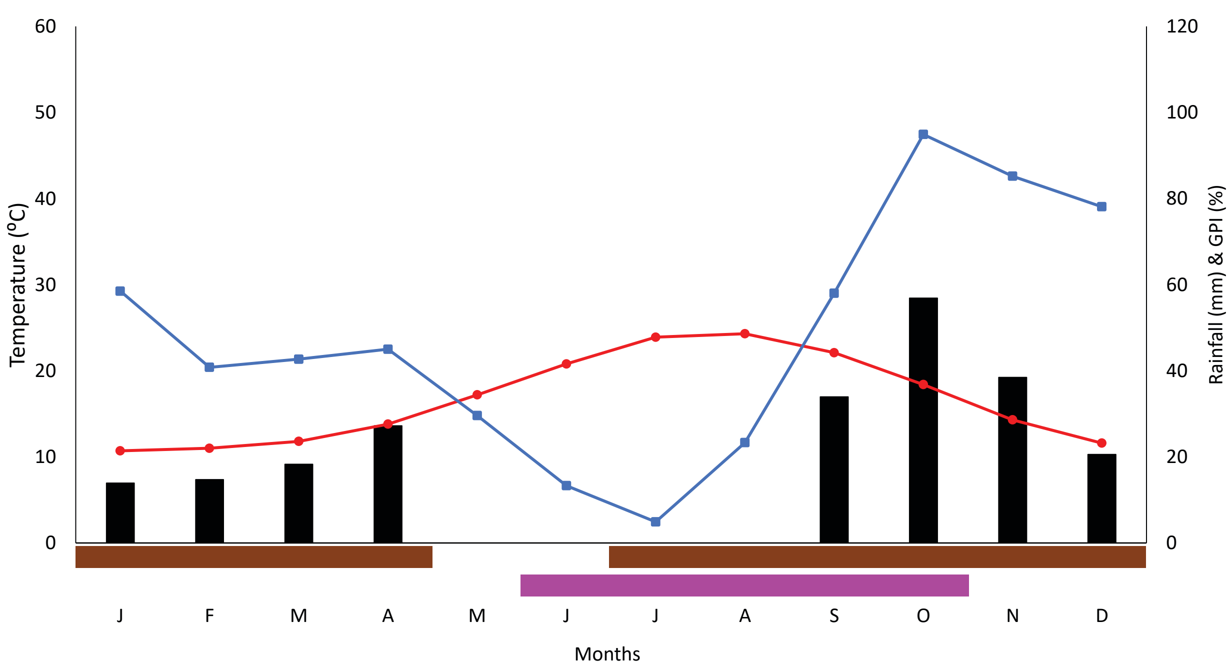

Figure 3 – Phenology of flowering and seed dispersal, and germination probability index (GPI) of *Limonium minutum* (horizontal bars: purple = flowering period; brown = seed dispersal period. Lines: red = temperatures in °C; blue = precipitation in mm; vertical bars: GPI). Seed germination only happens between September and April.

Vicente et al. 2007, 2009; Hameed et al. 2014; Santo et al. 2019). In addition, salt tolerance at the moment of germination can vary greatly between species, being specific for each species (Ungar 1982; Baskin & Baskin 2014; Santo et al. 2019).

In their natural habitat, the germination of *L. minutum* seeds mainly occurs in autumn (i.e. October and November), the months when the greatest number of seedlings are observed in the field (Gil 1994). Although spring conditions are also suitable for germination, seedings are less likely to establish, since this season is followed by a dry period that decreases their survival probability. Thus, the average temperature during autumn coincides with the optimum germination temperature found in this work. It is also the time of greatest rainfall in the Western Mediterranean. Rain leaches edaphic salts, in such a way that it allows germination. High water availability and adequate temperatures cause greater speed and germination percentage. This early germination confers an ecological advantage to this species, since the rapid germination, as soon as the wet period begins and precipitation promotes the leaching of edaphic salts, is an adaptive strategy that allows the development of the plants throughout the favourable season (Santo et al. 2017a). Thus, the seedlings can grow enough before the arrival of the next summer to be able to establish themselves in the habitat before other plants. The same behaviour is found in other *Limonium* species (Redondo-Gómez et al. 2008; Giménez Luque et al. 2013; Delgado et al. 2015; Delgado et al. 2016; Santo et al. 2017a; Melendo & Giménez 2019). Other non-Mediterranean *Limonium* species have higher optimum germination temperatures (Mahmoud et al. 1983; Zia & Khan 2004), corresponding to the temperatures found in the wet season of the habitats where they occur.

Germination inhibition observed in the salinity tests may be due to the osmotic potential of the medium or ionic toxicity (Ungar 1996; Pujol et al. 2000; Vicente et al. 2007). The results of the germination recovery tests are close to 100% in all treatments and show that the osmotic potential is responsible for the inhibition of germination in *L. minutum*. In some species, salt pre-treatment produces a “seed priming” effect (Ibrahim 2016), increasing germination when saline stress disappears. In the case of *L. minutum*, this increase does not occur, since the germination of the control reaches 100%. Although in the tests with lower FGP, we observed a synchronisation and increase in the speed of germination. One of the characteristics of halophytes is the ability to keep their seeds viable during exposure to hypersaline concentrations (Khan & Ungar 1997; Delgado et al. 2016). According to this definition, *L. minutum* should be considered as a halophyte. Moreover, according to Woodell’s (1985) classification, the germination pattern of this species would fall into type 1: “If germination occurred at all it was inversely proportional to salinity, when transferred to fresh water, germination rose to quite high levels”. Almost all the species he tested were dune or driftline plants, but *L. minutum* is a plant characteristic of coastal rocky shores, subjected to salt spray but rarely flooded, similar to Woodell’s (1985) dune plants, so the behaviour of these species he tested will be characteristic of habitat where sea spray occurs but is never inundated.

Limonium minutum may be affected by climate change in the Mediterranean, as the average temperature will be higher, the number of cold days will decrease, and droughts will become more common (IPCC 2014). These droughts can affect the germination of this species, since perhaps the salts accumulated in the soil would not leach out until winter and germination would therefore be delayed until after this time. Also, the increase in sea level might force this species to move further inland.

With regard to ecosystem restoration and species management of coastal rocky habitats with *L. minutum*, we recommend sowing the seeds in the field at the end of August, as their germination will not be affected by the salts present in the soil, and they will be able to germinate successfully. Determining the optimum temperature range for germination, the effect of salt concentrations on germination, and the germination predictability index can be helpful to accurately pin down an optimum sowing period for the restoration of any species, optimising the use of resources.

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