

Germination pattern of *Salicornia patula* as an adaptation to environmental conditions of the specific populations

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Abstract

Salicornia patula Duval-Jouve is a diploid species that usually shows a small bushy growth form. It is an important component of the halophytic annual pioneer vegetation of salt marshes. This hyper-saline vegetation is rare and threatened by a wide variety of pressures (tourist and industrial practices, coast management, invasive species) in the European Mediterranean area. *S. patula* is a characteristic species in the habitat 1310 "Salicornia and other annuals colonizing mud and sand". This paper shows an analysis (by GLM and PCA) on the germination response to temperature and salinity of three populations of *S. patula* from different localities (Italy, Spain and Croatia) in order to identify the key factors controlling germination phases for the implementation of projects on conservation and environmental restoration of the hypersaline environments. We assessed the inter-specific competitiveness of species related to several localities. The germination of *S. patula* was mainly influenced by temperature and salinity and secondly by the site of collection of seeds. In fact, even though *S. patula* optimal temperature range was between 25 and 35° C, the pattern of the germination response curve shows three distinct levels induced by the site of collection of seeds. For salinity, *S. patula* showed the ability to germinate at high salinities even in this case with distinct levels induced by the site of collection of seeds. This might suggest an adaptation of *S. patula* to the different sites bioclimatic conditions here estimated by summer and annual aridity. The population of Sentina Natural Regional Reserve had higher percentages of germination under saline conditions than the other populations. The seeds of the population from Blace were less salt tolerant than those of the populations from Sentina and Girona in the recovery responses. In conclusion, the local environmental conditions, especially I_o and I_{os_2} , are important variables to characterize and diversify the seed germination behavior in the three populations. In addition, *S. patula* seeds are able to germinate in high percentages in a rather wide range of temperature and salt concentration. Thus, it can be stated that the *S. patula* populations studied can be easily multiplied and successfully used for projects on conservation and environmental restoration of hypersaline environments.

Key words: Croatia, habitat restoration, hyper-saline vegetation, Italy, *Salicornia patula*, seed germination, Spain.

Introduction

The halophytes are highly specialized plants with great tolerance to salt. They can germinate, grow and reproduce successfully in saline areas that would cause the death of other plants. The halophytes play a very significant role in the salt coastal areas overcoming the salinity in different ways (Khan & Weber, 2006). The halophytes form grasslands that are a clear example of an ecosystem including poor species or monospecific communities with distributions related to environmental physical (influence of the tides, dynamics and the salinity of ground water etc.) and chemical gradients as well as to biological interactions (Adams, 1963; Andreucci *et al.*, 1996; Sánchez *et al.*, 1998). The success of halophyte populations mostly depends on the germination response of their seeds (Khan, 2003). The soils where halophytes normally grow become more saline due to evaporation of water particularly during the summer. Therefore, the soil surface tends to have higher soil salinity and higher water potentials (Khan & Gul, 2006). There is a great deal of variability in the response of halophytes to various levels of salinity, moisture, light, and temperature stresses and their in-

teractions. Species that live in highly specific habitats, such as salt marshes, often produce seeds with highly specialised adaptations (Redondo *et al.*, 2004). Due to their high salt tolerance, high growth rate, short generation time, capability of producing many seeds, glassworts can be representative species in terms of salt tolerance mechanisms (Katschnig *et al.*, 2013). In fact, glassworts can survive and grow in this selective environment thank to many mechanisms: succulence, fleshy articulated stem segment, ability to regain the metabolic status and osmotic adjustment through intracellular compartmentalization. They can sequester the toxic ions in vacuoles, away from cytoplasm, by an energy-dependent transport. The accumulation of sodium chloride in the vacuoles causes "salt succulence" involving the enlargement of the stem, linked to the absorption of significant amounts of water in order to dilute the concentration of salts and reduce their toxicity (Grigore *et al.*, 2014). The genus *Salicornia* L. includes annual glassworts of the tribe *Salicornieae* Dumort (*Chenopodiaceae*/*Amaranthaceae*). They are halophytic herbs apparently leafless with articulated succulent stems (Davy *et al.*, 2001). There is a great taxonomic complexity in this genus due to the few

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taxonomical characters, the possibility to inbreeding and the hybridization (Davy *et al.*, 2001; Kadereit *et al.*, 2006; Kadereit *et al.*, 2007; Vanderpoorten *et al.*, 2011; Katschnig *et al.*, 2013) and considerable phenotypic plasticity (Ingrouille & Pearson, 1987). As a result, many morphometric and taxonomical studies have been focused on defining European taxa (Huiskes *et al.*, 1985; Ingrouille & Pearson 1987; Géhu, 1992; Iberite, 1996; Lahondère, 2004). Recently, the taxonomy and phylogeny of glassworts employing different kinds of markers nrDNA (Papini *et al.*, 2004), point mutations in large cpDNA fragments (Vanderpoorten *et al.*, 2011) and ETS sequences (Kaligarič *et al.*, 2008) demonstrated a genetic separation between diploids and tetraploids of the *Salicornia* species.

According to the phylogenetic analysis (nrDNA) carried out by Papini *et al.* (2004) *Salicornia patula* has a basal chromosome number of $2n=18$ (diploid species). Then, Kadereit *et al.* (2012) in a review on the *Salicornia* genus simplified the classification of annual Euriasian glassworts which is based on morphology and geographical distribution at only four species and ten subspecies. Thus, *Salicornia patula* became a synonym for *S. perennans* Willd Sp. Pl., ed. 4, 1(1): 24 (Berolini) 1797. Lectotype designated by Freitag (2011).

In the coastal salt marshes of the Mediterranean region and the Black Sea, among the annual *Salicornia*, *Salicornia patula* occurs.

S. patula has typical small bush growth form (Wilkoń-Michalska, 1985; Géhu, 1992). The plant goes from a few centimeters to 40 cm high, poorly, to profusely branched, always from the base, almost patent. The main features are: fertile shoots with convex sides, flowers 18.7 - 2.9 mm long consisting of 7.4 - 1.1 fertile segments relatively short (3.1 - 0.2 mm) and thin (2.5 - 0.4 mm). Flowers in fertile segments are unequal, usually lateral and much smaller than the central (Kaligarič *et al.*, 2008). It produces two kind of seeds: the central larger and the lateral smaller, differing in their germination biology (Grouzis *et al.*, 1976; Berger, 1985). *S. patula* occurs on sandy-loamy soils in the clearings or on the edges of the areas colonized by the perennial glassworts mostly dry and semi-dry during the summer (Grouzis *et al.*, 1977; Biondi & Casavecchia, 2010).

The seed germination behavior of *S. patula* has been studied in some populations of the Mediterranean coast of S-France (Grouzis *et al.*, 1976; Grouzis *et al.*, 1977; Heim & Grouzis, 1978, Berger, 1985) and of some populations of Croatia and Slovenia (Šajna *et al.*, 2013).

This work focused on the seed germination pattern of *S. patula* related to the environmental conditions of the localities where the seeds of the three populations studied were collected (Italy, Spain and Croatia). Thus,

the aim is to analyze the germination response to temperature and salinity of these populations in order to identify the key factors controlling germination phases for the implementation of projects on conservation and environmental restoration of the hypersaline environments. We also assessed the inter specific competitiveness of this species related to the conditions of the different localities.

Materials and Methods

Site of collection

Seeds were collected from wild populations in three different localities (Fig. 1; Tab. 2).

The site 1 of seeds collection is Sentina Natural Regional Reserve (Marche, Italy). It occurs at the mouth of River Tronto in the northern side. The Reserve still has a particularly rich and interesting flora and vegetation, especially when compared to the regional coastal areas strongly altered by man (Brilli-Cattarini, 1970; Biondi *et al.*, 1988; Biondi *et al.*, 1989; Biondi & Formica, 2000; Conti *et al.*, 2013).

The site 2 is Blace in the Neretva Delta Valley (Dubrovačko-Neretvanska region, Croatia). The halophytic marshy vegetation is the most widespread and ecologically important in the Neretva delta (Horvat *et al.*, 1974; Lakušić *et al.*, 1977). It is a small bay almost completely closed and separated from the open sea with 500 m long (averagely 50 m wide) sickle-like sandy beach in the middle. Connection with the sea is restricted to one narrow passage. This situation provides very still water and unobstructed sedimentation of sand (Alegro *et al.*, 2004).

The site 3 is Sant Pere Pescador, Girona (Spain). It is the inner plain area of the River Fluvià, near to the Aiguamolls de l'Empordà Natural Park (Catalonia, North-East Iberian Peninsula). A general characteristic of this coastal plain is the abundant variations in mi-

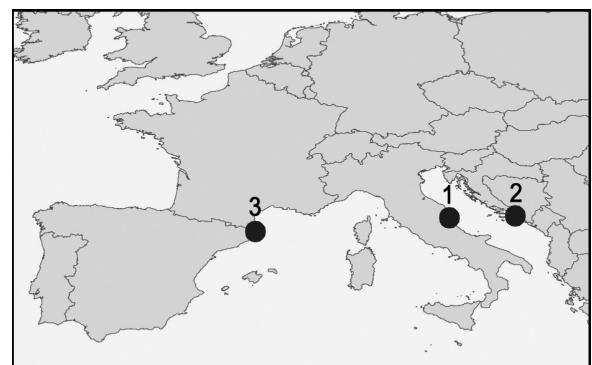


Fig. 1 - Map of the sites of collection of *Salicornia patula*: (1) Sentina Natural Regional Reserve (IT) [42° 90' N, 13° 90' E]; (2) Blace (CR) [43° 01' N, 17° 62' E]; (3) Sant Pere Pescador, Girona (ES) [42° 14' N, 3° 11' E]. System reference: Datum WGS84.

crotopography and sedimentation patterns that occur over relatively small areas producing a complicated mosaic effect in the vegetation, creating many halophytic communities. This mosaic is further emphasized by human activity.

Bioclimatic classification of the sites

According to the bioclimatic classification of Rivas-Martínez *et al.* (2011), site 1 and site 2 are in the Temperate oceanic (sub mediterranean variant) bioclimate. Site 1 belongs to the lower mesotemperate thermotype and lower sub humid ombrottype horizon, site 2 to the upper thermotemperate thermotype and lower humid ombrottype horizon whereas site 3 to the Mediterranean pluvisesional-oceanic bioclimate, of the low mesomediterranean thermotype and upper dry ombrottype horizon. For the subsequent analysis, the following bioclimatic indices (Rivas-Martínez *et al.*, 2011) were considered: the mean annual temperature (T), the annual ombrothermic index (Io) and the bimonthly summer ombrothermic index (Ios₂). The bioclimatic indices were derived from the bioclimate map of Italy (Pesaresi *et al.*, 2014) and the worldclim datasets (Hijmans *et al.*, 2005). Sites bioclimatic classification and bioclimatic indices are summarized in Table 1.

Phytosociological classification of site of collection

The only relevé of *Suaeda maritima*-*Salicornietum patulae* was carried out according to the phytosociological method of the Sigmatis School of Zurich-Montpellier, as proposed by Braun-Blanquet (1964), and successively integrated (Rivas-Martínez, 2005; Géhu, 2006; Biondi, 2011; Pott, 2011). The syntaxonomic classifications follow the Prodrome of Vegetation of Italy (Biondi *et al.*, 2014) and its online advancing version (<http://www.prodromo-vegetazione-italia.org/>).

Seed collection

Mature seeds were collected in the three sites (Fig. 1) between October and December 2013, 2014, 2015 (Tab. 1) following international protocols (Bacchetta *et al.*, 2006; International Seed Testing Association, 2006).

For each population studied there is a sample in the Herbarium Anconitanum (ANC) of the Polytechnic

University of Marche. The Herbarium codex is ANC Num. 27757 for *S. patula* of Sant Pere Pescador, Girona (Spain); ANC Num. 27758 for *S. patula* of Blace (Croatia); ANC Num. 27759 for *S. patula* of Sentina Natural Regional Reserve (Italy).

After the harvest, the seeds were extracted from the articulated segments and gently cleaned on a rubber mat.

The empty seeds and impurities were removed with a blower (Agricullex CB1 Column Seed Cleaner, T.A. Baxall and Co., Ltd). Then, the seeds were stored in paper bags at room temperature until the start of the experiments. All the seeds were dried in dry room at 15 °C and 15% relative humidity for some days before weighing.

Seed characterization data and analysis

Images of a hundred seeds were taken over millimeter paper in a binocular stereozoom microscope (Nikon SMZ800) and dimensions, width and length, were measured using the image analysis software ImageJ (Rasband, 1997-2012). Ten batches of ten seeds each were weighted using an Orion Cahn C-33 microbalance with a precision of 1µg.

Seed surface was analysed with a SEM Hitachi S-4800, file emission, from the SCSIE Department, Electronic Microscopy Section of the University of Valencia. Samples were examined at an accelerating voltage of 5 kV. Different levels of magnifications (x70, x600, x1000 and x20000) were used to determine the seed coat morphological features of *S. patula* from different populations. The terminology of Stearn (1992), Barthlott (1984) and Werker (1997) was used to describe seed features.

Seed volume was calculated with the equation of Dias & Ganhão (2012). We tested the population differences in terms of volume (log transformed) and weight by One Way ANOVA.

Germination assay and analysis

Seeds were sown on 1% distilled water agar held in 6 cm diameter transparent polyethylene Petri dishes. Germination response was tested in programmable-environmental chambers with controlled temperature and illumination for 30 days (Keifer & Ungar, 1997).

Tab. 1 - Collection sites with bioclimate analysis: annual precipitation (P), mean annual temperature (T), annual ombrothermic index (Io), bimonthly summer ombrothermic index (Ios₂). Sites of collection: (1) Sentina Natural Regional Reserve (IT), (2) Blace (CR), (3) Sant Pere Pescador, Girona (ES).

SITE OF COLLECTION	BIOCLIMATE	P (mm)	T (°C)	Io	Ios ₂
1	Temperate oceanic (submediterranean var.) - lower mesotemperate - lower subhumid	757	15	4.21	2.02
2	Temperate oceanic (submediterranean var.) - upper thermotemperate - lower humid	1194	16	6.18	1.7
3	Mediterranean pluvisesional-oceanic - low mesomediterranean - upper dry	579	16	3.02	1.36

Four replicates of 25 seeds were used for every treatment of *S. patula*. Seeds were considered to be germinated with the emergence of the radicle. Germination response to temperature was evaluated at 7 constant temperatures ranging between 5 and 35° C and at one alternating temperature regime of 25/15° C (Tab. 4). Illumination was provided for 12 hours each day by 30 W cool white fluorescent lights. Germination tests were conducted 1 to 13 months after the harvest (germination test date: 27/01/2015).

The germination salt tolerance was tested at 14 increasing NaCl concentrations: 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 1000 mM at constant temperature (25° C) with a 12-h photoperiod. The seeds that did not germinate in the salt experiments were transferred to distilled water and maintained for 30 days to check the recovery capacity. The recovery germination percentage was calculated from the total number of seeds sown. Samples were randomly selected. The tests at different temperatures and NaCl concentrations allowed the determination of t_{50} ; i.e. the days to achieve 50% germination (Thanos & Doussi, 1995).

Germination response data were analysed by generalized linear model (GLM) with a logit link function and a binomial error structure constrained to Temperature and Salinity and Site of Collection. At first a full model including all main factors (Temperature, Salinity and Site of Collection) and interactions was fitted, then the full model was reduced according to the deviance likelihood ratio test. To plot the germination response curves, an extended logistic regression model (with lower limit at 0 and upper limit estimated) were fitted by the 'drc' package (Ritz & Streibig, 2005).

The difference between the alternating temperature (25/15° C) and the constant temperature (20° C) and between the salt-treated groups (salinity and recovery) and the control group, were tested by one-way ANOVA. The germination percentages were previously arcsine – square root transformed.

In order to identify the principal gradient of germination variation both in terms of salinity and temperature, and then to evaluate the differences and similarities between the *S. patula* populations, a standardized Principal component analysis (PCA), was performed on the arcsin-square root transformed germination data. The mean bioclimatic and morphological characteristic of the three populations were fitted on the PCA biplot.

All analysis were performed in R (R Development Core Team 2011).

Results

Phytosociological classification

According to the Vegetation Prodrôme of Italy (Biondi et al., 2014), *S. patula* is a characteristic species of

the *Salicornion patulae* Géhu & Géhu-Franck ex Rivas-Martínez 1990 alliance (*holotypus*: *Suaedo maritimae-Salicornietum patulae* (Brullo & Furnari 1976) Géhu & Géhu-Franck 1984 Rivas-Martínez 1990 *corr.*) belonging to the order *Thero-Salicornietalia* Tüxen in Tüxen & Oberdorfer ex Géhu & Géhu-Franck 1984 and the class *Thero-Suaedetetea splendidis*.

In Italy, this annual community generally develops, in a catenal contact, inside the clearings of halophilous perennial formations of the class *Sarcocornietea fruticosae*. The alliance *Salicornion patulae* is in a catenal contact with the hemicryptophyte formations of the order *Juncetalia maritimi* where there is a little amount of salt. Sometimes, this annual community is also in catenal contact with cliff vegetation and with the plant communities of sand dunes. In the European Mediterranean area, this hyper-saline vegetation is rare and threatened by a wide variety of pressures (tourist and industrial practices, coast management, invasive species). *S. patula* is a characteristic species in the habitat 1310 "Salicornia and other annuals colonizing mud and sand" (Codex sensu Eunis Classification: A2.6513) (Biondi et al., 2013).

In the Sentina Regional Natural Reserve (Marche, Italy) *S. patula* forms the *Suaedo maritimae-Salicornietum patulae* (Fig. 2) community. This association is widely distributed in Italy and known for many localities of Mediterranean and Adriatic coasts (the mouth of the Po River, Gargano, Gulf of Taranto, Géhu et al., 1984; the mouth of the Candelaro River, Biondi & Casavecchia, 2010; Saline of Margherita di Savoia, Corbetta et al., 2006). Currently, it is the only stable station of *S. patula* in Marche Region. In the study on the coastal vegetation of Marche Region, Biondi et al. (1989) found the *Suaedo maritimae-Salicornietum patulae* at the tourist Port of Ancona, but the station has not been maintained over the years.

Moreover, in the Sentina Regional Natural Reserve



Fig. 2 - *Suaedo maritimae-Salicornietum patulae* in the Sentina Regional Natural Reserve (Marche, Italy) in the summer 2015.

(Marche Region, Italy) *Suaedo maritimae-Salicornietum patulae* also occurs with the subassociation *crypsidosum aculeati* (Tab. 4 in Biondi *et al.*, 1988). It is an open, therophytic and pioneer vegetation that develops on sandy-loamy substrates, covered by water during the winter and chapped in warm weather due to the strong evaporation.

Moreover, *S. patula* constitutes a variant of the *Spergulario-Puccinellietum distantis* Freekes 1934 (Tab. 10 in Biondi *et al.*, 1989) association related to the morphology of the basin and the presence of the high salt content. It develops in small clearings of this association with *Aster tripolium* and *Crypsis aculeata*. These formations have been found over the years.

In Blace (Croatia), *S. patula* forms a thick community in a ruderal retrodunal zone near a camping. It is attributed to the association *Suaedo maritimae-Salicornietum patulae* (Tab. 2; Fig. 3).

In seed collection site of Sant Pere Pescador, Girona (Spain), there were only a few plants of *S. patula* attributed to the *Suaedo maritimae-Salicornietum patulae* subass. *suaedetosum splendidis*, widespread in clayey saline soils in the Alt Empordá coastal plain (Perich & Vilar, 2002)

Seed characterization

Seeds of collection 1 were larger than seeds of collection 2 and 3 in terms of mean seed length, width and volume (Tab. 2). Mean seed volume was statistically different [F(2, 296) = 110.297; P<0.001].

Mean seed weight was also significantly different [F(2, 27) = 7.156; P = 0.03]. The seeds of collection 3 were heavier than seeds of collection 1 and 2 (Tab. 2).

The seed surface of *S. patula* was hairy and dull. The seeds showed an elongation of the outer epidermal cells of the testa to form hooked hairs. The embryo was bent. Endosperm (actually perisperm) was absent. They were not covered by salt efflorescence. Their ventral region was flat and smooth or slightly rough while in the dorsal region there was an invaginated protuberance arranged in long parallel row (Fig.4).

The seed coat was usually brown, commonly well developed and thick. The surface was smooth or minutely pitted. The epidermal cells exhibited a jigsaw puzzle-like pattern. We have not observed any kind of

Tab. 2 - *Suaedo maritimae-Salicornietum patulae* (Brullo & Fumari 1976) Géhu & Géhu-Franck 1984 Rivas-Martínez 1990 *corr.*

Relevè number	1
Cover (%)	30
Surface (m ²)	10
Salicornia patula Duval-Jouve	2.3
Suaeda maritima (L.) Dumort.	2.2



Fig. 3 - *Salicornia patula* in the bay of Blace (Croatia) in October 2014.

papilloid projections. The seed ornamentations were present over the whole surface that presented hook-shaped simple trichomes (Fig. 5).

Germination tests

Temperature effect

Germination of *S. patula* was quite high for seeds from all the three populations with maximum germination of 87.4 % for the population 3; 87.0 % for the population 1; 71.2 % for the population 2, all at 35° C (Tab. 4).

The analysis of deviance for the binomial GLM fitted to *S. patula* seed germination showed the significant effects of the main factors temperature and site of collection as well as their interaction with a total deviance explained of 61.25%. Especially the temperature, the site of seed collection and their interaction account respectively for 45%, 13% and 3% of the total deviance (Tab. 5). The significant interaction term means that

Tab. 3 - Mean and standard deviation values for length (n=100), width (n=100), volume (n=100) and weight (n=10 x 10) of seeds of the three populations of *Salicornia patula*. Population 1 from Sentina Natural Regional Reserve (IT), population 2 from Blace (CR) and population 3 from Sant Pere Pescador, Girona (ES).

pop	Length (mm, mean ± stdv)	Width (mm, mean ± stdv)	Volume (mm ³ , mean ± stdv)	Weight (µg, mean ± stdv)
1	1.09 ± 0.81	0.81 ± 0.13	0.23 ± 0.08	202 ± 36.8
2	1.05 ± 0.12	0.60 ± 0.08	0.11 ± 0.03	207 ± 33.2
3	1.03 ± 0.16	0.60 ± 0.12	0.11 ± 0.05	253 ± 29.3

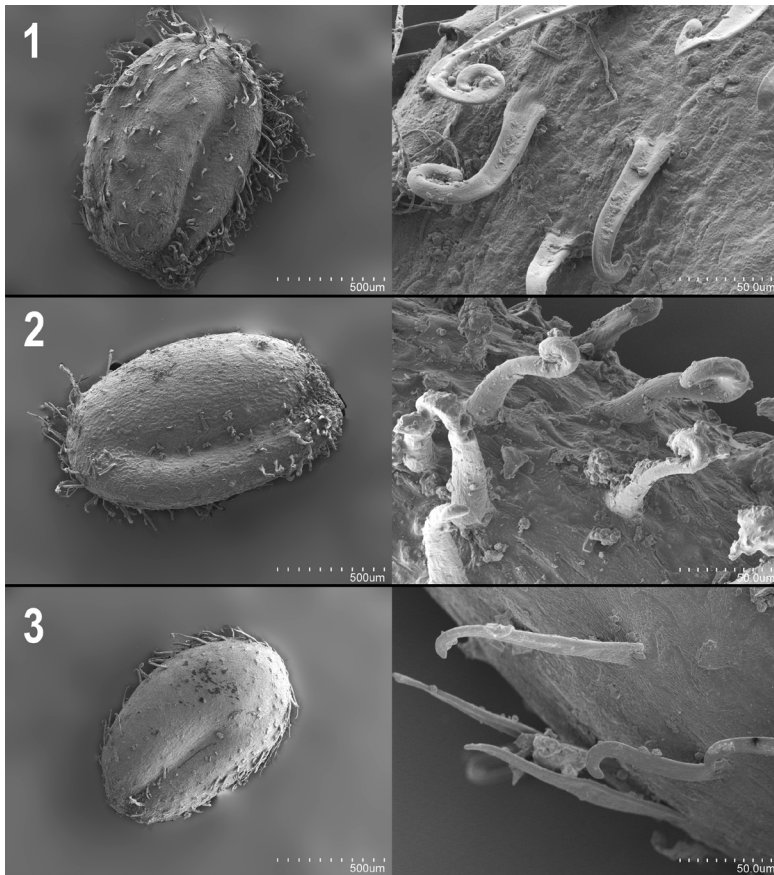


Fig. 4 - Seeds and hairs under SEM examination of *Salicornia patula* in the studied populations: (1) Sentina Natural Regional Reserve (IT); (2) Blace (CR); (3) Sant Pere Pescador, Girona (ES) (a) x70 and (b) x600.

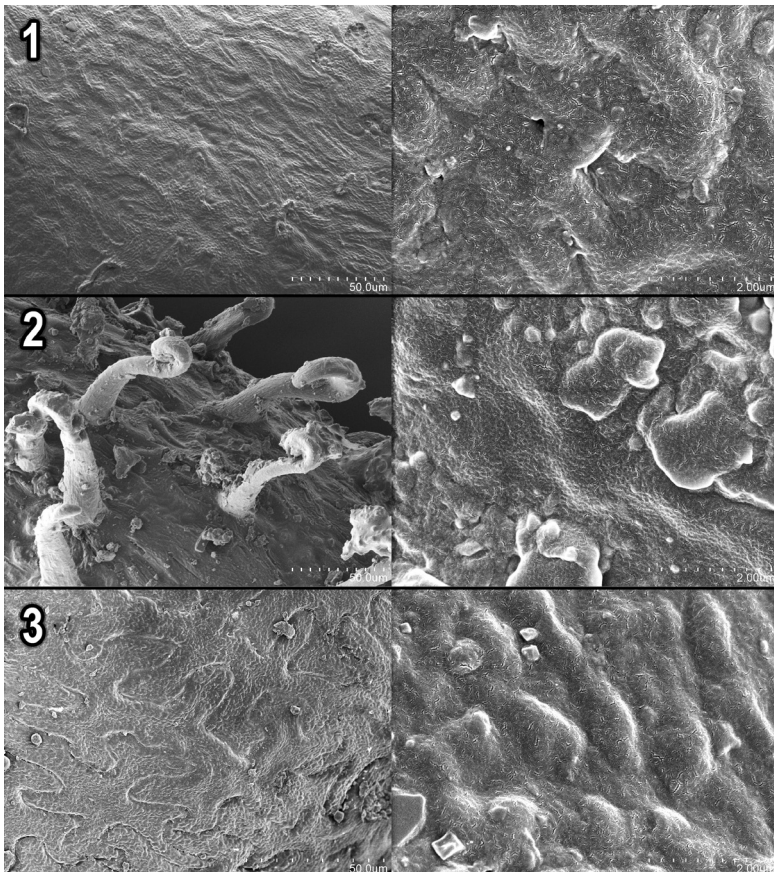


Fig. 5 - Cell surfaces under SEM examination of *Salicornia patula* in the studied populations: (1) Sentina Natural Regional Reserve (IT); (2) Blace (CR); (3) Sant Pere Pescador, Girona (ES) (a) x1000 and (b) x20000.

Tab. 4 - Final germination percentage (mean and st. dev.) and t_{50} for seeds from population 1: Sentina Natural Regional Reserve (IT); population 2: Blace (CR); population 3: Sant Pere Pescador, Girona (ES) at different temperatures.

pop	Germination (%)						
	5 °C	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C
1	45.0 ± 11.9	54.1 ± 4.4	42.5 ± 7.7	57.0 ± 12.7	83.7 ± 9.6	78.5 ± 10.1	86.7 ± 7.1
2	24.6 ± 5.1	22.3 ± 13.1	41.6 ± 17.3	61.6 ± 8.2	68.4 ± 9.5	64.9 ± 12.2	72.0 ± 18.5
3	77.9 ± 23.2	63.5 ± 7.7	71.7 ± 7.0	70.5 ± 15.6	78 ± 4.0	63.9 ± 13.8	85.6 ± 3.8
Time days to achieve 50% germination (t_{50})							
1	8.0 ± 1.9	5.2 ± 2.7	6.6 ± 1.0	5.8 ± 0.7	2.7 ± 2.1	4.9 ± 2.8	0.8 ± 0.1
2	10.5 ± 3.5	5.8 ± 3.1	8.9 ± 1.5	9.8 ± 3.9	7.6 ± 3.0	6.0 ± 1.3	4.7 ± 1.5
3	4.5 ± 0.8	5.2 ± 2.4	2.8 ± 2.3	1.8 ± 1.5	2.5 ± 1.2	0.8 ± 0.1	2.1 ± 0.7

Tab. 5 - Analysis of deviance table for the binomial GLM fitted to *Salicornia patula* seed germination constrained to temperature and site of seed collection. (Signif. codes: 0 '***'; 0.001 '**'; 0.01 '*'; 0.05 '.'; 0.1 ' '; 1).

Source	Df	Deviance	Deviance (%)	Resid.Df	Resid. Dev.	p-value
Deviance Tot.				80	375.18	
Temperature (T)	1	168.956	45	79	206.22	0.001 ***
Site of Collection (SC)	2	49.109	13	77	157.11	0.001 ***
T X SC	2	11.81	3	75	145.3	0.04 *

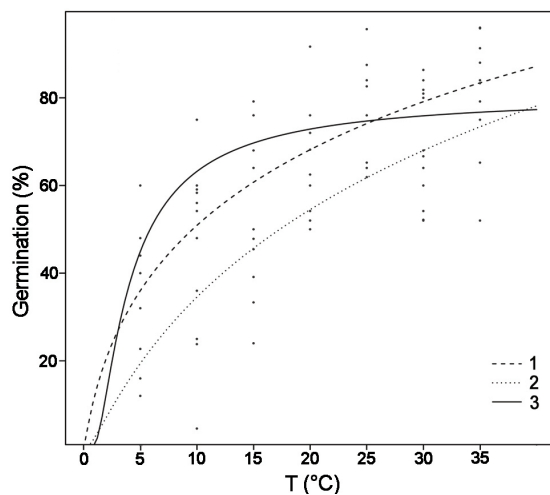


Fig. 6 - Germination response curves for the binomial GLM fitted to *Salicornia patula* seed germination (Germination) constrained to Temperature (T) and Site of seed collection. (Sites of collection: 1: Sentina Natural Regional Reserve (IT); 2: Blace (CR); 3: Sant Pere Pescador, Girona (ES)).

S. patula germination response and temperature relationship was different in one site of collection. In fact, the population from Girona showed a different pattern of the germinative response curve. This population reached a high germination at low temperatures (5, 10 and 15° C) while populations 1 and 2 had a similar and more gradual germinative trend but on two different average levels of germination (Fig. 6; Tabs. 4, 5).

The seeds of *S. patula* showed an optimal range of temperature between 25° C and 35° C.

S. patula exhibited no significant difference between constant and alternating temperature ($P < 0.05$).

Salt effect

Population 1 had the highest germination percentages on saline substrate than the other. The maximum germination for population 1 was 91.0% at salt concentration of 100 mM. Conversely, the minimum germination for population 1 was 24.1% at salt concentration of 800 mM. Population 2 showed the maximum germination (68.5%) at salt concentration of 100 mM. Conversely, the minimum germination for population 2 was 7.1% at salt concentration of 700 mM. Population 3 had the maximum germination (67.0%) at salt concentration of 50 mM. Conversely, the minimum germination for population 3 was 15.0% at salt concentration of 1000 mM. The three populations showed a germination up to 1000 mM and they had almost 50.0% of germination until 500 mM (Tab. 6).

The analysis of deviance for the binomial GLM fitted to *S. patula* seed germination showed the significant effects of the main factors "Salinity" and "Site" of seed collection (the interaction term was not significant) with a total deviance explained of 61.0%. Salinity and site of seed collection accounted for 56.0% and 5.0% respectively of the total deviance (Tab. 7). The seed response curve germination for the three sites of collection had a similar relationship with the salinity concentrations with a gradual decrease on different average levels of germination (Fig. 7; Tab. 7).

The seeds of *Salicornia patula* exposed to all NaCl concentrations exhibited good germination recovery after being transferred to distilled water (Fig. 8).

The seed germination behavior in the recovery phase varied in the three populations. Population 1 had the highest recovery percentages at 700 mM (42.6%; Fig. 8). The rate of germination recovery of popula-

Tab. 6 - Final salt germination percentage (mean and st. dev.) and t_{50} for seeds from population 1: Sentina Natural Regional Reserve (IT); population 2: Blace (CR); population 3: Sant Pere Pescador, Girona (ES) at different salt concentrations at 25 °C.

pop	Germination (%)														
	0 mM	50 mM	100 mM	150 mM	200 mM	250 mM	300 mM	350 mM	400 mM	450 mM	500 mM	600 mM	700 mM	800 mM	1000 mM
1	84.0 ± 9.6	77.0 ± 13.9	91.0 ± 7.4	68.0 ± 5.7	74.0 ± 10.8	48.0 ± 11.6	50.0 ± 9.2	58.0 ± 6.9	43.0 ± 16.1	36.0 ± 16.0	42.0 ± 19.2	32.0 ± 13.8	29.5 ± 13.8	24.1 ± 5.1	24.3 ± 8.4
2	68.4 ± 9.5	50.04 ± 7.1	68.5 ± 14.2	54.0 ± 6.6	53.29 ± 14.0	49.0 ± 4.5	35.07 ± 8.3	30.0 ± 6.5	32.0 ± 17.7	48.0 ± 19.1	44.91 ± 4.8	38.0 ± 16.5	7.1 ± 3.7	18.8 ± 9.4	9.94 ± 8.2
3	78.0 ± 4.0	67.0 ± 3.3	60.0 ± 5.5	51.0 ± 9.0	45.8 ± 18.2	48.0 ± 18.2	31.0 ± 11.8	57.54 ± 20.1	51.0 ± 17.7	40.4 ± 14.9	42.91 ± 5.8	31.0 ± 13.4	28.2 ± 5.4	17.1 ± 14.7	15.1 ± 5.1
pop	Time days to achieve 50% germination (t_{50})														
	0 mM	50 mM	100 mM	150 mM	200 mM	250 mM	300 mM	350 mM	400 mM	450 mM	500 mM	600 mM	700 mM	800 mM	1000 mM
1	2.7 ± 2.1	2.9 ± 1.1	3.8 ± 3.0	3.8 ± 1.7	2.7 ± 2.5	2.9 ± 3.2	5.7 ± 4.0	4.5 ± 5.0	2.0 ± 0.5	4.7 ± 3.8	9.5 ± 9.5	2.0 ± 1.8	3.5 ± 2.0	1.1 ± 0.6	0.9 ± 0.4
2	7.6 ± 3.0	2.6 ± 0.8	3.4 ± 0.8	4.8 ± 2.4	4.9 ± 1.1	5.5 ± 3.0	4.0 ± 1.8	5.0 ± 3.0	5.3 ± 3.7	4.7 ± 0.7	6.8 ± 6.0	3.6 ± 2.6	3.2 ± 1.9	2.0 ± 1.8	1.3 ± 1.0
3	2.5 ± 1.2	1.4 ± 0.5	4.7 ± 1.0	1.6 ± 0.9	1.8 ± 0.8	3.0 ± 1.8	2.9 ± 2.0	1.2 ± 0.6	2.3 ± 1.2	2.3 ± 1.4	6.3 ± 6.4	0.8 ± 0.2	2.7 ± 1.2	1.9 ± 1.8	1.3 ± 1.0

Tab. 7 - Analysis of deviance table for the binomial GLM fitted to *Salicornia patula* seed germination constrained to salinity concentration and site of seed collection. (Signif. codes: 0 ‘***’; 0.001 ‘**’; 0.01 ‘*’; 0.05 ‘.’; 0.1 ‘ ’; 1).

Source	Df	Deviance	Deviance (%)	Resid. Df	Resid. Dev.	p-value
Deviance Tot.				179	977.46	
Sal (S)	1	545.65	56	178	206.22	0.001 ***
Site of Collection (SC)	2	48.87	5	176	382.94	0.001 ***

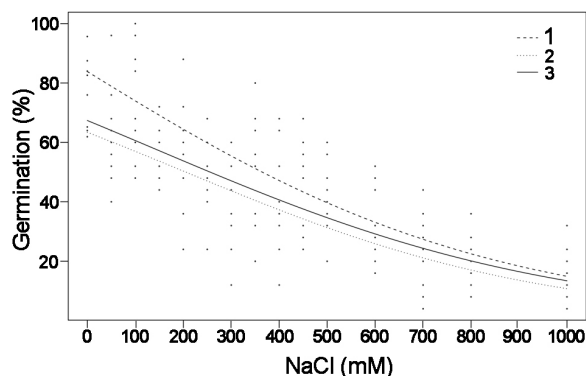


Fig. 7 - Germination response curves for the binomial GLM fitted to *Salicornia patula* seed germination (Germination) constrained to Salinity (NaCl) and Site of seed collection. Sites of collection: 1: Sentina Natural Regional Reserve (IT); 2: Blace (CR); 3: Sant Pere Pescador, Girona (ES).

tion 1 (IT) seemed to be unaffected by the pretreatment concentration of NaCl. Similarly, population 3 had the highest recovery percentage at 400 mM (32%; Fig. 8). The rate of germination recovery of population 3 seemed to be unaffected by the concentration of NaCl used for salinity tests. Population 2 had the highest recovery percentage at 700 mM (27.6 %; Fig. 8). Whereas, the rate of germination recovery of population 2 seemed to be affected by the concentration of NaCl used for salinity tests. In particular, the one way ANOVA ($P < 0.05$) performed on final germination of salt tests versus final germination obtained on neutral substrate shows that seed germination of population 2

seemed to be permanently inhibited by high salinities (700, 800 and 1000 mM). The permanent inhibition, even if it was not shown by all of the seeds sown, caused a significant decrease in germination [$F(14, 45) = 4.470, P < 0.001$].

The PCA ordering has clearly identified the main gradients of variation for the sites of seed collection (populations) of *S. patula* germination data, in terms of both temperature and salinity. The first two gradients (PC1 and PC2 axes) accounted for 52.0% of the total variation and have clearly underlined the main differences between the three sites of collection (Fig. 9). The first gradient (PC1 axes) was principally determined and directly linked to the germination at low temperatures (T5, T10, T15) and well separated the site 1 of Girona from the other two (Blace and Sentina). The second gradient PC2 was mainly determined by S100, S300 and S50 inversely with T20, S450, T25.15 and separated the site of collection of Sentina from the other two. The fitting of the site bioclimatic indices values (Tab. 1) and the seed morphological characteristics (Tab. 3) on the PCA biplot showed a direct correlation between weight (We) and PC1 and a inverse correlation between the annual ombrothermic index (Io) and PC1. While PC2 is directly correlated with the bimonthly summer ombrothermic index (Io_s), the seed volume (V) and seed length (L) and inversely correlated with the mean annual temperature (T) (Fig. 9).

Discussion and conclusions

Salicornia patula colonizes unstable transition zones between permanently flooded muds and perennial vegetation. The beginning of the flooding period and the dry period may vary from year to year, depending on the erratic distribution of autumn and winter rains on the Mediterranean coast (Berger, 1985). In hypersaline habitats with an extreme variation of environmental conditions, the phenomenon of seed dimorphism provides multiple opportunities for a population to maintain its continuity and it is a particular strategy for survival (Philipupillai & Ungar, 1984; Khan & Ungar, 1986). According to Khan & Ungar (1986), in the stu-

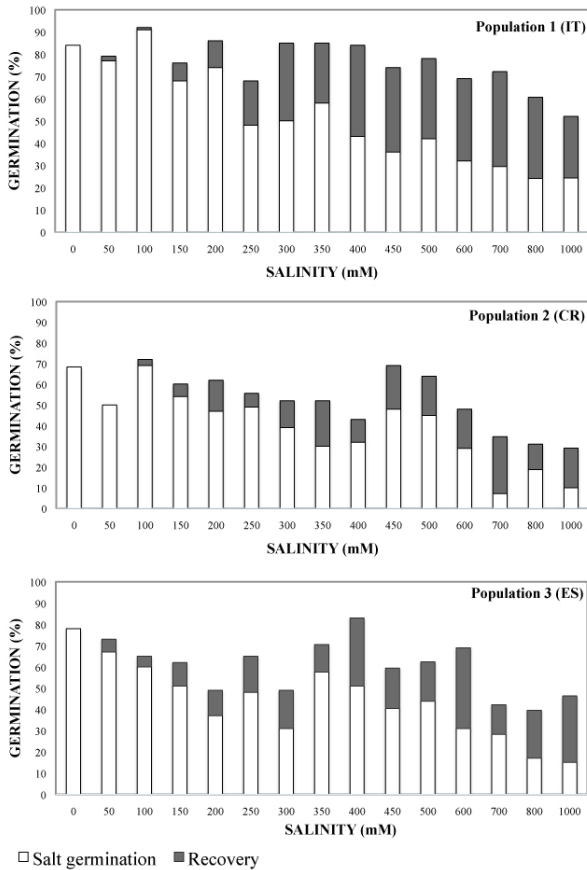


Fig. 8 - Germination percentages indicating the salinity effect (white) and total germination (light-grey) after recovery in seeds from population 1: Sentina Natural Regional Reserve (IT); population 2: Blace (CR); population 3: Sant Pere Pescador, Girona (ES) at 25° C.

dy about the life history and population dynamics of *Atriplex triangularis* the large seeds germinated early and rapidly in the growing season and were not part of the persistent seed bank. Small seeds were accumulated in the seed bank and did not germinate until the following spring (February to June).

According to several studies (Grouzis *et al.*, 1976; Berger, 1985; Šajna *et al.*, 2013), the seeds of *S. patula* showed heteromorphism: large central and small lateral seeds. The seeds of *S. patula* were not separated and randomly selected to germination test, to understand the response and the versatility of these short-lived plants related to the local environment. In fact, in this study we have not found any difference in germination response between small and large seeds, and according to Šajna *et al.* (2013) the populations studied did not need any pretreatment to germinate under saline conditions. This was in contrast with the findings of several authors who treated the seeds with scarification and cold stratification (Grouzis *et al.*, 1976), and the variation day and night (Philipupillai & Ungar, 1984). The morphological analysis carried out on *S. patula* demonstrated that the seed weight of population 3 was significantly different from those of population 1 and population 2 (Tab. 3). Such values were compared to the ones available in the literature for seeds of the same species. Published values were between 390 and 450 μg (Šajna *et al.*, 2013), considerably higher than those recorded for the three populations studied.

The seed ornamentations of the three populations were similar to each other. These were similar to those of the population of *Salicornia europaea* studied by

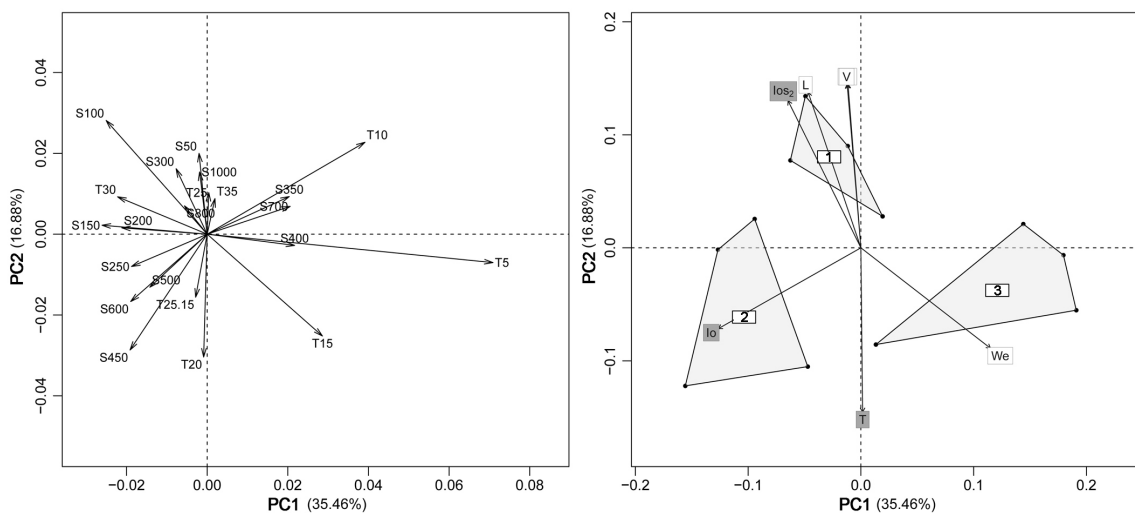


Fig. 9 - PCA ordering plots of the arcsine square root transformed germination test data for the three sites of collection *Salicornia patula*. A) plot of temperature and salinity scores of PCA axis 1 and 2. B) plot of the three site of collection of *S. patula* with the bioclimatic and morphological characteristics overfitted. Percentage of variance in the data explained by PCA axes 1, 2 are respectively: 35.46% and 16.88%. Labels: Salt concentrations: S50, 50 mM; S100, 100 mM; S150, 150 mM; S200, 200 mM; S250, 250 mM; S300, 300 mM; S350, 350 mM; S400, 400 mM; S450, 450 mM; S500, 500 mM; S600, 600 mM; S700, 700 mM; S800, 800 mM; S1000, 1000 mM; Temperatures: T5, 5 °C; T10, 10 °C; T15, 15 °C; T20, 20 °C; T25, 25 °C; T30, 30 °C; T35, 35 °C; T25/15, alternating temperature at 25/15 °C. Bioclimatic indices of the sites of collection: Io, Annual ombrothermic index, Ios₂, Summer bimonthly ombrothermic index; T, Mean annual temperature. Mean values of morphological seed characteristics: L, lenght; We, Weight; V, Volume. Sites of collection: 1, Girona; 2, Sentina; 3, Blace.

Sherpherd *et al.* (2005) and Gul & Weber (1999).

Moreover, the embryo of the seeds of the three populations can be characterized in line with the study of Martin (1946): large bent embryo without the starchy perisperm. According to Sherpherd *et al.* (2005) a larger embryo in annual species may confer an advantage, facilitating rapid germination and establishment to optimize growth before flowering and seed set.

The seeds of the populations of *S. patula* were not dormant and showed the ability to germinate at high percentages in a wide range of temperatures and salinity. The analysis of deviance (GLM regressions) (Tab. 4, 5, 6, 7; Fig. 6, 7) showed a significant effect of sites of seed collection on the response to the temperature and the salinity in germination tests. In particular, the effect of sites of seed collection was more important in the germination temperature test (13% of total deviance; Tab. 5) than in germination salinity tests (5% of total deviance, Tab. 7). Moreover, in the germination temperatures test, the site of collection interacted with the Temperature (Tab. 5). In fact, the population of Girona showed a different behavior of germination from the other two populations. The seeds from this population achieved immediately a high germination percentages at the low temperatures (5, 10 and 15 °C) while the other populations (Sentina and Blace) showed a more gradual trend (Tab. 4; Fig. 6).

The variability of germination in salinity tests induced by the 'site of collection' factor seems to be realized especially at low concentrations (0 to 500). The curves in response to salinity of the three sites were similar to each other. Therefore, the effect 'site' was significant for germination both in the salinity and temperature tests.

These trends were also confirmed by the PCA ordering: the gradient variation PC1 (the most important in terms of variation explained) was mainly determined by germination percentages at low-temperature and low salinity.

As expected, the effect 'site' was important in the germination response of this species, although seed germination of halophytic species is regulated by factors such as water, temperature, light, soil salinity, and their interactions (Noe & Zedler, 2000). There is a considerable variation in the halophyte seed germination response and in the different factors involved. Light and temperature conditions are insufficient to explain the germination differences registered in some cases, even in very closely related species. Several authors highlighted a close relationship between germination patterns of different species and environmental factors in their natural habitats (Baskin & Baskin, 1973; Bewley & Black, 1994; Estrelles *et al.*, 2010; Liu *et al.*, 2014; Estrelles *et al.*, 2015) and physiological response of seed germination of halophytes has evolved to adapt to specific environmental conditions (Khan &

Gul, 2006; Estrelles *et al.*, 2015).

The different percentages of germination at different levels of temperatures of the three site of collection appear to be related mainly to the different bioclimatic conditions in particular to the annual thermal ombrothermic index (Io). In fact, the PC1 axis order the three sites according to a descending humidity gradient: Blace (Io = 6.18), Sentina Regional Natural Reserve (Io = 4.21) and the driest locality Girona (Io = 3.02) (Tab. 2; Fig. 8). Moreover, the PC1 axis was related to the seed weight. The seed germination of the population of Girona shows an 'opportunistic' strategy of germination (Gutterman, 1993). The rapid germination response reported during the brief period of moistened soils might be an adaptation to arid environmental conditions (Estrelles *et al.*, 2015). The seeds are ready to germinate whenever there are favourable conditions for germination, giving the population a competitive advantage via early germination (Ross & Harper, 1972; Zia & Khan, 2004). This indicates that water availability, but not temperature, was a limiting factor for germination (Estrelles *et al.*, 2015). According to Shaikh *et al.* (2013) this behavior is especially necessary for the annuals to have viable seeds ready to germinate. So, as the seeds of the population *Suaeda corniculata* subsp. *mongolica* studied by Cao *et al.* (2012) in which seed germination occurred both in spring (low temperature, low precipitation moderate salinity) and mid - and late summer (high temperature, high precipitation, low salinity), the seeds of the population of Girona might also have a similar behavior.

In general, for the seeds of *S. patula* we can confirm that the optimal temperature range was between 25 and 35° C as reported in other studies carried out on the seeds of a population of *S. europea* from North-America that had the maximum germination at 25° C and the minimum at 10° C (Ungar, 1977). The germination augmented with increasing temperature in accordance with several studies on germination of *Salicornia rubra* and *S. pacifica* var. *utahensis* of Great Basin desert halophytes under various temperature regimes (Khan & Weber, 1986; Khan *et al.*, 2001). The seeds of *S. patula* did not show a significant difference between the germination percentages at constant temperature (20° C) and at fluctuating regime (25/15° C), according to the studies carried out by Berger (1985) on the seeds of the population of *S. patula* from Etang de Vaccares, Camargue, S France, where there was no significant difference between the maximum percentage obtained at optimal constant temperature of 23° C and the one obtained in alternating regime of 25/12° C.

The germination percentages variation in the salinity germination test induced by the sites of collection might suggest an adaptation to the summer aridity (bi-monthly summer ombrothermic index (Ios₂) and the annual average temperature T (Tab. 2, Fig. 8). In fact,

the PC2 axis separated the site of Sentina ($Ios_2 = 2.02$, $T = 15$) from the other two (Blace $Ios_2 = 1.7$, $T = 16.1$, Girona $Ios_2 = 1.36$, $T = 15.9$). Moreover, the PC2 axis is related to the seed mass.

In the recovery phase, the three populations studied differed from each other. According to Noe & Zedler (2000) and Estrelles *et al.* (2015), a variation in annual rainfall quantity and distribution causes significant seasonal changes in salt concentrations and modifies the degree of moisture and seed germination. The relationship between plant cover and soil moisture and salinity may change at other times of the year (Alvarez Rogel *et al.*, 2000) and this could have large effects on germination window and community dynamics because germinating and establishing plants respond to their immediate surrounding environment (Harper, 1977).

In fact, the seeds of population 1, where there is an average annual precipitation of 757 mm, Ios_2 of 2.02 and Io of 4.21 did not seem to be affected by a permanent inhibition. It had the best germination in the recovery phase. Similarly, the seeds of the population 3, with an average annual precipitation of 579 mm, Ios_2 of 1.36 and Io of 3.02 did not seem to be affected by a permanent inhibition after salt removal. Instead, the seeds of the population 2 where the average annual rainfall is 1194 mm, Ios_2 of 1.7 and Io of 6.18, had a permanent inhibition in the recovery phase at high salt concentrations (700/800/1000 mM).

According to Keifer & Ungar (1997), for annual plants the seed tolerance to salinity during germination is related mostly to the environmental conditions at the time of germination in natural habitats, while seed-bank response to extended salinity exposure is closely related to the environmental conditions during the seed dormancy period.

The reported results might actually demonstrate that the seeds of the population of Blace are less salt tolerant than those of Sentina and Girona. This suggests that *S. patula* was salt tolerant also at high salinities and it exhibited a germination behavior similar to other halophytes of *Salicornia* genus: *S. herbacea* (1700 mM; Chapman, 1960); *S. patula* (340 mM; Berger, 1985; Šajna *et al.*, 2013); *S. brachystachya* (240 mM; Huiskes *et al.*, 1985); *S. bigelovii* (1000 mM; Rivers & Weber, 1971); *S. europea* (850 mM; Ungar, 1962).

In conclusion, the local environmental conditions, especially Io and Ios_2 , are important variables to characterize and diversify the seed germination behavior in the three populations. In addition, *S. patula* seeds were able to germinate in high percentages at a rather wide range of temperature and salt concentrations. So, it can be stated that *S. patula* populations studied can be easily multiplied and successfully used for projects on conservation and environmental restoration of the hypersaline environments.

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Appendix: Relevè date and locality

Tab. 2: rel. 1, right side of ruderal retrodunal zone near to a camping in the Bay of Blace (CR) [43°01' N, 17° 62' E], 14.10.2014.
[Localities reference system: GAUSS-BOAGA (ROMA 1940) Est Zone]