

Research Paper

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Environmental predictors of seed germination in two *Halocnemum* species from Mediterranean (Balearic, Tyrrenic and Adriatic) and Red Sea coastal salt marshes

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Abstract

Reproductive strategies for specific populations are closely related to environmental factors. Consequently, they are fundamental for conservation plans and the management of threatened habitats like salt marshes. From this viewpoint, germination strategy under different temperatures and salt conditions, voltammetric parameters and molecular analysis were performed and compared in six *Halocnemum* populations (four of *H. cruciatum* and two of *H. strobilaceum*) growing on Mediterranean (Balearic, Tyrrenic and Adriatic) and Red Sea coasts to establish the relation to environmental variables. Significant interpopulation differences were found in all the evaluated parameters. The Mediterranean populations showed a variable opportunistic germination strategy that was directly related to the drought period length at the studied sites. Consequently, potential environmental predictors of seed response were identified. The most noteworthy were bioclimate, soil texture, continentality index, winter temperatures and summer precipitations. Additionally, voltammetric parameters were evidenced as indicators of maternal plant stress levels and, thus, as potential determinants of future seed responses. The phylogenetic analyses showed a split into two species that did not correspond to germination response. The phylogeographic analyses showed interpopulation differences in haplotype composition for *H. cruciatum*, but not for *H. strobilaceum*. In conclusion, the tight connection between seed responses and the ecological parameters of natural populations as an adaptation for successful seedling emergence was proved regardless of its phylogenetic relations.

Introduction

The link between plant populations, vegetation structure and composition, environmental factors, and the reproductive strategies that arise from it, particularly germination ecophysiology, are fundamental factors for maintaining the ecological balance between species. These aspects should be taken into account when planning the management of natural habitats (He et al., 2007; Gomez-Mercado et al., 2012; Gul et al., 2013; Zheng et al., 2013). Likewise, knowledge of genetic diversity and the phylogenetic ascription of species are crucial issues to contemplate when carrying out such conservation plans (Maxted et al., 2020). European Mediterranean halophytic vegetation currently faces a serious risk of extinction and is protected by Directive 92/43/EEC (1420 Mediterranean and thermo-Atlantic halophilous scrubs, *Sarcocornietea fruticosae*).

In this regard, not much information is available in the literature about the geographical variation of seed features and salt tolerance in the germination phase and their connection with the ecological factors in natural halophytic populations and, furthermore, linked with its variability and taxonomy in molecular terms. We studied this relation in different populations of two species of the genus *Halocnemum*, perennial shrubs living in the inland salt depressions and littoral marshes of the Mediterranean Basin with differences in salinity. Seeds do not present deep physiological dormancy, although Pujol et al. (2000) observed a certain degree of dormancy when the osmotic potential dropped, which disappeared during recovery. The previous analysis of two Mediterranean populations, situated in different climate regions, showed different germination strategies. Those located in an arid regime reveal an opportunistic germination strategy adapted to water availability, whereas the population in the temperate area presented notable adaptation to the season and duration of flooding, unless

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seeds had been previously exposed to high salt concentrations as an osmopriming effect (Estrelles et al., 2015). Indeed, different strategies have been previously detected in two populations of this genus, which were morphologically ascribed to distinct species, *Halocnemum strobilaceum* (Pall.) Bieb. and *H. cruciatum* (Forssk.) Tod. (Biondi et al., 2013). This gives rise to some questions about its causes and the role of genetic variation during the process. Therefore, it might be an intrinsic species character or an adaptation to environment variations, as proposed by Estrelles et al. (2015). It might be related to some genetic feature associated with either genetic variability or species division or lineage because both species were previously considered the same taxon (Papini et al., 2004).

Donohue et al. (2005) demonstrated the capacity of plants to adapt germination timing to diverse seasonal cues as an evolutionary response. Galloway and Etterson (2007) emphasized that maternal effects are a flexible evolutionary mechanism that allows plants to survive and colonize heterogeneous environments. However, several research works are about the characterization of seed traits, particularly germination patterns, of different taxa that are taxonomically related or not, for example, Song et al. (2005), Noumi et al. (2010), Chérifi et al. (2011), Kim et al. (2013) or Silva et al. (2014), among many others. Specific studies on *Halocnemum strobilaceum* (Pall.) Bieb. have been carried out on various territories by several authors (Pujol et al., 2000, 2001; Song et al., 2006; Qu et al., 2008; Hosseini and Shahmoradi, 2011), and some include scarce references about its autoecology. Gul et al. (2013) reviewed germination strategies in halophytes and found that wider variability in seeds provides numerous germination opportunities to ensure a population's continuity in a changing and stressful environment. Indeed, these authors further discussed how the perennial and annual halophytes living in different environments had developed particular strategies to maintain genetic diversity and to ensure enough variability to guarantee its survival in such a changing environment. Other studies relate fitness measured as germination success, among other parameters, to genetic diversity (Greimler and Dobeš, 2000; Ali et al., 2006; Crawford and Whitney, 2010; González-Varo et al., 2010; Li et al., 2020). Nevertheless, some authors report that seed responses are more related to environmental aspects than to genetic diversity (Heinicke et al., 2016; Rodríguez-Rodríguez et al., 2018), which might be mediated by epigenetic processes (Zhang and Ogas, 2009), whereas some specific genes have been associated with germination success in stressful environments (Liu et al., 2020; Rehman et al., 2020).

Otherwise, the *in situ* micro-extraction-assisted voltammetry of microparticles (VMP) assay has been recently revealed as an effective tool in detecting plant response to saline stress (Doménech-Carbó et al., 2019). This technique has been previously applied for taxonomic purposes to leaves (Doménech-Carbó et al., 2015; Ortiz-Miranda et al., 2016) and seeds (Doménech-Carbó et al., 2017). It exploits the electroactive character of many electroactive compounds in plant matter, ranging from lignins (Kilmartin and Hsu, 2003; Milczarek, 2009; Admassie et al., 2014) to flavonoids and flavones, etc. (Grygar et al., 2003; Doménech-Carbó et al., 2005, 2010a, 2010b; Gil and Couto, 2013).

The objectives of this study were to evaluate the correlation between seed parameters and the germination responses with environmental factors that are significantly involved in these processes; determine the ecological predictors responsible for behavioural strategies in natural populations; assess the role of different types of genetic features in seed responses related to taxonomical

ascription of species, phylogeographical relations and genetic diversity.

Furthermore, the voltammetric response of microparticulate films from ethanolic seed extracts has been studied in contact with aqueous acetate buffer to detect possible differences associated with the distinct stress experienced by mother plants in relation to the environmental conditions of the ripening year.

In order to clarify these issues, the following hypotheses about *Halocnemum* species were posed:

- Specific climatic and edaphic factors are determinant parameters that control variation in seed response to temperature and salt tolerance.
- Germinative strategy correlates with phylogenetic or phylogeographic relations.
- Germinative response correlates with genetic diversity.
- The voltammetric response of seeds is a good indicator of the stress level to which the seed-producing plant has been subjected in its natural habitat.

Materials and methods

Population characterization

Field sites description

Six populations were considered: (1) Parque Natural El Hondo, Crevillente, Spain; (2) Ravenna, Riserva Naturale Sacca di Bellocchio; (3) Toscana, Principina a Mare, Parco Naturale della Maremma; (4) Cagliari, Santa Gilla, Sardegna; (5) Saline di Trapani, Sicilia, Italy and (6) Sharm el Sheik, Ras Mohamed National Park, southern extreme of the Sinai Peninsula, Egypt. Populations 2 and 3 correspond to *H. strobilaceum*, while the rest correspond to *H. cruciatum*. The population distribution map for the studied populations is shown in Fig. 1.

Ecological characterization was done by the analysis of 38 parameters per population (Supplementary Appendix A). The bioclimatic diagnosis was typified from monthly temperature and precipitation averages, which were obtained from the Global bioclimatics, Phytosociological Research Centre, Spain (Rivas-Martínez, 2008) and the 'Servizio Meteorologico dell'Aeronautica Militare', Italy (<http://www.meteoam.it/>). The field method (Kew et al., 2004) was used to define soil texture. Phytosociological characterization was performed according to Biondi et al. (2013).

Molecular characterization

DNA extraction and PCR amplification. Four individuals per population were germinated to extract DNA for the molecular analysis. These analyses were conducted to prove the phylogenetic ascription and lineage relations of the species and as an approach to their genetic diversity levels. DNA was isolated from *Halocnemum* seedlings following the Doyle and Doyle (1987) protocol, modified by Soltis laboratory (2002; <https://www.floridamuseum.ufl.edu/wp-content/uploads/sites/95/2014/02/CTAB-DNA-Extraction.pdf>) and other minor modifications adapted to our organism (700 µl CTAB; 30-minute incubation at 65°C; longer centrifugations; Milli-Q water for DNA resuspension). The entire internal Transcribed Spacer (ITS1 and ITS2, including 5.8S) region of nuclear rDNA was amplified using the universal primer pair ITS1-ITS4 (White et al., 1990). PCR reactions were carried out following Molins et al. (2018). The *trnQ-5'rps16* region of the chloroplast genome T fragment from Shaw et al. (2007) was also amplified following the author's

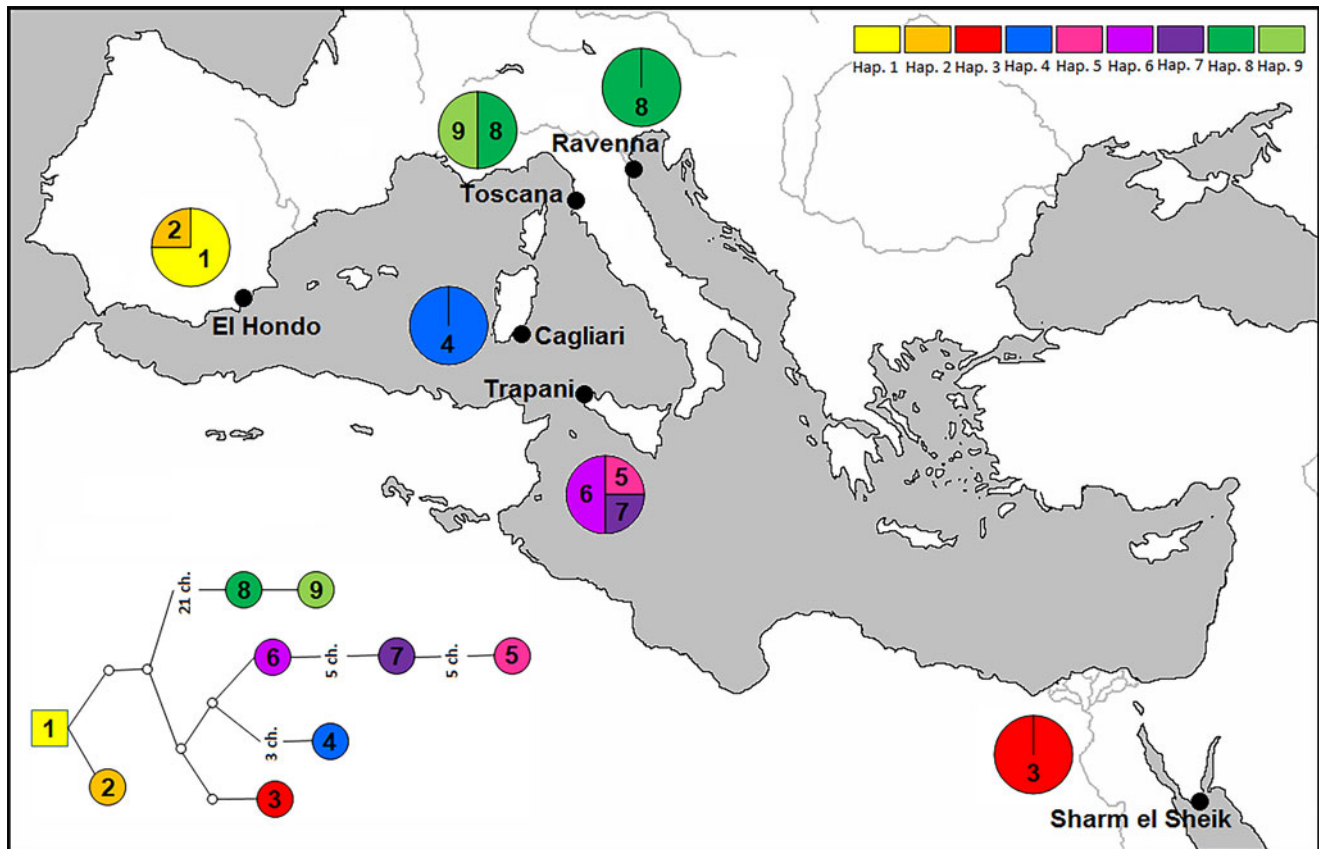


Fig. 1. Studied populations: (1) El Hondo, (2) Ravenna, (3) Toscana, (4) Cagliari, (5) Trapani and (6) Sharm el Sheikh. Sector graphs represent haplotype partition. The statistical parsimony haplotype network (bottom right): different haplotypes are coded with colours and numbers, where 1–5 haplotypes correspond to *H. strobilaceum* and 8–9 to *H. cruciatum*. Small white circles represent the single nucleotide intermediate changes. When these changes are multiple, the number of intermediate changes is specified, for example, 21 changes split the haplotypes corresponding to the two species.

instructions. PCR reactions were run in 25 μ l using the EmeraldAmp GT PCR Master Mix (Takara, Shiga, Japan), with the addition of *Halocnemum* template DNA, the specific primers explained above and water. The amplified PCR products were sequenced in an ABI 3100 Genetic Analyzer using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA).

Sequence alignment and phylogenetic analysis. MAFFT v. 7.402 (Kato et al., 2002; Kato and Standley, 2013) was used to generate a multiple sequence alignment. An automatic search for parameters was performed and the L-INS-I algorithm was selected, which is suitable for up to 200 sequence alignments. 200PAM/k = 2 scoring matrix, the gap open penalty of 1.53 and the offset value were the other parameters. Manual optimization consisting in trimming alignment ends of longer sequences or replacing gaps at the ends of shorter sequences with 'N' (IUPAC base representing any base) was carried out in MEGA v. X (Kumar et al., 2018). Ambiguously aligned regions were automatically dealt with using GBlocks v. 091b (Castresana, 2000) and implementing the least stringent parameters, but allowing gaps in 50% of sequences (NCBI accession numbers: MK928464–MK92848, ITS; MK937247–MK937270, *trnQ-5'rps16*).

A maximum likelihood (ML) phylogeny was estimated with RAxML-HPC2 (Stamatakis, 2006; Stamatakis et al., 2008). The analysis used the GTRGAMMA substitution model for three delimited partitions, two in nrITS (ITS1 + ITS2 and 5.8S) and

the *trnQ-5'rps16* chloroplast region, and 1000 pseudoreplicates were conducted to evaluate nodal support. Additionally, a Bayesian phylogenetic MCMC analysis was implemented using MrBayes v. 3.2.2 (Ronquist et al., 2012). Indels were coded by SeqState v. 1.4.1 (Müller, 2005) according to modified complex coding. Coded indels were considered a partition of standard data (states = 0, 1, 2) with a gamma rate and hyperprior set at 1.0 to allow different stationary state frequency proportions to be explored by the MCMC procedure. The optimal substitution models for the above DNA three partitions were inferred with PartitionFinder2 (Lanfear et al., 2016) by considering a model with linked branch lengths for ITS partitions and the Bayesian Information Criterion (BIC). This analysis favoured the model TRN for the ITS1 + ITS2 partition, JC for 5.8S and F81 for the *trnQ-5'rps16* chloroplast region. Then, the MrBayes analysis was conducted with two parallel and simultaneous four-chain runs executed over 2×10^6 generations starting with a random tree, and with sampling after every 500th step. The first 25% of the data was discarded as burn-in. The 50% majority-rule consensus tree and the corresponding posterior probabilities were calculated from the remaining trees. Chain convergence was assessed by ensuring that the average standard deviation or split frequencies (ASDSF) values were below 0.01 and the potential scale reduction factor (PSRF) values approached 1.00. FigTree v. 1.4.4 was used to construct the 50% majority-rule consensus tree. Programmes MAFFT, RAxML-HPC2, MrBayes and PartitionFinder2 were hosted on the CIPRES Science Gateway (Miller et al., 2010).

Table 1. Climate data of the closest meteorological stations of the studied populations

Stations	P	T	WR	SpR	SR	AR	PR	Io	Ic
El Hondo	286	18	78	68	30	110	AWSpS	1.3	15.4
Ravenna	584.2	13.7	106.8	144.2	143.9	189.3	ASpSW	3.6	20.4
Toscana	600	14	204	128	62	206	AWSpS	3.5	15.6
Cagliari	426	16	158.2	105.1	20.9	142.2	WASpS	2.2	14.5
Trapani	512	17	176	118	29	189	AWSpS	2.5	14.9
Sharm el Sheik	79	22	45.7	16.6	1.3	14	WSpAS	0.3	13.1

P (mm), mean annual rainfall; T (°C), mean annual temperature; WR, winter rainfall; SpR, spring rainfall; SR, summer rainfall; AR, autumn rainfall; PR, precipitation regime (W: Winter, Sp: Spring, S: Summer, A: Autumn); Io, Rivas-Martínez Ombrotermic Index ($I_o = 10 \times P_p/T_p$); Ic, continentality index ($I_c = T_{max} - T_{min}$). Data from the Global bioclimatics, Phytosociological Research Centre, Spain (Rivas-Martínez, 2008) and the ‘Servizio Meteorologico dell’Aeronautica Militare’, Italy (<http://www.meteoam.it/>).

Table 2. Bioclimatic diagnosis

Stations	Macrobioclimate	Bioclimate	Thermotype	Ombrotype
El Hondo	Mediterranean	Xeric-oceanic	Low Tm	Low Semiarid
Ravenna	Temperate	Xeric	Mesotemperate	Upper dry
Toscana	Mediterranean	Pluvistacional-oceanic	Low Mm	Upper dry
Cagliari	Mediterranean	Pluvistacional-oceanic	Upper Tm	Low dry
Trapani	Mediterranean	Pluvistacional-oceanic	Low Tm	Low dry
Sharm el Sheik	Mediterranean	Desertic-oceanic	Upper Im	Upper hyperarid

Data from the Global bioclimatics, Phytosociological Research Centre, Spain (Rivas-Martínez, 2008) (Tm, Thermomediterranean; Mm, Mesomediterranean; Im, Inframediterranean).

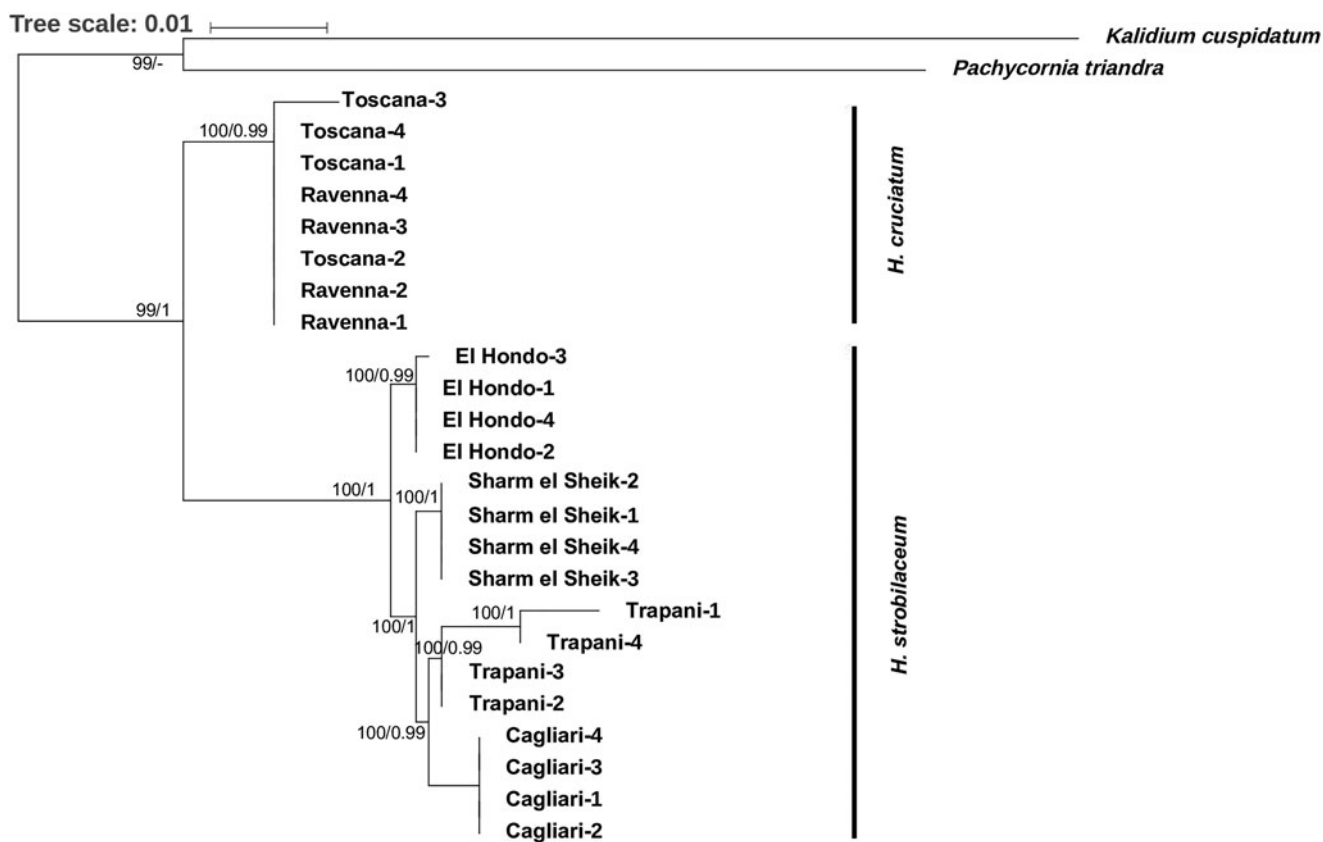


Fig. 2. Phylogram depicting the phylogenetic relations between *H. cruciatum* (El Hondo, Sharm el Sheik, Trapani and Cagliari) and *H. strobilaceum* (Toscana and Ravenna), obtained with RAxML and based on the nrITS and cpDNA data. Support values are given for each node (ML and BI analyses on the left and right, respectively).

Table 3. Polymorphism analyses for populations and species

	Gaps	s	π	k	h
El Hondo	2	1	0.000	0.500	2
Sharm el Sheik	2	0	0.000	0.000	1
Cagliari	6	0	0.000	0.000	1
Trapani	32	12	0.006	7.000	3
<i>H. cruciatum</i>	32	25	0.006	7.208	7
Ravenna	3	0	0.000	0.000	1
Toscana	3	5	0.002	2.500	2
<i>H. strobilaceum</i>	3	5	0.001	1.250	2
<i>Halocnemum</i>	32	52	0.014	16.275	9

Gaps, the number of gaps after removing ambiguously aligned positions in GBlocks v. 0.91b; s, the number of segregating sites; k, the number of nucleotide differences; π , nucleotide diversity; h, number of haplotypes.

Genetic diversity and haplotypes partition. Mafft original alignment without outgroups and excluding gaps was used to evaluate DNA polymorphism at the population and species levels. Four parameters were calculated with DnaSP v. 6 (Rozas et al., 2017): segregating sites (s), nucleotide diversity (π), the average number of nucleotide differences (k) and the number of haplotypes (h). Haplotypes partition of population is represented on the map in Fig. 1. The relations among haplotypes were calculated by TCS v1.21 (Clement et al., 2000).

Germination tests

Germination analyses were carried out for the seeds collected at the time of natural dispersion in six wild populations. Seeds were manually cleaned and stored in paper bags at 20°C and 40–50% relative humidity until the germination tests began (after 5 months of storage), where the seed were used directly dry, not previously imbibed.

Germination was checked within a constant temperature range from 5 to 35°C at 5°C intervals by considering the minimum and maximum average temperatures of the meteorological stations with the most extreme climate. Salt tolerance was analysed from 0 to 500 mM NaCl at 25°C and with a 12/12 h photoperiod. The percentage and mean germination time (MGT) were calculated for all the tests (Brenchley and Probert, 1998). The MGT was not considered when percentages were equal to or below 5% because, in such cases, it is not a consistent indicator of the germination response. The seeds that did not germinate after the different salt treatments were transferred to distilled water to study recovery effects. For the samples with low germination after these experiments, seed viability was checked following the standard tetrazolium staining protocol (AOSA/SCST, 2010) to discriminate non-viable seeds from potentially dormant ones.

All the tests were done on 5.5 cm-diameter Petri dishes, with four samples of 25 seeds and a substrate of 0.6% agar, prepared with the corresponding NaCl solutions to reach the indicated concentrations. Illumination was provided with daylight fluorescent tubes with a 12 h photoperiod and mean irradiance of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Germination tests, including recovery experiments, were maintained for 30 d. The germination index (GI) *sensu* Melville et al. (1980), which emphasizes both the percentage

Table 4. Generalised linear model (GLM) results for the effect on germination of different factors: Pop (seed population), Temp (incubation temperatures from 5 to 35°C) and their interactions

	Wald's chi-square	d.f.	P
Pop	789.942	5	0.000
Temp	154.165	6	0.000
Temp \times Pop	239.394	1	0.000

Significance levels: $P < 0.001$.

of germination and its speed, was calculated as $GI = \sum (T_i - (T_i - 1))N_i/S$, where T_i is the total time (days) spent on the germination test; T_i is the number of days until the i^{th} reading; N_i is the number of seeds germinated on day i and S is the total number of tested seeds (Ranal and Santana, 2006).

Recovery (germination percentage of the seeds transferred to distilled water) and total germination (TG) after recovery (expressed as the sum of the germination percentage at the different salt concentrations, plus recovery) were calculated. Base temperature (T_b), thermal time (S, to predict timing of germination under fluctuating environmental conditions) (García-Huidobro et al., 1982; Trudgill, 1995), the base water potential (Ψ_b) (Bradford, 1990) and hydrotime (Θ_H , to analyse germination rates at different water potentials) (Kebreab and Murdoch, 1999) were calculated from the regression line equation values of the inverse of germination velocity ($1/T50$) to provide information about potential germination process competitiveness. The base values should be treated cautiously because they were obtained from extrapolation beyond the range of experimental conditions. These calculated values were taken as theoretical figures for the comparisons among populations. The osmotic potentials (Ψ) of the different salt concentrations were calculated according to van't Hoff equation (Ben-Gal et al., 2009). All the obtained data shown in both the tables and graphs are expressed as the mean of four samples.

Voltammetric analysis

The voltammetric experiments were performed at 298 ± 1 K in a CH cell using a CH I660 potentiostat (Cambria Scientific, Llwynhendy, Llanelli, Wales, UK). A BAS (Bioanalytical Systems, West Lafayette, USA) MF2012 glassy carbon-working electrode (GCE) (geometrical area 0.071 cm^2), a platinum wire auxiliary electrode and an Ag/AgCl (3 M NaCl) reference electrode were used in a conventional three-electrode arrangement. To ensure repeatability, a voltammogram on the bare electrode after mechanical cleaning in a polishing clot with alumina slurry was performed before each voltammogram with a sample-modified electrode.

Voltammetric measurements were taken with freshly prepared sample-modified GCE using air-saturated 0.25 M aqueous acetic acid/sodium acetate buffer at pH 4.75 as a supporting electrolyte. For electrode preparation, 3–4 seeds were crushed in an agate mortar and pestle by adding 0.5 ml of ethanol (HPLC grade, Carlo Erba reagents, Sabadell, Spain) for 1 min. Next, 50 μl of the resulting suspension were dropped onto the GCE surface and the solvent was allowed to evaporate in air. The electrode was inserted into the electrochemical cell and electrochemical runs were performed.

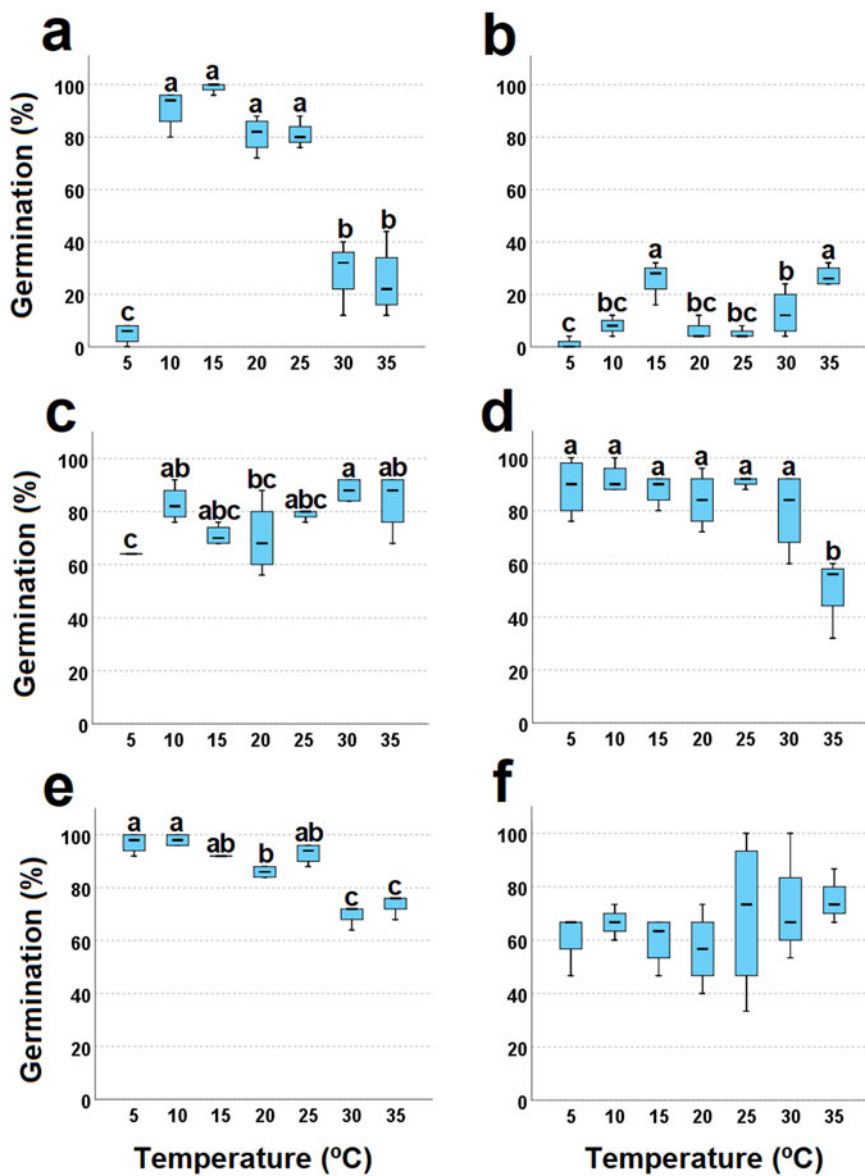


Fig. 3. Germination response to temperature for all the studied populations (0 mM NaCl). (a) El Hondo, (b) Ravenna, (c) Toscana, (d) Cagliari, (e) Trapani and (f) Sharm el Sheik. For each population, *p*-values are indicated and the same letters indicate homogeneous groups (Tukey $P < 0.05$).

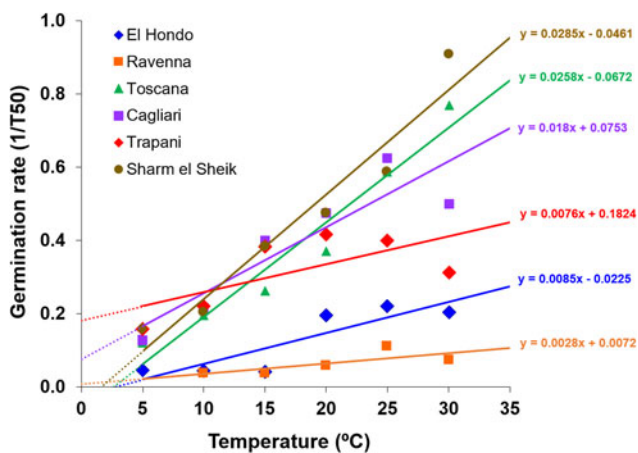


Fig. 4. Relation between temperature and the germination rate (1/T50) for each studied population. Regression lines are represented for the calculation of the thermal time requirements. The regression lines that are above correspond to the most competitive population within a range of temperatures.

The potential scan was initiated at -1.25 V in the positive direction with a potential step increment of 4 mV (25 mV square wave amplitude; 10 Hz frequency) to check the values of the current peaks at different potential values during scanning.

Statistical analysis

All the germination data used to analyse temperature and salt effects were expressed as mean values and were statistically assessed using IBM SPSS statistics software (v. 26.0). Germination percentage was modelled by fitting Generalised Linear Models (GLMs) with a logit link function and binomial error structure, to evaluate the effects of temperature or salt concentration and population and their interactions on final germination percentage. Considering the low amount of replicates (i.e. Petri dishes) and seeds per replicate, GLMs were carried out considering individual seeds as replicates (i.e. $n = 100$ or 60, depending on the population), attributing the value of ‘1’ when germinated or ‘0’ when not germinated.

Table 5. The mean germination time (MGT) in days within the range of studied constant temperatures with a 12/12 h photoperiod and 0 mM NaCl

T (°C)	MGT (days)					
	El Hondo	Ravenna	Toscana	Cagliari	Trapani	Sharm el Sheik
5	22.33 c	–	8.38 c	8.63 c	7.27 c	6.97 e
10	22.83 c	25.91	6.09 b	5.19 b	5.04 b	5.38 de
15	21.10 c	25.50	4.66 b	3.86 ab	4.54 ab	3.80 cd
20	6.29 ab	17.67	4.66 b	3.88 ab	5.01 b	3.26 bc
25	5.73 a	–	2.42 a	2.05 a	3.51 a	2.08 ab
30	6.89 ab	12.88	2.19 a	2.87 a	4.15 ab	1.61 a
35	10.18 b	10.88	2.19 a	3.65 ab	4.04 ab	1.05 a

The same letters indicate homogeneous groups (Tukey $P < 0.05$). When germination percentages were lower than 5%, the MGT was not calculated.

Table 6. Theoretical base temperature (Tb) and the thermal time (S) calculated for each population from linear regressions, and data relative to the regression analysis, R square (R^2), standard error (SE), F-test (F), coefficients (Coef) and P-value (P)

Localities	Tb (°C)	S (°C day)	R^2	SE	F	Coef	P
El Hondo	2.6	117.6	0.77	0.05	13.614	0.008	0.021
Ravenna	–2.6	357.1	0.57	0.02	3.946	0.003	0.141
Toscana	–2.6	38.8	0.94	0.07	68.636	0.026	0.001
Cagliari	–4.2	55.6	0.82	0.09	18.559	0.018	0.013
Trapani	–24.0	131.6	0.46	0.09	3.390	0.008	0.139
Sharm el Sheik	1.6	35.1	0.94	0.08	60.621	0.029	0.001

Table 7. Generalised linear model (GLM) results for the effect on germination of different factors: Pop (seed population), Osmpot (osmotic potential from 0 to 500 mM MPa) and their interactions

	Wald's chi-square	d.f.	P
Pop	916.196	5	0.000
Osmpot	422.425	10	0.000
Pop × Osmpot	42.191	1	0.000

Significance levels: $P < 0.001$.

Significant differences in MGT among temperature and salt treatments were calculated by applying one-way ANOVA according to Khan and Rayner (2003).

To detect homogeneous groups among all the data resulting from temperature effect, a HSD Tukey *post-hoc* analysis was applied, and to consider the effect of salt in the different populations the *post-hoc* comparisons respect to the control were established using Dunnett's test. The equality of variances was checked through a Levene's test.

The correlation between the environmental parameters, germination response, voltammetric measurements and seed features was established with Pearson's coefficients. To find the variables with the strongest influence on population behaviour, a factor analysis based on principal components, CATPCA, was applied. This kind of analyses also incorporates categorical variables and allows any non-linear relations between them to be discovered.

In order to confirm previous results, a cluster analysis (CA) for statistical data processing was carried out. The Ward method was

applied for grouping different variables and dissimilarity was defined by Euclidean distance. Ninety-four variables were considered for the analyses: 38 environmental parameters and 56 related to germination responses. The variables considered in these analyses are found in Supplementary Appendix A.

Results

Population characterization

Field sites description

The analysis of climate data and bioclimatic diagnosis of all the localities are compiled in Tables 1 and 2. The data of the meteorological stations allow include all the studied populations in the Mediterranean macrobioclimate, with the only exception being the Ravenna population, which corresponds to the temperate macrobioclimate. This one, along with Toscana, had the highest rainfall values and the lower mean temperature. In contrast, the Egypt population was the driest, with only 1.3 mm of summer precipitations, and where the highest mean temperature was also registered.

The considered populations showed diverse soil textures ranging from sandy (Sharm el Sheik) to clay (Ravenna). The textures measured for the other populations were clay loam (El Hondo), loamy sand (Toscana), clay loam (Cagliari) and loam (Trapani).

Plant communities were assigned to four different associations: *Frankenio corymbosae-Halocnematum cruciati* (El Hondo), *Arthrocnemo macrostachyi-Halocnematum cruciati* (Cagliari and Trapani), *Arthrocnemo glauci-Halocnematum strobilacei* (Ravenna and Toscana) and *Zygophyllo albi-Halocnematum cruciati* (Sharm el Sheik) (Biondi et al., 2013).

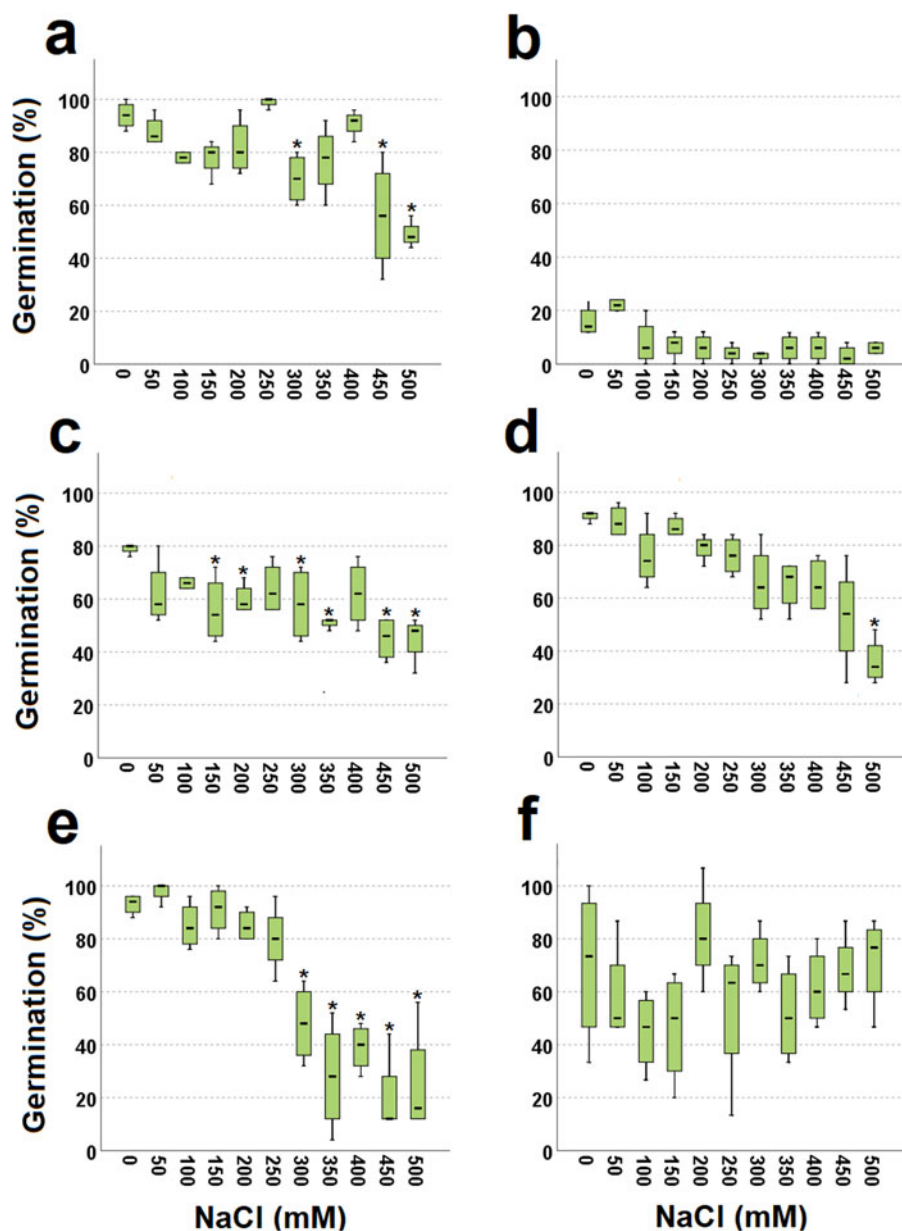


Fig. 5. Germination response to NaCl for all the studied populations (25°C). (a) El Hondo, (b) Ravenna, (c) Toscana, (d) Cagliari, (e) Trapani and (f) Sharm el Sheik. For each population, P -values are indicated and the asterisks indicate significant differences from 0 mM NaCl (Dunnett $P < 0.05$).

Molecular characterization

Phylogenetic analysis. When considering a molecular analysis, the dataset comprised new 24 nrITS and cpDNA concatenated sequences belonging to species *H. cruciatum* and *H. strobilaceum*. Phylogenetic analyses included two more concatenated sequences as the outgroups obtained from NCBI: *Kalidium cuspidatum* and *Pachyornia triandra*. The Mafft algorithm produced an alignment of 1195 bp with outgroups and 1187 without them. After the automatic removal of the ambiguously aligned positions in GBlocks v. 0.91b, 99% (1184 nucleotides) of the original length was left in five selected blocks after taking outgroups into account. This final alignment included 129 variable positions, of which 41 were parsimony informative and 78 singletons. The ML analysis resulted in a single best tree of $\text{Ln} = -2500.91$. The Mrbayes analysis reached an average standard deviation of split frequencies of 0.01 after 390,000 generations. No statistically supported conflict was observed among the *Halocnemum* branch topologies obtained

by these two methods. The RAxML-resulting topology is seen in Fig. 2. It shows a better resolution of the outgroups topology.

The phylogenetic relations inferred with RAxML and Mr Bayes agree with the species division proposed in previous works based on morphology characters (Biondi et al., 2013) and a high clade support (BP = 99%, PP = 1) defined both species. The *H. cruciatum* populations were also separated with high clade support (BP = 100%, PP ≥ 0.99). In contrast, the two *H. strobilaceum* populations were clustered in a single group.

Genetic diversity and haplotypes partition. The genetic diversity approach revealed low intrapopulation variation, except for Trapani, which showed 12 polymorphic sites (s) and 7 was the average number of nucleotide differences (k). This resulted in three haplotypes separated by intermediate nucleotide changes, which indicated that more extensive sampling could discover a higher diversity. On the contrary, Sharm el Sheik, Cagliari and

Table 8. Mean germination time (MGT) expressed in days under the range of studied salt concentrations (mean) at 12/12 h photoperiod

NaCl (mM)	MGT (days)					
	El Hondo	Ravenna	Toscana	Cagliari	Trapani	Sharm el Sheik
0	2.94	24.50	2.42	2.05	3.51	2.08
50	3.38	24.40	6.34*	3.69	5.43	6.85*
100	3.72	21.83	6.29*	4.20*	4.84	5.08*
150	4.16	23.67	6.01*	4.25*	4.92	5.75*
200	5.19	22.78	7.43*	5.46*	6.89	5.59*
250	6.90*	–	5.71*	6.48*	7.69	5.69*
300	7.67*	–	6.83*	6.14*	14.56*	6.32*
350	8.88*	21.17	6.19*	8.26*	12.29*	5.94*
400	10.36*	21.56	7.21*	9.04*	12.91*	5.52*
450	12.05*	–	5.92*	9.91*	10.80*	6.72*
500	12.41*	21.88	8.29*	11.97*	7.62	5.99*

Asterisks indicate significant differences from the control (Dunnett $P < 0.05$). When germination percentages were lower than 5%, MGT was not calculated

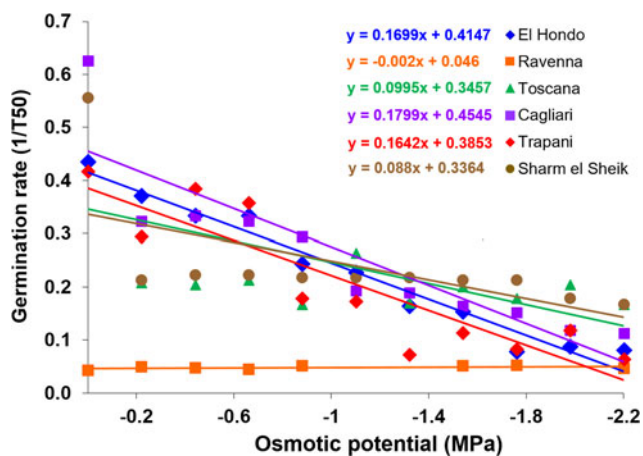


Fig. 6. Relation between the osmotic potential and germination rate (1/T50) for the studied populations. Regression lines are represented for the calculation of the thermal time requirements. The regression lines that are above correspond to the most competitive population at the determined osmotic potentials or salt concentrations.

Ravenna did not show any diversity (Table 3) in the analysed samples.

Trapani presented three haplotypes, El Hondo and Toscana two, and the rest one per population. All the haplotypes were private for one population, except 8 that was shared between Ravenna and Toscana. Haplotypes distribution and the relations among them are shown in Fig. 1. The haplotype network revealed a specific lineage for each *H. cruciatum* population. Therefore, specific features could be associated with each one, for example, germination response. Another clearly differentiated lineage, separated by 21 changes of the main network, was found for *H. strobilaceum*.

Germination tests

Regarding temperatures, germination percentages and velocity, measured as the MGT, were significantly different (Table 4),

showing an effect that depends on the population (Figs. 3 and 4; Table 5). All the studied populations reached high germination percentages, even at the extreme temperatures tested, except El Hondo and Ravenna. The less limiting and more competitive response was observed in the seeds collected from Sharm el Sheik, the most restrictive in the material from Ravenna. The moderate values and high standard deviation observed for Sharm el Sheik were associated with poor seed quality, as the tetrazolium test confirmed.

Trapani showed greater competitiveness at temperatures below 10°C. The Egypt population displayed the strongest reaction to increasing temperatures, the most opportunistic strategy and the most competitive response for temperatures higher than 11.6°C (calculated from intersection in regression lines, Fig. 4). In comparison to this strategy, the Ravenna and El Hondo populations showed less competitiveness at most of the temperatures. The Tb and S data were obtained from regression lines (Table 6), which numerically reflected the presented results.

The base values shown in Fig. 4 and Table 6 should be cautiously interpreted because they were obtained from the extrapolation beyond the range of experimental conditions. These calculated values were taken as theoretical figures.

Regarding salt response, germination percentages and velocity (as MGT) were significantly different (Table 7) depending on osmotic potential and populations. The overall effect in the studied populations was negative when the salt concentration increased (Fig. 5). Two exceptions were observed: Ravenna due to the low germination of the control, and Egypt, the most tolerant without significant differences between the control and the highest tested salt concentration. However, the MGT significantly increased under all the salt conditions in relation to 0 mM NaCl (Table 8).

When the MGT and GI values were analysed, greater competitiveness and better tolerance of the Sharm el Sheik population were once again noted under the most stressful conditions when the osmotic potential was very low (Figs. 6 and 7; Table 9).

For recovery after salt exposure, the case of the seeds from Ravenna was remarkable (Fig. 8), with a strong stimulation effect observed at all the tested salt concentrations in relation to the low

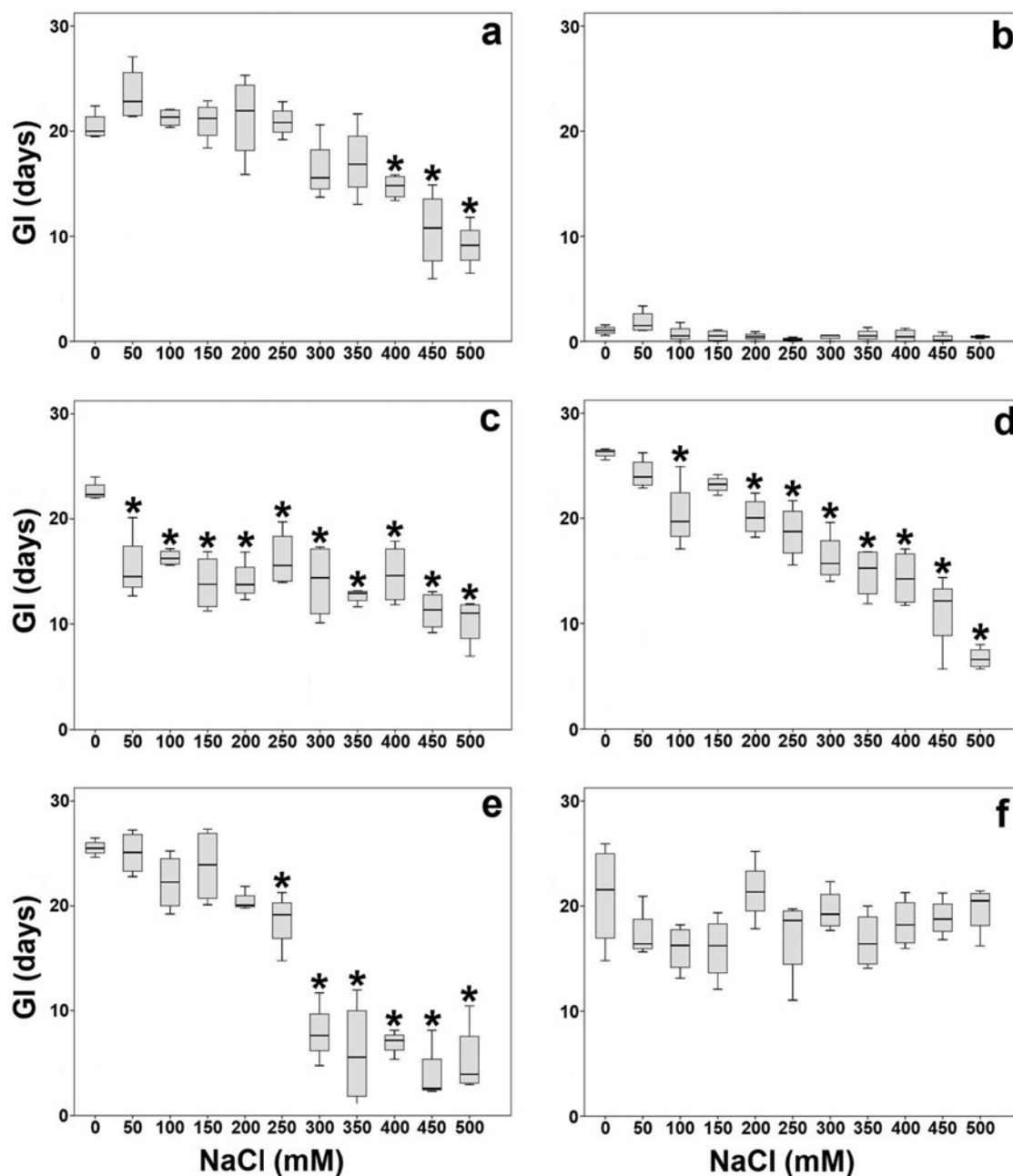


Fig. 7. Germination response to the different salt concentration for all the studied populations, and represented by the germination index (GI), which combines both the germination percentage and speed. (a) El Hondo, (b) Ravenna, (c) Toscana, (d) Cagliari, (e) Trapani and (f) Sharm el Sheik. Asterisks indicate significant differences in relation to the control ($P < 0.05$).

germination percentage obtained in the control. Indeed, the TG values for this population, expressed as the sum of the germination percentages at the different salt concentrations, plus recovery, showed a significant increase in TG after recovery with rising NaCl concentration. In the other populations, this priming effect is not observed on the TG after recovery.

Regarding the correlation between the considered environmental parameters and germination, when we looked for the potential predictors of specific seed response, Pearson's coefficients of the bioclimatic variables revealed a higher correlation with the germination parameters, as summarized in

Supplementary Appendix B. From these results, we conclude that macrobioclimate, continentality index (I_c) based on the warmest and coldest temperatures of the year, winter temperatures and summer rainfall (Psm), were the climate factors that showed a higher correlation with the germination parameters, mainly with S , velocity, expressed as the MGT in the salt treatments and final germination after recovery (TG).

The germination percentages exhibited an inverse correlation with I_c , and also with Psm. MGT, showed a higher correlation with the same bioclimatic parameters in the response to both temperature and salt concentration.

Table 9. Base water potential (Ψ_b) and hydrotime (Θ_H) calculated for each population through the linear regressions, and data relative to the regression analysis, R square (R^2), standard error (SE), F -test (F), coefficients (Coef) and P -value (P)

Localities	Ψ_b (MPa)	Θ_H (MPa day)	R^2	SE	F	Coef	P
El Hondo	-2.4	5.9	0.96	0.03	242.256	0.170	0.000
Ravenna	23.0	-500.0	0.22	0.00	1.654	-0.002	0.246
Toscana	-3.5	10.1	0.30	0.12	3.910	0.100	0.079
Cagliari	-2.5	5.6	0.78	0.07	32.204	0.180	0.000
Trapani	-2.3	6.1	0.80	0.06	36.677	0.164	0.000
Sharm el Sheik	-3.8	11.4	0.36	0.09	5.153	0.088	0.049

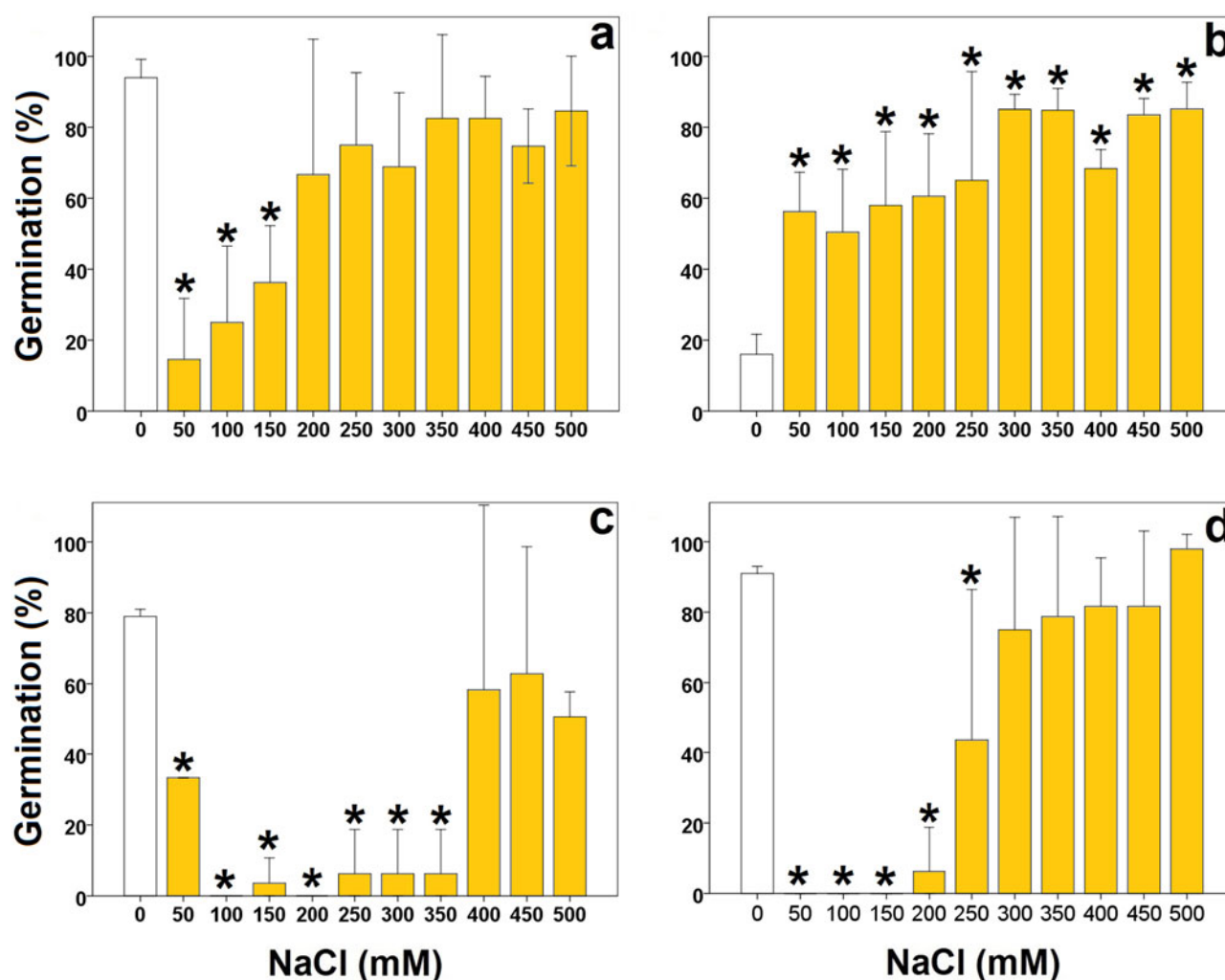


Fig. 8. Germination recovery (yellow bars) after salt exposure and the germination percentage of the 0 mM from the initial salt experiment (white bar). (a) El Hondo, (b) Ravenna, (c) Toscana and (d) Cagliari. Asterisks indicate significant differences in relation to the control ($P < 0.05$).

Of the thermal parameters (T_b and S), only S revealed a positive correlation with I_c and P_{sm} . TG also showed a strong positive correlation with the same climatic parameters, mainly at lower salt concentrations.

Neither phylogenetic relations nor genetic diversity could be correlated with germination strategy, but the CATPCA analysis provided relevant data when comparing environmental factors to germination response. This analysis indicated that the

considered variables were represented by two new variables or principal components. These two new components accounted for 78.9% of total variance. Therefore, two vectors were used for the statistical analysis. Of the total variance given by these first two components, 58.7% corresponded to the first component and 20.2% to the second component. Cronbach's alpha values for each component were 0.986 and 0.921, respectively, with a total of 0.995 that indicated good internal consistency.

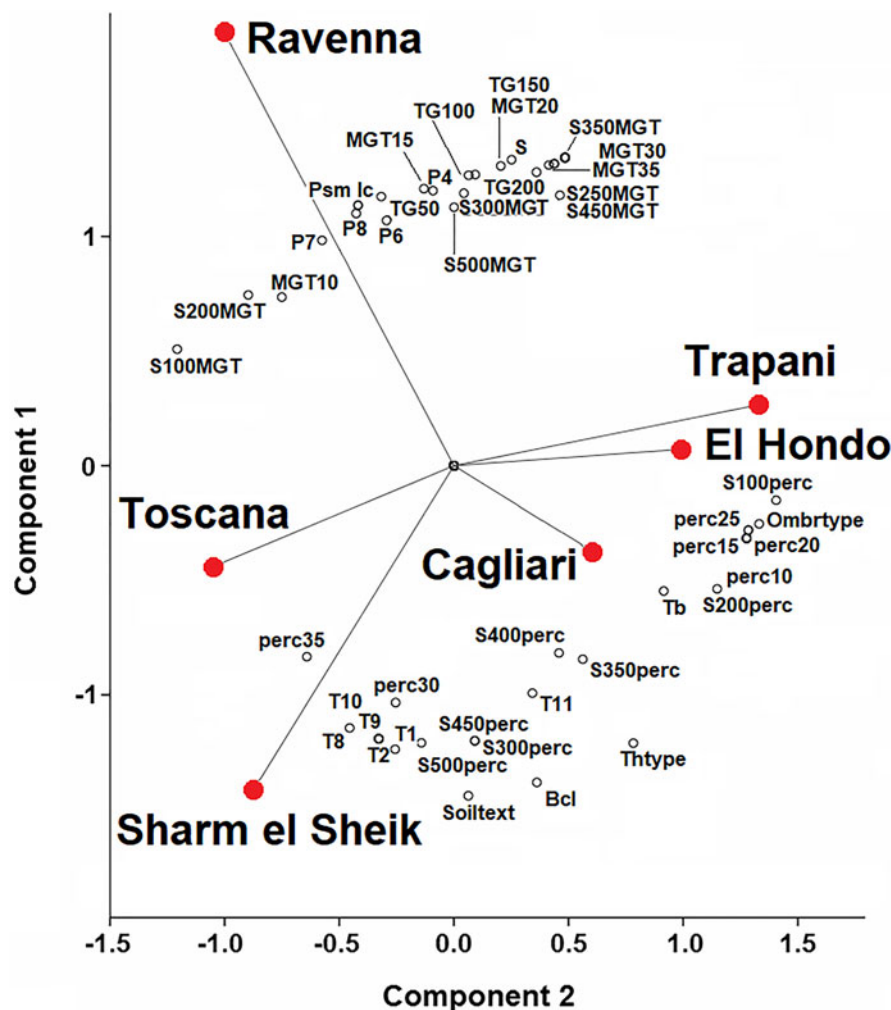


Fig. 9. Biplot of the component loadings from CATPCA for the first two components. Populations are indicated by vectors. The acronyms for the variables are described in Supplementary Appendix A.

The graphic representation of the loadings and scores in Components 1 and 2 shows the distribution of the studied populations according to the considered variables, seed features, climate factors, soil texture and germination variables (Fig. 9). To simplify the graphic, only the most correlated variables, based on the Pearson coefficient results, are represented.

The variables with the highest weight value in each component were:

- Component 1: Recovery from 300 to 500 mM NaCl, MGT 350–400 mM and S; with negative values: Bioclimate and germination percentage at 500 mM.
- Component 2: Germination percentage at 10, 20 and 25°C, and percentage at 50 and 150 mM; with negative values, MGT at 100 mM NaCl.

Based on these findings, the Ravenna population showed a considerable distance from the others, mainly due to the low germination (percentage and velocity) and greater recovery at high salt concentrations. Sharm el Sheik was on the other extreme of the graph because of its broad germination response under the high-salinity conditions.

The other four populations, with intermediate behaviour in relation to these variables, were separated into two groups according to the variables with the highest weight values in

Component 2. Toscana differed from El Hondo, Cagliari and Trapani given by the response under low salt concentrations and at moderate temperatures, but mostly by climate factors.

The CA analysis results agree with those previously described for CATPCA and showed three clusters (Fig. 10) based on the different environmental factors and germination response. The analyses indicated that only 70 of the 94 examined parameters had a significant effect on the separation of groups.

Group I, represented by a single population (Ravenna), showed a long distance from the other groups. This sample point displayed substantial differences for both environmental conditions (Ic, winter and autumn temperatures and summer rainfalls) and germination response, mainly the seeds with the lowest germination percentage and the slowest velocity, as well as the greatest recoveries.

As expected, the Sharm el Sheik population in cluster II showed an intermediate distance between clusters I and III as a result of the special environmental factors (higher winter temperatures, and less summer, spring and autumn rainfall). This environment gave rise to a different germination strategy, a high germination percentage and a high velocity value, with no recovery after salt exposure.

The four remaining populations formed the other group (cluster III). It obtained intermediate values for the above-mentioned climatic parameters between the other two groups, and also an intermediate response to salt exposure and recovery.

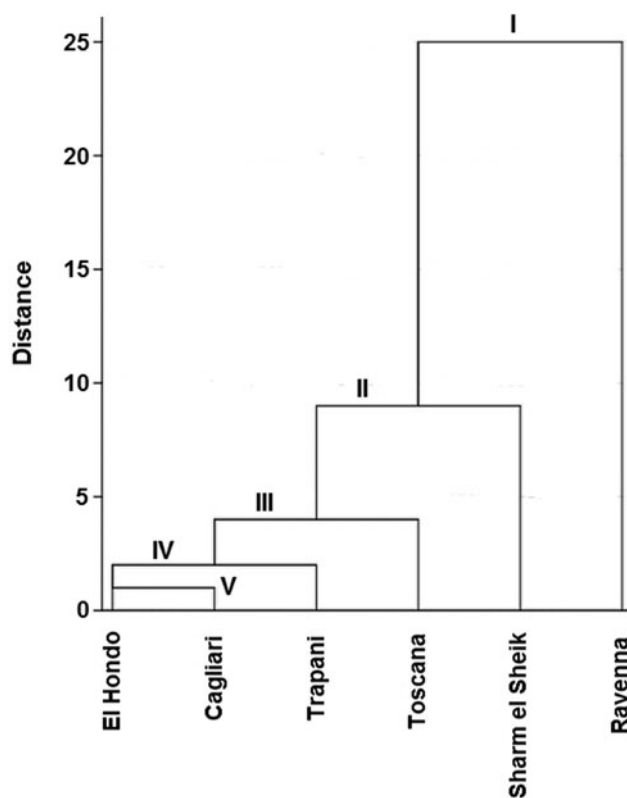


Fig. 10. Dendrogram for the cluster analysis conducted with 94 variables. Dissimilarities are defined by Euclidean distance and the combination of clusters is based on the Ward method.

Voltammetric analysis

In relation to the voltammetric study, Fig. 11 shows the square wave voltammograms recorded for the seeds of studied populations. The values of the intensity measured at the potentials of 400 mV ($i(400)$), 750 mV ($i(750)$) and 950 mV ($i(950)$) provided useful information, but their absolute values could not be considered because they depend on the amount of extract deposited on the electrode. However, the general voltammogram profile was precisely repeated for each sample. This means that the ratios between the different peak intensities were correctly reproduced regardless of the amount of extract. The ratio between the signals at +0.40 and +0.75 V significantly varied for the different samples.

As shown in Fig. 12, where a two-dimensional diagram containing the $i(950)/i(750)$ and $i(400)/i(750)$ peak current ratios measured from the base lines depicted are in Fig. 11. Two groups from the averaged values of the $i(950)/i(750)$ ratio were discerned: (1) the samples from Toscana, El Hondo and Sharm El Sheik, characterized by higher values for the above ratios (0.88, 0.754 and 0.947, respectively); (2) the samples from Cagliari, Ravenna and Trapani with values of around 0.5.

This ratio ($i(950)/i(750)$) correlated with specific environmental parameters and seed response. The Pearson analysis between the voltammetric and germination parameters showed a higher correlation of $i(950)/i(750)$ with the germination percentage and recovery at high salt concentrations, that is, 450 and 500 mM NaCl (Supplementary Appendix C). The same analysis carried out, but by considering the voltammetric and environmental parameters, gave higher correlations between $i(950)/i(750)$, (Supplementary Appendix D) and spring and autumn

temperatures and spring and summer rainfall. In our case, ratio $i(950)/i(750)$ provides us with a good indicator tool.

Discussion

Germination behaviour is a complex process dependent on multifactorial causes. In most cases, and especially under extreme conditions, it is somewhat determined by the local intrinsic and environmental factors interacting in a complex manner. The genetic factors, such as local alleles or epigenetic modifications, lead the process in the last term. Finally, all these factors allow plants to be in the right place at the right time to reach germination success (Li et al., 2015).

The germination of the studied populations differs considerably in response to the tested temperature and salinity conditions, and depending on the geographical distribution of populations. Sharm el Sheik in Egypt proved to be the most tolerant population, just the opposite of Ravenna in northern Italy. In the present article, some predictors associated with these responses have been identified for *Halocnemum* species. In general, these responses appear to be directly related to ecological factors rather than phylogenetic ascription or genetic variability, as previously observed in other species (Estrelles et al., 2010; Gul et al., 2013; Rasool et al., 2017). Additionally, a voltammetric tool has been successfully tested as an indirect technique to predict seed response in stressful environments.

Environmental parameters

The first group of predictors of germination studied, which affect both percentage and speed, are those related to environmental parameters. The differences in germination behaviour in relation to them have been described as a survival mechanism characteristic of species growing in areas subjected to extreme water stress, with unpredictable amounts and rain distribution (El-Keblawy and Al-Shamsi, 2008; El-Keblawy et al., 2009; Liu et al., 2014). For *Halocnemum* species, the CATPCA analysis points out the fundamental role of the environmental variables, such as soil texture, bioclimate, thermotype and ombrotype, in the distribution of the populations in both the extracted components. However, macrobioclimate comes over as the key factor for seed germination in the studied species. The populations belonging to the Mediterranean macrobioclimate display an opportunistic strategy, which is the characteristic of drought stress environments in which germination depends on water availability. This behaviour is even more pronounced in places with lower precipitation rates. Conversely, the seeds collected at Ravenna show the opposite response, with specific requirements to reach optimal germination levels. The climate of this locality is considered a sub-Mediterranean variant of the temperate macrobioclimate according to Biondi et al. (2013), that is, a higher and more regular precipitation regime. Additionally, the analyses conclude that the use of the specific climatic data of the collection year can be proposed to obtain more accurate correlations with the analysed germinative parameters, instead of taking average from different year values (T and P). In our study, the values of winter temperatures and summer precipitation recorded in the collecting localities have been revealed as determining factors.

In addition, soil texture acts as another fundamental factor predicting seed response given the interpopulation differences when exposed to different salt concentrations, since both are directly related due to water holding capacity. In this sense, the clay

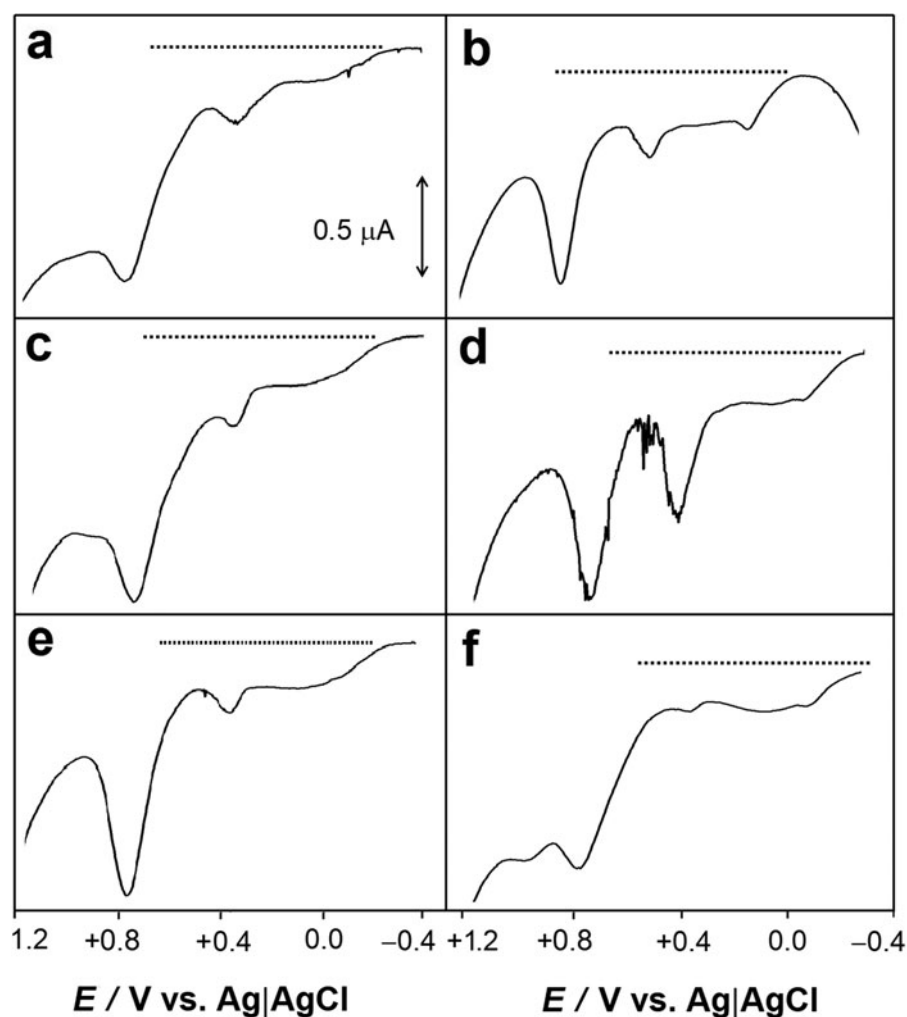


Fig. 11. Square wave voltammograms of the microparticulate films deposited on glassy carbon electrode from the ethanolic extracts of *Halocnemum strobilaceum* seeds from (a) El Hondo, (b) Ravenna, (c) Toscana, (d) Cagliari, (e) Trapani and (f) Sharm el Sheik immersed into air-saturated 0.25 M aqueous acetate buffer, pH 4.75. Only the region of the potentials between -0.40 and $+1.20$ V is depicted. Dotted lines represent the base lines adopted for the peak current measurements.

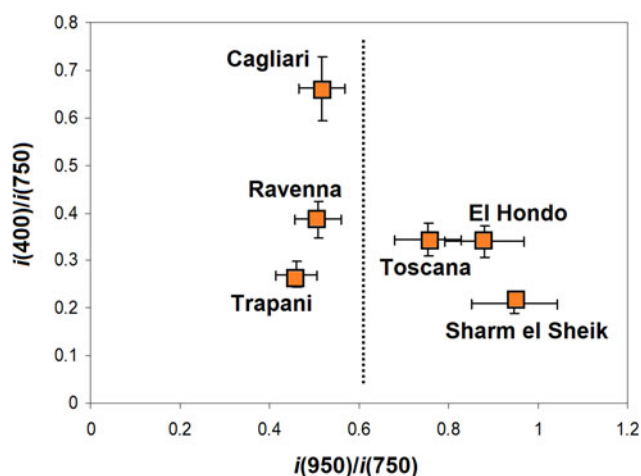


Fig. 12. Two-dimensional diagram showing the $i(950)/i(750)$ and $i(400)/i(750)$ peak current ratios recorded from the voltammograms in Fig. 11.

soil had the highest water holding capacity and the sand soil had the least, the latter enhancing the stress due to osmotic potential. Thus texture and, therefore, water retention capacity, are among the most determining variables in the PCA analysis for the

separation between populations. Previous studies into *Halocnemum* populations have pointed out the influence of soil features on germination behaviour (Estrelles et al., 2015). These results also agree with not only Rubio and Escudero (2000), who highlighted the relevance of this soil property for the vegetation response in dry gypsum environments, but also with Ferriol et al. (2006), who found a correlation between sand proportion in soil and germination velocity.

Therefore, the seeds from Sharm el Sheik are exposed in a natural environment to water stress conditions, which result from the low rainfall and high temperatures registered all year long, plus sandy soils make water retention difficult. In this sense, the effect of maternal conditions to improve salt tolerance for offspring of different species has also been evidenced by several authors (Mohamed et al., 2020; Shah et al., 2020). An opposite response is seen for the seeds collected from Ravenna, where clay is the main soil component and is responsible for high water retention capacity, long flooding periods and low salt concentrations.

Although Ravenna proved to be the least tolerant population in relation to the low salt concentration of its maternal location, its results in terms of recovery rates allow to confirm a halopriming effect for this species. The recovery test showed that germination was stimulated after exposure to a high concentration of NaCl. This response has been formerly verified in other species living under similar environmental conditions (Khan and Gul, 2006;

Estrelles et al., 2018). Natural priming confers these plants major advantages over others, mainly better germination process initiation control, others like a quick cellular defence response against abiotic stress (Jisha and Puthur, 2014) and a strategy for increasing antioxidant enzyme activities to achieve osmotic adjustment to control salt stress effects (Saha et al., 2010).

Genetic features

The other group of predictors analysed is related to the genetic characteristics of species: genetic variability and phylogenetic ascription. On the one hand, higher levels of genetic diversity may offer adaptive advantages in changing environments (Dekker, 2003; Crawford and Whitney, 2010) and, on the other hand, species divisions, or even local lineages, could be determined by specific adaptations. However, these intrinsic features do not seem to be clearly related to germination responses.

Genetic diversity does not seem to affect the current germination success herein determined and similar results have been obtained in other plants, such as *Sambucus palmerensis* (Rodríguez-Rodríguez et al., 2018), *Stippa pennata* (Heinicke et al., 2016), among others. Likewise, the phylogenetic characterization shows a clear split into two species, as Biondi et al. (2013) concluded using morphologic data, which cannot be associated with germination strategies. Alternatively, specific lineages appear in the haplotype characterization of populations that could be related to these strategies, at least in *H. cruciatum*. Although the studied genetic regions are not related to ecophysiology plant responses, they could develop in parallel with other genetic adaptations to the conditions of their particular area due to environmental selection pressure. In fact, some studies report gene alleles related to germination responses and drought or salt stress (Mumtaz et al., 2010; Liu et al., 2020; Rehman et al., 2020). However, the two *H. strobilaceum* populations belong to the same lineage, but the ecological conditions of their localities and, therefore, their germinative responses, significantly differ. This means that different responses cannot be explained by the existence of these lineages alone, and genetic characterization appear to be a partial predictor, which only function in one of the species. In this sense, other genetic processes related with priming and epigenetics could determine the different responses observed (Chinnusamy et al., 2008; Zhang and Ogas, 2009; Roy, 2016; Bowers and McCullough, 2017; Al-Shamsi et al., 2018; Godwin and Farrona, 2020).

The voltammetric tool

The study of the voltammetric profiles proves an effective technique that can act as an indirect behaviour predictor based on maternal plant stress. Plants under abiotic stress conditions exhibit an increased synthesis of polyphenols, such as phenolic acids and flavonoids (Sharma et al., 2019). Water deficit or salt stress, and some stress types in general, trigger the metabolic mechanisms related to the chemical group of flavonoids and other polyphenolics (Winkel-Shirley, 2002; Hernández et al., 2009). The presence of this kind of compounds in seeds is widely referenced in the literature and also in Amaranthaceae (Oliveira-Alves et al., 2017; Adegbola et al., 2020), mainly in relation to their use as antioxidants, but also in association with stress tolerance (Sahitya et al., 2018). Thus, voltammetric profiles provide valuable information about the oxidation of polyphenolic compounds in plants (Doménech-Carbó et al., 2015). Indeed,

these techniques have been applied to different aspects related to germination response and offer interesting results (Doménech-Carbó et al., 2019). In this study, the parameter $i(950)/i(750)$ ratio obtained from the voltammetric profile measured from seeds can be specifically associated with plant stress by showing the maternal effect related to drought in *Halocnemum* species. Although other factors influence the observed electrochemical response, the data summarized in Fig. 12 can only be analysed after considering the local environmental factors found where specimens grow.

Conclusion

The multidisciplinary approaches that encompass different aspects of halophytes are important to help to gain a better understanding of these species' ecophysiology. From this research, the following conclusions can be drawn:

- Microbioclimate, the continentality index, summer precipitation and winter temperatures, and soil texture, are determinant parameters that are directly associated with the differences in seed response.
- The specific climatic data of the collection year are fundamental in germination and should be considered by future research.
- Germination strategies cannot be associated with the taxonomic rank and genetic diversity of the studied species.
- The existence of local lineages can be related to seed responses only in *H. cruciatum*.
- Some voltammetric parameters are good indicators of the water stress that plants undergo in their natural habitats, which support the applicability of these techniques as a tool to predict germination response.

The determination of key parameters related to the timing of emergence events provides effective tools for optimizing management programmes in natural habitats.

Supplementary material. To view supplementary material for this article, please visit: <https://doi.org/10.1017/S0960258522000253>.

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Conflict of interest. The authors declare no conflict of interest.

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