



Unravelling reproductive resilience and genetic characteristics of *Cistus heterophyllus* subsp. *carthaginensis* and their role in limiting recruitment on natural populations

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Abstract

This study compares reproduction and genetic traits of *Cistus heterophyllus* subsp. *carthaginensis* in two Iberian Peninsula populations (Murcia and Valencia), alongside *C. albidus*, the main competitor responsible for hybridisation in these populations. The former taxon is critically endangered (IUCN CR), justifying the conservation efforts. Thermal time, base temperature, hydrotime, base water potential, and seed longevity (through accelerated ageing) were used to assess competitiveness during germination. Six pollination treatments evaluated reproductive success in both populations. Three molecular markers analysed the phylogenetic affiliation of the populations. The Valencian population showed a stronger competitive response than *C. albidus* in temperature and water stress tests, while the Murcian seeds exhibited intermediate behaviour, reflecting their hybrid genetic traits. Longevity tests revealed higher P50 values in the hybrid population, suggesting potential hybrid vigour. Reproductive success was higher in the Murcian population, also associated to its hybrid nature; both populations exhibited low but positive self-compatibility. Our findings indicate that the germinative phase does not drive the regression of *C. heterophyllus* in Spain, compared to the advance of *C. albidus*. Instead, reduced fruit and seed production, linked to very low genetic variability, contribute to this regression.

Keywords *Cistus* reproductive success · Hybridisation · Seed longevity · Thermal response · Water stress

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Introduction

Mediterranean territories are climate change hotspots, specially threatened by global warming, and, thus, affected by drought and wildfires (Cos et al. 2021). The rockrose plant group has its own essence within Mediterranean vegetation. In fact, Cistaceae are plants highly adapted to typical Mediterranean climate conditions, with dry and hot summers and recurrent fires. Actually, this family shows the highest genus and species diversity in the Mediterranean floristic region (Scuderi et al. 2010), being the genus *Cistus* one of the most representative in the Mediterranean scrubland vegetation (Guzman and Vargas 2005).

One of the most threatened taxa of this group is *Cistus heterophyllus* subsp. *carthaginensis* (Pau) M.B.Crespo & Mateo. This perennial shrub is endemic to South-eastern territories of Spain; including one natural population in Valencia, another in Murcia and a new population that has just been described for the isle of Cabrera Gran in Cabrera Archipelago National Park (Cardona and Capó 2023). The species grows in the thermomediterranean areas with dry to semi-arid ombroclimate, on limestone substrates (Crespo and Mateo 1988). In Spain, it is categorized as critically endangered taxon (Güemes et al. 2003) and legally protected at regional and national levels. This statement justified the huge efforts and resources devoted to its conservation by multiple research and work groups during the last twenty years.

This taxon pertains to the purple-flowered lineage of the genus and is genetically close to *C. albidus* L. and *C. creticus* L. (Guzman and Vargas 2010; Lukas et al. 2021). Several authors indicate the slight reproductive barriers that exist between *C. heterophyllus* Desf. and one of the most common species in Southern Spain, *C. albidus*, responsible for the non-rare citations of hybrid individuals in the field (Fernández-Mazuecos and Vargas 2018; Jiménez et al. 2007; Jiménez-Martínez et al. 2018). Indeed, hybridisation has been considered as one of the main evolutionary forces of the genus (Dansereau 1940; Demoly 1996). Additionally, over the last twenty years, a lack of natural recruitment has been observed (Ferrando-Pardo et al. 2018; Navarro-Cano 2018a; Navarro-Cano and Robles-Sánchez 2018). Therefore, the few natural individuals of *C. heterophyllus* subsp. *carthaginensis* together with the reproductive difficulties due to the presence of self-incompatibility (Boscaiu and Güemes 2001), may lead to a dilution of the species by introgressive hybridisation into *C. albidus*, unless an accurate management is conducted in its natural habitat. This management should include a genetic verification of the individuals to not facilitate the introgression process unintentionally.

In situ conservation programmes require specific plant culture protocols to provide pure material for reinforcement actions. Reproductive biology studies of translocated populations are important to improve knowledge on population's persistence ability (Picó and Van Groenendael 2007) and develop effective conservation programmes (Anderson et al. 2015). Some studies provide relevant data related to seed germination of the taxa under study that evidence the presence of physical dormancy and optimal protocols to obtain high percentages of germination (Escriba-Baeza et al. 2007; Navarro-Cano 2018b).

Seed response is narrowly dependent on temperature and water availability. Consequently, climate change is a relevant factor affecting the natural recruitment of wild plant populations. Increases in temperatures and/or a decrease in rainfall or a displacement of the rainy season, lead to greater water stress in plants. It might not only affect the production of vigorous seeds, but also the stress tolerance at germination and longevity of the seeds constituting the soil seed bank.

To date there has been no information about the resilience of the germinative response of this threatened plant to the pressure of competitors on known natural populations. Therefore, this work studied the variability in germination capacity under controlled conditions of both Iberian populations of *C. heterophyllus*, and compared them with those of *C. albidus*, the main competitor in its natural habitat. Our efforts are focused mainly on competitive aspects of seed responses of these seriously threatened populations of *Cistus*, including the recommended verification of the genetic adscription and the study of their genetic variability. In addition, reproductive success in the Valencian and the Murcian populations of *C. heterophyllus* was also tested. In this sense, the research aimed to determine whether germination response, reproductive success and/or genetic characteristics could be directly related to the regression of the natural populations of *C. heterophyllus* in the Iberian Peninsula, but also to obtain an assessment of the effect of the ongoing climate change on this endemic species. In this study, the following questions were addressed:

- (1). Could the different level of dormancy be a potential factor limiting recruitment in natural populations?
- (2). Has *C. heterophyllus* subsp. *carthaginensis* higher temperature requirements for germination than *C. albidus*?
- (3). Are there differences in dormancy release between species and populations, when seeds are exposed to equal thermal shock pretreatment?
- (4). Could a different tolerance to water stress be one of the responsible factors of the low recruitment observed in Spanish populations?
- (5). Do seeds from Spanish populations of *C. heterophyllus* have lower longevity compared to *C. albidus*?
- (6). Given the ease of hybridisation between the two species, could there be any relationship between the genetic characteristics of the populations studied and the germination responses observed?
- (7). Is the reproductive biology of the species one of the factors of the low recruitment observed in Spanish populations?

Material and methods

Species and populations

Two Spanish populations of *C. heterophyllus* were considered for this study (Table 1, Fig. 1). The current status of natural plants from Murcia and Valencia provinces was checked. Both populations were subjected to an extreme regression. Valencian population was composed by a unique individual with inconsistent seed set. Thus, seeds from this origin were provided by the CIEF Centre (Generalitat Valenciana) where an *ex situ* plant collection was maintained for conservation purposes, cultivated from seeds produced by the original plant in 2013 (Ferrer-Gallego et al. 2018). The natural population in Murcia was also seriously diminished after the fire of 1996, and only three individuals were found in optimal conditions to produce seeds. In this case the staff of the Consejería of Murcia helped to collect enough seeds for the experiments from plants introduced in a neighbouring area in 2017 and 2019, within the conservation programme developed in this region.

Table 1 Details of the origin localities of seed studied samples (climatic parameters from climate-data.org). The climatic data for the plants from original population of “La Pobra de Vallbona” corresponds to the location of CIEF centre

Species	<i>C. albidus</i>	<i>C. heterophyllus</i> subsp. <i>carthaginensis</i>	
Acronym	Ca	ChV	ChM
Ref. number	UVEG-JBVAL-BG 07B2020	UVEG-JBVAL-BG 35B2020	UVEG-JBVAL-BG 28B2020
Population	Valencia, Bétera	Valencia, La Pobra de Vallbona	Murcia, Cartagena
UTM	30SYJ1692	30SYJ1289	30SXG8964
Altitude	184 masl	145 masl	215 masl
P (mm)	427	427	272
T (°C)	16.7	17.3	18.3

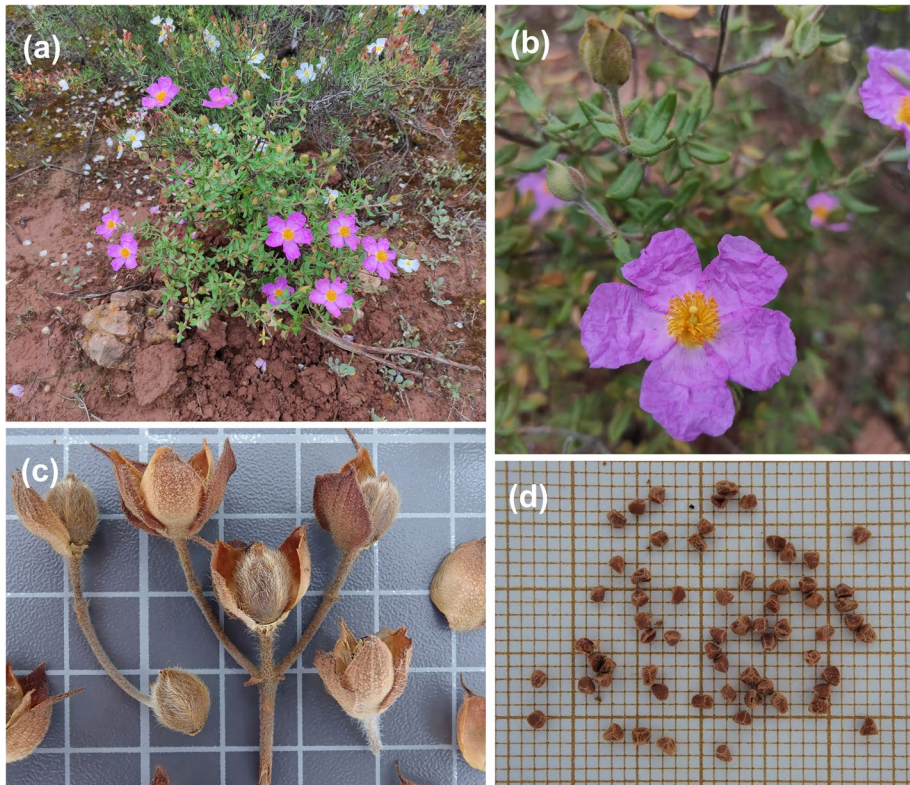


Fig. 1 Different traits of *Cistus heterophyllus* subsp. *carthaginensis*. **a** and **b** Adult flowering individual and flower detail, from the Valencian population. **c** and **d** Mature fruits collected and obtained seeds from the Murcian population

The reproductive studies were conducted on these Murcian introduced individuals and on one of the translocations conducted by the staff of Wildlife Service, Generalitat Valenciana at CIEF in Valencia, near the natural population.

To establish a comparison with the main competitor of this species, seeds were also collected from a natural population of *C. albidus*. Details of the locations are indicated in Table 1. In all cases, seeds were collected at complete maturity.

Seed features: morphological characterisation

The seeds were photographed on graph paper and dimensions, width and length were measured using the image analysis software ImageJ (Rasband 1997–2018). Seed weight was determined using an Orion Cahn C-33 microbalance. Batches of 100 seeds from each population were used for all measures. Data were expressed as mean $\pm$ standard deviation.

Seed surface was analysed with a SCIOS 2 FIB field emission scanning electron microscope, from the SCSIE Department, Electronic Microscopy Section of the University of Valencia. Samples were mounted on aluminium stubs with a carbon double-sided tape, then sputter-coated with a 100–200 Å-thick layer of gold and palladium by an Ion Sputter (Bio-Rad SC500) and finally examined at an accelerating voltage of 5 kV. Seed micrographs at two different magnifications, $\times 200$ and $\times 1000$, were used to determine the seed coat morphological features of the species. Seed descriptions were according to Werker (1997). The terminology of Stearn (1992) and Barthlott (1984) was adopted for the scanning electron microscopy (SEM) aspects of the seed coat.

Genetic characterisation

DNA extraction and PCR amplification

Ten individuals of *C. heterophyllus* (four belonging to the Valencian and six to the Murcian populations) and four individuals of *C. albidus* were collected from the same locations as the seeds used in this study (Table 1). Sampled leaves were dried in silica gel (Chase and Hills 1991) and stored at room temperature until DNA extraction. DNA was isolated 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. Three ad hoc molecular markers (Table S1) were designed from the data extracted of a TruSeq LT genomic library that was constructed from a paired-end de novo DNA sequencing using Illumina NextSeq 550 platform (Illumina Inc. San Diego, CA, USA) by the sequencing service from the University of Valencia (SCSIE, Spain). These markers were applied to verify the genetic ascription of the individuals belonging to the different populations. PCR amplifications were performed in a 25 μ L mix containing 1 $\times$ EmeraldAmp® GT PCR Master Mix (Takara), 5 μ M (final concentration) of each primer, and 20 ng of template DNA. Touchdown cycling was used: 95°C for 5 min, 10 cycles of 95°C for 40 s, 61°C decreasing to 51°C at 1°C per cycle for 30 s, 72°C for 40 s, followed by 30 cycles of 95°C for 40 s, 50°C for 30 s, and 72°C for 40 s, concluding with a 10 min extension at 72°C. 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) (Genbank-NCBI accession numbers: OQ331165—OQ331209).

DNA data analyses

MEGA v. X (Kumar et al. 2018) was used to generate a multiple sequence alignment (ClustalW algorithm, default options). The alignment was used to evaluate DNA polymorphism at the population level. Polymorphic and genetic differentiation parameters were calculated with DnaSP v.6 (Rozas et al. 2017): number of segregating sites (s), number of haplotypes (h), nucleotide diversity (π), haplotype diversity (H_d), average number of nucleotide differences (k) and fixation index between populations (F_{st}). The relationships between the individuals of the three populations were captured in a principal coordinate analysis (PCoA), with a superimposed minimum spanning tree (MST, Gower and Ross 1969), based on pairwise genetic distances calculated in MEGA-X, using composite maximum likelihood nucleotide substitution model that included transitions and transversions, 10,000 Bootstrap replicates, heterogeneous Gamma inter-site distribution rate and gaps removed only in pairwise comparisons (Kumar et al. 2018). PCoA was plotted in PAST v. 4.11 (Hammer et al. 2001) where eigenvectors and eigenvalues were computed from pairwise genetic distances explained above.

Germination assays

General germination tests were conducted at 20°C and 12/12 h photoperiod (100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 30 days. Four replications of 25 seeds were sown on agar medium (0.6%) for all the tests. Petri dishes of 5.5 cm diameter were used and held in temperature-controlled incubators within zip-closure plastic bags to avoid desiccation of the medium during the experiments. Germination was checked daily, except during weekends, when germination time was assigned on the following Mondays based on radicle length. Radicle protrusion ≥ 2 mm was the considered seed germination criterion.

Germination response was expressed as final germination percentage and speed as mean germination time (MGT) following Brenchley and Probert (1998).

Seeds of *Cistus* have a characteristic physical dormancy due to hard seed coats. The dormant seeds proportion was determined, and diverse methods to release dormancy were tested to determine the best method to use during the study when necessary. Before the main experiments, screening tests, comparing mechanical scarification with sandpaper, chemical scarification with sulphuric acid and hot water at different times, were performed. All treatments used had positive effects on the rupture of the seminal coat impermeability, and thus on physical latency. However, moist heat treatment, has proven to be one of the most effective, homogeneous and easiest to apply to large seed samples. Therefore, hot water, specifically immersion in water at 80 °C for 5 min, was the pretreatment chosen to remove seed dormancy when necessary for further analysis. After the pretreatment the seeds were dried over a sheet of filter paper and cooled down for 5 min at room temperature. This condition has been used to compare the response to different factors of the studied *C. heterophyllus* and *C. albidus* populations. Moreover, all these preliminary assays that require the use of a large number of seeds were carried out only with *Cistus albidus* due the limited amount of *C. heterophyllus* subsp. *carthaginiensis* seeds available.

Effects of incubation temperature on seed germination

The response, germination percentage and MGT, to constant temperatures was checked over a range of 10 to 35°C in 5°C increments. Base temperature (T_b) and thermal time (S) were calculated to determine the relationship between temperature, time and germination behaviour and compare thermal responses between the selected species and populations (García-Huidobro et al. 1982; Trudgill et al. 2005). Base Temperature (T_b) corresponds to the theoretical temperature in degrees Celsius below which germination is not possible and the thermal time reflects the thermal requirement in temperature per time to reach the germination. Both parameters were calculated from the linear regression on the plot of the inverse time to germination, expressed in this work as the mean germination time ($1/MGT$), as a function of temperature. The regression line was calculated considering the speed values for temperatures up to the optimal condition. T_b was calculated as the hypothetical case in which $1/MGT$ is zero and S as the inverse of the slope.

Effects of heat shock

Dry heat shock, specifically at 100°C for 10 min, was applied for the comparative study of potential stimulation by fire temperatures. This condition was determined following the considerations of Luna et al. (2019), complemented with a previous screening on different temperatures (80, 100 and 110°C) and exposure times (5 to 30 min, in 5-min increments). The heat shock was finally applied using a thermoblock because of the more effective and homogeneous results when compared with those obtained using a pre-heated oven. These tests lasted 60 days, until germination stabilised, to obtain reliable data.

Effects of PEG on seed germination

Water stress tolerance was compared through germination tests in decreasing osmotic potentials (−0.025, −0.05, −0.1 and −0.2 MPa) using different concentrations of polyethylene glycol 6000 (PEG) solutions on filter paper. The concentrations of PEG were calculated following Michel and Kaufmann (1973) to obtain the specific osmotic potentials (Ψ) for tests at 20°C and checked with a WP4-T dewpoint potentiometer (Decagon Devices, Inc.).

In all treatments, a control (untreated seeds) was also included, and the reduction of germination percentage was calculated as $RGP = [1 - (\text{number of germinated seeds in the PEG treatment} / \text{germinated seeds in the control})] \times 100$.

The germination-time curves for the different PEG concentrations were analysed applying the hydrotime model to determine the relationship between osmotic potential, time and germination response. The hydrotime modelling provides an approach to predict seed germination in soil under water deficit conditions, according to the equation of Gummerson (1986): $\theta_H = (\psi - \psi_b(g))t_g$, where: θ_H is the hydrotime constant, $\Psi_b(g)$ is the threshold or base potential below which germination is zero and Ψ is the actual water potential. Base water potential (Ψ_b) for both species and populations studied, was calculated by extrapolating the regression line on the plot of the inverse of the time to radicle emergence, expressed in this work as the mean germination time ($1/MGT$), against

osmotic potential to the x-axis intercept (Bradford 1990). Hydrotime (θ_H) was estimated as the inverse of the slope of these regression lines.

Comparative accelerated ageing experiment

Seed longevity comparisons between samples were completed using accelerated seed ageing method. After the pre-treatment to remove the dormancy, seeds were maintained in an atmosphere with 60% of relative humidity within hermetic glass jars, and at 45°C in an oven, following recommendations of Hay et al. (2019). The relative humidity was controlled using a LiCl solution (Hay et al. 2008). Samples were removed after different periods of time. The total time of exposure to observe loss of viability was determined also in a previous screening, where seeds remained fully viable after 2.5 months of exposure to this ageing conditions. In the light of these results, the accelerated ageing experiment was planned during a total period of 8 months.

A probit analysis was applied to fit the viability equation of Ellis and Roberts (1980) and seed viability (percentage germination) against the ageing period (days). The result was plotted to create a seed survival curve. P50 (the time for viability to decrease to 50%) was also determined as an estimation for relative seed longevity in order to facilitate the comparisons between populations and species.

Reproductive success

The reproductive experiments started in mid-April 2021 at the beginning of anthesis. The breeding system was tested on 71 plants from Valencia and 27 from Murcia. Six pollination treatments were conducted: control and autonomous self-pollination applied in both populations, and facilitated self-pollination, geitonogamy, xenogamy, and supplementary pollinations only in the Valencian population. Flowers from autonomous and facilitated self-pollination, geitonogamy and xenogamy treatments were bagged with a fine-mesh bag to exclude pollinators. The number of replicates in the Valencian population were: 64 for control, 35 for autonomous self-pollination, 56 for facilitated self-pollination, 63 for geitonogamy, 76 for xenogamy and 62 for supplementary. The replicates in the Murcian population were 25 and 22 for control and autonomous self-pollination, respectively. When mature, fruits were collected and taken to the lab to analyse seed production. The reproductive success of the pollination treatments was tested by fruit set, being the ratio of produced fruits to flowers treated; seed set, as the ratio of seeds to ovules; mean 10 seed weight and seed viability, and MGT as indicated above on the germination assay.

Statistical analysis

Statistical analysis of seed features parameters was performed using one-way analysis of variance (ANOVA) with a 95% confidence interval with Tukey post hoc analysis.

The significance of the differences among germination treatments was tested by applying a nonparametric test, Kruskal–Wallis one-way ANOVA. One-way ANOVA is a very robust method that provides good approximations for small samples when model assumptions are not fully satisfied (Khan and Rayner 2003). Generally, a post-hoc Tukey test was used to estimate homogeneous groups, except for PEG-treated groups that was evaluated by Dunnett's t-test in order to determine differences with respect to the control. $P < 0.05$ was considered significant.

Results

Seed features: morphological characterisation

The woody loculicidal capsules, characteristic of the genus *Cistus*, are, more or less globose in the studied species, usually with five seed filled fusiform locules. Full developed seeds are compactly disposed within the locules, and consequently seeds growing in the same locule have variable shape, that frequently was defined as polyhedric. When seed traits were analysed, significant differences were found in the parameters measured. Seeds of *Cistus albidus* are longer and wider than those of *Cistus heterophyllus*. Conversely, seeds of *C. albidus* are heavier than one of the *C. heterophyllus* studied populations but lighter than the other. Regarding the differences between the two *C. heterophyllus* populations, seeds from Murcia are longer ($L=1.51$ mm), wider ($W_i=1.14$ mm) and heavier ($W_e=1.27$ mg) than seeds collected in Valencia ($L=1.36$ mm, $W_i=1.05$ mm, $W_e=0.87$ mg; Table S2).

Figure 2 shows details of seed surface in the three samples examined. Primary sculpture, associated to the shape of cells constituting the outermost layer of the testa, is not clearly observed in the examined seeds. But differential features between samples are observed at this level due the morphology of the outer cell wall. Seeds from Valencian population of *Cistus heterophyllus* subsp. *carthaginensis* show a densely and long flattened papillae collapsed inward, providing a characteristic soft appearance when they are observed with stereoscopic light microscopy. These papillae are formed by the protrusion of the outer cell wall (Fig. 2). However, seeds of *Cistus albidus* show a rugose pattern with intercellular holes. Seeds from Murcian population have a rugose surface with some small flattened papillae. When the seeds have angular edges, they always tend to show here longer papillae. Secondary sculpture at the cuticle level is not observed.

Genetic characterisation

The sequence lengths of the analysed DNA regions ranged 184–196 bp, 107 bp and 172–190 bp, for Ch15, Ch18 and Ch20, respectively. The combined matrix of the three regions (481 bp) accounted for fifteen polymorphic sites, including five indels. The combined matrix rendered eleven haplotypes: three for *C. albidus*, seven for *C. heterophyllus* from Murcia and two for the Valencian population. Genetic diversity parameters were high for the Murcian population ($s=23$, $\pi=0.025$, $H_d=0.952$ and $k=11.238$) and low for the Valencian ($s=1$, $\pi=0.001$, $H_d=0.500$ and $k=0.500$). *C. albidus* showed an intermediate level in these parameters ($s=4$, $\pi=0.005$, $H_d=0.833$ and $k=2.500$). Pairwise population differentiation was high between *C. albidus* and *C. heterophyllus* from Valencia ($F_{st}=0.905$), but *C. heterophyllus* from Murcia presented an almost equidistant differentiation in relation to the other two samples, even higher from the Valencian population of the same species ($F_{st}=0.610$) than from the *C. albidus* population ($F_{st}=0.405$).

The PCoA analysis (Fig. 3) shows an intermediate position of *C. heterophyllus* individuals from Murcia with respect to Valencian individuals of the same species and those belonging to *C. albidus*, as well as a wide range of distribution between the two, i.e., showing hybrid genetic characteristics between both species. Besides, the Murcian population connects the other two in the MST superimposed on the PCoA plot.

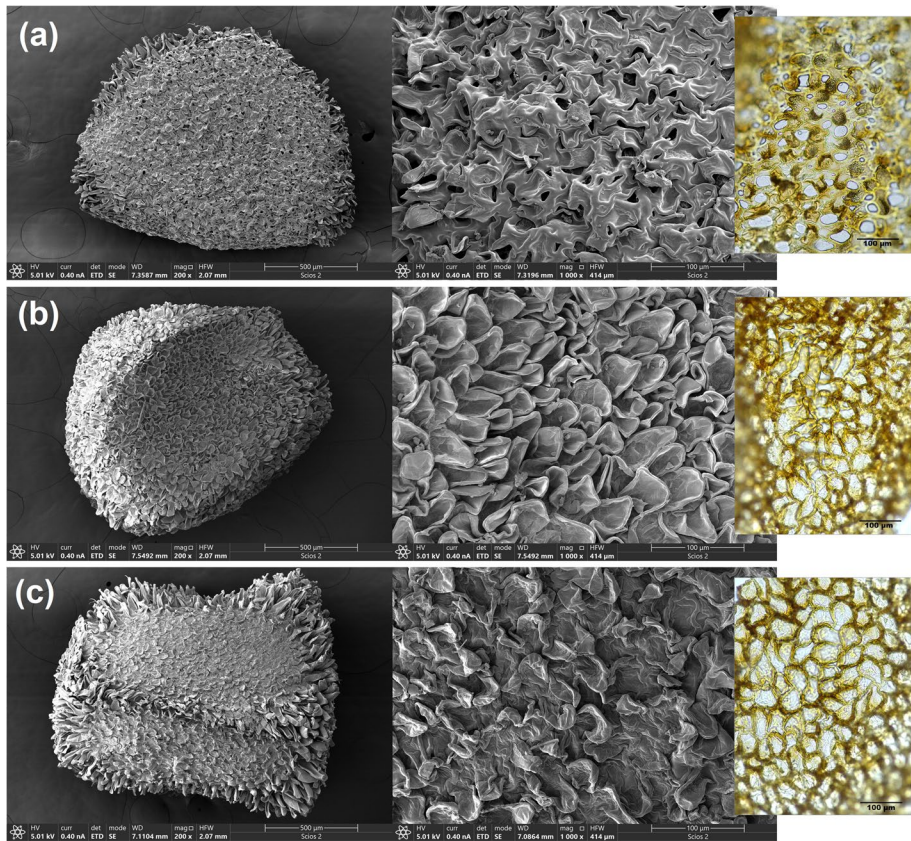


Fig. 2 General aspects of seed coat surface: the whole seed and ornamentation detail through scanning electron microscopy and detail of the cell disposition with optical microscopy; from left to right in each row. **a** *Cistus albidus*, **b** *Cistus heterophyllus* subsp. *carthaginensis* from the Valencian population and **c** *Cistus heterophyllus* subsp. *carthaginensis* from the Murcian population

Germination assays

Seeds of both species showed an important level of physical dormancy, characteristic of the Cistaceae. In all samples germination percentages were below 26%, when seeds germinated at 20 °C. In any case germination behaviour in the studied populations showed important differences in dormancy level between the two species. Seeds of the two populations of *C. heterophyllus* reached higher values in germination percentages (Chv 25.2% and Chm 22%; Table S3) than *C. albidus*, which saw a higher level of dormancy with only 5% of the sown seed germinated. In the case of speed, seeds from *C. heterophyllus* from Valencia were faster than the other two tested populations (Table S3).

Moist heat treatment, specifically at 80 °C for 5 min, was applied to break the physical dormancy and compare the response of the different *C. heterophyllus* and *C. albidus* populations. When comparing both species, seeds of all populations reached high germination percentages, above 85% (Table S3). Populations of *C. heterophyllus* reached the best response in terms of speed.

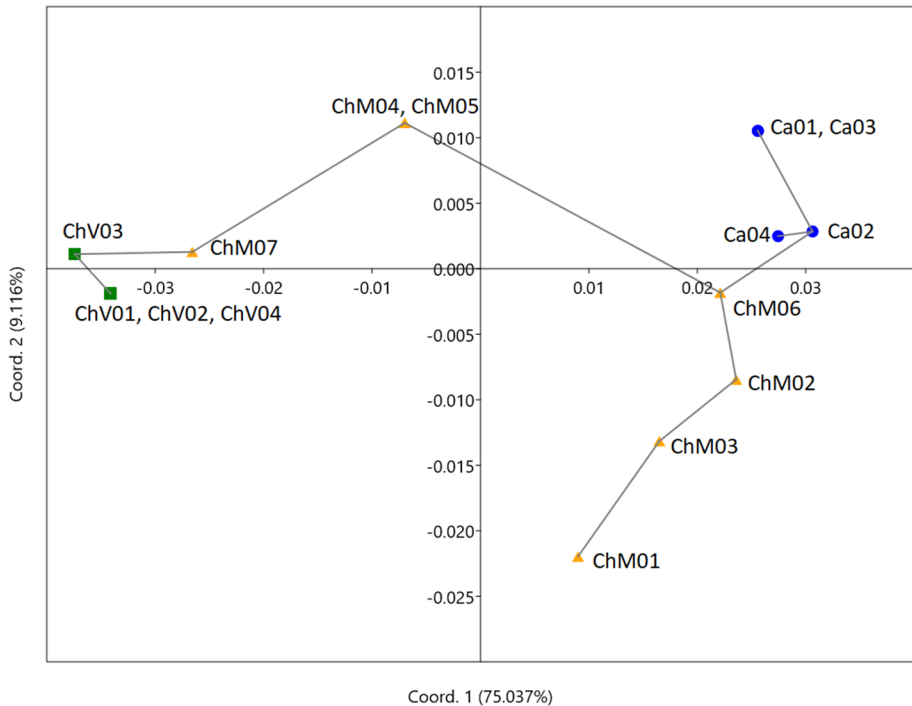


Fig. 3 PCoA analysis based on pairwise genetic distances including individuals from the Murcian (orange triangles) and Valencian (green squares) populations of *Cistus heterophyllus* and individuals of *Cistus albidus* (blue dots). Coordinate axis 1 explains 75.037% of the variance and axis 2 explains 9.116%, therefore between the two axes up to 84% of the variance due to genetic distances between individuals is shown. A MST connecting genetically closest individuals is superimposed on the PCoA plot

Effects of incubation temperature on seed germination

When the sensitivity to incubation temperature in pretreated seeds with moist heat treatment (80 °C, 5') was analysed, statistically significant differences among species were found. The optimal temperature for *C. albidus* was 15 °C, while in the sample of *C. heterophyllus* subsp. *carthaginensis* from Valencia the optimal response was achieved in a wider range of temperatures, from 10 to 30 °C (Table S4). Optimal values of germination percentage from Murcian population of *C. heterophyllus* were achieved at 10 and 25 °C. An exceptional tolerance to temperature has been observed in *C. albidus*, reaching high germination percentages above 70% at 35 °C (Table S4). Concerning germination speed, no differences were found in seeds of *C. albidus*. In case of *C. heterophyllus* from Valencia the fastest germination was obtained from 15 to 25 °C and in the Murcian population at 20 and 25 °C (Table S5).

In summary, both populations of *C. heterophyllus* are more tolerant in terms of germination percentage and speed to an increase of the temperature incubation than the population of *C. albidus* (Table S4 and S5).

Figure 4a shows the representation of the inverse of the MGT values at the different temperatures to fit the linear regression for thermal time constants calculation for each population. Population of *C. heterophyllus* from Valencia showed the most competitive

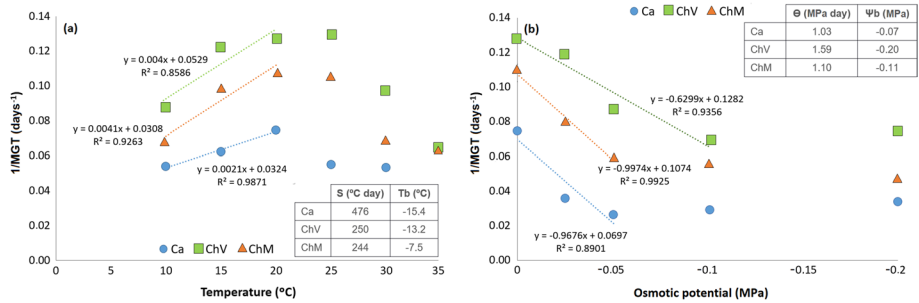


Fig. 4 Effects of Incubation temperature and PEG concentration on mean germination rate for each studied species and populations. **a** Relationship between temperature and germination rate (1/MGT) where the regression lines are represented for the calculation of the thermal time requirements. The table embedded shows the estimate of the base temperature (Tb) and the thermal time (S) from the linear model. **b** Progression of the germination speed in relation to the osmotic potential of the germination medium, generated by the applied increasing PEG concentration. The table indicates the Hydrotime (Θ) and base water potential (Ψ_b). In both cases, the regression lines that are above correspond to the most competitive population, within the temperature range in the former and in a range of PEG concentrations in the latter

response in all the tested temperatures. On the contrary, that of *C. albidus* was the least competitive of the three tested populations. Data of base temperature (Tb) and thermal time (S) were obtained from regression lines (Fig. 4a, embedded table).

These data reflect numerically the exposed results. The base values showed in Fig. 4a and the embedded table should be treated with caution as they were obtained from extrapolation beyond the range of experimental conditions. These calculated values were considered as theoretical figures.

The thermal time required for germination at suboptimal temperatures was similar among *C. heterophyllus* populations, and higher for *C. albidus*. Thus, based on the results, seeds from this population took longer to germinate than the other ones. Tb value was lower in the population of *C. albidus* than the other two populations of *C. heterophyllus* (Fig. 4a, embedded table).

Effects of heat shock

The thermoblock method was used to carry out the test of thermal shock to simulate fire effects, as it has been revealed as the most effective in a former test.

Based on a previous screening, 100 °C and 10 min of exposure was the treatment selected to compare the response between the two populations of *C. heterophyllus* and that of *C. albidus*. Germination response, in terms of percentage, was higher in the populations of *C. heterophyllus* from Valencia with a 95.5% of germination (Fig. 5a). The populations from Murcia and *C. albidus* reached lower germination percentages without significant differences between them. In terms of speed (MGT), seeds of the two populations of *C. heterophyllus* were faster, and without significant differences, than the one of *C. albidus*. (Fig. 5b). Germination percentages in all populations were higher in seeds previously exposed to heat shock than in controls (Table S3). Conversely, results of germination speed were slower in case of seeds subjected to thermal shock than in controls in all the tested seeds.

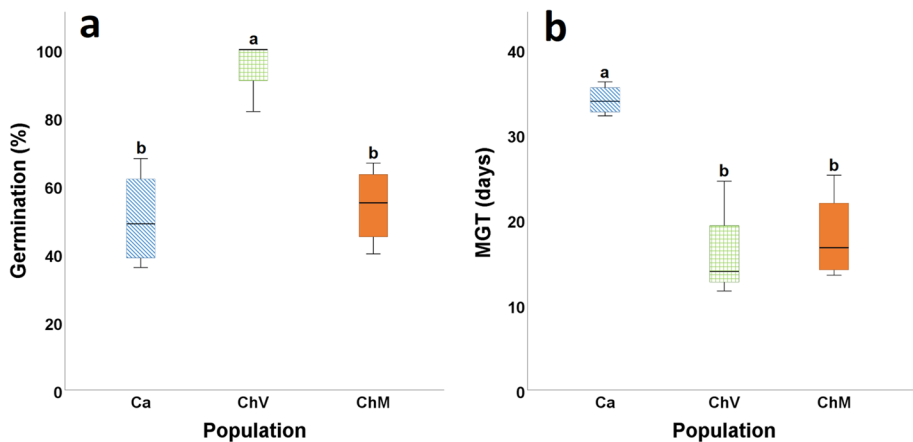


Fig. 5 Germination parameters, **a** percentage and **b** mean germination time (MGT), with seeds previously exposed to thermal shock, of *Cistus heterophyllus* and *Cistus albidus* populations. The germination tests were conducted at 20°C and 12/12 h photoperiod ($100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 60 days, four replications of 25 seeds on agar medium (0.6%)

Effects of PEG on seed germination

Under the water stress conditions generated by the different concentrations of PEG applied to the pretreated seeds, the population of *C. heterophyllus* from Valencia was the most tolerant to water stress, reaching high germination percentages at all osmotic potentials, even at -0.1 and -0.2 MPa (Table S6), and higher speed at all osmotic potentials (Table S7). This trend is also observed when analysing the reduction of germination percentage with respect to the control, where the *C. albidus* population is seriously affected, showing a higher reduction at all tested concentrations. On the contrary, the effect is slight in the population of *C. heterophyllus* from Valencia, especially at lower values of osmotic potential (Fig. 6).

The hydrotime model was applied to predict the germination of both species and populations under reduced water potentials, and to determine the more drought-tolerant of them. Figure 4b shows the inverse of the MGT values obtained at the different osmotic potentials tested. The purpose of this figure is to fit the linear regression for calculation of the hydrotime constant for each population. In this sense, seeds from Valencian population of *C. heterophyllus* were the most competitive in drought conditions and germinated faster at all tested osmotic potentials. On the contrary, seeds of *C. albidus* were the least competitive while the seeds of *C. heterophyllus* from Murcia showed an intermediate response.

Comparing the different species and populations to each other, the lower hydrotime corresponds to the *C. heterophyllus* from Valencia and therefore, the shorter time required for seed germination in reduced water potentials. In case of base water potential, the most negative values correspond to *C. heterophyllus* from Valencia, the most drought tolerant population with a more rapid germination at a given water potential (Fig. 4b, table embedded).

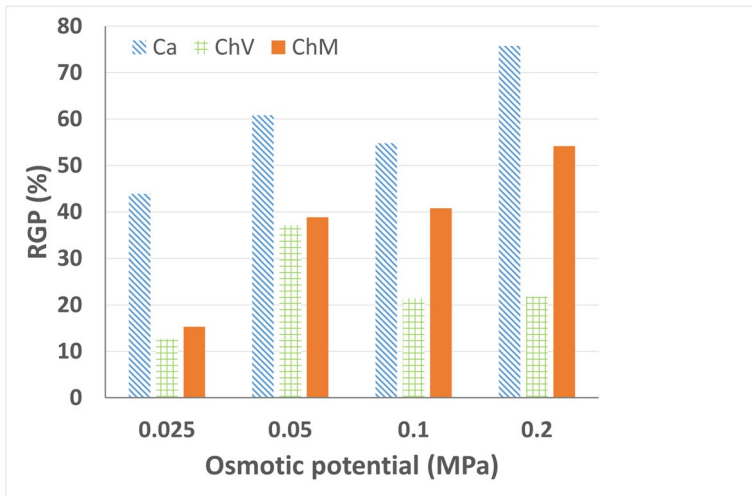


Fig. 6 Reduction of germination percentage (RGP) of the species studied and populations at increasing osmotic potentials respect to the control

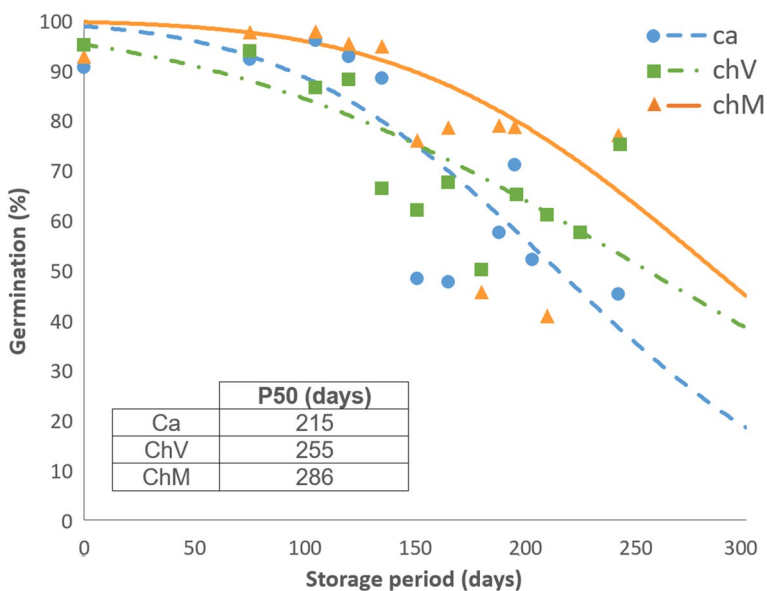


Fig. 7 Seed survival curves fitted by probit analysis showing the pattern of viability loss during storage under controlled conditions. The table embedded shows the ageing coefficients calculated by probit analysis for each tested population

Comparative accelerated ageing experiment

The seed germination from the ageing experiment provided estimated viability data to evaluate relative seed longevity on the three populations (Fig. 7 and the embedded table).

The curves obtained showed a similar pattern in the three studied populations. All the tested populations reached high germination percentages from the beginning to the middle of the experimental duration. As predictable, seed viability progressively diminished as the length of experimental ageing increased for the two species and the three populations (Fig. 7). However, there was a variation across species and populations in the time in which viability falls to 50%. The P50 values for the Murcian population of *C. heterophyllus* were higher than the Valencian population and, specially, than *C. albidus* (Fig. 7, table embedded). Seeds of the latter taxon were the shortest-lived, an important decline in germination was observed from 215 days of storage while the decline was smoother in the other two.

Reproductive success

In total, 356 crosses of six different pollination treatments in the Valencian population and 47 of two treatments in the Murcian were carried out. All treatments produced fruits in both populations, but the results were highly different. In the Valencian population, only 13.40% of the treated flowers produced fruits, whereas in the Murcian population this was achieved in 53.19% of the cases. Due to the scarce production of fruit and seeds in the Valencian population, the statistical analyses were conducted by treatment not having the replicates (production per plant) in account. The results are shown in Table 2. Xenogamy was the treatment that obtained the best results on fruit set and seed set followed by the control treatment in the Valencian population. The other treatments achieved results under 7%. The results from this assay should be treated with caution as very few fruits and seeds were obtained. Regarding seed viability, excluding the geitonogamy treatment (tested only once due to the low number of seeds), all the treatments presented high viability without significant differences. Instead, the MGT obtained when analysing self-pollination was significantly lower than the other treatments. Xenogamy, the treatment with higher fruit and seed production, took more time to germinate, but without significant difference with

Table 2 Fruit set (%), seed set (%), mean weight of 10 seed (mg), seed viability and speed (MGT) of the two studied populations. Seed germination tested at 20 °C and 12-h photoperiod. The seeds were pretreated with a humid heat shock of 80 °C for 5 min. Same letters indicate homogeneous groups (Tukey $p < 0.05$).

**Tested in only one replicate

Population	Pollination treatment	Fruit set (%)	Seed set (%)	Seed weight (mg)	Seed viability (%)	MGT
ChV	Control	12.5	28.5	12.4	95.28a	7.5 cd
	Autonomous self-pollination	5.7	3.1	-	0.0	-
	Facilitated self-pollination	5.4	24.8	14.0	96.7a	4.8a
	Geitonogamy	3.2	9.3	-	70.0b**	5.3ab
	Xenogamy	19.7	54.3	12.7	84.1ab	8.6d
	Supplementary pollination	6.5	30.2	15.1	95.5a	6.3bc
ChM	Control	84.0	66.1	11.2	82.7	9.0
	Autonomous self-pollination	18.2	50.1	12.0	88.4	8.8

the control. The data obtained were so poor that statistical analysis was not possible to conduct. Plants from Murcia produced significantly more fruits and seeds than the Valencia population. Contrary to that population, Murcian seeds did not presented significant differences among the tested treatments.

Discussion

Spanish populations of *C. heterophyllus* are situated on the northern border of the distribution area of the species. Therefore, the environmental conditions of these populations are suboptimal, and they could be more sensitive to changes and less competitive than other co-occurring species.

The seed characterisation showed differences in both morphological and genetic traits between the species and populations. On the one hand, significant differences in size and weight were also found by Navarro-Cano et al. (2009) among batches of *C. heterophyllus* collected in different years. They proved interannual variation within a single population. In this sense, our work coincides with previous observations of seed variability among species sharing the same habitat, as well as interpopulational variability within *C. heterophyllus*, as occurs in other species (Galiè et al. 2015; Gasparri et al. 2016). Notwithstanding, the samples of *C. albidus* and *C. heterophyllus* from Valencia showed a clear differentiation in their genetic features whereas the sample from the Murcian population presented a wide range of genetic diversity with mixed characteristics. The former two populations presented the expected results regarding two different species, but the hybridisation process is fully evident in the latter, in agreement with previous studies (Jiménez et al. 2007; Werner et al. 2021). The hybrid character of this population has been evidenced, as well, by the intermediate characters observed in seed coat surface examination. Therefore, not only expected intraspecific variability in seeds may explain these differences, but also their hybrid genetic composition. However, seed traits interact in some species in various aspects of germination response, such as temperature and light requirements (Gairola et al. 2021), germination rate under stress conditions (Steiner et al. 2019; Sharma Koirala and Neff 2020) and others such as seedling survival or resilience to climate change (Samarasinghe et al. 2022).

Species of the genus *Cistus* characteristically possess seeds with some degree of physical dormancy, due to hardness of the seed coat, which confers a certain impermeability even under optimal conditions for germination. In general, the higher dormancy level observed with increasing temperatures could be related to an adaptive behaviour to minimise seed germination during summer, the most unfavourable season for seedling survival (Escriba-Baeza et al. 2007). The combination of physical dormancy, high longevity, and small size and mass, characteristic of *Cistus* seeds, results in persistent soil seed banks (Thanos et al. 1992).

When germination response was analysed after dormancy release across temperature ranges, adaptive differences were found for some specific habitats. Thanos et al. (1992) indicated an optimal germination range for some *Cistus* species between 15 and 20°C. The range of optimum temperatures observed in the species studied, in general, coincided with that described for this Mediterranean species. However, the Valencian population of *C. heterophyllus* subsp. *carthaginensis* presented a wider optimal range of temperatures for germination, showing greater tolerance to higher temperatures. Nevertheless, the Murcian

population displayed intermediate results compared to both the former and *C. albidus* at 25°C and above.

Species with a narrow range of temperature requirements would be most at risk from climate change, while those with a wider range would be less vulnerable (Luna and Moreno 2010). A wider range of tolerance is a characteristic response of plants distributed in arid territories, as this behaviour can offer certain advantages when rainy periods are scarce. This is a frequent circumstance for *C. heterophyllus*, whose most important populations are mainly distributed in Algeria and Morocco. In an environment with greater water stress, the factor that primarily controls the timing of germination is water availability rather than temperature (Moruno et al. 2011; Estrelles et al. 2015).

The analysis of the germination speed under conditions of different constant temperatures allows comparison the relative competitiveness among the samples studied (Trudgill et al. 2005). The higher germination speed of *C. heterophyllus*, especially in the Valencian population under all tested conditions, indicates a theoretical advantage for seedling establishment. The calculated thermal time values provide evidence of theoretically higher competitiveness of *C. heterophyllus* compared to *C. albidus* under increasing temperatures.

Dry heat treatment increased germination in most Cistaceae, although the rate of increase was species-dependent, reflecting the differential role of fire within this plant family (Moreira et al. 2010; Luna et al. 2019). For all species, the effectiveness of heat treatment in stimulating germination has been widely demonstrated. At the same time, the effects of heat are usually reflected in germination level but not in germination rate. Fire is an important mechanism for *Cistus* species to colonise heterogeneous environments, particularly in habitats degraded by recurrent fires (Scuderi et al. 2010).

The samples studied of our target species exhibited dormancy release when dry heat shock was applied. However, a very slow germination response was observed compared with other methods tested for the physical dormancy breaking. We consider this behaviour an adaptation to avoid germination during summer dry conditions, the characteristic fire season, in fire-prone Mediterranean ecosystems. This behaviour is especially evident for the seeds from the Valencian population of *C. heterophyllus* subsp. *carthaginensis* which reached germination percentages close to 100% but they delay germination by an average of one month. The potential ecological advantage of a slow germination rate has already been noted for some leguminous seeds by Herranz et al. (1998), and even for *Cistus* by Thanos et al. (1992).

Regarding drought tolerance, our results agree with the conclusions of Luna and Chamorro (2016) concerning the reduced and delayed germination of Cistaceae under water stress once physical dormancy is removed. This response functions as a water-availability control of germination to maximise seedling survival after seed activation during summer fires. Plants inhabiting Mediterranean fire-prone ecosystems, particularly in semiarid environments, require precise regulation of germination processes to ensure successful seedling establishment.

The sensitivity, thermal and water stress, observed in the seeds of *C. albidus* in our experiments is comparable to the results of Chamorro and Moreno (2019). This species appears to be especially sensitive to water stress, in a polyethylene glycol (PEG) medium, when compared with other Mediterranean *Cistus* species. *C. heterophyllus* subsp. *carthaginensis* exhibits higher tolerance to osmotic stress induced by increased PEG concentration in germination medium. Again, the Murcian population shows intermediate tolerance between the Valencian samples and *C. albidus*, reflecting its genetically mixed character.

This response is related to its adaptation to semiarid environments, common in its main distribution area in north-west Africa. In the Iberian Peninsula, where the environment

is less extreme, this adaptation may not confer a competitive advantage over other, more widespread species. However, this situation may change in the future due to global warming, as temperatures are expected to rise and water availability to decline. Under these conditions, *C. heterophyllus* could become significantly more competitive.

The tests of accelerated ageing indicated very similar longevity patterns for the three populations of *Cistus* analysed. The P50 values obtained, always greater than 200 days, provide strong evidence of the high longevity exhibited by species of this genus, particularly compared to other species under the same conditions, where seed viability declines in a few tens of days. For example, a species that also presents physical dormancy, *Mimosa polydactyla* Humb. & Bonpl. ex Willd., requires 61.5 days under these accelerated ageing conditions to reduce its viability by 50% (Davies et al. 2016). According to Mondoni et al. (2011), these taxa may be considered as having long seed longevity, with P50 values ranging from >100 to ≤ 1000 days. Other species, lacking a hard seed coat and considered short- or medium-lived by these authors, showed lower P50 values, for example 4.7 days for *Veronica alpina* and 37.0 days for *Silene armeria* L. It should be noted that this response was obtained after removing the seed coat impermeability. For this reason, it can be confirmed with strong evidence that the soil seed bank constituted by these species is persistent.

In view of the results related to germination competitiveness, it cannot be confirmed that there is an evidence-based relationship between the decline of the *C. heterophyllus* Iberian Peninsula populations and a reduction of the soil seed bank.

Nevertheless, most of the flowers studied in the pollination assay aborted at early stages. The higher results for xenogamy compared to the control could suggest pollen limitation (Knight et al. 2005), but as observed the supplemental pollination treatment, adding more pollen did not improve the fruit set. These results could be due to a negative effect of pollinators visiting both *C. heterophyllus* subsp. *carthaginensis* and co-flowering species (Morales and Traveset 2008). All translocated plants came from the only natural plant in Valencia, which proved to be self-incompatible (Boscaiu and Güemes 2001). However, some translocated plants have at least partially lost the self-incompatibility and have demonstrated the ability of *C. heterophyllus* to self-pollinate, which improves the conservation prospects of the species. Even if the fruit and seed production are low, they are producing a soil seed bank and the viability tests yielded positive results. The higher fruit and seed production in Murcia might be influenced by hybridisation, as was observed by Boscaiu and Güemes (2001) and Ferrer-Gallego et al. (2017) when pollinated *C. heterophyllus* from Valencia with pollen of *C. albidus*. The viability of seeds obtained in the reproductive assay was similar to that obtained in the germination assay, lasting longer than the Valencian population, but shorter than *C. albidus*.

In this regard, the two populations presented some degree of self-pollination by both autonomous and facilitated methods. The results obtained for the autonomous self-pollination treatment were scarce but showed some degree of self-pollination. This is in accordance with the observations conducted on other translocated populations by García et al. (2020).

Overall, the evidence of genetic hybridisation seems to be supported by the germinative responses and reproductive success observed. In general, our seed results in the laboratory are consistent with the conclusions reached by Navarro-Cano et al. (2017), who presented the hybridisation as an advantageous strategy for these taxa, which might be reflected in some seed traits. In fact, we could affirm that there is a certain hybrid vigour linked to a greater longevity in the seeds of the Murcian population, at least during the initial stage of the ageing study, and particularly in terms of higher reproduction success. Although

this hybrid vigour has been proved for the reproductive success of this plant (Boscaiu and Güemes 2001; Ferrer-Gallego et al. 2017), further research on seed longevity would be valuable. In fact, there are multiple studies that describe effects on the germinative response due to hybridisation processes. As early as 1960, Finlay demonstrated the genetic control of the germinative response in *Hordeum vulgare* L. (Finlay 1960). Liebst and Schneller (2008) demonstrated the influence of genetic factors, in addition to environment factors, on germination response of some *Euphrasia* species and their hybrids. Schröder and Prasse (2013) found altered germination parameters, both total germination and speed, in wild plants when hybridised with cultivars propagated for restoration purposes. Winkler and Massatti (2020) highlight the importance of considering patterns of genetic variation to take decisions in restoration actions for biodiversity conservation.

In summary our results support that the germinative phase is not a determining factor for the regression of the natural populations of *C. heterophyllus* in the Iberian Peninsula, especially when compared to the most dominant plant within its natural distribution area, *C. albidus*. We absolutely agree with the statement that the major concern in threatened species with low effective population size is the reduced genetic diversity and increased inbreeding (Crow and Kimura 1970; Keller and Waller 2002), which seriously affects their survival capacity. Finally, other factors, related to reproductive aspects of this rare plant, such as pollinators, as well as the vigour of seedlings and juvenile plants, must be studied, to clarify the causes for the regression of this plant in our territory.

Conclusions

The genetic diversity, germination and reproductive responses observed during this study indicate that:

- The germinative behaviour seems not to have a key role for the low fitness of wild populations of *C. heterophyllus* subsp. *carthaginensis* in the Iberian Peninsula but reproductive success does.
- Seeds of the Iberian populations of *C. heterophyllus* show high competitiveness at higher temperatures during the germination phase.
- Both species of *Cistus* show fire adapted germination traits typical on obligate-seeder shrub species growing in Mediterranean regions.
- *C. heterophyllus* is a more competitive species under water stress conditions. It could be not expected that its seed germination will be more affected by climate change than that of *C. albidus*.
- A reduced longevity of the seeds is not a clear reason for the decline of the natural populations of *Cistus heterophyllus* on the Iberian Peninsula.
- The intermediate response in most germinative experiments of *C. heterophyllus* from Murcia, in relation to *C. albidus* and *C. heterophyllus* from Valencia, corresponds with the hybrid origin of their individuals.
- The higher reproductive success observed in the plants from Murcia can correspond with the hybrid origin of their individuals and can also be influenced by pollinators.
- The Valencian translocated population present very low reproductive success, but the production of viable seeds contributes to the regeneration of the soil seed bank.

- The positive results of seed germination and the seed bank reinforcement are a good prognosis for the future of these populations and thus also for the conservation of the species.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s44473-025-00050-z>.

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Author contributions Conceptualization: Elena Estrelles and Pilar Soriano; Experimental work: Elena Estrelles, Pilar Soriano, Josefa Prieto-Mossi, M. Isabel Martínez-Nieto and Anna Nebot; Formal analysis and investigation: Elena Estrelles, Pilar Soriano, M. Isabel Martínez-Nieto and Anna Nebot; Writing—original draft preparation: Elena Estrelles, Pilar Soriano, M. Isabel Martínez-Nieto and Anna Nebot; Writing—review and editing: Elena Estrelles, Pilar Soriano and M. Isabel Martínez-Nieto; Resources (seeds): Elena Estrelles, Pilar Soriano and Josefa Prieto-Mossi.

All the authors contributed to data discussion and conclusions and reviewed and approved the final manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

The genetic dataset is available in Genbank (NCBI). Accession numbers: OQ331165 – OQ331209.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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