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Induction of Seed Germination in *Cistus heterophyllus* (Cistaceae): A Rock Rose Critically Endangered in Spain

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Abstract: Seed germination from the only European population of the Iberian-North African endemism *Cistus heterophyllus* Desf. was studied by using germination inducing pretreatments. A seeding-cultivation method with flowerpots in a greenhouse was also tested and compared with the usual germination method using Petri dishes in a growth chamber. Seeds were collected in three different years. Germination percentages ca. 43% were obtained without pretreatment. This can be considered a high percentage in the genus *Cistus* and abnormally large for an isolated population composed by only nine individuals. A large variability in seed germination was found among the samples from the same population collected in different years. The dry-heat pretreatment (87±3°C for 12 min and soaking in water at 20°C for 48 h) significantly increased the germination percentage, reaching 81.5%. Germination in greenhouse was as effective as in growth chamber. The seeds maintained their viability until at least 6 years after collection and storage in a dry atmosphere at laboratory temperature. These results mean the first contribution to the germination ecophysiology of this species and they are fundamental to the recovery plan of the last remnant population of *C. heterophyllus* in Spain.

Key words: Cultivation method, intrapopulational variability, scarification pretreatment, seed size

INTRODUCTION

The species of *Cistus* have been traditionally considered as pyrophytes since they establish massively after fire (Tárrega *et al*., 2001). However, seedlings of *Cistus* can be also found in unburnt areas (Thanos *et al*., 1992; Trabaud, 1995), suggesting that these species do not exclusively depend on fire for their emergence (Delgado *et al*., 2001). Therefore, they can be considered as opportunistic species and colonizers of soils that have undergone impacts in their structure or plant cover (Trabaud, 1995; Pérez-García, 1997).

Seeds require either physical (mechanical and thermal scarification) or chemical treatments (acid scarification) before they imbibe water and germinate (Troumbis and Trabaud, 1987; Trabaud and Oustric, 1989; Thanos *et al*., 1992; Pérez-García, 1997; Hanley and Fenner, 1998). Since the seed coat is impermeable to water, gibberellic acid (GA_3) is not successful in promoting germination (Castro and Romero-García, 1999). Several researchers have proposed that in the absence of fire and under natural conditions, the weakening of the seminal coat can take place as the effect of multiple ecological factors, such as microbial degradation or changes in soil humidity and temperature (Baskin and Baskin, 1998).

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Cistus presents an intrapopulational heterogeneity in its germinative behavior (Thanos and Georghiou, 1988). Currently, it is accepted that only a small proportion of seeds of each population can germinate annually under natural conditions (Trabaud and Renard, 1999). These are called soft-coated seeds and generally represent less than 25% of the total seed bank (Thanos and Georghiou, 1988; Trabaud, 1995).

Cistus heterophyllus Desf. is an Iberian-North African purple-flowered rock rose (Guzmán and Vargas, 2005) whose European distribution is limited to an old individual in Valencia province (E Spain) and 24 individuals in Murcia province (SE Spain), which are descendants of a population of nine mature individuals that suddenly disappeared after a fire in April 1998 (Navarro-Cano and Rivera, 2001). The taxon is one of the most endangered plants of Europe and is included in the IUCN Red List of Threatened Species as critically endangered under the name of *C. heterophyllus* Desf. ssp. *carthaginensis* (Pau) M.B. Crespo and G. Mateo (Güemes *et al*., 2006). The Valencian individual has a Plant Micro-Reserve (Laguna *et al*., 2004). It was subjected to an *in vitro* micropropagation programme (Arregui *et al*., 1993), from which numerous individuals were obtained that showed problems of sexual reproduction due to self-incompatibility. Moreover, Boscaiu and Güemes (2001) studied the development and fertility of either pollen or ovules produced by individuals from Murcia and Valencia finding no problems in their breeding system. This survey analyzes the Murcian population, from which a seed stock has been collected since 1994 (Navarro-Cano, 2002). We pursue to know both: i) the behavior and germinative variability of the seeds collected in three different years, by means of the experimentation with pretreatments that stimulate germination and ii) to compare a germination - cultivation method in pots with substratum in a greenhouse with the classic method of incubation in a growth chamber in Petri dishes.

MATERIALS AND METHODS

Seed Collection, Seed Size and Climatic Data of the Years of Collection

Fruits of *C. heterophyllus* were collected in Calblanque, Monte de las Cenizas y Peña del Águila Regional Park (Murcia, SE Spain) on 21/01/94, 13/09/95 and 4/09/97. Seeds were sieved and examined for predation or deformity by using a binocular microscope. Morphologically normal seeds were measured and kept in paper envelopes in a dry atmosphere and laboratory temperature until analyses. The average weight of samples consisting of 100 seeds was calculated in triplicate for each sampling date. The average diameter of the seeds was obtained as (L+W)/2, L and W being the greatest and smallest diameters, respectively. For this purpose, groups of 30 seeds were measured in triplicate.

Table 2 shows the climatic data for the period between the months of maximum blossoming and fruit collection, for each sampling year. The climatic data were taken from the meteorological station El Algar, which is located 5 km north of the sampling site.

Germination Pretreatments

Preliminary germination tests were carried out both by dry heat over 100°C, immersion in hot water at different temperatures and times of exposure and acid scarification. None of these methods increased significantly the germination percentage; furthermore, preliminary tests showed that temperatures of 120±3°C for 10 min or 140±3°C for 2 min killed the seeds (Navarro-Cano, 2002), so we tested the following pretreatments:

- **Immersion in distilled water (I):** The seeds were soaked in distilled water at 20°C for 48 h.

- **Scarification with dry heat (S):** Seeds of the three collections were wrapped in aluminum paper, oven-heated at 87±3°C for 12 min and soaked in water at 20°C for 48 h.
- **Control (C):** Unsoaked and unheated seeds of the three collection dates.

Germination and Growth Conditions

Two methods of seed germination were tested, which were performed either in a greenhouse or in a growth chamber.

- **Greenhouse (GH):** Seeds were sown in a peat + sand substratum (4:1) at 0.5 cm depth, inside a greenhouse with a polycarbonate covering. Automatized watering was performed by spraying with decalcified water. A hydrothermograph was used to record the conditions of humidity and temperature inside the greenhouse. The germination criterion was the emergence of the cotyledons. The experiment was monitored for 90 days.
- **Growth chamber (GC):** Seeds were placed in Petri dishes on two filter paper disks moistened with 2 mL of distilled water. Seeds were incubated in a growth chamber (Model Microclima 1000, ASL-Snijders), under alternating temperatures of 15/25°C with a 12 h photoperiod, 80% relative humidity and 66 μE m^{-2} sec^{-1} photosynthetic photon flux, the lowest temperature coinciding with the period of darkness. A seed was considered to have germinated when at least 1 mm of radicle had emerged. The experiment was monitored for 60 days.

Given the reduced number of available seeds, the samples varied between 60 and 100 seeds for GC and GH experiments, respectively.

Statistical Analyses

One-way ANOVA was used for the statistical analysis of the seed weight and size. Post hoc comparisons were done with Tukey Test, $p<0.05$. Germination data were analyzed using binary logistic regression that is suitable for analysis of the effect of independent variables on a dependent variable with binomial distribution. Pretreatment, collection date, method of incubation and the interactions between them (independent variables) were entered as factors to evaluate their effect on the seed germination (dependent variable), that exposed a binary response (0 = no germination; 1 = germination). All statistical analyses were performed with the SPSS 11.5 statistical package (SPSS Inc, Chicago, IL).

RESULTS

The average seed weight was significantly larger in lots of 1994 than in lots of 1997 (Table 1). The seed size was significantly different among the three lots tested. We have not found any correspondence between climatic data of the collection years and the size and weight seeds, since all the parameters present a maximum value for the collection of 1994 and a minimum value for 1995, being the values of 1997 among these extremes (Table 2).

Table 1: Average weight and size (± SD) of three lots of 100 seeds of *C. heterophyllus* gathered in different dates

Collection date	Weight/lot (mg)[1]	Size/seed (mm)
21/01/94	120 ± 0^{a}	1.507 ± 0.129^{a}
13/09/95	117 ± 6^{ab}	1.423 ± 0.133^{b}
4/09/97	100 ± 10^{b}	1.371 ± 0.128^{c}

[1]Average values in each column sharing the same letter (upper index) are not significantly different (Tukey Test, $p<0.05$)

Table 2: Climatic data for the period between the months of maximum blossoming and fruit for each sampling year

Period of reference	P (mm)	TM (°C)	DA	PM (mm)	TD (°C)	AR (mm)
1/04/93-21/01/94	326.9	23.0	34	222.6	24.5	28
1/04/95-13/09/95	12.7	23.2	11	9.3	21.1	0
1/04/97-4/09/97	115.8	24.8	17	115.8	23.9	1

P: Rainfall; TM: Monthly average maximum temperature; DA: Days of rainfall; PM: Rainfall from April to July; TD: Daily average maximum temperature from April to July; AR: Rainfall during the month before seed collection

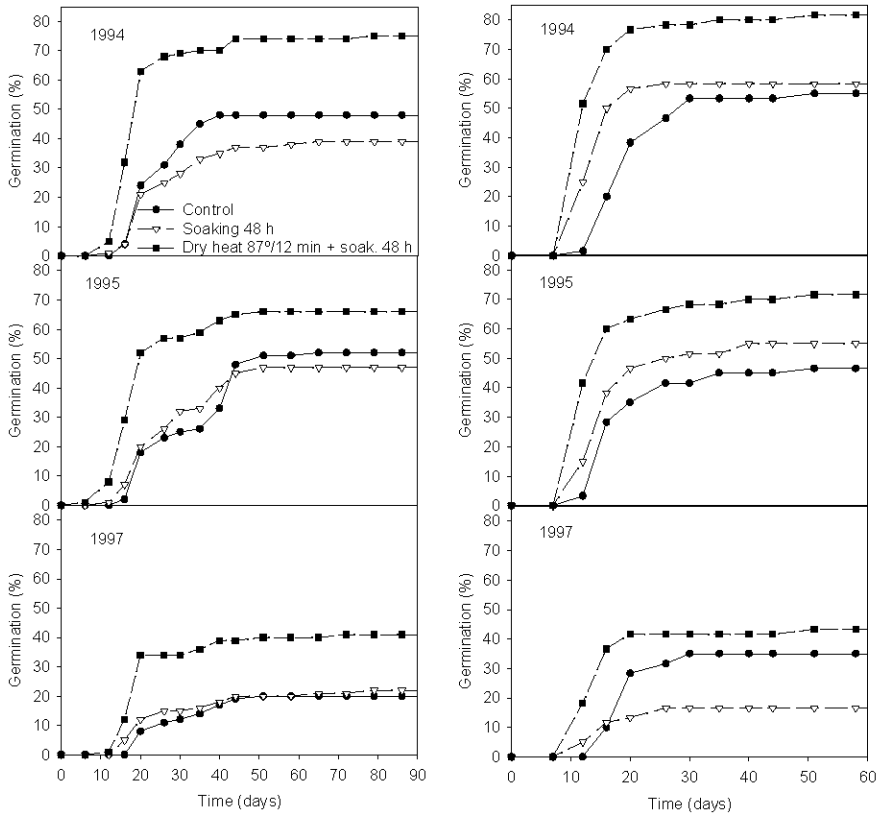

Fig. 1: Curve of accumulated germination of seeds collected in 1994, 1995 and 1997. In the left, germination of the seeds sown on substratum in a greenhouse; in the right, the seeds incubated in Petri dishes in a growth chamber

The germination percentages of the seeds gathered in 1994 were significantly larger than those of the ones collected in 1995 ($p = 0.021$) and 1997 ($p<0.001$). The binary logistic regression analysis indicated that the germination of S-pretreated seeds was the largest (Table 3). GH cultivation was as effective as GC to promote seed germination in *C. heterophyllus* ($p = 0.087$). The combined use of seeds collected in 1994 + S-pretreatment + GC cultivation yielded up to 81.5% germination (Fig. 1). After grouping together the cultivation method and the collection date, S-pretreated seeds reached (mean±SE) 63±7% germination, whereas control and I-pretreated seeds showed 42.8±5.4% and 39.8±7%, respectively. Non-significant interactions were found between the factors (Table 3).

Table 3: Results of the binary logistic regression analysis, showing the effect of the incubation method, collection date, pretreatment and their interactions on seed germination of *Cistus heterophyllus* ($p<0.05$)

Variables[1]	Coeff.	Z_{Wald}	p-value
Constant	1.550	5.75	<0.001
Cultivation method			
Greenhouse	-0.4776	-1.71	0.087
Seed collection year			
Collection 95	-0.7288	-2.31	0.021
Collection 97	-1.7617	-5.58	<0.001
Seed pretreatment			
Control	-1.2370	-3.95	<0.001
Soaking 48 h	-1.3595	-4.32	<0.001
Cult. method×Coll. year			
Greenhouse×collection 95	0.3795	1.35	0.176
Greenhouse×collection 97	0.2906	0.99	0.324
Cult. method×seed pretreatment			
Greenhouse×control	0.0180	0.06	0.950
Greenhouse×soaking 48 h	-0.0706	-0.24	0.809
Coll. year×seed pretreatment			
Collection 95×control	0.4658	1.37	0.171
Collection 95×soaking 48 h	0.6437	1.89	0.059
Collection 97×control	0.4871	1.40	0.160
Collection 97×soaking 48 h	0.3412	0.96	0.339

[1]The constant term of the regression includes the following levels of the three variables: growth chamber, collection 1994 and dry-heat pretreatment. The coefficients of the other levels of each variable show differences regarding the level included in the constant

DISCUSSION

Present results suggest that the biometric parameters of the seeds of *C. heterophyllus* vary interannually within a single population and together to the interpopulational variability detected in other species (Michaels *et al*., 1988; Corral *et al*., 1989) made advisable to carry out germination studies for each taxon comparing lots of seeds of different origin and collection date (Perez-García *et al*., 2003).

The S pretreatment significantly improved seed germination in *C. heterophyllus*, as indeed happens in most *Cistus* species (Thanos *et al*., 1992). Navarro-Cano (2002) tested hot water and acid pretreatments in *C. heterophyllus* without satisfactory results. On the contrary, other rock roses like *C. albidus* (Vuillemin and Bulard, 1981), *C. osbeckiifolius* (Pérez-García, 1997) or *C. clusii* (Castro and Romero-García, 1999) showed a higher germination rate when their seeds were submitted to these scarification pretreatments.

The high germination percentages reached with the control seeds, are relevant since they contradict the results of most surveys concerning the eco-physiology of the germination of *Cistus*. Nevertheless, Pérez-García (1997), Hanley and Fenner (1998) and Thanos *et al*. (1992) showed the existence of germination percentages about 50% in some tests performed with seeds without treatment in species of *Cistus*. Pérez-García (1997) demonstrated the existence of intrapopulational heterogeneity, even within the same individual, in the germination of seeds without pretreatment of *C. ladanifer*, questioning both the genetic control and the influence of environmental factors when explaining these high germination percentages. On the contrary, present results, obtained from a single population studied along the time, as well as those carried out with different populations (Thanos *et al*., 1992), seem to indicate that this behavior could be constant and exclusive of each population.

From an applied perspective, results support the idea that it is effective to propagate *C. heterophyllus* directly from seeds sown in containers with substratum and grown in a greenhouse, what is of great interest for the propagation applied to the recovery of the taxon. It should be

remembered that when working with threatened species with minimal populations, the tests of seed viability and germination improvement should be connected with their propagation in nursery for restitutions in the natural environment.

The relationships between the viability of each lot of seeds collected in different dates and the biometric data should be explained on the base of temporary environmental factors, since all the seeds belong to a single population. Troumbis and Trabaud (1986, 1987) found differences in the number of fruits and seeds per capsule of *C. villosus* and *C. salviifolius* in two groups within a single population growing on distinct soils and followed during several contrasted years. In this sense, Pérez-García *et al*. (2003) found differences of germination in several populations of some Labiatae species that were related both to the seed weight and various geographic parameters.

Seeds of *C. heterophyllus* of different ages were tested for their viability, showing at least 6 years of maintenance after their collection and storage in a dry atmosphere at laboratory temperature.

These results mean the first contribution to the germination ecophysiology of this Iberian-North African endemism and they are a key factor to the recovery plan of the last remnant population of this species in Spain.

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