In vitro germination of four herbaceous species endemic to the Azores archipelago

MARIA JOÃO PEREIRA, ELISABETE DIAS & SAIOA RAMOS

Pereira, M.J., E. Dias & S. Ramos (*in press*). In vitro germination of four herbaceous species endemic to the Azores archipelago. *Arquipelago*. Life and Marine Sciences 29: 07-14.

Current projects aimed at the conservation of endemic species and restoration of native habitats require information about seed viability, held in a germplasm bank, and *ex situ* seed and plant production. In this study, we investigated: a) the viability of *Spergularia azorica* Lebel seeds after 18 years of storage, and the ability of the developed plants to produce viable seeds in *ex situ* conditions; b) the viability of *Leontodon filii* (Hochst. ex Seub.) Paiva & Ormonde seeds after seven years of storage; c) the germination characteristics of *in situ* harvested seeds of *Luzula purpureo-splendens* Seub.; and d) the feasibility of harvesting seeds from transplanted flowering *Bellis azorica* Hochst. plants to initiate *in vitro* cultures on Murashige and Skoog (MS) medium. For each of these four species, we estimated the percentage of germination, the number of days for radicle emergence, and the mean time to germination in different experimental conditions. The best germination percentages obtained were: a) 81.6 % for *Spergularia azorica* 18 year-old seeds and 97 % for the next generation of *ex situ*-produced seeds; b) 91% for *Leontodon filii* after seven years' storage; c) 73% for *in situ* harvested seeds of *Luzula purpureo-splendens*; and d) 81% for *Bellis azorica ex situ*-produced seeds. Also, *in vitro* cultures of *Bellis azorica* were initiated on MS medium.

Key words: *Bellis azorica,* germplasm bank, *Leontodon filii, Luzula purpureo-splendens, Spergularia azorica*

Maria João Pereira (e-mail: mjpereira@uac.pt), Elisabete Dias & Saioa Ramos, Department of Biology, University of the Azores, 1422, PT-9501-855 Ponta Delgada, Açores, Portugal.

INTRODUCTION

Germplasm banks – collections of genetic material, principally in the form of seeds – are widely used for *ex situ* conservation and management of plant species. Therefore, information about viability of stored seeds, and *ex situ* plant and seed production from stored and non-stored seeds, is of great importance in the conservation management of endemic species and restoration of native habitats. In this study, we selected three endangered species (*Bellis azorica* Hochst., *Leontodon filii* (Hochst. ex Seub.) Paiva & Ormonde, and *Spergularia azorica* Lebel), and one species of 'least concern' (*Luzula purpureo-splendens* Seub.), all based on a recent classification (Corvelo 2010) of Azorean endemic species using the International Union for Conservation of Nature (2001) categories (Fig. 1).

Bellis azorica (Asteraceae) is a protected species under the Berne Convention (Council Decision 82/72/EEC). This species is recorded as present on all the Azorean islands, with the exception of the islands of Graciosa, São Miguel and Santa Maria (Silva et al. 2010). Nevertheless, in the last decade, this species was only spotted on the islands of Flores, Faial and Pico (Schaefer 2003; Pereira et al. 2005). *Bellis azorica* grows in *Juniperus* and *Laurus forests* and on slopes of craters and ravines, at altitudes between 600 and

800 m (Schaefer 2003, 2005). *Spergularia azorica* (Caryophyllaceae) is a protected species under the Habitats Directive (Council Directive 92/43/EEC). This species is present in all the Azorean islands and occurs in scattered locations on coastal rocks and landslides, at altitudes between 0 and 50 m (Schaefer 2003, 2005; Silva et al. 2010). Finally the Azorean endemics *Leontodon filii* (Asteraceae) and *Luzula purpureo-splendens* (Juncaceae) are not protected by the Berne Convention or the Habitats Directive. *Leontodon filii* is recorded as present in all the islands, with the exception of the islands of Santa Maria, Graciosa and Corvo; while *Luzula purpureo-splendens* occurs in all the islands with the exception of the islands of Santa Maria and Graciosa (Silva et al. 2010). *Leontodon filii* is found on grassy slopes, usually above 500 m of altitude, while *Luzula purpureo-splendens* is usually found at altitudes between 200 and 900 m on grassy slopes, in natural pastures, and forests of *Juniperus* and *Erica* (Schaefer 2005).

Previous data on *Spergularia azorica*, *Leontodon filii* and *Bellis azorica* germination, with seeds sown on Petri dishes with moistened filter paper, were obtained by Maciel (2004) under different temperature (10ºC, 15ºC, 20ºC or 20ºC day/10º night) and light (0, 8 or 16h photoperiod) regimes. Maciel (2004) using one seed lot of *Spergularia azorica* from S. Miguel Island performed seven germination trials over a period of 9 years. This author found that regardless of light and temperature regimes used or seed age, all the trials resulted in germination percentages above 94.5%, mean germination times between 5.7 and 17.4 days (Harrington 1963), and between 4 and 9 days to first radicle emergence. Using two seed lots of *Leontodon filii* from S. Miguel Island, Maciel (2004) performed eight germination trials over a period of 8 years and a half. The best germination percentages, 94-99%, with correspondingly mean germination times of 182.9–133 days and 9.5–8 days to first radicle emergence, were obtained with seeds that had been stored for ten days, regardless of light and temperature regimes. Maciel (2004) also found that *Leontodon filii* seeds lose their germination capacity after 8 years and a half of storage at room temperature. Using one seed lot of *Bellis azorica* from Pico Island Maciel (2004) performed two germination trials, with seeds stored for 5 and 17 months. The best germination percentage was obtained with seeds stored for five months; 73% of the seeds germinated under a regime of 8 hours' photoperiod at 20ºC and 16 hours dark at 10ºC; the corresponding mean time for germination was 130 days and it took 82 days to the first radicle emergence. Finally, no published data was found on *Bellis azorica in vitro* culture or on *in vitro* germination of *Luzula purpureo-splendens.*

The present study aimed to test: a) the germination characteristics of *Spergularia azorica* seeds after 18 years' storage, the development of the plants produced, and the germination characteristics of the next generation of *ex situ*-produced seeds; b) the germination characteristics of *Leontodon filii* seeds after seven years' storage; c) the germination characteristics of *Luzula purpureo-splendens seeds*; and d) the production of *Bellis azorica* seeds and plants *ex situ.*

MATERIAL AND METHODS

Seeds of *Spergularia azorica*, collected by Pereira on São Miguel island, which had been stored for 18 years at room temperature in a test tube with silica gel, were washed for 30 min under a stream of tap water, and placed on Petri dishes with Whatmann® no.1 filter paper moistened with distilled water, under different regimes of light (either dark, continuous light or eight hour photoperiod) and temperature (continuous 15ºC or 20ºC during the light period alternating with 10ºC during the dark period). All the plants produced were potted and kept in outdoor conditions. Flourishing plants were checked daily and the ripe seeds were collected between July and September. The *ex situ* produced seeds were then placed in Petri dishes with moistened filter paper, under a regime of a temperature of 20ºC for eight hours of light alternating with a temperature of 10ºC for 16 hours of dark. In addition, ripened seeds were harvested in August on São Miguel Island and placed immediately under the same conditions as the *ex situ* produced seeds. Seeds of *Leontodon filii* collected by Pereira on São Miguel Island, stored for seven years at room

Figure 1. a) *Spergularia azorica* Lebel., b) *Leontodon filii* (Hochst. ex Seubert) Paiva & Ormonde, c) *Luzula purpureo-splendens* Seub., d) *Bellis azorica* Hochst. (b-c, courtesy of Mathias Ogonovsky)

temperature in a test tube with silica gel were washed for 30 minutes under a stream of tap water and then placed on Petri dishes with-Whatmann® no.1filter paper moistened with distilled water, under conditions of an eight hour photoperiod and continuous temperature of 20ºC. Seeds of *Luzula purpureo-splendens* collected by Pereira on São Miguel island, were kept on a tray for two months at room temperature, and were washed for 30 minutes under a stream of tap water and placed on Petri dishes with moistened Whatmann® no.1filter paper, under conditions of an eight hour photoperiod and two different temperature regimes: continuous 20ºC or 10ºC during the dark period alternating with 20ºC during the light period. Three flowering plants of *Bellis azorica* were collected by Pereira, together with the soil they were growing in, on 12 June 2005 during a scientific expedition to Pico Island, and immediately potted in one single pot. Plants were

kept in sheltered outdoor conditions and were checked daily, and the ripening seeds collected between July and August. The bigger and more substantial seeds were selected, and 400 seeds were given the following treatment: washing for 30 minutes under a stream of tap water; soaking for 30 min in 1% benomyl (Benlate®) solution; rinsing three times with sterile distilled water; soaking for 10 min in 10% commercial bleach solution (a final concentration of 0.6% sodium hypochlorite) containing 0.02% Tween-20 (Sigma®); and, finally, rinsing six times with sterile distilled water. Seeds were incubated *in vitro* on moistened Whatmann® no.1 filter paper for germination, under a regime of alternating temperatures of 20ºC during the eight hours of light and 10ºC during the 16 hours of dark. Seedlings with no visible contamination were used as sources of explant for *in vitro* culture without further disinfection. The roots were severed and the remaining section, with two cotyledons, inoculated in an autoclaved (121ºC for 20 minutes) Murashige & Skoog (1962) medium supplemented with 20 gl^{-1} sucrose, solidified with 8 g^{-1} Bacto Agar Difco® and pH of 5. After four weeks of *in vitro* culture, counts were made of the number of contaminated explant cultures, the number of shoots (>3 mm long) per explant culture, and the number of rooted explant cultures.

Growth cabinets with temperature control (± 1ºC) and a light intensity of ~56 $\mu mol.m^{-2}.s^{-1}$ were used for all the germination trials and cultures. The number of seeds showing radicle emergence was recorded every day for all the germination trials. The number of days to first cotyledon and first leaf appearance was only recorded for *Bellis azorica* germination trials. For each different temperature and light regime, four replicates of 100 seeds were set up (ISTA 2005). The mean time to germination or emergence (MT) was calculated according to the formula $MT = \sum (ni*di) /N$ (where *n* is the number of germinated seeds on day *i*, *d* the number of days from the beginning of the test, and N is the total number of germinated seeds) (Harrington 1963). For the variable 'percent of germination', a χ^2 test was used to analyse the contingency tables and, when the replicate results were homogeneous, the total χ^2 was used. A Levene test was used to test the homogeneity of the variables 'number of days to first radicle emergence', and 'mean time to germination'. When homogeneity was encountered, data were statistically compared using the t-student test or the one–way analysis of variance (ANOVA), and, when the null hypothesis was rejected, the Tukey multiple comparison test was used. When homoscedasticity was not verifiable, data were statistically compared using the Mann-Whitney test or the Kruskal-Wallis test for non-parametric analysis of variance; and, when the null hypothesis was rejected, a non-parametric Tukey-type test for multiple comparisons was used.

All the *Spergularia azorica* plants were used to produce seeds for the Germplasm Bank of the Biology Department (Azores University). A number of the produced *Bellis azorica* plants were offered to Faial Botanical Garden and to the Garden of the Sant'Ana Palace, and the remaining plants were used as a source of explants for *in vitro* culture. All the produced plants of *Leontodon filli* and *Luzula purpureo-splendens* were offered to the EU 'LIFE Priolo' project.

RESULTS

The effect of temperature regime on the germination characteristics of *Spergularia azorica* seeds (18 years old).

The continuous regime of 15ºC significantly decreased the mean time to germination and increased the germination percentage when compared with the regime of alternating temperatures 20ºC-10ºC ($P<0.05$) (Table 1).

The effect of light regime on the germination characteristics of *Spergularia azorica* seeds (18 years old)

A significantly larger percentage of plants germinated under the dark regime in comparison with the eight hour and 24 hour photoperiod regimes ($P<0.05$) (Table 1); the number of days to first radicle emergence and the mean time to germination were also both reduced under the dark regime when compared with the continuous light regime.

The effect of seed age and seed origin on the germination characteristics of *Spergularia azorica*

The 18 years old seeds revealed significantly longer times to first radicle emergence and mean time to germination and a significantly smaller germination percentage when compared with seeds that had not been stored (P<0.05) (Table 1). No differences were found between any germination characteristic of fresh seeds collected *in situ* and fresh seeds collected *ex situ* from plants with origin in 18 years old seeds (P<0.05) (Table 1).

The effect of seven years' storage on the germination characteristics of *Leontodon filii* seeds

The seven years old seeds showed a slight but statistically significant decrease in germination percentage and a significant decrease in the mean time to germination (P<0.05) (Table 2).

Table 1. The effect of temperature regime on the germination characteristics of *Spergularia azorica* 18 years old seeds, under a light regime of 8h per day; the effect of light regime on the germination characteristics of *Spergularia azorica* 18 years old seeds, at 15ºC; and germination characteristics of non stored seeds and 18 years stored seeds harvested *in situ* and the produced *ex situ* seeds (from plants with origin on 18 years old seeds) under a photoperiod of 8h with alternating temperatures of 20ºC/10ºC. Mean values ± standard deviations. Groups that showed significant differences (p<0.05) are indicated with different letters.

Temperature	N	Days to first radicle emergence	Mean time to germination (days)	Percent of germination at 35th day
15ºC	4x100	8.5 ± 0.6 (a)	12.3 ± 0.8 (a)	75.6 ± 3.9 (a)
20ºC/10ºC	4x100	8.6 ± 2.6 (a)	13.8 ± 0.5 (b)	64.7 ± 9.9 (b)
Hours of light *per day*				
0	4x100	10.0 ± 0 (a)	12.3 ± 0.1(a)	81.6 ± 4.1(a)
8	4x100	8.5 ± 0.6 (a)	12.3 ± 0.8 (a)	75.6 ± 3.9 (b)
24	4x100	6.0 ± 2 (b)	10.3 ± 0.5 (b)	73.0 ± 5.6 (b)
Seeds' origin				
Stored 18 years	4x100	8.6 ± 2.6 (b)	13.8 ± 0.5 (b)	64.7 ± 9.9 (b)
Harvested *in situ*	4x100	4.5 ± 0.57 (a)	6.1 ± 0.2 (a)	99.0 ± 1.3 (a)
Harvested *ex situ*	4x100	4.75 ± 0.5 (a)	5.9 ± 0.1 (a)	97.0 ± 1.2 (a)

Table 2. *Leontodon filii.* Germination characteristics of non stored seeds and 7 years stored seeds from the same seed lot under a photoperiod of 8h at 20ºC. Mean values ± standard deviations. Groups that showed significant differences (p<0.05) are indicated with different letters.

Storage period (years)	N	Days to first radicle emergence	Mean time to germination (days)	Germination at 35th day (%)
0*	4x100	9.3 ± 2.1 (a)	125.8 ± 18.3 (a)	97.8 ± 2.1 (a)
7	16x100	6.9 ± 0.9 (a)	19.2 ± 2.8 (b)	91.8 ± 4.6 (b)

* Data from Maciel (2004)

Table 3. Germination characteristics of *Luzula purpureo-splendens* under a light regime of 8h per day and two different temperature regimes. Mean values ± standard deviations. Groups that showed significant differences (p<0.05) are indicated with different letters.

Temperature	N	Days to first radicle emergence	Mean time to germination (days)	Germination at 35th day (%)
20ºC	4x100	10,0 ± 2,3 (a)	25,3 ±3,6 (a)	71,5 ±12,6 (a)
20ºC/10ºC	4x100	12,3 ± 2,2 (a)	23,5 ±3,4 (a)	73.0 ±4,7 (a)

Determination of *Luzula purpureo-splendens* seed germination characteristics

For the two temperature regimes tested (either continuous 20ºC or alternating 20ºC and 10ºC, as described above), no significantly differences were found in the germination characteristics ($P<0.05$) (Table 3); the number of days to first radicle emergence was respectively 10 and 12 days, the mean times of germination were 24 and 25 days, and the germination percentages were 72% and 73%.

***Ex situ* production of *Bellis azorica* seeds and plants**

All the plants transplanted in June survived and produced new flowers until August, and all the flowers successfully produced seeds. Seeds started to germinate on the second day of the germination trial 81% had germinated in 34 days. Germinated seeds took an average of 5.7 days for cotyledons to be distinguishable, and a further 2.9 days to develop the first true leaf (Table 4). After four weeks of the *in vitro* initiation stage, 11% of the explants were contaminated (N = 300). From the remaining 267 explants, 66% developed leaves, and 0.5% rooted.

Table 4. Mean values ± standard deviations for germination and developmental characteristics of *Bellis azorica* Hochst. seeds (N = 4x100, 8h photoperiod, 20ºC/10ºC alternating temperature regime)

***Bellis azorica* Hochst. seeds**	**Mean ± SD**
Days to first radicle emergence	3.5 ± 3
Mean time to germination	17.2 ± 0.8
Percent of germination at 34th day	81 ± 1
Days to cotyledons emergence	5.7 ± 16.6
Days to first true leave	2.9 ±4.5

DISCUSSION

Maciel (2004) found that the germination ability of *Spergularia azorica* seeds does not significantly decrease after a period of nine years of storage. In our work, doubling the time of storage to 18 years allowed us to detect a significant decrease in the germination percentage and the speed of germination. In addition, while non-stored seeds do not present a clear preference for a specific light or temperature regime (Maciel 2004), seeds stored for longer periods start to differentiate in their reactions to different light and temperature regimes. Nevertheless, even after 18 years of storage, we could still obtain germination percentages of over 80% when selecting the optimum light and temperature regimes. We interpreted this as indication that the seeds of this species are suitable for long-term storage in a germplasm bank. Finally, our results also showed that the seed germination characteristics of the progeny of plants produced from 18 year old seeds were not different in quality from seeds directly harvested *in situ*. In the case of *Leontodon filii,* our data extended the known admissible storage period in a germplasm bank from 62 months (Maciel 2004) to 84 months (seven years). In spite of a slight but significant decrease in the germination percentages, the obtained mean value of above 90% showed that *Leontodon filii* seeds can be maintained in the germplasm bank at room temperature with silica gel for as long as seven years. The much shorter mean time for germination of stored seeds could be explained by the physiological maturation that seeds underwent during storage, allowing seed physiological synchronization. In fact, exogenous addition of gibberellic acid to non-stored seeds decreases the mean time to germination (Maciel 2004) resulting in a more synchronous response. For *Luzula purpureo-splendens*, no information was found on seed germination. In the genus *Luzula*, seed dormancy is discussed by Bell & Amen (1970) with respect to the alpine species *Luzula spicata* L. and the subalpine species *Luzula parviflora* (Ehrh.). In our experiments, we did not detect either seed dormancy of *Luzula purpureo-splendens* seeds, or a preference for any particular temperature regime. Further studies are needed to ascertain if the germination of this species is a light-dependent process and to improve understanding of its habitat adaptation strategy. Fresh seeds of *Bellis azorica* germinated quickly,

attaining a final germination percentage of over 80%. When comparing these results with those obtained by Maciel (1994) for seeds stored for five months, harvested from the same population and under the same temperature and light regime, we concluded that seed storage significantly increased both the number of days to first radicle emergence (from 3.5 days to 82 days) and the mean time to germination (from 17.2 days to 130 days). However the germination percentage decreased slowly with the seed age: 81% for fresh seeds and, as found by Maciel (1994), 73% and 67% for seeds with 5 and 17 months of storage, respectively. In spite of the lack of knowledge about the reproductive biology of this species, the minimum number of plants used, and being restricted to using those plants flowering at the time of the scientific expedition visit to the island, we obtained good quality seeds and plants in *ex situ* conditions and in a short period of time. The produced plants could quickly be returned and were able to integrate with the original population and therefore replace the used plants and reinforce the population. The first results of *in vitro* culture showed that it is possible to develop a micropropagation protocol for this species.

Our conclusions were that: a) *Spergularia azorica* seeds can be successfully maintained in a germplasm bank (at room temperature in silica gel) for at least 18 years, and it is possible to activate 18 year old seeds to produce new seeds *ex situ*; b) *Leontodon filii* seeds can successfully be maintained in a germplasm bank (at room temperature in silica gel) at least for seven years; c) for the tested conditions, *Luzula purpureosplendens* seeds presented a good germination percentage (73%) and a mean time to germination of less than a month; and d) for the very difficult to find *Bellis azorica* populations: if a new and very small population of *Bellis azorica* were discovered, even without seeds, we could easily increase the size of the population through micropropagation and establish new (but genetically identical) populations as an insurance measure against local destruction.

ACKNOWLEDGEMENTS

To the Azorean 'Regional Secretariat for the Environment and the Sea', for permits to collect seeds and plants from their natural habitats. The experimental work involving *Spergularia azorica* and *Bellis azorica* was undertaken in the classes of 'Plant Physiology' and financially supported by Azores University. The experimental work involving *Leontodon filii* and *Luzula purpureosplendens* was carried out under the Azorean programme, 'Estagiar-L', and financially supported by the Azores Regional Government and the University of the Azores.

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Received 25 Sep 2011. Accepted 09 Jan 2012, Published online 15 February 2012.