Article

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Effect of storage conditions on seed germination of eight Tyrrhenian endemic vascular plant species of conservation interest

Abstract

Porceddu, M., Cuena-Lombraña, A., Boi, M. E., Podda, L., Sarigu, M., Dessì, L., Meloni, F., Atzeri, P. & Bacchetta, G.: Effect of storage conditions on seed germination of eight Tyrrhenian endemic vascular plant species of conservation interest. — Fl. Medit. 32: 421-433. 2022. — ISSN: 1120-4052 printed, 2240-4538 online.

The conservation of endemic and endangered plant species is of great interest to the scientific and research community. In this frame, seed banks play a crucial role when biodiversity preservation and climate change are considered. The study of seed viability and germination during storage conditions provides basic and useful information to ensure successful *ex situ* conservation. The aim of this study was to evaluate whether storage time and conditions (i.e., base collection at -25°C and active collection at +5°C) affect seed germination in the long term. For these purposes, eight Tyrrhenian endemic vascular plant species (mostly endangered) with orthodox seeds were studied: *Brassica insularis*, *Centranthus amazonum*, *Dianthus morisianus*, *Digitalis purpurea* var. *gyspergerae*, *Ferula arrigonii*, *Helicodiceros muscivorus*, *Iberis integerrima* and *Verbascum plantagineum*. These species were stored in the Sardinian Germplasm Bank (BG-SAR) at -25°C and at +5°C for a time ranging from 2 to 12 years. Germination tests were carried out following the optimal conditions reported in the literature for each species. The results showed, in general terms, the high seed germination capacity of all species stored at both conditions; regarding the time of seed storage, germination in some tested species (such as *B. insularis* and *C. amazonum*) slightly decreased over time. We argued that seed dehydration, low seed moisture content during storage and the use of hermetic glass containers can be considered key factors for long-term conservation of these orthodox seeds. In conclusion, this study showed that the conservation of these endemic species is ensured by seed bank storage, according to the general assumption that seed longevity depends on seed lot quality, on well-sealed storage containers and conditions before and during storage.

Key words: *ex situ* conservation, endangered species, mediterranean flora, seed banking, seed longevity.

Introduction

Sardinia is considered a priority region for biodiversity conservation due to its high number of endemic species (Cañadas & al. 2014). According to Bartolucci & al. (2018)

and Galasso & al. (2018), the Sardinian flora consists of 2922 taxa, of which 2441 are autochthonous and 481 alien. Recently, Fois & al. (2022) reported that the Sardinian vascular native flora consists of ca. 2300 taxa, of which 341 are considered Sardinian endemics. Most of these species are facing several threats, e.g., land-use and land-cover change, habitat fragmentation, climate warming and impact of invasive plant species (Fenu & al. 2015). In this context, seed banks in general and, in particular, the Sardinian Germplasm Bank (BG-SAR) play a central role in the conservation of Sardinian plant diversity and protection of the most sensitive species of the island (Porceddu & al. 2017a). Accordingly, BG-SAR contributes to guaranteeing the long-term conservation of the genetic diversity of Sardinian threatened taxa on the basis of the regional responsibility criterion by also investigating and providing conservation strategies for these species at the local level (Bacchetta & al. 2012a; Mattana & al. 2012; Fenu & al. 2015).

It is an unquestionable fact that the study of seed germination of endemic and/or endangered taxa has a pivotal role in their preservation. Indeed, seed germination is a crucial stage in the life cycle of a plant wherein the metabolic mechanisms that lead to the growth and emergence of the radicle and plumule are reactivated (Baskin & Baskin 2014).

Seed harvest, seed dehydration, storage conditions and storage periods play a key role in seed viability, germination and growth (Hay & Probert 2013). Seed harvest is the first step in acquiring and ensuring a high-quality seed lot, and germplasm collection of threatened endemic plants is often difficult (Porceddu & al. 2017b). Another key step is the evaluation of the tolerance to drying and the low temperatures of a specific taxon. Indeed, seeds, depending on their tolerance to drying and low temperatures, are commonly classified as orthodox (i.e., dehydration tolerant; include seeds of most native Mediterranean vascular plant species), recalcitrant (i.e., dehydration sensitive) or intermediate between them (Bacchetta & al. 2006b, 2008a).

All seed samples should be dried to equilibrium in a controlled environment of 5–20°C and 10–25% relative humidity (RH), depending on the species (Bacchetta & al. 2006b, 2008a). After hermetic sealing, the seed batches must be stored in a range between -25 and -18°C (base collection) and/or at 5–10°C (active collection) to guarantee their conservation with an estimated viability of decades (Bacchetta & al. 2006b, 2008a). In general, the combination of 3–5% moisture content and storage temperature below 8°C permits long-term seed preservation; however, viability may decrease because of deterioration processes (FAO/IPGRI 1994). Consequently, studies about long-term viability are needed to determine the storability of seed materials in seed banks and to provide conditions that will maintain the viability of each accession above a minimum value (FAO/IPGRI 1994).

Several studies have shown how the storage times, seed traits, environmental conditions at the site of collection and methods of preservation can affect the viability and longevity of accessions stored in seed banks (Probert & al. 2009; Godefroid & al. 2010; Mondoni & al. 2011; Royal Botanic Gardens Kew 2022). For instance, Pérez-García & al. (2007, 2009) showed that seeds of Brassicaceae stored for 40 years at sub-zero temperatures (-5 to -10°C) and low moisture content (ca. 3%) can provide a successful technology for *ex situ* plant conservation. Furthermore, these authors highlighted that the seeds stored at room temperature for 34–39 years showed germination percentages comparable to those of a cold room. These results underlined that a temperature under zero might not be as important a factor in seed conservation, as expected, at least for medium-term preservation, and

suggested the possibility of using ultra-dry methods as valid tools to effectively preserve germplasm in seed banks (Singh & al. 2003; Pérez-García & al. 2007). Moreover, currently, very few studies concerning the times and methods for seed preservation on Mediterranean endangered vascular plant species are available. *Hypericum scruglii* Bacch., Brullo & Salmeri and *Senecio morisii* J.Calvo & Bacch., two exclusive and endangered plant species from Sardinia, had been recently evaluated after long-term conservation in the seed bank (Cuena-Lombraña & al. 2020; Porceddu & al. 2020). These studies showed the high viability of long-term stored seeds and the possible use of germplasm collections when fresh seeds are not available or limited.

The main aims of this research were to determine how storage times and conditions (i.e., base collection at -25°C and active collection at +5°C) can affect seed germination of eight endemic and endangered vascular plant species of Tyrrhenian islands: *Brassica insularis* Moris, *Centranthus amazonum* Fridl. & A.Raynal, *Dianthus morisianus* Vals., *Digitalis purpurea* var. *gyspergerae* (Rouy) Rouy, *Ferula arrigonii* Bocchieri, *Helicodiceros muscivorus* (L.f.) Engl., *Iberis integerrima* Moris and *Verbascum plantagineum* Moris. Seed collections of these taxa were stored at BG-SAR for a time ranging from a minimum of two to a maximum of 12 years, and seed germination requirements for each taxon were already known.

Materials and Methods

Study species

Among the eight selected Tyrrhenian endemic plant species under study (Fig. 1), four are exclusive to Sardinia, two to Sardo-Corsican, one to Sardo-Corsican-Balearic and one to Sardo-Corsican-Sicily-Tunisian (Table 1). Information on the taxa selected for this study is shown in Table 1. Each accession for each taxon tested for seed germination came from the same collection site, as reported in Table 1. To evaluate the differences in germination percentages of the different accessions for each taxon tested in this study, we used the data reported in Table 1 (see the "Germination percentage; optimal temperature and photoperiod in hours" column) based on the assumption that the initial germination percentages (i.e., before storage) for each species collected in the same locality remained constant or very similar.

Sardinian Germplasm Bank (BG-SAR)

BG-SAR, active since 1997, preserves, studies and manages the germplasm of Mediterranean plant species with particular focus on Sardinian endemic, threatened and policy species, as well as Crop Wild Relatives (CWR), landraces, useful plants and archaeological plant remains. In detail, BG-SAR preserves approximately 3500 seed lots from different Mediterranean areas, many of which are endemic to the Tyrrhenian islands, and ensures the preservation of several Sardinian taxa listed in the Italian national Red Lists (Porceddu & al. 2017a), especially those consider threatened according to the criteria of the IUCN (Orsenigo & al. 2018, 2021) or inserted in the attention list as the Top 50 species of the Mediterranean islands (Pasta & al. 2017). As of 2015, BG-SAR stored the germplasm of ca. 47% of policy species and ca. 42% of the strict Sardinian endemics (Fenu

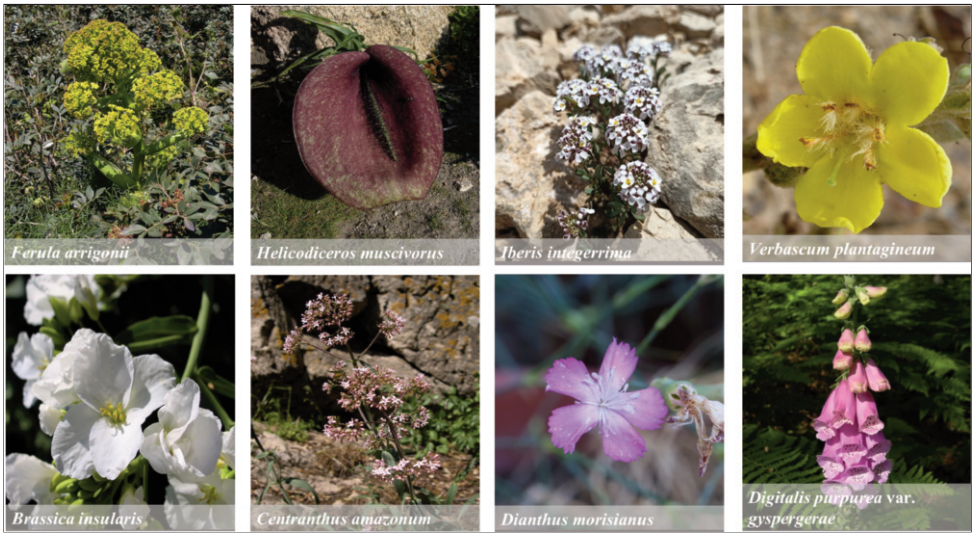

Fig. 1. Taxa preserved at BG-SAR and selected for this study.

Table 1. Study species details and information about the best seed germination protocols reported in the literature. References for IUCN category: [1]Dulloo & al. (2011); [2]Orsenigo & al. (2018); [3]Orsenigo & al. (2016); [4]García Murillo (2018). References for germination protocols: [1]Bacchetta & al. (2007);[2]Mattana & al. (2010); [3]Cogoni & al. (2012); [4]Bacchetta & al. (2008b); [5]Bacchetta & al. (2006a); [6]BG-SAR unpublished data.

Taxon	Family	Collection site	N° of years of storage	Distribution	IUCN category	Germination percentage; optimal temperature and photoperiod in hours
Brassica insularis	Brassicaceae	Isola dei Cavoli-Serpentara (Villasimius)	11, 10, 8, 5	SA-CO-SI-TN	NT[1]	97 %; 20°C, 12/12.[1]
Centranthus amazonum	Caprifoliaceae	Monte Corrasi (Oliena)	9, 8, 6, 5	SA	CR[2]	85 %; 10°C, 12/12.[2]
Dianthus morisianus	Caryophyllaceae	Portixeddu (Buggerru)	10, 8, 7, 6, 2	SA	CR[2]	97 %; 15°C, 12/12.[3]
Digitalis purpurea* var. *gyspergerae	Plantaginaceae	Monte Lattias (Uta)	12, 11, 4	SA-CO	NE	89 %; 20°C, 12/12. [4]
Ferula arrigonii	Apiaceae	Isola dei Cavoli-Serpentara (Villasimius)	11, 10, 8	SA-CO	LC[3]	57 %; 10°C, 12/12.[1]
Helicodiceros muscivorus	Araceae	Isola dei Cavoli-Serpentara (Villasimius)	11, 10, 8, 6, 3	SA-CO-BL	NT[4]	84 %; 15°C, 0/24.[5]
Iberis integerrima	Brassicaceae	Valle Rio San Giorgio (Iglesias)	12, 11, 9, 8, 3	SA	NT[2]	99 %; 20°C, 12/12.[6]
Verbascum plantagineum	Scrophulariaceae	Zona diga Riu Monte Nieddu (Pula)	11, 4	SA	VU[2]	89 %; 15°C, 12/12.[4]

& al. 2015). Currently, the seed bank preserves more than 50% of policy species and Tyrrhenian Island endemics (unpublished data; work in progress). Among them, most are taxa listed in the Habitats Directive (such as *Astragalus maritimus* Moris, *A. verrucosus* Moris, *B. insularis*, *Gentiana lutea* L. subsp. *lutea*, *Helianthemum caput-felis* Boiss., *Lamyropsis microcephala* (Moris) Dittrich & Greuter, *Linum muelleri* Moris, *Ribes sardoum* Martelli, *Daucus rouyi* Spalik & Reduron and *Silene velutina* Pourr. ex Loisel.), and several accessions of the 10 most threatened exclusive endemic vascular plant species (*sensu* Bacchetta & al. 2012b) of Sardinia, such as *R. sardoum*, *D. morisianus*, *A. maritimus* and *A. verrucosus* (Porceddu & al. 2017a).

Seed processing carried out at BG-SAR

The seed conservation processes carried out at BG-SAR follow internationally recognised protocols and guidelines for seed bank standards (Bacchetta & al. 2006b, 2008a). The collected germplasm was added to the seed bank after a quarantine period in an isolated room set at controlled environmental parameters (20°C, 40% of RH). This procedure allows slow and gradual post-ripening and permits the evaluation of the phytosanitary state (identification of fungal infections, phytophagous insects, or other harmful parasites). After this period, the germplasm is cleaned, quantified, selected, weighed, prepared for morpho-colorimetric characterisation by image analysis and processed for preservation.

Concerning long-term conservation, all seed lots are dried at 15°C and 15% RH to reduce the internal seed moisture content to ca. 3–5% (according to the oil content) and stored at -25°C (as base collections under long-term conservation) and/or at +5°C (as active collections under medium-term conservation) in hermetic glass vials and Bormioli glass containers.

Germination test

Prior to germination tests, the hermetic Bormioli glass containers were stabilised within the dehydration room for six hours, and then the glass vials were opened, while the right seed amounts for testing were collected and stored for 24 hours at 15°C and 15% RH. For each species, four replicates of 25 seeds were sown on the surface of 1% water agar in 90 mm diameter plastic Petri dishes and incubated in a 12 h light/12 h dark photoperiod (except *Helicodiceros muscivorus* in full darkness) for a maximum of three months at constant temperatures, following the optimal conditions for each species from the literature (Table 1).

The experiments were carried out using growth chambers (SANYO MLR-351, SANYO Electric Co., Ltd.), equipped with white fluorescent lamps (FL40SS.W/37 70–10 μmol m^{-2} s^{-1}). At the end of the germination tests, when no additional germination occurred for two consecutive weeks, non-germinated seeds were cut with a scalpel to determine the number of filled, viable and empty seeds (Bacchetta & al. 2008a).

Data analysis

For each germination trial, the final germination percentage (FGP) and the germination rate (T_{50}) were calculated. The FGP was calculated as the mean of four replicates (± SD) based on the total number of filled seeds (empty seeds were excluded). The T_{50} was determined as the time (expressed in days) required to reach 50% of the germination percentage; this value was only calculated when 50% germination was reached (Cuena-Lombraña & al. 2016).

Statistical analysis

Generalised linear models (GLMs) were used to evaluate the effect of the storage conditions (i.e., stored at -25° and +5°C) and the years of storage (see Table 1) on FGP. A quasibinomial error structure with logit link function and F-tests with an empirical scale parameter instead of chi-squared on the subsequent ANOVA were used to overcome residual overdispersion. To test the correlation between FGPs and years of storage, a linear regression analysis was performed. All statistical analyses were carried out using R v. 3.0.3 (R Development Core Team 2014).

Results

Seed germination

The GLM analysis highlighted, for the "Storage condition" (SC) factor, no statistically significant differences ($P > 0.05$) on seed germination, whereas statistically significant differences ($P < 0.05$) were found for the "Years of storage" (YS) factor, as well for the two-way interaction SC × YS (Table 2).

In general, all seeds of the tested species stored from 2 to 12 years, both belonging to +5°C and -25°C, germinated at percentages > 50% (Fig. 2; Electronic Supplementary File 1: Table S1).

In detail, *Brassica insularis* showed different germination responses, in particular when seed storage time was considered; in fact, seeds stored for 10 years highlighted germination percentages of ~2% and ~29% at +5°C and -25°C, respectively; among the ungerminated seeds, ~88% and ~68% were viable/imbibed, and ~ 10% and ~ 3% were dead at +5°C and -25°C, respectively (Fig. 2). *C. amazonum* showed an FGP > 80% with a percentage of dead seeds < 14% and a percentage of viable/imbibed seeds of ~4%. *D. morisianus* had an FGP > 90% with a percentage of dead seeds < 4%, and no viable/imbibed seeds (Fig. 2). *D. purpurea* var. *gyspergerae* had an FGP > 90% with a percentage of dead seeds < 13% (Fig. 2). *F. arrigonii* showed an FGP > 60% with a percentage of dead seeds < 30% and a percentage of viable/imbibed seeds < 6% (Fig. 2). *H. muscivorus* had an FGP > 70% with a percentage of dead seeds of ~0% and a percentage of

Table 2. GLM results for the effect on seed germination of the following factors: "Storage condition" (SC, +5°C and −25°C) and "Years of storage" (YS, from 2 to 12) and their interaction.

	d.f.	Deviance	Residual d.f.	Residual deviance	*F*	*P* (> F)
Null			359	9822.8		
Storage condition (SC)	1	23.2	358	9799.6	2.2001	0.3189
Years of storage (YS)	11	1655.9	347	8143.6	14.2501	$< 2.2^{e-16}$ ***
SC × YS	9	290.6	329	324.3	3.0569	0.0015 **

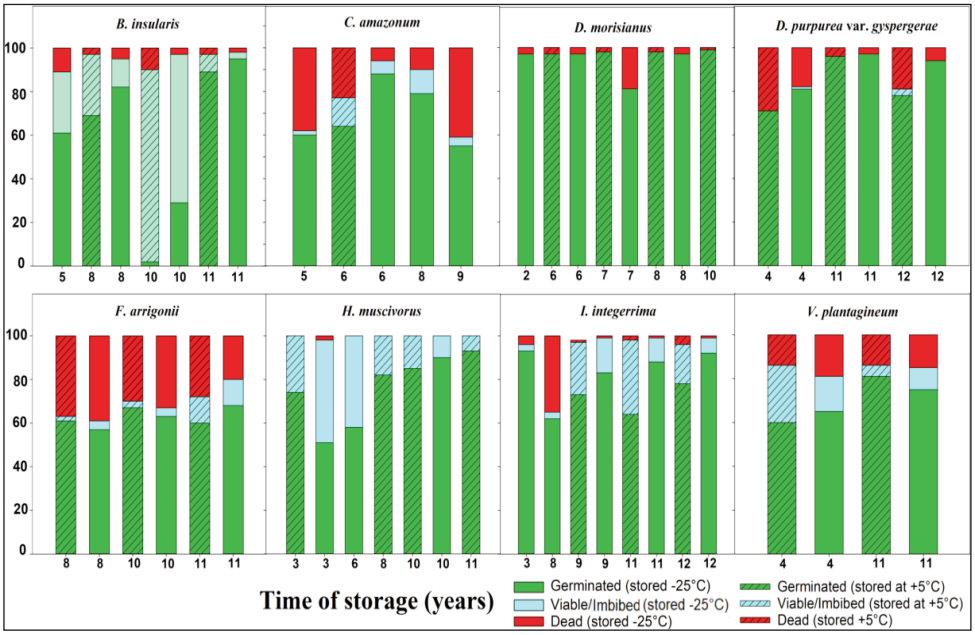

Fig. 2. Effects of time of storage (in years) and storage conditions (+5°C and −25°C) on final germination percentage for the selected Tyrrhenian plant species seeds stored at Sardinian Germplasm Bank (BG-SAR) and their percentage of viable/imbibed and dead seeds.

viable/imbibed seeds < 16% (Fig. 2). *I. integerrima* showed an FGP > 90% with a percentage of dead seeds < 4%. A seed lot storage time for 9, 11 and 12 years highlighted FGPs of 83%, 88% and 92%, respectively, at -25°C, showing higher percentages of germination than seeds stored at +5°C for 9, 11 and 12 years (73%, 64% and 88%, respectively) (Fig. 2). *V. plantagineum* had an FGP > 80% with a percentage of dead seeds < 15% and a percentage of viable/imbibed of ~14% (Fig. 2).

Effects of storage time

Linear regression analysis was carried out to verify if the years of conservation were correlated with the FGP. Figure 3 highlights a general positive linear trend over the years for most of the species tested. *B. insularis* showed a decreasing trend in both storage conditions, probably due to the low germination percentage recorded in seeds stored for 10 years (Fig. 3). *C. amazonum* showed a decreasing trend with years of storage at -25°C. In contrast, *D. morisianus* showed a positive linear trend over the years of storage at +5°C, while a decreasing trend was observed for the storage conditions at -25°C. In the case of *D. purpurea* var. *gyspergerae, F. arrigonii* and *H. muscivorus,* the FGPs increased with the years of storage in both conditions (+5°C and -25°C). *V. plantagineum* showed a growing trend for the storage conditions at +5°C (Fig. 3).

In general, as indicated by the R^2 and *P* values, there were no linear correlations between FPG and years of storage (Fig. 3) for any tested species. In addition, given the

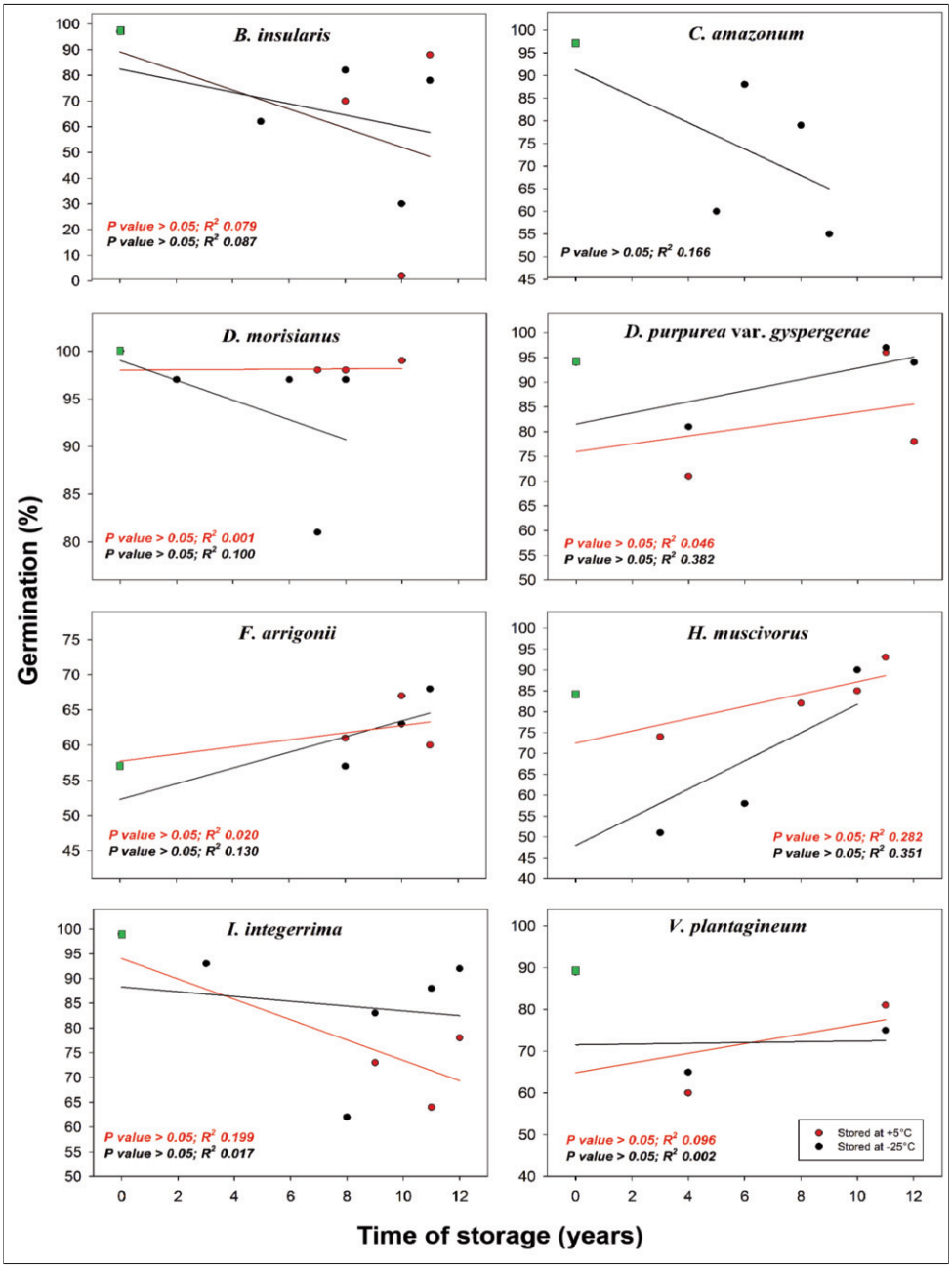

Fig. 3. Correlation between final germination percentages and years of storage represented by linear regression for all selected Tyrrhenian plant species stored at +5°C and at -25°C. Green squares correspond to the optimal germination percentage reported in the literature (see Table 1). Red dots and straight lines represent the storage conditions at +5°C and black dots and straight lines represent storage conditions at -25°C.

lack of statistical significance obtained for storage conditions highlighted by GLM (Table 2), the analyses were repeated by joining the dataset of final germination percentages obtained at +5°C and at -25°C. The R^2 and *P* values obtained for the unified/combined dataset showed that no linear correlations between FPG and years of storage were present.

Discussion

The Tyrrhenian endemic vascular plant species analysed in this study, in accordance with the optimal germination protocols from the literature, showed high percentages of germination, both for accessions stored at +5°C and for those at -25°C. *Brassica insularis* overall showed a germination capacity slightly lower than the attested one from the literature equal to 97% (Bacchetta & al. 2006a); in particular, low percentages of germination were obtained only at 10 years of storage, for causes that were not determined in this study. Godefroid & al. (2010), working on the species stored in the seed bank of the National Botanic Garden of Belgium, indicated that the loss of viability during storage and low germination percentage could be seed lot-specific.

Centranthus amazonum showed a decline in germination percentage compared with the 97% reported in the literature (Mattana & al. 2010). *Digitalis purpurea* var. *gyspergerae, Ferula arrigonii* and *Helicodiceros muscivorus* showed an increment in the germination response compared with their values reported in the literature at 89% (Bacchetta & al. 2008b), 57% and 84% (Bacchetta & al. 2008b), respectively, whereas *Iberis integerrima* and *Verbascum plantagineum* had a slight drop in the germination percentage compared to 99% and 89%, respectively (Table 1), known from the literature (BG-SAR unpublished data; Bacchetta & al. 2008b).

As shown in Fig. 2, the analyses carried out on each species confirm, in general terms, the high germination capacity of seeds stored at both temperatures (+5°C and -25°C). The high germination percentages recorded after several years of storage demonstrated how the preservation methods implemented in BG-SAR are effective for long-term storage. However, the statistical analysis (see Table 2) showed that both storage temperatures (+5°C and -25°C) seemed to be effective since there were no statistically significant differences. Based on these results, it was not possible to determine which storage temperature between +5°C and -25°C was the most advantageous for preserving the eight analysed species, at least after 12 years of conservation.

BG-SAR stores the dried seeds in transparent and hermetic glass containers, which fully prevent water uptake and allow the use of silica gel with moisture indicators as an excellent monitoring system of internal moisture conditions. In accordance with Gómez-Campo (2007), when containers are not perfectly tight, seeds tend to become balanced with the environmental air humidity and any potential benefit of low temperatures will be offset by the increased moisture content, which adversely affects seed longevity. As far as the time of storage is concerned, the germination percentage seems to decrease with the years of conservation in some tested species (e.g., *B. insularis* and *C. amazonum*). Such behaviour has also been detected in other species; for example, out of 124 accessions of 72 native Australian species, only 12 accessions of 10 species showed a significant decline after 5–12 years of seed bank storage at -20°C (Crawford & al. 2007). However, based on our

analyses (see Fig. 3), no correlation between the final germination percentage and the time of storage was proven. Hence, the percentage decrease in seed germination may depend on other factors, such as harvest time and/or climatic conditions during seed ripening and may differ among families (Godefroid & al. 2010).

The seed quality also plays an important role in conservation, and the Food and Agriculture Organization of the United Nations (FAO/IPGRI 1994) has set several standards that aim at ensuring maximum seed quality; focal features are, for instance, the harvest time (as close as possible to fruit/seed ripening and prior to dispersal), the time between harvest and storage under controlled dry conditions (3–5 days or as short as possible), the prompt cleaning and pest check (FAO/IPGRI 1994).

Particularly important to ensure long-term storage is the state of dehydration, and specifically the ultra-drying condition. In fact, low humidity is certainly the key factor for the long-term storage of orthodox seeds (Singh & al. 2003). This was remarked by Pérez-García & al. (2007), who proved high seed viability, with germination percentages close to 100%, in 37 species of the Brassicaceae family after 40 years of ultra-dry storage (moisture content ranged from 0.3 to 3% of fresh weight basis after storage). Notwithstanding, seed desiccation to less than 3% of moisture content (ultra-drying of orthodox seeds) has rarely been used in seed banking. Furthermore, according to Pérez-García & al. (2007), the results of our study suggest that temperature might not be a unique factor for seed preservation, at least within 12 years of storage.

Conclusions

Our study revealed that seed bank storage ensures the conservation of these Tyrrhenian endemic vascular plant species. Stored seeds at germplasm banks can be useful for translocation actions when there is little or no fresh material available. To ensure the *ex situ* conservation of these species, it is important to test seed viability every 5–10 years (FAO/IPGRI 1994) for monitoring seed longevity over time and to continue with the collection and preservation of the species of high conservation value. Similar efforts should be addressed in other Mediterranean endemic/endangered vascular plant species from the perspective of climate change and related issues on biodiversity conservation.

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