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High seed viability recorded in an endangered endemic species, *Isoplexis isabelliana* (Scrophulariaceae), after more than 30 years of storage in a conservation seed bank

Miguel Ángel González Pérez[1], Nereida Cabrera-García[1] & Isabel Cayon-Fernández[1,2]

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Abstract. Conservation seed banks are essential for *ex-situ* conservation of genetic biodiversity. These institutions are especially relevant for threatened species and play a vital role in their conservation by preserving genetic material. However, samples deposited in the seed banks must germinate when necessary to use them (i.e., recovery plans, etc.). This study uses four accessions of the endemic endangered species from Gran Canaria Island (Canary Islands), *Isoplexis isabelliana* (Webb & Berthel.) Masf. (Scrophulariaceae). Germination tests were carried out to measure seed viability through time and the possible impact of seed storage on their viability. These accessions have been kept in the seed bank for four months to thirty years under different storage conditions. Germination results differed for seeds after 45 days of exposition using 16 hours light and 8 hours darkness at 17 °C. Accessions kept in the seed bank, independently of storage, showed a high germination percentage (89%). Whereas the accessions with rough storage conditions showed a 0% germination rate. The results highlighted the good state of conservation of the material deposited in the Seed Bank of the Botanical Garden "Viera y Clavijo" and the reliability of the temperature and humidity conditions in which the seeds of *I. isabelliana* have been stored. We consider these results as momentous since several natural populations of *I. isabelliana* has been affected by the last forest fire on the island.
Keywords. Canary Islands; endangered species; germination; *Isoplexis isabelliana*; long-term storage.

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Introduction

Recent studies show that current global plant diversity extinction is 100 to 1000 times higher than during the recent geological past (Pimm *et al*., 1995; Humphreys *et al*., 2019). An average of 2.3 species has become extinct each year for the past 2.5 centuries. The ongoing plant extinction is of particular concern to isolated oceanic islands due to the island biota's vulnerability (Humphreys *et al*., 2019).

Conservation seed banks (genebanks) have become essential for the genetic biodiversity conservation of natural ecosystems. Seed preservation is especially important for threatened species since seeds are the primary genetic material used for reintroducing plant species (Broadhurst *et al*., 2008). The maintenance of *ex situ* seed viability over long periods in gene banks is a key element for conserving plant genetic resources (Fu *et al*., 2015). In this sense, it is essential to establish germination protocols for each of the species stored in the bank (pretreatment, seed dormancy). Besides, storage conditions (humidity and temperature) must be optimized to guarantee the seeds' viability (Harrington, 1972; Bacchetta *et al*., 2008). Germination is a highly decisive stage in the plant life cycle, and its study is fundamental for the conservation of species (Melo *et al*., 2004). Germination studies are essential for the re-introduction of plant populations of threatened species. Over recent years, an increasing number of germplasm banks have been established to support the future recovery of species that might become extinct in the near future (Myers *et al*., 2000).

Isoplexis isabelliana (Webb & Berthel.) Masf. is an endemic species from Gran Canaria Island (Canary Islands, Spain). It is cataloged as 'endangered' by the International Union for Conservation of Nature and Natural Resources (Salas-Pascual *et al*., 2011). Its distribution is limited to the western, northern and northeastern part of the island, between 790 and 1600 m of altitude. It grows in the Laurel forest's sunniest and rocky areas and the humid native pine forest (Salas-Pascual *et al*., 2004). There are three natural locations that present isolated and fragmented populations with very few individuals: Tamadaba (75 individuals), San Mateo (232 individuals) and Valleseco (156 individuals). Moreover, there is one reintroduced population in Llanos de la Pez with 662 individuals, after having disappeared from the east of the island (Bañares *et al*., 2004; Salas-

[1] Jardín Botánico Canario "Viera y Clavijo"-Unidad Asociada CSIC (Cabildo de Gran Canaria), Ap. de correos 14 de Tafira Alta, 35017, Las Palmas de Gran Canaria, Spain. Email: miguelglezprez@gmail.com
[2] Gibraltar Botanic Gardens, "The Alameda", Red Sands Road PO Box843. Gibraltar GX11 1AA

Pascual *et al*., 2011). There are 1125 individuals, with an area of occupancy of 19 km² (Santana López & Naranjo Suárez, 2007). Negative trends have been reported for its distribution and some subpopulations have disappeared (Bañares *et al*., 2004). Threats to this species are the presence of introduced herbivores (e.g., goat, rabbits), as well as the anthropogenic pressure and forest fire. Notably, the localities of Tamadaba and Valleseco were affected by the last forest fire in 2019 (J. Naranjo, pers. comm).

The Seed Bank of the Botanical Garden "Viera y Clavijo" (JBCVC) was created in 1983 and currently hosts more than 5000 accessions, including more than 30 accessions of this endangered endemic species, which have been sampled in different years in all its localities. These samples have been cleaned, dried in a drying room at 15°C and 15% relative humidity. When seed moisture content dropped to 5–7%, they were stored in a glass tube with silica gel at -10 ºC. (González Pérez & Cabrera García, 2019).

In this context, this study aims to evaluate the effect of time and storage conditions on the germination of *Isoplexis. isabelliana* seeds by addressing the following questions: (i) do the storage time limit the germination percentage of *I. isabelliana*?, (ii) what factor has an influence on seed viability of this endangered endemic species?, (iii) can the resazurin reagent determine the seed viability in Scrophulariaceae?, (iv) are the current seed storage conditions in the Seed Bank of JBCVC favorable for the long time storage (up to 30 years) of *I. isabelliana* seeds?

Material and Methods

Plant material

In order to test the effect of storage time and conditions, four accessions with different storage history were selected from the Seed Bank JBCVC (Table 1); (1) an accession from the natural population of Tenteniguada, which has been stored for 31.5 years following the protocol of the Seed Bank JBCVC (529/B); (2) an accession belonging to the reintroduced population of Llano de la Pez and stored for 22.3 years at -10 °C in the Seed Bank (1236/B); (3) an accession sampled in the Natural Park of Tamadaba in September 2002 (2204/B) and stored at room temperature (15.8 – 22.1ºC) and ambient humidity (80.5%); (4) a recent accession, from the natural population of Cazadores (5183/B), which has been stored at room temperature and humidity for four months.

Table 1. Collection data of seeds from four populations of *Isoplexis isabelliana* used in the present study.

Accession number	Collection date	Collection site	Altitude (m asl)	Time of storage (years)	Anual precipitations (mm)	Storage conditions
529/B	19/08/1987	Tenteniguada	1.000	31.5	329	-10ºC
1236/B	26/10/1996	Llanos de la Pez	1.600	22.3	792	-10ºC
2204/B	12/09/2002	Tamadaba	1.200	16.4	541	Room temperature (16–22ºC)
5183/B	01/10/2018	Cazadores	1.400	0.4	856	Room temperature (16–22ºC)

Complementarily, annual precipitations in each sampling locality and year for each accession were obtained from the State Meteorological Agency (AEMET, 2019) (Figure 2).

Morphological study

Polar and equatorial axes of the seeds were measured using a stereoscopic microscope (Olympus SZX12). Pictures were taken with a digital camera (Nikon DS-Fi2) and resulting images were analyzed using NIS Elements v 4.0 software to calculate the seeds' area. Seed weight was determined during this study by weighing four replicates of 100 seeds on an analytical balance with 0.001 g-precision (Sartorius Quintix 65-1S).

Viability test

Germination and viability trials consisted of four batches of seeds or four treatments, each belonging to a different storage period. A germination test was performed to measure seed viability for each of the treatments. Four replicates of 25 seeds per accession were pretreated with hydrogen peroxide for three minutes, rinsed with distilled water for three minutes and sown on 1% water agar in Petri dishes. Seeds were incubated at 17 °C under a photoperiod of 16 hours of light and 8 hours of darkness. Seeds with an emerged radicle were counted every day, removed from the Petri dishes and later cultivated in a greenhouse. Seeds were considered to have germinated when the radicle was 1–2 mm long. The final germination percentage was recorded after 30 days of incubation, as well as the number of seedlings that gave viable plants in the greenhouse after ten months. The germination percentage (%G), mean germination time (MGT), time to 50% of final germination (T_{50}), germination speed (GS) and germination speed coefficient (CV), was calculated according to Coolbear *et al*., 1984 and Gavassi *et al*., 2014. Percentage of seedlings viability after ten months was also estimated.

Additionally, a resazurin reagent test (Min & Kang, 2011; Mohammed *et al*., 2019) was carried out in seeds of the four accessions analyzed. Single seeds were placed into a 96-well PCR plate contained 150 µL of resazurin reagent in each well. The plate was incubated at 35 °C for 4 hours. Visual color and absorbance were evaluated at 570 nm with a microvolume spectrophotometer (Thermo Scientific™ NanoDrop 2000).

Data analyses

Seed biometric data and germination parameters were subjected to ANOVA and multiple range test analysis using Excel software (Microsfot Office Standard 2016) in order to test for significant differences between different accessions.

Pearson's correlation between annual precipitation and altitude, annual precipitation and germination percentage, and between storage time and germination percentage were calculated using XLSTAT ver. 3.02, 2008.

Results

Isoplexis isabelliana flowers from June to September and fruits in September and October. The fruits of this species are brown capsules, harboring a multitude of tiny seeds. Seeds are dispersed by ballistic dispersal. The shape of seeds varies, ranging from D-shaped to ovate and with a visible furrow (hilum) on one side. Seeds present a reticulated primary and irregular ornamentation on its surface with open fields and a single thin wall. The colour of the seeds is egg-yellow (Figure 1).

Figure 1. *Isoplexis isabelliana* seeds (x10).

Meteorological data showed a rainfall period from September to March with rainfall peaks in different months for each of the different localities. The annual precipitations in each locality ranged from 329 (529/B) to 826 mm (5183/B) (Table 1, Figure 2), and this was correlated with altitude (r = 0.909).

The polar axes length of the seeds ranged from 0.722 (5183/B) to 0.859 mm (529/B), while the equatorial axes ranged from 0.521 (5183/B) to 0.580 mm (529/B). Besides, the seed area of *Isoplexis isabelliana* ranged from 0.333 (5183/B) to 0.413 mm^2 (529/B). In addition, the weight of 100 seeds ranged from 0.008 g (2204/B) to 0.013 g (529/B) (Table 2). ANOVA analysis detected significant differences among accessions with respect to polar and equatorial axes, as well as the area (Supplementary Material, Table S1). Significant differences in seeds morphological parameters (polar axes, equatorial axes, or area) were detected between the two oldest accessions (529/B and 1236/B) and the other two analyzed accessions (5183/B and 2204/B) (Supplementary Material, Table S2). However, no significant differences were found between the two oldest accessions (529/B and 1236/B). Overall, the oldest accessions showed a larger seed size considering polar and equatorial axes size, as well as area. On the other hand, the most recent accession (5183/B) showed a smaller seed size (Table 2).

Table 2. Seed morphological parameters (mean ± standard deviation) of *Isoplexis isabelliana* used in the present study.

Accession number	Weight of 100 seeds (g)	Polar axes (mm)	Equatorial axes (mm)	Area (mm²)
529/B	0.013	0.859 ± 0.097	0.580 ± 0.080	0.413 ± 0.059
1236/B	0.011	0.855 ± 0.108	0.569 ± 0.070	0.406 ± 0.069
2204/B	0.008	0.773 ± 0.097	0.544 ± 0.084	0.337 ± 0.069
5183/B	0.009	0.722 ± 0.101	0.521 ± 0.073	0.333 ± 0.080

Germination started in all accessions, except the 2204/B, on the eighth day of imbibition through rupture of the seed coat and protrusion of the primary root. Normal seedlings have a fully developed hypocotyl and a foliaceous cotyledonary sheath, from which the first leaves emerge 10 days after sowing (Figure 3). The mean germination time (MGT) ranged from 10.82 (529/B) to 12.29 days (1236/B), while the germination speed (GS) ranged from 7.03 (5183/B) to 8.70 seeds/day (529/B). On the other hand, time to 50% of final germination (T_{50}) was approximately ten days in all tested accessions, and the germination speed coefficient had a maximum value of 8.14%/day (1236/B) and a minimum of 9.24%/day (529/B) (Table 3).

Germination ranged from 0% (2204/B) to 89% (529/B) of germination after 30 days (Figure 3). In addition, accession 529/B (the oldest accession analyzed) showed the lowest mean germination time (10.82 days) and the lowest T_{50} value (10.35 days), as well as the highest germination speed (GS) of the three accessions of *I. isabelliana* germinated. However, the ANOVA analysis and the multiple range test did not show significant differences between these germinated accessions. Five days after seed germination, when the cotyledons, hypocotyl and radicle were visible, the seedlings were cultivated in a substrate with peat moss, vermiculite and coconut coir (5:1:1). After ten months in the greenhouse, the percentage of viable seedlings ranged from 37.97% (1236/B) to 59.21% (5183/B) (Table 3).

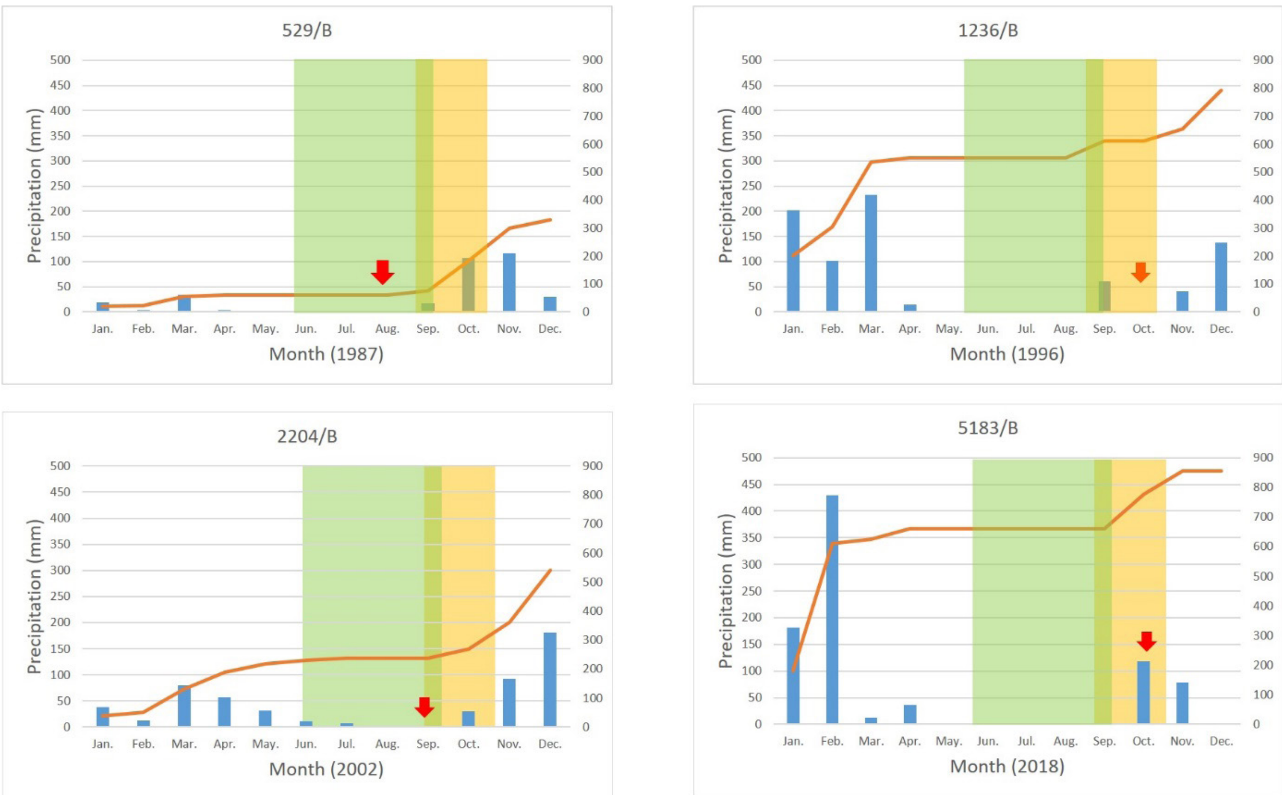

Figure 2. Monthly rainfall corresponding to the localities and year of sampling of the four accessions analyzed. Orange lines characterize accumulated rainfall over a year. The green and orange squares represent the flowering and fruiting periods of the *Isoplexis isabelliana*, respectively. The red arrow indicates the sampling date.

Resazurin reagent test did not show color or absorbance (570 nm) differences among the accessions analyzed. Average absorbance recorded after 4 hours at 35 °C were 0.640 (529/B), 0.557 (1236/B), 0.547 (2204/B) and 0.609 (5183/B) (Table 3).

Table 3. Germination parameters and viability test of *Isoplexis isabelliana* seeds. Percentage of germination (%G), mean germination time (MGT), germination speed (GS), germination speed coefficient (CV), time to 50% of final germination (T_{50}), percentage of viable seedlings after ten months (%VS) and absorbance (570 nm) after the resazurin reagent test.

Accession number	%G	MGT (days)	GS (seeds/day)	CV (%/day)	T_{50} (days)	% VS	Absorbance (570 nm)
529/B	89.00	10.82	8.70	9.24	10.35	48.31%	0.640
1236/B	79.00	12.29	7.08	8.14	10.67	37.97%	0.557
2204/B	0.0	-	-	-	-	-	0.547
5183/B	76.00	11.89	7.03	8.41	10.54	59.21%	0.609

Discussion

Seed moisture, storage temperature, environmental factors during seed maturation and harvest, as well as genetic characterization of the material, determine the seed longevity. Interactions among these factors contribute to the wide variation in quality or viability over time observed within and among seed accessions (Walters *et al*., 2010). Viability equations of longevity of the seeds in storage are focused on crops (Royal Botanic Gardens Kew, 2008). The longevity of the wild species seed storage is likely to be unknown (Hay & Probert, 2013). Therefore, knowledge about accessions of wild species in long-term storage is relevant, especially in threatened species. Our results showed that storage time, at least in the first thirty years, is not a factor that limits germination in *Isoplexis isabelliana*. In this sense, the two oldest accessions exhibited the highest germination percentage and germination speed (Figure 3, Table 3). The same accession (529/B) was assessed by Maya *et al*. (1988). It showed only a slightly higher germination percentage (100%) and speed germination coefficient (CV = 9.82 %/day) than the current study (89% and CV = 9.24%/day), suggesting that the viability of these seeds has only decreased by 10% since they were stored thirty-one years ago.

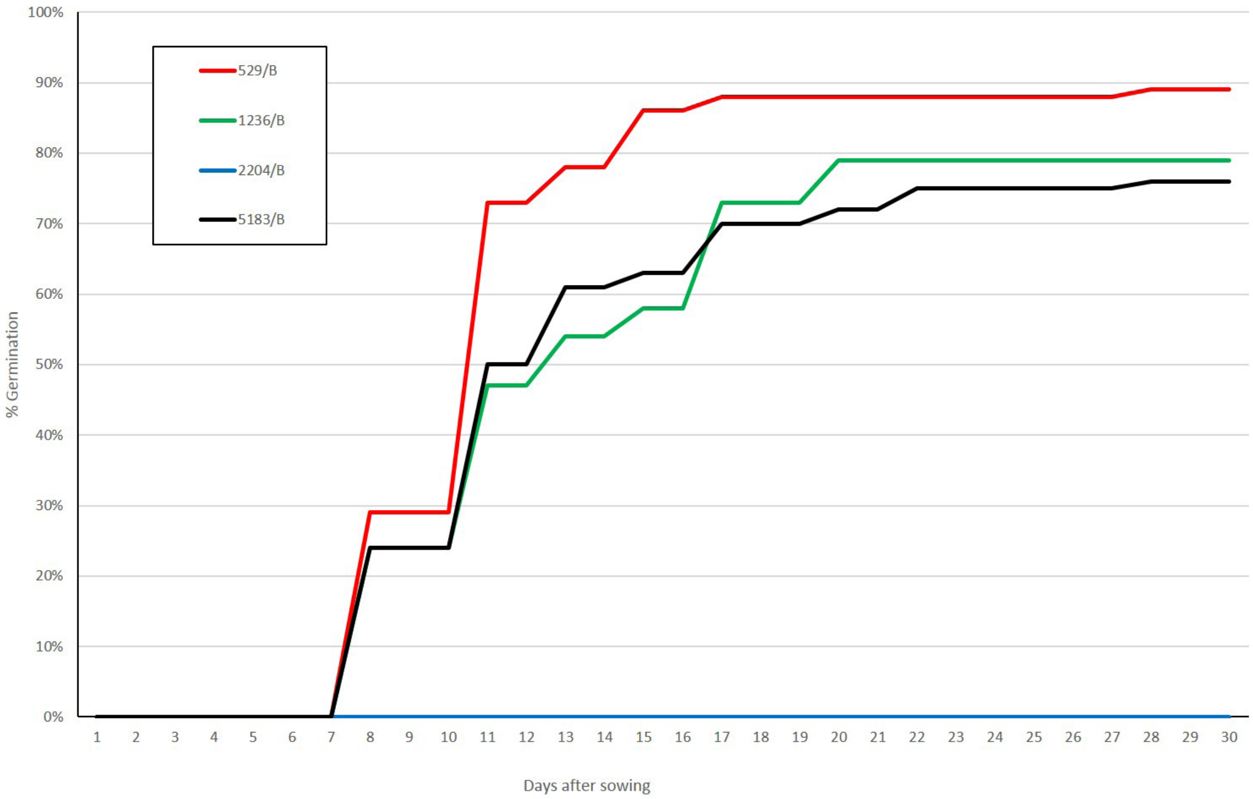

Figure 3. Germination time course of the four *Isoplexis isabelliana* accessions analyzed.

The only data about germination speed of the endemic flora of Canary Islands date back to the 1980s (Ayerbe & Ceresuela, 1982; Maya *et al*. 1988; Maya & Ponce, 1989; Pita, 1988, 1989). In these works, the germination speed coefficient values ranged from 1%/day (*Convolvulus scoparius*, Maya *et al*., 1988) to 33.33%/day (*Helianthemum thymiphyllum*, Maya *et al*., 1988). Overall, *Isoplexis isabelliana* seeds showed a similar germination speed coefficient (average CV = 8.59%/day) compared to other endemic species from the Canary Islands (*I. chalcantha,* CV = 7.69; *Cistus osbeckaefolius*, CV = 8.28; *Helianthemum juliae*, CV = 7.86; Maya *et al*. 1988).

Although long-time stored accessions of *Isoplexis isabelliana* showed lower seedling viability than a recently stored accession, there was not a significant correlation between seed storage time and seedling viability. Therefore, our result suggests that seed storage for long periods does not reduce seed germination, seedling growth and survival of *I. isabelliana*, which is in agreement with other studies (Silveira *et al*., 2014)

Germination of stored seeds depends on pre-storage factors, such as the age of the material and the method of processing. Also, storage factors, comprising moisture content, temperature and storage time, are influential on seed viability (Pritchard & Dickie, 2003). In this sense, our results showed that seeds of *Isoplexis isabelliana* stored at room temperature and humidity (5183/B) exhibited a significant decrease in germination rate, compared to accessions stored at -10 ºC and 8–9% RH. These results suggest that pre-storage factors and storage conditions are more relevant in the seed viability of *I. isabelliana* than the time of storage. Seed storage conditions have a strong influence on seed quality and, therefore, seedlings (Wyse & Dickie, 2017; Wawrzyniak *et al*., 2020). In this sense, the importance of identifying the optimal harvest time for maximum seed viability has been recognized (Hay & Probert, 2013). Although the accession 2204/B was collected within the fruiting period described for the species (September to October), seeds acquired the ability to withstand drying after having the ability to germinate when fresh the time they gained maximum dry weight (Hay & Probert, 2013). It is relevant, given that a high fruiting time variability, from March to November, has been recorded for this species in the Seed Bank of the JBCVC.

Other factors that could produce a loss of seed quality or viability over time are genetic and environmental interactions during seed maturation and harvest (Walters *et al*., 2010). However, we cannot establish any correlation between germination and annual precipitations. Besides, the average annual rainfall does not seem to be a key factor in seed viability since there is no significant correlation between annual rainfall and germination percentage (Table 1, 2; Figures 2, 3). In this sense, the accession 529/B gave the highest germination percentage (89%) even though this location recorded low annual precipitation (329 mm) for 1987. The results showed that the lack of germination of the accession 2204/B cannot be attributed to the low yearly rainfall since the annual rainfall data does not support it. Germination tests carried out in other taxons in the same locality (Tamadaba) and year (2002) have shown a high germination percentage (unpubl. data, database from the Seed Bank of the JBCVC). Therefore, there must be physical and physiological features and biotic and abiotic factors in the environment that determined the viability of seeds (Long *et al*., 2015). On the other hand, seed dormancy most often decreases during storage, but it may also increase or remain unchanged, which is very important when evaluating seed longevity in wild species (Pérez-Garcia *et al*., 2007).

The resazurin reagent test was not effective in the detection of non-viable seeds in the accession 2204/B. Furthermore, there were no significant differences in absorbance between healthy and non-viable or damaged seeds of the *Isoplexis isabelliana* accessions analyzed. The lack of response to resazurin in aged seeds could be attributed to the fact that seeds do not leak enough ethanol or sugar, perhaps due to a hard seed coat or small seed size (T. Min, pers. comm., November 2019). The resazurin reagent test has shown its use for species from different families and has not yet been tested in a Scrophulariaceae species (Mohammed *et al*., 2019).

We conclude that sampling and storage conditions following the Seed Bank protocol from the JBCVC showed a high potential for producing seedlings from seeds stored over long periods, essential for creating an *ex-situ* collection contributing to the species conservation. In this sense, our Seed Bank currently has more than thirty accessions of *Isoplexis isabelliana* available from all the natural populations described for this endemic endangered species. Five of these accessions are seeds sampled in the localities affected by the last forest fire declared in Gran Canaria Island (Natural Park of Tamadaba and Valleseco) in 2019. Therefore, if the soil seed bank has lost its viability, these accessions could be potentially used in reforestation campaigns. However, accessions from 30 years ago may be genetically too different from current populations. In this regard, when we carry out reinforcements of natural populations, we should be careful with the seed source origin (time and space) to avoid outbreeding depression, which commonly manifests through the decrease in the value of fitness-related traits such as fruit production, survivorship or seed germination (Storfer, 1999; Fenster & Galloway, 2000).

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Supplementary Material

Table S1. ANOVA analysis of seeds biometry (polar axes, equatorial axes and seed area) from the four accessions of *Isoplexis isabelliana* analyzed.

Table S2. Multiple range test among four accession of *Isoplexis isabelliana* analyzed for the three seeds biometric parameters.