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Effects of *In Situ* Burial and Sub-Optimal Storage on Seed Longevity and Reserve Resources in Sub-Tropical Mountain Cloud Forest Tree Species of Mexico

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Abstract

To design conservation and management strategies for sub-tropical moist forests, it is important to know how storage affects the longevity and germination of desiccation-sensitive seeds. These variables are controlled by a physiological process that is mainly associated with the seeds' moisture content and reserve content, which can be significantly affected by environmental conditions. The objectives of this study were: (i) to evaluate the relationship between longevity and reserve content of sensitive and non-sensitive desiccation seeds (ii), and to describe how the *in situ* burial and sub-optimal storage (inside growth chambers) can affect seed longevity. In seeds of six mountain cloud forest tree species, germination and seed reserve content were quantified in seeds collected recently (control) and at five different times, along with storage for 31 or 35 days *in situ* and under suboptimal conditions. Freshly harvested seeds with higher soluble carbohydrate and lipid content and the presence of thermo-stable proteins had the longest-expected ecological longevity and mean germination time. In contrast to *in situ* burial, germination was negatively affected by storage time. Burial time significantly affected the rate and mean germination time in most of the species. For two species, a greater soluble carbohydrate or a lower starch and lipid content in seeds was found to be related to the high germination rate in storage. Our results show that the sub-optimal storage reduces the lifespan in most of the study species and increases seed damages through dehydration.

Keywords

seed lifespan, germination rate, seed water content, water stress, seed reserve

Introduction

Knowledge of seed longevity (seed lifespan) and the physiological and environmental variables that affect it is important for the implementation of appropriate conservation strategies and for the management of desiccation-sensitive species. Seed lifespan plays an important role in the spatial and temporal dynamics of plant populations, because it determines the period in which seeds can produce new individuals into the community (Joët et al., 2013; Kitajima, 2002). Seed lifespan is related to seed moisture content (SMC), which is represented in the community by a gradient between orthodox and recalcitrant seeds (Farrant et al., 1993). Recalcitrant seeds present a high respiration rate, short lifespan and high moisture content, but no dormancy and are desiccation-sensitive (Murdoch, 2014; Pammenter & Berjak, 2000). In this type of seed, the loss or decrease of lifespan is significantly affected by even small losses in seed moisture content

(Hill et al., 2012; Wen & Cai, 2014; Yu et al., 2008); Therefore, standard storage at low moisture and temperature can be lethal. Desiccation-sensitive seeds can

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decrease their SMC, even after 8 days of storage inside glass jars at 25°C (Becerra-Vázquez et al., 2018). Although cryo-preservation has been successfully used for long time storage of desiccation-sensitive seeds (Berjak & Pammenter, 2008), this method is expensive and impractical for the management of large volumes. In this context, to improve seed management for restoration practices, it is important to assess the seed lifespan under sub-optimal conditions (*sensu* Vázquez-Yanes & Orozco-Segovia, 1993).

Desiccation-sensitive seeds have mainly been reported from humid and tropical rain forests, where they are common (Daws et al., 2005; Pammenter & Berjak, 2000; Vázquez-Yanes & Orozco-Segovia, 1993; Wyse & Dickie, 2017). As many as 89 and 44% of Asian and Neo-tropical species, respectively, produce this type of seeds (Tweddle et al., 2003; Umarani et al., 2015; Wyse & Dickie, 2017). Mountain cloud forest (MCF) is a subtropical ecosystem that comprises a mixture of temperate and tropical species (Cuevas & Jardel, 2004). Seed lifespan information for this ecosystem is scarce; however, a recent study reported a wide variation in the onset of germination after seed dispersal (8 to 122 days) (Toledo-Aceves et al., 2018). Similar results have been reported for rainforest species, with mean germination times of 6 days to 5 months (Hill et al., 2012; Vaz et al., 2016; Wen & Cai, 2014). The time that a seed remains quiescent in the soil, from dispersal to germination or natural death (ecological longevity -EL-, Vázquez-Yanes & Orozco-Segovia, 1993), can be related to the environmental conditions in which seeds are frequently dispersed (Hill et al., 2012). Therefore, the time since the seed dispersal and the beginning of the rainy season can be an estimator of seed lifespan for desiccation-sensitive seeds (expected ecological longevity- EEL). Other important traits related to the lifespan are the seed morphological and physiological processes (seed water content, depth and dormancy type) and seed reserves composition (Hamilton et al., 2013; Hill et al., 2012; Orozco-Segovia & Sánchez-Coronado, 2009; Satyanti et al., 2018; Walck et al., 2011; Wyse & Dickie, 2017).

Carbohydrates are the main reserves in recalcitrant seeds. The soluble carbohydrate provides the energy necessary to maintain a high respiration rate and fast germination (Berjak & Pammenter, 2008; Farrant et al., 1993), which are physiological traits of recalcitrant seeds; and starch is the main energy source that supports the seedling growth (Zeeman et al., 2010). In natural conditions, seed reserves composition is frequently related to seed lifespan (Vázquez-Yanes & Orozco-Segovia, 1993). Starch is possibly the most important reserve related to a long seed lifespan, due to its slow mobilization rate, which can be related to a germination delay (Farrant et al., 1993). On the other hand, increases in sugar storage in the embryo have been related to the

desiccation tolerance of the seed (Vertucci & Farrant 1995) or as a protective mechanism for reducing the loss of viability of the seed by drying (Connor & Sowa, 2003). Lipid content may negatively affect the lifespan of recalcitrant seeds, since it is positively related to cellular membrane integrity, while lipid peroxidation follows the opposite trend (Connor & Sowa, 2003; Long et al., 2015). Other important seed reserves related to seed desiccation tolerance and greater longevity are the thermo-stable proteins; however, their specific role in recalcitrant seeds is under discussion (Berjak & Pammenter, 2008).

Seed lifespan, reserve metabolism, and germination processes are highly regulated by environmental factors (Bewley & Black, 1994). In recalcitrant seeds, high temperatures can negatively affect germination rates and seed viability (Pritchard et al., 2004; Vinayachandra, 2012). Moreover, relatively high temperatures (35–45°C) can affect the status of the lipids and soluble carbohydrates through the accumulation of unsaturated fatty acids (Mira et al., 2015; Nkang, 2002) and depletion of soluble carbohydrates (Vinayachandra, 2012), respectively, leading to viability loss.

The mountain cloud forest is considered one of the most diverse ecosystems worldwide (Ponce-Reyes et al., 2012), where the high environmental moisture is related to high precipitation and frequent incidence of fog (Foster, 2001; Ponce-Reyes et al., 2012). However, the annual increase in the number of dry days recorded for this ecosystem is expected to have a negative impact on the regeneration capacity of mountain cloud forest populations (Foster, 2001; Hu & Riveros-Iregui, 2016; Ooi et al., 2009; Walck et al., 2011). On the other hand, because a cloud forest is a mixture of temperate and tropical species, we expect to find a high variability in the moisture stress tolerance and seed- longevity among species.

The objectives of this study were (i) to evaluate the relationship between longevity and seed reserve content of seeds of ten mountain cloud forest tree species and (ii) to describe the effect of seed burial in the forest soil (storage *in situ*) and sub-optimal storage inside growth chambers (25°C, RH 40%, constant airflow) on seed longevity, germination and seed reserve content of six tree species. We expected that (i) seeds with a longer EEL and mean germination time will present a greater content of starch, lipids, and thermo-stable proteins that those with a short EEL, (ii) *in situ* burial will favor a higher germination percentage and germination rate as well as a shorter mean germination time, compared to sub-optimal storage treatment, (iii) since high molecular weight reserves such as starch and lipid content require a higher amount of energy to be metabolized, our prediction is that starch and lipid content will be positively related to mean germination time. In the context of climate change, the results of this study will also contribute

toward a better understanding of the potential climate change effects on the viability, EL, and germination in tolerant and desiccation-sensitive seeds. This knowledge is crucial for *in situ/ex situ* seed conservation.

Methods

Study Area

Seed collection was performed in the mountain cloud forest of “Las Joyas” Scientific Station (LJSS), located between 19°34′14”–19°37′30”N and 04°14′49”–104°18′16”W in the core zone of the Sierra de Manantlán Biosphere Reserve, Jalisco, México. The reserve area is 3600 ha and its elevation range is 1600–2220 m. The predominant climate is temperate sub-humid, and the mean annual temperature and precipitation are 15.7°C ± 1.5°C and 1768.1 ± 203.42 mm, respectively. The wettest months are from mid-June to October (rainy season), and the dry season from April to mid-June, with the lowest rainfall occurring in November–December (Zuloaga-Aguilar, 2018). The main vegetation cover types and their common species in the LJSS are (1) cloud forest: *Dendropanax arboreus*, *Carpinus tropicalis*, *Cinnamomum pachypodum*, *Persea hintonii*, *Quercus nixoniana*, and *Cornus disciflora*; (2) mixed forest: *Pinus douglasiana*, *Q. candicans*, *Q. salicifolia*, *Q. obtusata*, and an understory with saplings of cloud forest species; (3) pine forest; *P. douglasiana*, *P. herrerae*, *P. oocarpa*, and *P. maximinoi*; (4) oak forest; *Q. magnoliifolia*, *Q. resinosa*, *Q. scytophylla*, and *Q. candicans*; and (5) pine-oak forest, characterized mainly by the species of pine and oak mentioned previously (Cuevas & Jardel, 2004).

Selected Species

To ensure the inclusion of seeds with a range of EL and sensitivity to dehydration, two groups of tree species were selected based on their seed dispersal time (during the dry and/or rainy seasons). In this study, EL is considered the interval between seed maturation till seed germination or death under natural conditions (Vázquez-Yanes & Orozco-Segovia, 1993). Seeds of 10 species: *Carpinus tropicalis*, *Citharaxylum mocinni*, *Cleyera integrifolia*, *Fraxinus uhdei*, *Magnolia iltisiana*, *Dendropanax arboreus*, and *Persea hintonii* seeds were collected during the dry season or the end of the dry season and *Quercus nixoniana*, *Nectandra lundellii*, and *Cinnamomum pachypodum* seeds were collected at the beginning of the rainy season (Table 1). Based on a bibliographic search, we have an approximate description of the storage behavior of the studied species: most of the 93 *Quercus* species reported by the Royal Botanic Gardens Kew Seed Information Database (Royal Botanic Gardens Kew [RBGK], 2020) are recalcitrant or probably recalcitrant; of the seven species of *Nectandra* genera, only two have been reported as

Table 1. Freshly-Harvested Seed Description (at Dispersion Time) of 10 Mountain Cloud Forest Species From “Las Joyas” Scientific Station in Western Mexico.

Species	Family	Date	DW (mg)	MC (%)	Volume (mm ³)	EEL (days)	EL (days)	CARB (%)	STARCH (%)	LIP (%)	TP (%)
<i>From dry season</i>											
<i>Carpinus tropicalis</i> ^f	Betulaceae	27 May , 08–22 Jun.	6.5 ± 0.1 ^g	6.2 ± 0.4 ^c	1.48 ⁱ	29 ± 4.0 ^{ab}	32.6 ± 2.3 ^{cd}	29.60 ± 1.39 ^a	7.47 ± 0.24 ^d	21.1 ± 1.01 ^a	0.0001 ± 0.00 ^e
<i>Citharaxylum mocinni</i> ^f	Verbenaceae	27 May , 10 Jun.	28.16 ± 1.5 ^f	6.5 ± 0.4 ^c	60.08 ^f	8 ± 3.0 ^b	26.4 ± 2.7 ^d	10.32 ± 0.34 ^d	3.2 ± 0.05 ^e	24.5 ± 2.3 ^a	0.28 ± 0.04 ^b
<i>Cleyera integrifolia</i>	Pentaphylacaceae	18 Apr., 22 May.	4.42 ± 0.6 ^h	4.45 ± 0.2 ^c	0.88 ⁱ	61 ± 120 ^a	122.3 ± 2.3 ^a	21.71 ± 0.45 ^b	5.8 ± 0.13 ^f	13.2 ± 2.61 ^{ac}	0.0016 ± 0.00 ^d
<i>Dendropanax arboreus</i> ^{fs}	Araliaceae	27 May , 07 Jun.	21.5 ± 0.6 ^{ef}	5.5 ± 0.4 ^c	28.48 ^g	23 ± 8.83 ^{ab}	25.6 ± 1.3 ^d	17.2 ± 0.39 ^{bc}	5.1 ± 0.07 ^f	23.0 ± 1.96 ^a	1.22 ± 0.3 ^a
<i>Fraxinus uhdei</i> ^f	Oleaceae	30 Apr., 06 Jun.	8.5 ± 0.3 ^g	0.7 ± 0.02 ^d	6.56 ^h	39 ± 2.9 ^a	112.6 ± 0.3 ^a	33.39 ± 1.8 ^a	8.0 ± 0.42 ^d	18.2 ± 2.06 ^a	0.013 ± 0.01 ^c
<i>Magnolia iltisiana</i> ^{fs}	Magnoliaceae	11–16 May , 20.7 ± 0.9 ^e	20.7 ± 0.9 ^e	6.1 ± 0.02 ^c	103.33 ^g	61 ± 1.4 ^a	36.3 ± 0.3 ^c	31.39 ± 1.65 ^a	2.0 ± 0.07 ^g	18.9 ± 0.71 ^a	0.50 ± 0.3 ^b
<i>Persea hintonii</i> ^{fs}	Lauraceae	23 May, 22 Jun.	790 ± 5.8 ^d	44.27 ± 3.2 ^{ab}	789.34 ^c	23 ± 1.4 ^a	99.3 ± 2.02 ^a	17.6 ± 1.12 ^{bc}	34.6 ± 1.15 ^c	7.4 ± 0.98 ^c	0 ± 0.00
<i>From rainy season</i>											
<i>Cinnamomum pachypodum</i> ^{fs}	Lauraceae	20 Jun. , 03–18 Jul.	53.4 ± 1.2 ^c	51.4 ± 0.4	694 ^d	5 ± 3.1 ^c	14.3 ± 0.6 ^e	31 ± 0.63 ^a	56.7 ± 0.32 ^a	7.6 ± 1.05 ^c	0 ± 0.00
<i>Nectandra lundellii</i> ^{fs}	Lauraceae	23 Jun., 20 Jul.	2379 ± 207 ^b	44.2 ± 0.1 ^a	4088.74 ^a	10 ± 10 ^c	47.6 ± 0.3 ^b	12.0 ± 1.01 ^d	43.7 ± 1.38 ^b	8.9 ± 0.63 ^c	0.002 ± 0.00 ^d
<i>Quercus nixoniana</i> ^{fs}	Fagaceae	20 Jun. , 11 Jul.	1153 ± 11.4 ^a	36.1 ± 1.1 ^b	837.76 ^b	4 ± 2.3 ^c	11.3 ± 0.3 ^f	13.7 ± 0.31 ^{cd}	10.8 ± 0.09 ^d	0.9 ± 0.03 ^d	0.35 ± 0.04 ^b

Most frequent collection date (Date) by species, dry weight (DW), moisture content (MC), volume, ecological longevity (EEL) and seed reserves are presented. Soluble carbohydrates (CARB), lipids (LIP) and thermo-stable proteins (TP). Different letters denote significant differences ($P < 0.05$). Genus with species reported as recalcitrant (§) and orthodox (£) (RBGK, 2020). Last collection date is marked in bold. Super index letters indicate significant differences at $\alpha < 0.05$.

recalcitrant (RBGK, 2020). Finally, for the genera *Persea* (21 sp), *Magnolia* (10 sp), and *Cinnamomum* (14sp), there is no clear pattern reported in the storage behavior. On the other hand, *Dendropanax arboreus* can be recalcitrant (Daws et al., 2005; Sautu et al., 2006), and *Fraxinus uhdei*, *Carpinus tropicalis*, and *Citharexylum mocinni* are probably orthodox (Becerra-Vázquez et al., 2018; RBGK, 2008), the storage behavior of *Cleyera integrifolia* is unknown (Table 1). We calculated the theoretical minimal value of EEL, which we defined as the number of days that we can expect the seeds to remain viable between dispersal and the onset of the rainy season. The EEL average was estimated using historical records of dates of collection carried out between 1998 and 2018 (N=10 years, only dates during peak germination were included). In contrast to EL, EEL does not include the time until the germination or death of the seed.

Seed Processing and Chemical Analysis

We collected mature seeds directly from at least 10 individuals of each species, during the fruiting peak (year 2016). The empty seeds were removed by the flotation method. Immediately after collection (fresh seeds), we measured the width and length of 75–90 individual seeds; later, we calculated the volume of the ellipsoid ($V = \frac{4}{3} \times \pi \times a \times b \times c$) from the three basic dimensions (length = a, width = b, and thickness = c). The seeds were then transported to the laboratory, where the fresh (FW) and dry (DW) weights of 30 individual seeds were measured using a microbalance (Precisa XT 120A, Precisa Gravimetrics AG, Switzerland). We recorded the seed dry weight (DW) after drying the seeds at 60°C, until we established a constant weight. Wet basis moisture content (MC) was calculated as $MC = [(FW - DW) / FW] \times 100$. The reserve content in fresh seeds was evaluated in all the 10 species. The seeds were preserved in an ultra-low freezer until the chemical analysis (So-LowU85-13, Environmental Equipment Co, Cincinnati, OH) at -40°C. We determined the total non-structural carbohydrates (soluble sugars and starch) of 5–30 seeds (depending on seed size) following the phenol-sulfuric acid method (Dubois et al., 1956). Lipid content was determined for the species using the method of Bligh and Dyer (1959). The thermo-stable protein (TP) content in fresh seeds was determined using the Bradford method, using bovine serum albumin as the standard (Bradford, 1976). The chemical analyses were done in triplicate.

Treatments

Due to limitations in seed availability, these treatments were carried out only for six species: *Carpinus tropicalis*, *Cinnamomum pachypodum*, *Citharexylum mocinii*,

Dendropanax arboreus, *Magnolia iltisiana* and *Quercus nixoniana*.

In Situ Burial. To evaluate the effect of natural environmental variation on viability and seed reserve resources, we buried three nylon mesh bags with 30–90 seeds each (depending on seed availability) at 3 cm depth in mountain cloud forest soil. This treatment was replicated in three sites separated by 100 m in the same stand of forest. We selected sites with slopes < 30% and tree cover between 87 and 90%. The slope was recorded with a compass with a clinometer and tree cover with hemispheric pictures taken with a digital camera with a fisheye lens (Nikon D5100) and processed in the Gap Light Analyzer (ver. 2.0). The reserve content was evaluated in fresh seeds (T0, control), and in unburied seeds dispersed during the dry season every seven days for 35 days, and for seeds dispersed in the rainy season, after 1, 4, 7, 15, and 31 days (5 times for both groups: T1 to T5, plus T0). For the *in situ* treatment, in total, we buried 15 mesh bags for each species (3 sites \times 3 bags \times 5 unearthing times = 45 bags). After each exposure time, for each treatment, time and species, a set of 5–30 seeds (depending on seed size) was preserved in an ultra-low freezer until the determination of their reserves (see section 2.3). The *in situ* burial date varied in relation to the collection date of each species (see Table 1). During the experiment, temperature and humidity in the soil were recorded with the sensors S-SMD-M005 and S-TMB-M006 (Onset HOBO), connected to a micro-station H21-002 (Onset Computer Corporation, Massachusetts).

Sub-Optimal Storage Treatment. To assess the effect of a temperature and relative humid constant in the viability and seed reserve content, three repetitions of 30–90 seeds in paper bags of each species were placed inside an environmental chamber at 25°C, in darkness, RH = 40% and constant airflow. The seed reserve content was evaluated at the same intervals as the *in situ* burial treatment. The loss of moisture from the seeds due the sub-optimal storage was not recorded, due to the lack of sufficient seeds.

Germination. To assess the germination capacity, we took three repetitions of 30–90 seeds of each species, for each treatment (*in situ* burial and sub-optimal storage) and for each time indicated above. The seeds were sown on bacteriological agar (1%, 10 g/L, agar/water) and incubated in a controlled environmental chamber (PERCIVAL, GR41VL, Percival Scientific, Inc. USA) at 25°C, with a photo period of 12/12 h (light/dark). We recorded germination daily for 60 days; thereafter, the viability of non-germinated seeds was tested with 2,3,5-triphenyl tetrazolium chloride 1% (Lakon, 1949). Germination was considered to have taken place when the radicle protruded 3 mm. For all the species, the variables evaluated were germination rate (seed \times day⁻¹),

mean germination time, and the final germination percentage. After each storage time, and per species, we fitted an exponential sigmoid curve to the number of accumulated germinated seeds, using the Table Curve 2D ver. 5.01 (SYSTAT Software, Inc). The exponential sigmoid curve allowed estimation of the germination rate (velocity) as the maximum first derivative and the mean germination time as the time to reach the maximum first derivative (González-Zertuche et al., 2001).

Ecological Longevity Estimation. According to Vázquez-Yanes and Orozco-Segovia (1993), we calculated the ecological longevity by considering the number of days from seed collection until the *in situ* germination beginning. To assess this, for each species we buried three bags of wire mesh with 30 fresh seeds on the soil surface (2–3 cm in depth), close to the sites used for the *in situ* burial treatment. The germination was recorded every seven days until germination occurred or the seeds were rotten.

Statistical Analysis

Freshly Harvested Seed Description. To analyze the variation in moisture content, dry weight, volume, ecological longevity (EL), expected ecological longevity (EEL), and reserve content of freshly-harvested seeds between species (10 species), a linear mixed-effects (LME) model was performed for each variable with a maximum likelihood-ratio test (LRT). In the model, the random factors were season ($n=2$)/replicates ($n=3$) and the species was the fixed effect. Paired differences between species were tested by least-squares means with the “contrast” and “emmeans” functions (“emmeans” package is part of R Core Team). Multiple linear mixed-effects (LME) model was used to test the relationship between soluble carbohydrates + starch + lipid + thermo-stable protein concentration and EL, EEL (in 10 species), mean germination time (MGT), and germination rate (GR, in 9 species). The fixed factor was the seed reserve content ($n=4$), and the random factor structure was as follows: season ($n=2$)/date of collection ($n=8$)/replicates ($n=3$ with 27–30 seeds each). The LME were fitted using the LME function in the NLME library (Pinheiro et al., 2018). Significant differences for the LME were tested with the maximum likelihood-ratio test using the function Drop 1 (“stats” package is part of R Core Team). To reduce the over dispersion all the variables were log10-transformed [$\log_{10}(\text{data} + 1)$] before carrying out the analysis. The normal distribution of model and residuals, homoscedasticity and linearity were tested.

Treatment Effects on Germination Capacity. A two-way generalized linear model (GLM) with binomial error was conducted to determine significant differences in the number of germinated and non-germinated seeds

between treatments ($n=2$, *in situ* burial and sub-optimal storage) and times ($n=5$ plus control) for each species. Tests for over-dispersion were performed before conducting the model.

Treatment Effects on Seed Reserve Content During Germination. We fitted linear regressions and quadratic curves to evaluate the relationships between mean germination time and germination rate with respect to buried and sub-optimal storage time ($n=6$ time) and the concentration of the reserve. Previously, normal distribution and homoscedasticity were confirmed. We used R software ver. 3.4.4 (R Development Core Team, 2018) to perform all the analyses.

Results

Freshly-Harvested Seed Description: Relationship Between Longevity and Seed Resources

The ten species differed significantly in terms of dry weight ($\chi^2 = 190.47$, $df=9$, $P < 0.0001$), moisture content ($\chi^2 = 27.22$, $df=9$, $P < 0.0001$), volume ($\chi^2 = 293.05$, $df=9$, $P < 0.0001$), ecological longevity ($\chi^2 = 152.63$, $df=9$, $P < 0.0001$), and expected ecological longevity ($\chi^2 = 33.46$, $df=9$, $P < 0.0001$) (Table 1). The seeds dispersed during the rainy season were larger in size with higher dry weight (53–1184 mg) and moisture content (36–51%) values, compared to those dispersed in the dry season. Our records showed that the EL of the seeds dispersed during the dry season was 2.6 times longer (25.6–122.3 days) than the seeds dispersed in the rainy season (2–47 days) (Table 1). In all the cases, the longevity was ended by germination. The seeds dispersed during the dry season had a longer EEL (8–83 days before the rainy season began) than those dispersed during the rainy season (4–24 days).

The soluble carbohydrate ($\chi^2 = 105.27$, $df=9$, $P < 0.0001$), starch ($\chi^2 = 185.89$, $df=9$, $P < 0.0001$), lipid ($\chi^2 = 86.67$, $df=9$, $P < 0.0001$), and TP ($\chi^2 = 38.05$, $df=7$, $P < 0.0001$) percentage values varied significantly between the species (Table 1). The highest soluble carbohydrate content was recorded in seeds of *Cinnamomum pachypodum*, *Fraxinus uhdei*, and *Magnolia iltisiana* (31.1–33%), while the starch content was the highest in *C. pachypodum*, *Persea hintonii*, and *Nectandra lundellii* (56.7, 43.7, and 34.6%, respectively). The highest percentage values of lipids were recorded in *Dendropanax arboreus*, *Carpinus tropicalis* and *M. iltisiana* seeds (18.2–23%). The TP content was low (0–1.2%) in seeds of four of the ten species studied (Table 1).

The low fit of the multiple linear mixed-effects model and the non-normal distribution of residuals ($\alpha < 0.05$) showed that reserve content was insufficient to explain the variation in ecological longevity. However, our

results showed that seeds with higher soluble carbohydrate (31–34%) ($\chi^2 = 46.64$, $P = 8.51^{-12}$, $r = 0.97$; Figure 1A) and TP ($\chi^2 = 11.24$, $P = 7.97 \cdot 10^{-4}$, $r = 0.28$) had a longer EEL (29–62 day). The opposite was seen with the relationship among reserves and germination parameters. A high percentage of soluble carbohydrate (31–34%) in fresh seeds of nine species was found to be related to lower germination rates (2–5 seed \times days $^{-1}$) ($\chi^2 = 16.93$, $P = 3.87^{-05}$, $r = 0.53$; Figure 1B). In contrast, longer mean germination times (14–17 days) were related to high percentage of soluble carbohydrates ($\chi^2 = 7.81$, $P = 5.10^{-03}$, $r = 0.63$; Figure 1C) and lipids ($\chi^2 = 3.97$, $P = 0.046$).

In Situ Burial and Sub-Optimal Storage Effect on Germination Capacity

Exposure to *in situ* burial and sub-optimal storage treatments significantly affected the germination percentage of most species (Figure 2 and Table 2). The averages of

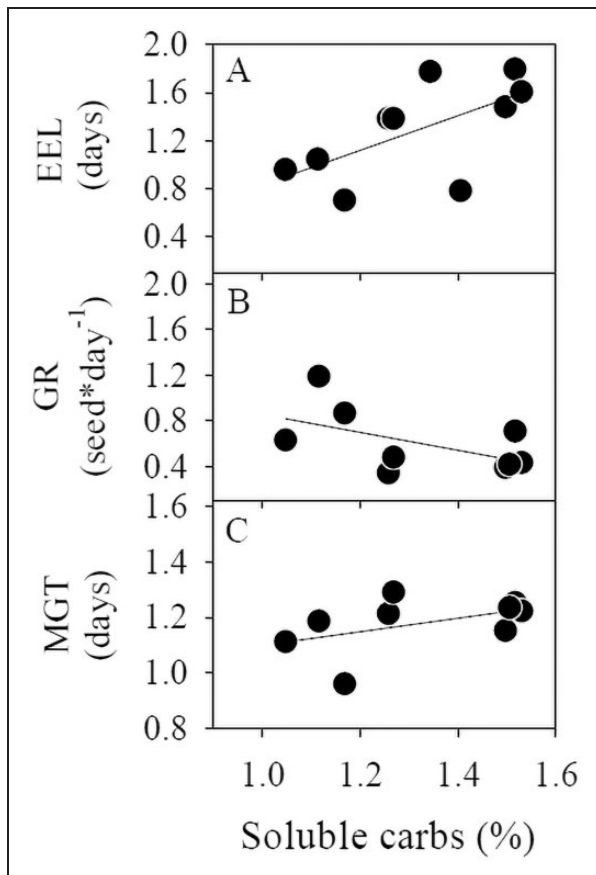


Figure 1. Relationship between content of soluble carbohydrates (carbs) and expected ecological longevity (EEL, $n = 10$ species), germination rate and mean germination time (GR, MGT, $n = 9$ species) of fresh seeds of species from the mountain cloud forest in “Las Joyas” Scientific Station in Western Mexico. The line represents the mixed model correlation. The data are presented in logarithmic scale.

maximum and minimum temperatures and soil moisture content during seed burial in the mountain cloud forest soil were 17.45–15.27°C and 21.25–3%, respectively, in May, 17.61–15.84°C and 34.1–17.5%, respectively, in June, and 16.83–15.43°C and 45.5–33.4% in July of 2016. For most of the species, the germination percentages had a higher reduction when the seeds were under sub-optimal storage, compared to those in the burial treatment (11–56.67%, 0–24.58%, respectively). After 7 to 21 days *in situ* burial, *Citharexylum mocinni*, *C. tropicalis*, and *D. arboreus* seeds recorded an increase of 1.8 to 7 times in germination percentage (Figure 2), compared to fresh seeds. A similar pattern was recorded under sub-optimal storage.

For most species, the mean germination time and the germination rate were affected by both *in situ* and sub-optimal storage time (Supplementary 1). The longer the seeds of *C. pachypodum* remained buried, the higher their germination rate was ($F_{2, 4} = 21.8$, $P = 0.01$, $r^2 = 0.87$, Figure 3A) but the shorter their mean germination time ($F_{2, 4} = 17.11$, $P = 0.01$, $r^2 = 0.85$, Figure 3B). A longer burial time decreased the germination rate in *M. iltisiana* seeds ($F_{2,5} = 323.96$, $P < 0.001$, $r^2 = 0.98$, Figure 3C). On the contrary, an increase in the burial time of *M. iltisiana* seeds decreased the germination rate (Supplementary 1, Figure 3E), but the mean germination time of *D. arboreus* ($F_{2, 3} = 57.12$, $P = 0.004$, $r^2 = 0.95$), *C. tropicalis* ($F_{2, 3} = 10.95$, $P = 0.04$, $r^2 = 0.79$), and *C. mocinni* seeds increased with the length of burial time ($F_{2, 3} = 22.87$, $P = 0.01$, $r^2 = 0.89$; Figure 3D).

In Situ Burial and Sub-Optimal Storage Effects on Seed Reserve Content During Germination

In most of the species studied (four to six species) the soluble carbohydrates, starch, and lipid content in the seeds varied significantly among exposure times to *in situ* burial and sub-optimal storage (Supplementary 2). Soluble carbohydrates, starch, and lipids in *C. pachypodum* seeds decreased to 84% and 66% after seven days under *in situ* burial and sub-optimal storage, respectively, with respect to the values found in fresh seeds (T0). An increase in the starch content was recorded towards the end of both *in situ* burial and sub-optimal storage in *M. iltisiana* seeds (from T0 = 2.0% to T5 = 4.4% and 7.3%, respectively), and soluble carbohydrates and lipids decreased by up to 8 and 2 times, respectively (Supplementary 2). Regardless of the exposure time, the soluble carbohydrate content was positively related to the germination rate in *M. iltisiana* seeds under sub-optimal storage ($F_{2,15} = 166.61$, $P < 0.001$, $r^2 = -0.97$, Figure 4A). When the starch and lipid content were lower, the germination rates of *M. iltisiana* and *C. pachypodum* seeds increased under *in situ* burial ($F_{2,15} = 34.22$, $P < 0.0001$, $r^2 = 0.82$; $F_{2,13} = 11.46$, $P = 0.005$, $r^2 = 0.79$, respectively) (Figure 4B and C).

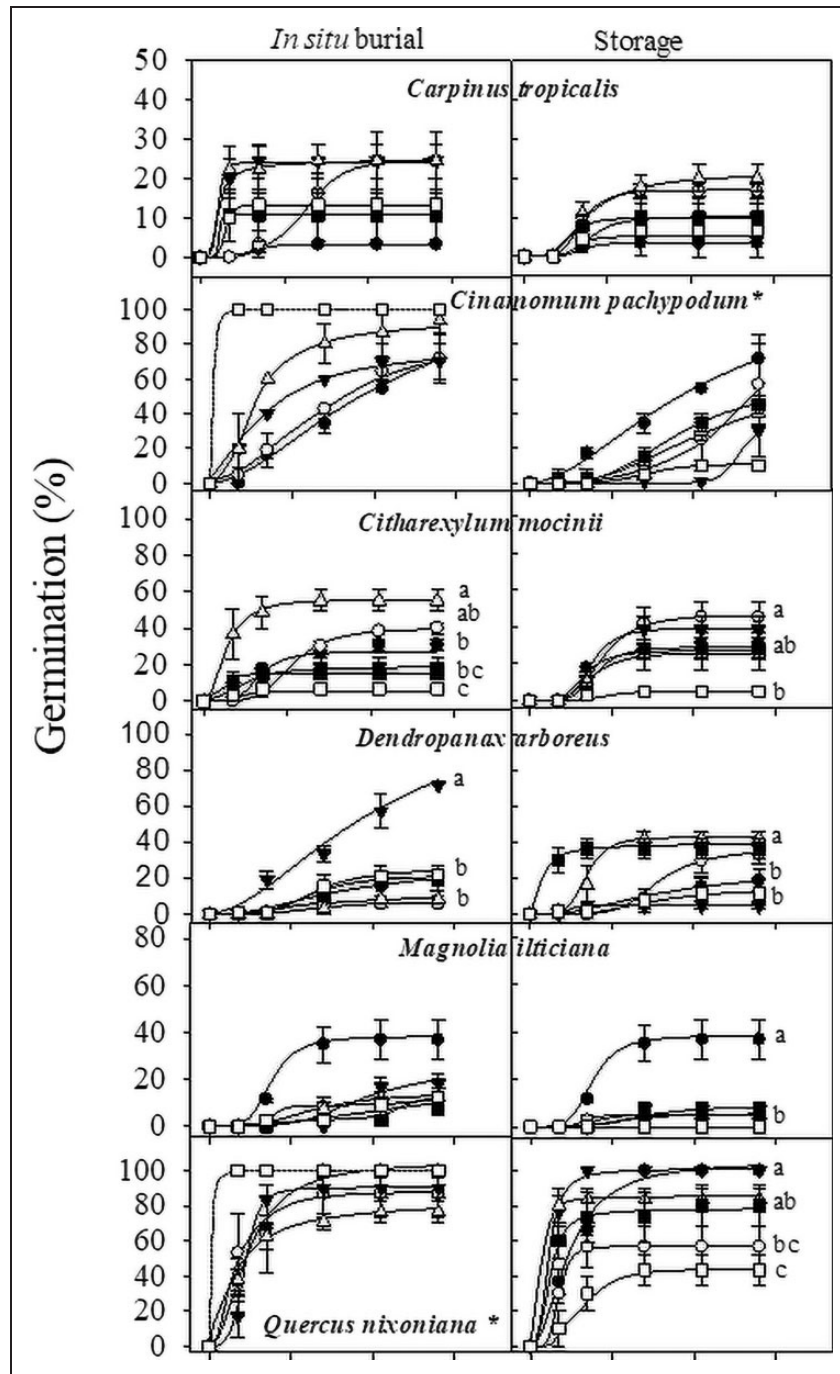


Figure 2. Accumulated germination (%) of seeds of six species stored by burial in natural conditions and suboptimal storage for the following periods: T0 (black circle), T1 (white circle), T2 (black triangle), T3 (white triangle), T4 (black square) and T5 (white square). Seeds were collected from the mountain cloud forest in “Las Joyas” Scientific Station in Western Mexico. It is to be noted that in the *Carpinus tropicalis* and *Magnolia iltisiana*, the axis (Germination) is different. Data were fitted to exponential sigmoid curves: $y = a/[1 + b^{(-cx)}]$.

Discussion

Relationship Between Longevity and Seeds' Resources

Our first hypothesis was partially confirmed because the soluble carbohydrates, lipid and protein, but not starch content, were found to be related to the expected

ecological longevity in fresh seeds. The species with higher soluble carbohydrate content in their seeds had greater expected ecological longevity, longer mean germination time, and lower germination rate. In our study, seeds with such characteristics were dispersed mainly during the dry season, had a small size and low water

Table 2. Analysis of Deviance for a GLM (Binomial Error) to Test the Effect of Treatments of Storage Condition and *In Situ* Burial ($n=3$) for Different Exposure Times ($n=6$) on Seed Germination of Six Species From the Mountain Cloud Forest in “Las Joyas” Scientific Station in Western Mexico.

Species	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
<i>Carpinus tropicalis</i>					
Treatment	2	1.77	51	87.94	0.41
ET	5	45.46	46	42.47	1.16^{-08}
Treatment:ET	10	5.68	36	36.79	0.84
<i>Cinnamomum pachypodum</i>					
Treatment	2	11.19	42	46.46	3.69^{-03}
ET	4	9.53	38	36.92	0.04
Treatment:ET	8	5.97	30	30.95	0.65
<i>Citharexylum mocinii</i>					
Treatment	2	13.41	51	104.4	1.2^{-03}
ET	5	11.37	46	93.03	0.04
Treatment:ET	10	70.5	36	22.52	3.54^{-11}
<i>Dendropanax arboreus</i>					
Treatment	2	104.81	51	293.69	$< 2.2^{-16}$
ET	5	16.28	46	277.4	6.04^{-03}
Treatment:ET	10	215.15	36	62.25	$< 2.2^{-16}$
<i>Magnolia iltisiana</i>					
Treatment	2	66.97	51	189.138	2.86^{-15}
ET	5	89.65	46	99.479	$< 2.2^{-16}$
Treatment:ET	10	55.12	36	44.357	2.99^{-08}
<i>Quercus nixoniana</i>					
Treatment	2	4.79	42	101.693	0.09
ET	4	36.46	38	65.225	2.31^{-07}
Treatment:ET	8	27.4	30	37.818	6.01^{-04}

ET = Exposure time; Df = degrees of freedom; Resid. Df = Residual degrees of freedom; Resid. Dev = Residual deviance; Pr(>Chi) = chi-squared value.

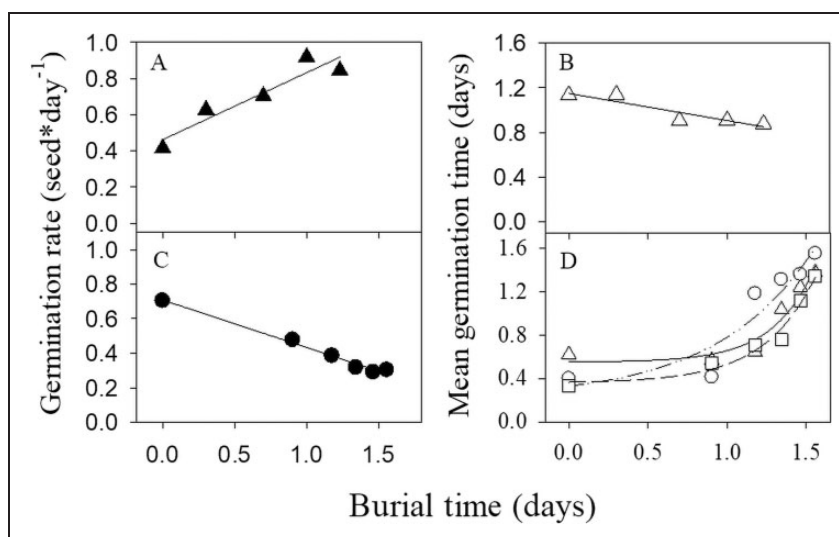


Figure 3. Relationship between *in situ* burial time and germination rate (black symbols) and mean germination time (white symbols) in *Cinnamomum pachypodum* (A, B), *Magnolia iltisiana* (C), *Carpinus tropicalis* (circles), *Citharexylum mocinii* (triangles) and *Dendropanax arboreus* (squares) (D). Data were fitted to lineal regression in A ($y=0.46+0.37x$), B ($y=1.14+0.24x$) and C ($y=0.70+0.27x$), and to exponential growth fit in D (*C. tropicalis*: $y=0.22+0.11 \times \exp(1.62 \times x)$, *C. mocinii*: $y=0.55+0.002 \times \exp(3.78 \times x)$, *D. arboreus*: $y=0.36+0.008 \times \exp(3.11 \times x)$). The data are presented in logarithmic scale.

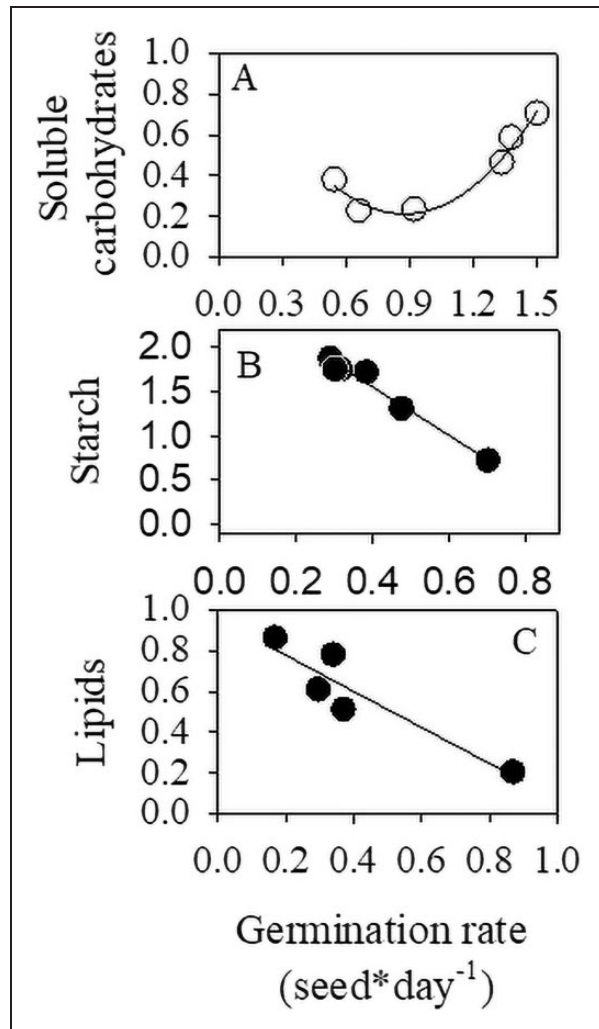


Figure 4. Relationship between soluble carbohydrates, starch and lipid contents and the germination rate of *Magnolia iltisiana* (A) and *Cinnamomum pachypodum* seeds in dry storage (B, white) and *in situ* burial (B, black). A quadratic regression was fitted in the figure A ($y = 1.19 - 2.24 \times x + [1.30 \times x^2]$) and a linear regression was fitted in the figures B ($y = 0.958 - [0.360 \times x]$) and C ($y = 0.955 - [0.885 \times x]$). The data are presented in logarithmic scale.

content, and the lipid and thermo-stable protein content were higher than those dispersed in the rainy season. In this group, we could include *Carpinus tropicalis*, *Citharexylum mocinni*, *Cleyera integrifolia*, *Dendropanax arboreus*, *Fraxinus uhdei* and *Magnolia iltisiana*. Only *Fraxinus uhdei* has been reported as orthodox. There is no specific information about the remaining species. However, within these genera (*Carpinus*, *Citharexylum* and *Magnolia*) some species have been reported as orthodox (RBGK, 2020). It is necessary to assess accurately the storage behavior of the studied species. For orthodox seeds, the carbohydrate concentration during drying is

related to desiccation-tolerance (Bewley & Black, 1994). Lipid content has been related to the cellular membrane integrity and higher tolerance to low moisture and high temperatures that are prevalent in the season of greater stress (Hamilton et al., 2013; Long et al., 2015; Mello et al., 2010; Soriano et al., 2014). Therefore, high lipid content in seeds with a longer EEL can enhance tolerance to moisture stress. However, in our species and others, further studies are required to fully understand the role of lipids in the longevity of water-stress-sensitive seeds.

Our observations showed that seeds dispersed close to the rainy season were larger, had higher moisture content at the time of dispersion, and presented faster germination rates than seeds dispersed in the dry season. In contrast to the seeds dispersed in the dry season, these species have fewer lipids; however, the starch content is six times higher than the soluble carbohydrate. As they have a faster germination rate, higher starch content has been associated with energy requirements for seedling development and establishment (Zeeman et al., 2010). *Nectandra lundellii*, *Cinnamomum pachypodum*, and *Quercus nixoniana* seeds have shorter EEL and are negatively affected by the condition during the storage. Although inside the environmental chamber the HR was 40%, the constant airflow may favor seed dehydration faster compared to buried seeds. In fact, the seeds inside the environmental chamber looked dehydrated (the seed cover was cracked). The seed traits of *Persea hintonii* are similar to those of *N. lundellii*, *C. pachypodum*, and *Q. nixoniana*; however, as the dispersal time varied widely in the last ten years in the area, their EEL and EL can be up to six times longer than those of other species.

In Situ Burial and Sub-Optimal Storage Effect on Germination Capacity

Most of the species studied were sensitive to the sub-optimal storage (25°C and RH 40% and airflow). At the end of the sub-optimal storage time (35 days), the germination percentage decreased in five of the six species studied. Based on previous experiments in desiccation-sensitive seeds of *Symplocarpon purpusii* (seed water content = $37.5 \pm 0.98\%$), we know that after 30 days under sub-optimal storage, the seeds can lose on average more than 14% of their fresh weight (unpublished data). Therefore, the viability loss of most of the species can be related to the dissection of the seeds. On the other hand, we observed that germination can be more effectively improved by a burial period. This means that the occurrence of natural-priming (*sensu* González-Zertuche et al., 2001) can be important in increasing the germination rate of species from moist environments that require fluctuating temperatures (Becerra-Vázquez et al., 2018; Hill et al., 2012;

Orozco-Segovia & Sánchez-Coronado, 2009). Moreover, we found that the germination rate, mean germination time, and reserve resource content of most of the species in this study were affected by the environmental fluctuations in *in situ* burial and sub-optimal storage. More time in *in situ* burial can improve the germination rate and lower mean germination time of seeds with higher moisture content, such as in *C. pachypodum* seeds, or increase the mean germination time, as in *C. tropicalis*, *C. mocinni*, and *D. arboreus*; these results confirm our second hypothesis.

In general, desiccation-sensitive seeds have a higher moisture content and greater mass compared to desiccation-tolerant seeds (Daws et al., 2005; Hamilton et al., 2013; Hill et al., 2012; Satyanti et al., 2018). The moisture content of *P. hintonii*, *N. lundellii*, *C. pachypodum*, and *Q. nixoniana* seeds (36–51%) is within the ranges reported for humid tropical forest species and recalcitrant species (20–67%) (Bonner, 1996; Hamilton et al., 2013; Hill et al., 2012; Umarani et al., 2015; Vaz et al., 2016; Wen & Cai, 2014; Yu et al., 2008); therefore, we can describe these species as sensitive to damage under storage. For several species with desiccation-sensitive seeds, moisture content loss exceeding 8% can be lethal (Hill et al., 2012; Wen & Cai, 2014; Yu et al., 2008). For these species, seed dispersal during the rainy season can help avoid this dehydration risk (Daws et al., 2005; Hill et al., 2012; Pritchard et al., 2004). Our results confirm this strategy; the germination percentages and the germination rate of seeds exposed to the *in situ* burial increased compared to those in sub-optimal storage, since the seeds undergoing burial *in situ* require a shorter imbibition time (Vázquez-Yanes & Orozco-Segovia, 1993). This means that under natural conditions in the mountain cloud forest, environmental humidity is necessary to maximize seed lifespan (Escobar et al., 2018; Toledo-Aceves, 2018).

In Situ Burial and Sub-Optimal Storage Effects on Seed Reserve Content During Germination

In general, reserve content in the seeds varies due to longer exposure time in *in situ* burial and sub-optimal storage which, in most of the species, either decreases or is similar to freshly-harvested seeds. In *M. iltisiana* and *C. pachypodum* seeds, exposure to *in situ* burial, a higher starch or lipid content was found to be related to slow germination rates. Starch and lipid content depletion can be related to the onset of germination, as these are the main resources used during germination and seedling growth (Satyanarayana et al., 2011; Zeeman et al., 2010). However, in recalcitrant seeds, the role of starch has been related to seed germination delay, due to its slow depletion rate (Farrant et al., 1993). On the other hand, we found higher soluble carbohydrate content to be associated with a higher germination rate of

M. iltisiana seeds in sub-optimal storage. Our third hypothesis was partially supported, since we found the germination rate rather than the mean germination time to be related to reserves.

Taking into account the traits of seeds (orthodox type), increase in the mean germination time under *in situ* burial, and the need for sub-optimal storage time to increase the germination rate, we expect the germination of species such as *F. uhdei*, *C. integrifolia*, *C. tropicalis*, *C. mocinni*, and *D. arboreus* will be less affected by the decrease in soil moisture due to changes in the seasonal rainfall pattern.

Implications for Conservation

In the context of climate change, the conservation of mountain cloud forest presents a great challenge, because most of its abundant and frequent species have seeds that are sensitive to *non-standard* storage conditions. The germination percentages of the studied species were negatively affected by storage at 40% RH and 25°C; therefore, long-term storage under these conditions is not recommended. For several species with seeds that are sensitive to moisture stress, *in situ* burial is the pre-condition that aims to improve germination and enable those seeds to remain vigorous for a longer time. In the context of climate change, reduced precipitation and increased temperatures will have a negative effect on the regeneration of species with recalcitrant seeds and, consequently, on the conservation of this type of community.

Author's Contribution

C. Garcias-Morales carried out the experiment and collected data from the field, analyzed the data and wrote the manuscript. S. Zuloaga provided overall guidance for the project and the experimental design, analyzed data and contributed to the manuscript. A. Orozco-Segovia and D. Soriano contributed to the experimental design, writing and editing of the manuscript.

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Supplemental material

Supplementary material for this article is available online.

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