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ORIGINAL ARTICLE

Embryo growth and seed germination of four taxa of *Narcissus* (section *Pseudonarcissi*) conserved under non-recalcitrant seed conditions in germplasm banks

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Abstract

The aim of this study was to determine the germinative ability of the seeds of four *Narcissus* taxa belonging to Section *Pseudonarcissi* after they had been conserved under the conditions of non-recalcitrant seed storage protocols. For each taxon (*N. alcaracensis*, *N. longispathus*, *N. radinganorum* and *N. pseudonarcissus* subsp. *munozii-garmendiae*), one seed lot was desiccated to 4% moisture content (MC) and stored under laboratory conditions (22°C, 40–50% relative humidity (RH), whereas another was dehydrated to 3% MC and stored at −10°C. The latter treatment simulated standard conservation conditions for non-recalcitrant seeds. After 26 months, embryo growth and germination were evaluated. Seed responses were correlated with their MC upon dispersal. Seeds of *N. alcaracensis*, *N. longispathus* and *N. radinganorum* left to dry on the mother plant during maturation had 8–10% MC when dispersed, tolerated non-recalcitrant seed conservation and germinated to >90% under the most favorable incubation conditions. *Narcissus pseudonarcissus subsp. munozii-garmendiae* seeds did not undergo maturation drying and had 46.7% MC upon dispersal. They reached 100% germination after being desiccated to 4% and stored at 22°C, were not recalcitrant, but failed to germinate when stored at −10°C under non-recalcitrant seed conservation conditions. Therefore, *N. alcaracensis*, *N. longispathus* and *N. radinganorum* seeds can be conserved under non-recalcitrant seed conditions in germplasm banks, whereas those of *N. pseudonarcissus* subsp. *munozii-garmendiae* are moderately recalcitrant. Seed storage behavior is influenced primarily by the extent of maturation drying of the seeds on the mother plant.

KEYWORDS

Amaryllidaceae, Iberian endemism, seed-storage behavior, morphophysiological dormancy, recalcitrance

1 | INTRODUCTION

For some time now, the classical distinction between orthodox and recalcitrant seeds has been blurred, because the more species that are studied, the more difficult it is to place their seeds into one of these categories. Many researchers propose new classifications based on the existence of different degrees of recalcitrance (e.g., Barbedo, Centeno, & Figueiredo-Ribeiro, 2013; Berjak & Pammenter, 2008; Walters, 2015). Considering the above, the preservation of non-recalcitrant seeds in germplasm banks is a vital strategy for the conservation of phytogenetic resources (Chin, 1994). Non-recalcitrant seeds can be dried down to 2–5% on a fresh mass basis and stored in airtight containers at subzero temperatures (optimum: −18°C) for extended periods without losing viability. In contrast, recalcitrant seeds have high moisture content (MC) at maturity. Drying them below ~20–30% is fatal. Moreover, recalcitrant seeds are sensitive to cold and will die when subjected to subzero temperatures (Roberts, 1973; Roberts & Ellis, 1989). Moderately recalcitrant seeds tolerate dehydration and low water content but become chilling sensitive in this state (Berjak & Pammenter, 1994).

Lately, a model of recalcitrance has been proposed that relates to the degree of maturation that seeds have when they are separated from the mother plant. In this way, the dispersion of non-recalcitrant seeds would occur at the end of maturation drying, coinciding with the highest values of germination capacity, dry matter content and desiccation tolerance, and the lowest value of MC. If a premature disconnection occurs, the seed would move away from the non-recalcitrant behavior, approaching the recalcitrant one. Different times of releasing seeds from the mother plant would produce different degrees of recalcitrance. Seed-storage behavior could also change as a function of environmental conditions at each new seed formation: in each different year or in different soil and climatic conditions (i.e., the phenotypic variation) (Barbedo, 2018).

It is known that it is difficult to germinate the seeds of certain rare, threatened plant species of the Ranunculaceae, Liliaceae, Lobeliaceae, Dioscoreaceae and Menyanthaceae. Godefroid, Van De Vyver, and Vander Borght (2010) pointed out that seeds conserved in germplasm banks should be evaluated for their viability rather than their germinative ability. Most germplasm banks test the seed conservation process by periodically measuring changes in seed germination capacity and viability (Pérez-García, Gómez-Campo, & Ellis, 2009; Pérez-García, González-Benito, & Gómez-Campo, 2007).

It has been difficult to germinate seed accessions of *Narcissus pseudonarcissus* L. (Amaryllidaceae) conserved in the Millennium Seed Bank collections (Royal Botanical Gardens, Kew, UK). The seeds have high MC (>50%) at dispersal time and suffer a drastic loss of viability when they are desiccated to 6–7%. Newton, Hay, and Ellis (2013) postulated that there is no concrete evidence that these seeds are non-recalcitrant. In fact, maturation drying is a non-recalcitrant seed development phase. In it, the seeds lose water and tolerate desiccation (Bewley & Black, 1994). At the other extreme, maturation drying is markedly reduced or absent altogether in recalcitrant seeds and there is minimal water loss at dispersal time (Newton et al., 2013).

The Amaryllidaceae are extremely variable in terms of seed storage behavior. The American clades are predominantly non-recalcitrant, whereas the African clades are mainly recalcitrant. However, the seed storage status has not been determined for Eurasian lineages, including *Narcissus* (Berjak & Pammenter, 2001; Newton et al., 2013). This genus comprises perennial geophytes that are geographically concentrated in the Mediterranean region, especially in the Iberian Peninsula, southern France and Morocco (Blanchard, 1990). Many wild seed banks collect *Narcissus* accessions. Thus, it is important to establish the degree to which their seeds tolerate non-recalcitrant seed storage conditions for conservation, especially for threatened taxa such as those analyzed herein, for which *ex situ* conservation actions can crucially complement *in situ* strategies (Schoen & Brown, 2001).

The present study evaluated the germinative responses of four *Narcissus* taxa subjected to various seed conservation conditions. These species included *N. longispathus* Pugsley, *N. alcaracensis* Ríos et al., *N. radinganorum* Fern. Casas and *N. pseudonarcissus* L. subsp. *munozii-garmendiae* (Fern. Casas) Fern. Casas. All of these belong to Section *Pseudonarcissi*, produce seeds with underdeveloped embryos at dispersal time and morphologically resemble *N. pseudonarcissus*. Recently, Aedo (2013) subordinated the first three species into one. This disposition together with the wide variability in the MC of mature seeds supported the evaluation of seed storage behavior and partially resolved the aforementioned uncertainty proposed by Newton et al. (2013).

The overall aim of this work was to determine whether: (a) seeds undergoing maturation drying tolerate desiccation and conservation at freezing temperatures after dispersal (non-recalcitrant seeds); and (b) seeds not subjected to maturation drying are (i) sensitive to desiccation after dispersal (recalcitrant seeds) or (ii) unable to be conserved at freezing temperatures even when they are desiccation-tolerant (moderately recalcitrant seeds).

Here, we compared embryo growth and germination percentages among various *Narcissus* seeds stored for 2 years under laboratory conditions (22°C, 40–50% relative humidity (RH), seed MC ~4%) or desiccated with silica gel to 3% MC and stored in flame-sealed glass vials for 2 years at −10°C.

2 | MATERIAL AND METHODS

2.1 | Plant materials

2.1.1 | *Narcissus longispathus* Pugsley

This Iberian endemic species has a naturally fragmented distribution over the Baetic System (Cazorla and Mágina Mountains) in southeastern Spain (Medrano & Herrera, 2008). It is a strict habitat specialist occupying permanent stream banks and waterlogged meadows. It is very sensitive to natural habitat disturbances. Aedo (2013) regarded this species as a synonym of *N. pseudonarcissus* subsp. *nevadensis* (Pugsley) A. Fern. Nevertheless, it should be considered an independent taxon based on its unique morphology and well-defined germination ecology identity (Herranz, Copete, & Ferrandis, 2013a). At dispersal, *N. longispathus* seeds are dormant and have underdeveloped embryos. Embryos grow to a critical length of 3.80 mm and attain germination capacity after the seeds are moderately warm stratified for 2 months (1 month at 20/7°C + 1 month at 15/4°C), cold stratified at 5°C for 2 months, and incubated at 15/4°C for 30 days. These seeds have non-deep complex morphophysiological dormancy (MPD) (Herranz et al., 2013a).

2.1.2 | *Narcissus alcaracensis* Ríos, Rivera, Alcaraz and Obón

This Iberian endemic species is geographically restricted to the Baetic System (Alcaraz Mountains). It grows on the borders of calcareous peat bogs and seasonal lagoons covered by large sedge communities (Ríos-Ruiz, Rivera-Núñez, Alcaraz-Ariza, & Obón De Castro, 1999). *Narcissus alcaracensis* seeds have underdeveloped embryos and exhibit MPD. Stratification at 5°C breaks the MPD and enables the seeds to germinate over a wide range of temperatures. Germination percentage increases with seed age. The seeds have intermediate complex MPD. Moderate warm stratification is not essential for dormancy break but increases germination percentage when it precedes cold stratification (Herranz, Copete, & Ferrandis, 2013b).

2.1.3 | *Narcissus radinganorum* Fern. Casas

This species is endemic to the mountains of central-eastern Spain (Sierras de Palomera and Caroch, southern Iberian System, Valencia province), where it grows on grasslands with seasonal moisture. Unlike *N. longispathus* and *N. alcaracensis*, embryo growth in the seeds of *N. radinganorum* occurs during warm stratification (28/14°C, 25/10°C). Radicles emerge when the temperature decreases (15/4°C in the dark). However, shoot emergence requires a cold (5°C) 30-day period to break dormancy. Its seeds have deep simple epicotyl MPD (Herranz, Copete, Copete, & Ferrandis, 2015).

2.1.4 | *Narcissus pseudonarcissus L. subsp. munozii-garmendiae* (Fern. Casas) Fern. Casas

This Iberian endemic species is geographically limited to central-western Spain (Ciudad Real province), where it grows on the banks of seasonal streams predominantly covered by alder trees (*Alnus glutinosa* (L.) Gaertn.) and seasonally flooded streambeds in *Quercus pyrenaica* Willd. patches. Temperature and light conditions promoting its embryo growth and radicle/shoot emergence resemble those required by *N. radinganorum*. Its seeds also have deep simple epicotyl MPD (unpublished data). This taxon flowers in early February. In contrast, the three aforementioned species bloom in early April. Its seeds mature in late April to early May, about 1 month before the other three species. Its capsules turn dark yellow when ripe. They quickly open and release seeds with high MC (>40%). In contrast, the other three taxa produce capsules that are light brown upon ripening and retain their mature seeds for 2–3 weeks. The capsules of all the preceding taxa contain 40–45 seeds on average.

As they have narrow geographical distributions, only a few small populations and vulnerability to habitat disturbance, all four taxa are threatened and currently on the Red List of Spanish Vascular Flora (Moreno, 2008). The first three species are in the EN (In Danger of Extinction) category, whereas the fourth is classified as Vulnerable (VU).

2.2 | Seed sources

The *N. longispathus* seeds were collected from a population in Valdeazores (Cazorla Mountains, Jaén, southern Spain; 37° 56′ N, 2° 50′ W; 1,330 m.a.s.l.) on stream banks consisting of deep marly limestone soils. On June 17, 2014, ∼200 mature capsules (∼8,000 seeds) were collected.

The *N. alcaracensis* seeds were collected from a core population in Peñascosa (Albacete, southeastern Spain; 38° 39′ N, 2° 18′ W; 1,280 m.a.s.l.) on the borders of a small seasonal pond colonized by sedge communities. On

June 6, 2014, ~200 mature capsules (~8,000 seeds) were collected.

On May 22, 2014, ~200 mature capsules (~8,000 seeds) of *N. radinganorum* were collected from a core population in La Hunde (Ayora, Valencia, southeastern Spain; 39° 5′ N, 1° 12′ W; 980 m.a.s.l.).

The mature capsules of the first three species were light brown. They opened at the apical region to ~1/3 of the fruit length and retained most of the seeds. No closed, dark yellow capsules were collected. The harvested capsules were stored in sealed plastic bags to avoid desiccation and transported to the laboratory 2–4 hr later. Most of the seeds were released from the capsules in transit.

About 200 capsules (~8,500 seeds) of *N. pseudonarcissus* subsp. *munozii-garmendiae* were collected on May 2, 2014, in Sierra Madrona (Solana del Pino, Ciudad Real, central Spain; 38° 25′ N, 4° 2′ W; 820 m.a.s.l.) on the quarzitic borders of a stream colonized by ferns (*Pteridium aquilinum* (L.) Kuhn), covered by an *Alnus glutinosa* tree canopy and adjacent to the Robledillo River confluence. The dark yellow capsules were open and no seeds were retained. Thus, 200 40-cm fruiting stems were collected. They bore closed yellow capsules. Upon arrival in the laboratory, the stem bases were immersed in beakers filled with water and cultivated to maturity in trays large enough to intercept all falling seeds. Capsule opening and seed collection continued for 3 days.

All seeds were desiccated under laboratory room conditions (22°C, 40–50% RH) from the collection date until July 1, 2014. Then ~2,000 seeds from each taxon were stored in glass jars at room temperature until analysis on October 1, 2016. The remaining seed lots were desiccated with silica gel inside a hermetic chamber for 1 months. On August 1, 2014, the seeds were transferred to flame-sealed glass vials containing 2 mL dehydrated silica gel isolated from the seeds by a layer of hygrophilous cotton (Gómez-Campo, 1985) and stored in a cold chamber (Euroclima, Córdoba, southern Spain) at −10°C until October 1, 2016.

We only tested seeds collected in the same year to minimize interannual variability (Herranz, Ferrandis, & Martínez-Duro, 2010). The seeds were not stored for >2 years as extended storage could reduce seed viability under laboratory conditions.

2.3 | Seed moisture content

The seed MC was determined by a standard drying method as follows: three replicate lots of 50 seeds were weighed, desiccated in a standard oven (103°C, 17 hr; ISTA, 2003), and reweighed to obtain the mean dry weight. The seed MC was calculated as a percentage of the fresh seed mass.

The MC was measured (a) for mature or nearly mature seeds in unopened yellow capsules, (b) for recently collected seeds (2–4 hr after field harvest) or immediately after release (*N. pseudonarcissus* subsp. *munozii-garmendiae*), (c) 7 days after collection or dispersal, (d) at the end of laboratory seed desiccation on July 1, 2014, and (e) on August 1, 2014, 1 month after desiccation with silica gel. Differences in seed MC between (a) and (b) were used as indicators of seed maturation drying.

2.4 | Germination tests and embryo growth

2.4.1 | General conditions

Four replicates of 25 seeds were assigned to various temperatures and light conditions. Each replicate was placed in a 9-cm Petri dish on a double layer of filter paper moistened with a distilled water solution of Benomyl® (2,000 mg /L) and sealed with Parafilm® to avoid water loss.

The experiments were conducted under temperature and light-controlled conditions in germination chambers (Ibercex Model F-4; Madrid, Spain) fitted with a digital temperature- and light-control system (±0.1°C; cold white fluorescent light; 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ [1,350 lx]). The seeds were stratified and incubated under a 12-hr light/12-hr dark photoperiod (light) and in constant darkness (dark). The latter was achieved and maintained by wrapping the Petri dishes with a double layer of aluminum foil. Studied temperatures were a constant 5°C and several 12-hr/12-hr temperature regimes of 15/4°C, 20/7°C, 25/10°C and 28/14°C. For the 12-hr/12-hr alternating temperature treatments, the high temperature coincided with the light phase and the low temperature coincided with the dark phase to simulate day/night conditions.

The fluctuating temperatures simulated natural outdoor mean maximum and minimum monthly temperatures. The annual climate cycle in the Mediterranean mountains at 900–1,200 m.a.s.l. was as follows: 15/4°C corresponded to March and November, 20/7°C corresponded to April and October, 25/10°C corresponded to May and September, and 28/14°C corresponded to June, July and August. The 5°C treatment simulated the mean temperatures for December, January and February.

2.4.2 | *Narcissus longispathus* and *N. alcaracensis*

To promote embryo growth and germination, the seeds of these species were exposed to moderately warm wet stratification and then cold stratification as follows:

1 month at 20/7°C + 1 month at 15/4°C + 2 months at 5°C. The seeds were incubated at 15/4°C in the light and dark. These conditions were determined by Herranz et al. (2013a, 2013b) to be optimal for germination.

On October 1, 2016, before stratification, 10 lots of 100 seeds each were prepared for each species and conservation temperature (22°C and −10°C). Each lot was further subdivided into four 25-seed replicates. Five lots were stratified in the light and the other five in the dark. At the end of each month, the germination percentage was recorded for each seed lot. These data were referred to as the numbers of viable seeds, namely, those whose embryo and endosperm were white and firm (Ryu et al., 2019). Twenty-five healthy seed embryos were evaluated. The embryo lengths of the germinated seeds were assumed to equal the critical embryo length (Table 1). This parameter is the length of the embryo at the time the seed coat splits open but before radicle emergence (Vandelook & Van Assche, 2008).

2.4.3 | *Narcissus radinganorum* and *N. pseudonarcissus* subsp. *munozii-garmendiae*

Seeds of these species were exposed to two warm treatments in the light (3 months at 25/10°C and 3 months at 28/14°C). At the end of each treatment, they were incubated at 15/4°C in the light and in the dark for 45 days. Warm stratification was performed only in the light as fungal infection commonly occurs in Petri dishes at high temperatures (≥25/10°C). Thus, the seeds had to be washed weekly under a stream of cold water during the first month of stratification. During warm stratification in the dark, it is difficult to shield the seeds from light while washing them. In contrast, warm stratification in the light and incubation at 15/4°C in the dark achieved 100% radicle emergence in previous studies on *N. radinganorum* (Herranz et al., 2015).

On October 1, 2016, before stratification, 10 lots of 100 seeds each were prepared for each species and

TABLE 1 Seed size, embryo length upon seed dispersal and critical embryo length in mm (mean ± standard error; $n = 25$) of the *Narcissus* taxa in the present study

	Seed length	Embryo length upon seed dispersal	Critical embryo length	Critical embryo length range
N. alcaracensis	3.99 ± 0.04	1.42 ± 0.03	3.30 ± 0.06	2.8–3.9
N. longispathus	4.09 ± 0.05	1.50 ± 0.05	3.80 ± 0.06	3.3–4.2
N. radinganorum	3.38 ± 0.04	1.36 ± 0.02	2.62 ± 0.04	2.2–3.0
N. pseudonarcissus subsp. *munozii-garmendiae*	3.36 ± 0.03	1.53 ± 0.02	2.52 ± 0.05	2.2–3.1

TABLE 2 Embryo growth (mm; mean ± standard error; $n = 25$) and germination percentage (brackets; mean ± standard error; $n = 4$) of *Narcissus radinganorum* seeds dry-conserved at various temperatures and stratified during specific months

		Temperature during previous storage			
		22°C		−10°C	
Temperature during stratification→		25/10°C Light	28/14°C Light	25/10°C Light	28/14°C Light
Duration of stratification	1 month	1.61 ± 0.03^{a} (0)	1.68 ± 0.04^{a} (0)	1.58 ± 0.04^{a} (0)	1.60 ± 0.04^{a} (0)
	2 months	1.82 ± 0.05^{a} (0)	1.78 ± 0.04^{a} (0)	1.75 ± 0.04^{a} (0)	1.81 ± 0.04^{a} (0)
	3 months	1.92 ± 0.04^{a} (0)	1.83 ± 0.03^{a} (0)	1.88 ± 0.05^{a} (0)	1.84 ± 0.04^{a} (0)
	Treatment A	2.06 ± 0.06^{a} (11 ± 1.6)^{A}	2.20 ± 0.06^{a} (12 ± 1.3)^{A}	2.06 ± 0.05^{a} (9 ± 2.5)^{A}	2.18 ± 0.05^{a} (10 ± 2.2)^{A}
	Treatment B	**2.54 ± 0.04^{a} (90 ± 2.2)^{A}**	**2.60 ± 0^{a} (100 ± 0)^{B}**	**2.52 ± 0.04^{a} (90 ± 1)^{A}**	**2.60 ± 0^{a} (100 ± 0)^{B}**

Note: Treatment A: incubation at 15/4°C in the light for 45 days after 3 months stratification at 25/10°C or 28/14°C in the light. Treatment B: incubation at 15/4°C in the dark for 45 days after 3 months stratification at 25/10°C or 28/14°C in the light. Different lowercase (embryo length) and uppercase (germination percentage) letters in a row denote significant differences ($p < .05$).

conservation temperature (22°C and −10°C). Each lot was further subdivided into four 25-seed replicates. Five lots were stratified at 25/10°C in the light and the other five at 28/14°C in the light. At the end of each monthly period and at the end of the 45-day incubation at 15/4°C in the light and dark (Tables 2 and 3), the germination percentages were recorded and 25 embryos were measured under the assumption that the germinated seeds had reached their critical embryo length (Table 1).

2.5 | Statistical analysis

The effects of seed conservation condition, temperature, length of stratification and light conditions upon embryo length and germination percentage were analyzed by multifactorial ANOVA. When the effect of a factor was significant, treatment differences were compared by multiple-comparison Tukey's test. Significant interactions were evaluated by contrasting confidence intervals. Before the analyses, data normality (Cochran's test) and homoscedasticity (David's test) were assessed. The germination percentages were root-squared arcsine-transformed to fit the data to a normal distribution. In the tables, the untransformed germination percentages are shown.

3 | RESULTS

3.1 | Seed moisture content

At maturity (unopened yellow capsules), the seed moisture levels were 47.4 ± 0.4% for *N. alcaracensis*, 46.4 ± 0.2% for *N. longispathus*, 47.8 ± 0.3% for *N. radinganorum*, and 49.5 ± 0.2% for *N. pseudonarcissus* subsp. *munozii-garmendiae*.

For the recently dispersed seeds, *N. pseudonarcissus* subsp. *munozii-garmendiae* had the highest MC (46.7 ± 0.6%). After 5 days at 22°C, however, this value decreased to 4.8 ± 0.1%. For the other *Narcissus* taxa, the MC of the recently dispersed seeds ranged from 8.2 ± 0.1% in *N. radinganorum* to 10.1 ± 0.1% in *N. longispathus*. MC also ranged from 3.9 ± 0.2% to 4.2 ± 0.1% after 5 days at room temperature (Figure 1). These values were slightly different during the period when the seeds were stored in the laboratory at 22°C. After 1 month desiccation with silica gel, the seed moisture

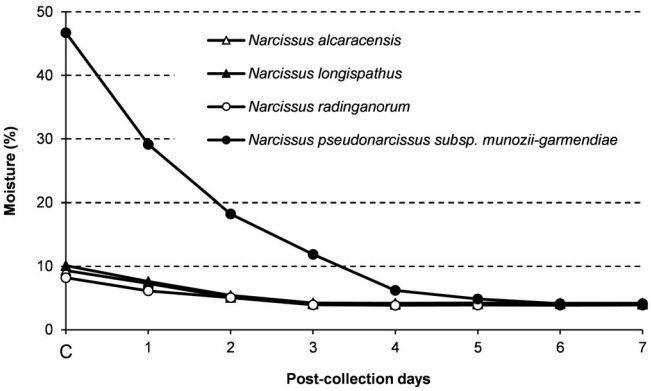

FIGURE 1 Changes in seed moisture content (fresh weight basis) between collection (C) and 1 week after desiccation under laboratory conditions (22°C; 40% relative humidity). For *Narcissus pseudonarcissus* subsp. *munozii-garmendiae*, C represents dispersal time. Standard error bars are not shown as they were all <1%

TABLE 3 Embryo growth (mm; mean ± standard error; $n = 25$) and germination percentage (brackets; mean ± standard error; $n = 4$) of *Narcissus pseudonarcissus subsp. munozii-garmendiae* seeds dry-conserved at various temperatures and stratified during specific months

		Temperature during previous storage			
		22°C		−10°C	
Temperature during stratification→		25/10°C Light	28/14°C Light	25/10°C Light	28/14°C Light
Duration of stratification	1 month	1.72 ± 0.03[a] (0)	1.65 ± 0.03[a] (0)	Non-viable seeds	
	2 months	1.77 ± 0.03[a] (0)	1.80 ± 0.03[a] (0)		
	3 months	1.95 ± 0.03[b] (0)	1.86 ± 0.03[a] (0)		
	Treatment A	2.35 ± 0.04[a] (52 ± 2.45)[A]	2.35 ± 0.04[a] (63 ± 2.96)[B]		
	Treatment B	2.48 ± 0.02[a] (96 ± 1.41)[A]	2.50 ± 0[a] (100 ± 0)[B]		

Note: Treatment A: incubation at 15/4°C in the light for 45 days after 3 months stratification at 25/10°C or 28/14°C in the light. Treatment B: incubation at 15/4°C in the dark for 45 days after 3 months stratification at 25/10°C or 28/14°C in the light. Different lowercase (embryo length) and uppercase (germination percentage) letters in a row denote significant differences ($p < .05$).

TABLE 4 Embryo growth (mm; mean ± standard error; $n = 25$) and germination percentage (brackets; mean ± standard error; $n = 4$) of *Narcissus alcaracensis* seeds dry-conserved under various temperature regimens and stratified during specific months in the light or dark

		Temperature during previous storage			
		22°C	**−10°C**	**22°C**	**−10°C**
Illumination during stratification→		Light	Light	Dark	Dark
Stratification conditions	1 month at 20/7°C	1.68 ± 0.03^{ab} (0)	1.62 ± 0.02^{a} (0)	1.85 ± 0.07^{c} (0)	1.79 ± 0.05^{bc} (0)
	1 month at 20/7°C + 1 month at 15/4°C	1.91 ± 0.04^{a} (0)	1.93 ± 0.04^{a} (0)	2.46 ± 0.07^{c} (0)	2.19 ± 0.06^{b} (0)
	1 month at 20/7°C + 1 month at 15/4°C + 1 month at 5°C	2.30 ± 0.05^{a} (0)	2.28 ± 0.06^{a} (0)	2.63 ± 0.06^{b} (0)	2.57 ± 0.05^{b} (0)
	1 month at 20/7°C + 1 month at 15/4°C + 2 months at 5°C	2.56 ± 0.08^{a} $(0)^{A}$	2.57 ± 0.05^{a} $(0)^{A}$	3.50 ± 0.07^{b} $(32.2 \pm 2.6)^{C}$	2.89 ± 0.07^{b} $(20 \pm 2.83)^{B}$
	1 month at 20/7°C + 1 month at 15/4°C + 2 months at 5°C + 1 month at 15/4°C	**3.14 ± 0.05^{b} $(72.7 \pm 2.2)^{B}$**	**2.87 ± 0.07^{a} $(24.2 \pm 1.4)^{A}$**	**3.25 ± 0.05^{b} $(92.9 \pm 2.9)^{C}$**	**3.28 ± 0.02^{b} $(96 \pm 1.4)^{C}$**

Note: Different lowercase (embryo length) and uppercase (germination percentage) letters in a row denote significant differences ($p < .05$).

TABLE 5 Embryo growth (mm; mean ± standard error; $n = 25$) and germination percentage (brackets; mean ± standard error; $n = 4$) of *Narcissus longispathus* seeds dry-conserved under various temperature regimens and stratified during specific months in the light or dark

		Temperature during previous storage			
		22°C	**−10°C**	**22°C**	**−10°C**
Illumination during stratification→		Light	Light	Dark	Dark
Stratification conditions	1 month at 20/7°C	1.86 ± 0.05^{a} (0)	1.86 ± 0.04^{a} (0)	1.86 ± 0.03^{a} (0)	1.89 ± 0.06^{a} (0)
	1 month at 20/7°C + 1 month at 15/4°C	1.92 ± 0.05^{a} (0)	1.92 ± 0.06^{a} (0)	2.28 ± 0.07^{b} (0)	2.33 ± 0.06^{b} (0)
	1 month at 20/7°C + 1 month at 15/4°C + 1 month at 5°C	2.51 ± 0.07^{ab} (0)	2.30 ± 0.05^{a} (0)	2.92 ± 0.10^{c} (0)	2.66 ± 0.06^{b} (0)
	1 month at 20/7°C + 1 month at 15/4°C + 2 months at 5°C	2.87 ± 0.06^{a} $(0)^{A}$	2.68 ± 0.06^{a} $(0)^{A}$	3.23 ± 0.09^{b} $(24.1 \pm 2.3)^{B}$	3.30 ± 0.09^{b} $(20.1 \pm 2.7)^{B}$
	1 month at 20/7°C + 1 month at 15/4°C + 2 months at 5°C + 1 month at 15/4°C	**3.25 ± 0.10^{b} $(36.2 \pm 2.6)^{B}$**	**2.84 ± 0.09^{a} $(4 \pm 1.4)^{A}$**	**3.76 ± 0.03^{c} $(92.8 \pm 2.2)^{C}$**	**3.67 ± 0.06^{c} $(88.8 \pm 2.9)^{C}$**

Note: Different lowercase (embryo length) and uppercase (germination percentage) letters in a row denote significant differences ($p < .05$).

level was 2.5 ± 0.2% for *N. radinganorum*, 2.9 ± 0.1% for *N. alcaracensis*, 3.1 ± 0.1% for *N. pseudonarcissus* subsp. *munozii-garmendiae*, and 3.2 ± 0.1% for *N. longispathus*.

3.2 | Germination and embryo growth

For *N. alcaracensis* and *N. longispathus*, embryo growth and germination percentage after stratification and incubation in the light were significantly lower in seeds stored under non-recalcitrant conservation conditions (3% moisture; −10°C) than in those under laboratory conditions (4% moisture; 22°C) (Tables 4 and 5). However, seeds of both species that were stratified and incubated in the dark broke dormancy and germinated more readily than those maintained in the light. There were no significant differences between seed conservation methods in terms of the aforementioned parameters. The final germination percentages of the *N. alcaracensis* seeds conserved at room temperature and at −10°C were 92.9% and 96%, respectively. For *N. longispathus*, they were 92.8% and 88.8%, respectively (Tables 4 and 5).

For *N. radinganorum*, embryo growth and germination percentage were similar for the seeds conserved at 22°C and −10°C when they were stratified at 25/10°C or 28/4°C in the light and when they were incubated at

15/4°C in the light (Treatment A) or at 15/4°C in the dark for 45 days (Treatment B). After Treatment B, the germination percentages were ≥90% (Table 2).

In the *N. pseudonarcissus* subsp. *munozii-garmendiae* seeds desiccated to 4% MC and stored at 22°C, germination percentages were 52% and 63% at 15/4°C in the light (Treatment A), after 3 months of stratification at 25/10 or 28/14°C, respectively, and were 96% and 100% at 15/4°C in the dark (Treatment B), after the same stratification conditions, respectively (Table 3). However, the seeds desiccated to 3.1% MC and stored at −10°C lost viability despite the fact that the silica gel remained orange throughout seed storage and effectively intercepted any moisture entering the glass vials. After 1 week of warm stratification at 25/10°C or 28/14°C in the light, the seeds were infected by fungi and rotting. After 3 weeks of warm stratification, many seeds disintegrated after being washed under a water stream and gently pressed with tweezers. A subsequent tetrazolium test on a seed lot conserved at −10°C and not subjected to warm stratification confirmed that the seeds had lost viability.

4 | DISCUSSION

Germplasm banks around the world constitute a strategical tool for the conservation of plant diversity and crops. According to our results, some factors, such as seed MC at the time of dispersal, strongly influenced by its maturation degree, might adversely affect the conservation success in germplasm collections. *Narcissus alcaracensis* and *N. longispathus* seeds stratified and incubated in the dark had greater embryo lengths and germination percentages than those stratified and incubated in the light. This finding corroborates the results of previous studies (Herranz et al., 2013a, 2013b), conducted with seeds of these species dry-stored between 0 and 12 months; storage, stratification and incubation conditions were similar to those in this study. Seeds of both species tolerated non-recalcitrant seed conservation conditions (∼3% moisture; −10°C). Embryo growth and germination percentage after stratification and incubation in the dark resembled those obtained for the seeds stored at 22°C. Embryo growth and germination percentages of seeds stratified and incubated in the light were lower for those previously stored at −10°C than for those stored at 22°C. These results show a certain degree of recalcitrance and must be considered when the ultimate goal of seed conservation is to produce plants for population-reinforcement programs because conservation efforts may adversely affect the physiological characteristics of seeds even when they remain viable. For example, *Alyssoides utriculata* (L.) Medik. and *Matthiola sinuata* (L.) R. Br. were induced to secondary dormancy when they were conserved at −10°C (Pérez-García et al., 2007).

Narcissus radinganorum seeds warm stratified under light conditions had higher germination percentages in the dark than in the light. This finding aligns with those reported in an earlier study (Herranz et al., 2015). These seeds were well conserved under non-recalcitrant seed storage conditions (2.5% moisture; −10°C) and their embryo growth and germination percentages were similar to those seeds conserved at 22°C.

All three *Narcissus* species tolerating non-recalcitrant seed conservation conditions had low seed MC (8–10%) at the time of dispersal (Figure 1) because they underwent maturation drying on the mother plant. The seed MC prior to the opening of the mature yellow capsule was 46–48%. In these species, the seeds were retained in the apical-opened capsules for several weeks and seed dispersion occurred after the standard maturation process.

In contrast, *N. pseudonarcissus* subsp. *munozii-garmendiae* seeds did not undergo maturation drying. In fact, their MC was 46.7% at the time of dispersal (Figure 1). Nevertheless, their storage behavior resembled that of *N. radinganorum* seeds when they were desiccated down to 4% MC and stored at room temperature (22°C). At 15/4°C, in the dark their germination percentage was 100%, and in the light it was higher than for *N. radinganorum* seeds (Tables 2 and 3). However, they lost viability during storage at −10°C and 3.1% MC. Thus, *N. pseudonarcissus* subsp. *munozii-garmendiae* seeds were moderately recalcitrant. They tolerate dehydration and low MC but are chilling sensitive in this state; some authors have classified this storage behavior as intermediate (e.g., Berjak & Pammenter, 1994). Seeds of *Caesalpinia echinata* Lam. behave in a similar way because they are highly tolerant of desiccation, surviving even when the water content is as low as 5%, but with low storage capacity (Martini-Neto & Barbedo, 2015). Ellis, Hong, and Roberts (1990) stated that most seeds with an intermediate degree of recalcitrance tolerate lower MC and temperatures than tropical recalcitrant seeds but are not as tolerant of desiccation and chilling as non-recalcitrant seeds. Newton et al. (2013) suggested that neither *Galanthus nivalis* L. nor *N. pseudonarcissus* seeds had intermediate degrees of recalcitrance as they can both withstand drying down to 6–7%. However, Berjak and Pammenter (2001, 2008) indicated that there is a wide range of seed storage behaviors that comprise a continuum or spectrum of relative desiccation and temperature tolerance/sensitivity. In the same way, according to Walters (2015), the classical distinction between orthodox and recalcitrant seeds limits a better understanding of the broad spectrum of physiological responses presented by seeds, which should be understood as a gradient of responses. One way of interpreting this gradient could be the degree of maturation at which

seeds are detached from the mother plant, with the recalcitrant ones in a very immature stage (Barbedo, 2018; Barbedo et al., 2013). Our results confirm that *N. pseudonarcissus* subsp. *munozii-garmendiae* seeds, which did not undergo maturation drying on the mother plant and did not tolerate freezing temperatures, are moderately recalcitrant, unlike those of the other species studied in this paper. It could be deduced that *N. pseudonarcissus* subsp. *munozii-garmendiae* seeds shortened their whole process of maturation, anticipating the dispersion. This might have affected their vigor and longevity, and increased their degree of recalcitrance in comparison with the other species studied. In practice, the collection of *N. pseudonarcissus* subsp. *munozii-garmendiae* seeds close to maturity was very difficult in nature and the required seeds for this study were obtained in laboratory conditions from fruiting stems (see Material and Methods: seed sources).

Here, the three species whose dried seeds tolerated storage at −10°C (*N. alcaracensis*, *N. longispathus* and *N. radinganorum*) differed in terms of their environmental dormancy breaking and germination requirements. They also had various MPD levels (Herranz et al., 2013a, 2013b, 2015). Thus, the ability of a seed to tolerate non-recalcitrant conservation conditions depends on its maturation drying on the mother plant rather than its MPD level.

The dormancy break, embryo length and germination requirements of the *N. pseudonarcissus* subsp. *munozii-garmendiae* seeds were similar to those for *N. radinganorum* (this study; unpublished data). The latter also had deep simple epicotyl MPD. However, *N. pseudonarcissus* subsp. *munozii-garmendiae* seeds do not tolerate storage under non-recalcitrant seed conservation conditions as they do not undergo pre-dispersal maturation drying (Bewley & Black, 1994). The relative habitat differences between these *Narcissus* species may also explain the comparative differences in their seed storage behavior (Barbedo et al., 2013; Berjak & Pammenter, 1994). *Narcissus pseudonarcissus* subsp. *munozii-garmendiae* seeds ripen in mid-spring (late April to early May), almost 1 month before *N. radinganorum* seeds. Moreover, the former requires higher rainfall and RH and the dense tree canopy shadow created by *Alnus glutinosa* and/or *Quercus pyrenaica*. As a consequence of cooler temperatures during seed development, the *N. pseudonarcissus* subsp. *munozii-garmendiae* seeds could be shed at a less developed stage than those of *N. radinganorum*, as seen in the germination responses of the recalcitrant seeded species *Aesculus hippocastanum* L. (Daws et al., 2004). Thus, studies performed with seeds maturated under different environmental conditions are essential to clarify the influence of the habitat. In addition, important information could be obtained from studies on the immature stages of non-recalcitrant seeds.

Our results strongly suggest that seed storage behavior is influenced primarily by the degree of maturation drying of seeds on the mother plant. Further studies are necessary to verify whether the model herein may be validated using closely related species.

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