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Non-deep complex morphophysiological dormancy in *Narcissus longispathus* (Amaryllidaceae): implications for evolution of dormancy levels within section *Pseudonarcissi*

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Abstract

Narcissus longispathus (Amaryllidaceae) is a perennial geophyte and a Mediterranean narrowly endemic species. At dispersal time, *N. longispathus* seeds are dormant and have underdeveloped embryos. This work aimed to determine requirements for dormancy break and germination and to compare dormancy traits with those of the two endemic Iberian congeners. Phenology of embryo growth and germination were studied by regularly exhuming seeds sown in near-natural conditions. Temperature and light requirements for embryo growth, breaking of dormancy and germination were determined by incubating seeds under controlled laboratory conditions. Mean embryo length in fresh seeds was 1.50 mm, and embryos had to grow to 3.80 mm before radicle emergence. Embryos grew to full size and seeds germinated when they were warm stratified for 2 months (optimum 1 month at 20/7°C + 1 month at 15/4°C), then cold stratified at 5°C for 2 months, and finally incubated at cool temperatures (15/4°C) for 30 d. However, in seeds only subjected to either warm or cold stratification, the embryos hardly grew and did not germinate. In natural conditions, the embryos elongate in autumn–winter, and in late winter–early spring (March) almost all radicles and seedlings emerged. Velocity of embryo growth and germination percentages increased with seed storage duration. Seeds of *N. longispathus* have non-deep complex morphophysiological dormancy (MPD). This is the first report of such a level of MPD in *Narcissus*. Our data suggest that non-deep complex MPD may have been derived from intermediate complex MPD in the section *Pseudonarcissi*.

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Introduction

Many species in temperate forests of the northern hemisphere produce seeds with an underdeveloped embryo that must elongate to a critical length inside the seed before the radicle emerges. These seeds are defined as morphologically dormant (MD). If an additional physiological mechanism preventing embryo growth and germination occurs, seeds have morphophysiological dormancy (MPD) (Nikolaeva, 1977). There have been numerous studies on germination ecology of seeds with MPD for species of North America (Baskin and Baskin, 1998; Forbis and Diggle, 2001; Scholten *et al.*, 2009), eastern Asia (Kondo *et al.*, 2004; Phartyal *et al.*, 2009) and western Europe (Vandelook *et al.*, 2007; Vandelook and Van Assche, 2008a, b). However, in the circum-Mediterranean region, where 25,000 species of flowering plants occur, half of them being endemic (Medail and Quezel, 1997), studies on seeds with MPD are scarce. So it is necessary to fill a gap in our knowledge about MPD in Mediterranean species, especially those belonging to families such as Amaryllidaceae, the germination ecology of which is little known (Baskin and Baskin, 1998). On the other hand, in a previous study (Herranz *et al.*, 2013) we found that seeds of *Narcissus alcaracensis* Ríos have intermediate complex MPD. The present work focuses on verification of the hypothesis that morphological differences between *N. alcaracensis* and the phylogenetically closely related species *Narcissus longispathus* Pugsley are associated with differences in ecophysiological traits such as dormancy and germination characteristics, since *N. longispathus* inhabits environments with warmer temperatures and more

abundant rainfall than those in which *N. alcaracensis* is found (Valle *et al.*, 1989). We also hypothesize that the two species have different levels of MPD.

Narcissus is a genus of perennial geophytes in the Amaryllidaceae, and it is geographically concentrated in the Mediterranean region, particularly in the Iberian Peninsula, southern France and Morocco (Blanchard, 1990). The taxonomy of *Narcissus* is unsettled and substantial revisionary work is needed for some taxa in the genus (Mathew, 2002). Thirty-six of the 65 species recognized by Blanchard (1990) are endemic to the Iberian Peninsula (Moreno and Sainz, 1992), thus revealing the importance of this geographic area in the evolution of *Narcissus* and in the development of studies on seeds with MPD.

N. longispathus belongs to the section *Pseudonarcissi*, the most diversified section in the genus. The Iberian Peninsula is the geographical centre of origin of the *Pseudonarcissi* section, where around 20 new taxa have been described during the past five decades. However, morphological differences between them are often not clearly delimited due to a recent and ongoing speciation process (Dorda and Fernández-Casas, 1994; Ríos-Ruiz *et al.*, 1999). *N. longispathus* is a good example of a Mediterranean narrowly endemic species with a naturally severely fragmented distribution, and it is restricted to a few contiguous mountain ranges in the Baetic System (Cazorla and Mágina mountains) in south-eastern Spain (Moreno and Sainz, 1992; Herrera *et al.*, 1999). The species is a strict habitat specialist, occupying poorly drained, deep soils around springs, permanent stream banks and waterlogged meadows. Throughout its small geographical range, *N. longispathus* occurs as discrete, small populations varying in size from dozens to a few thousand plants. For all these reasons, as well as for its vulnerability to natural habitat disturbances, the species has been included in the Red List of Spanish Vascular Flora (Aizpuru *et al.*, 2000; Moreno, 2008), as well as in the *Atlas and Red Book of Endangered Vascular Flora in Spain* (Bañares *et al.*, 2003) in the category EN 'In Danger of Extinction'.

Although *N. longispathus* has a high capacity of vegetative propagation with bulbs (Herrera *et al.*, 1999), its cultivation for ornamental purposes must be done with seeds to avoid damaging impacts on natural populations. However, the growth of a threatened species from seeds requires knowledge about its germination ecology. In addition, the reinforcement of wild plant populations using individuals raised *ex situ* must also be achieved with seeds, to maximize genetic diversity (Cerabolini *et al.*, 2004). Thus, to conserve threatened plant species it is necessary to have basic information on seed dormancy break, germination ecology and seedling establishment. Such information is crucial for both *ex situ* propagation and management programmes in nature, and is required to understand the ecological life cycle of the species and adaptations to its habitats (Navarro and Guitián, 2003; Giménez-Benavides *et al.*, 2005).

The *N. longispathus* embryo is linear, with the radicular end touching the base of the seed. In freshly matured seeds, the embryo length is 1.50 ± 0.05 mm versus a seed size of 4.09 ± 0.05 mm (mean $\pm$ SE, $n = 25$), suggesting that the embryo is underdeveloped. The small embryo and the lack of germination during 1 month of incubation at temperatures occurring in its natural habitat during the course of the year (5, 15/4, 20/7, 25/10, 28/14 and 32/18°C) strongly suggest that seeds of *N. longispathus* have some level of MPD.

The conditions necessary for germination have been studied extensively in only a few species of *Narcissus*, and all of them belong to section *Pseudonarcissi* which have underdeveloped embryos at the time of seed dispersal. In *N. pseudonarcissus* L. (Vandelook and Van Assche, 2008a) and *N. hispanicus* (Copete *et al.*, 2011a), growth of the embryo is continuous throughout summer. However, in these two species radicle emergence occurs in early autumn, but shoot emergence is delayed until late winter–early spring, thus corresponding to the phenology of deep simple epicotyl MPD. In *N. alcaracensis* (Herranz *et al.*, 2013) embryo growth occurs during cold stratification, and radicle and shoot emergence happen at almost the same time in late winter–early spring. Seeds of this species have intermediate complex MPD.

In this work, we study the seed dormancy and germination ecology of *N. longispathus*. Specifically, the objectives were to analyse and determine: (1) the phenology of germination and embryo growth; (2) the germination responses of seeds buried in soil and exhumed periodically; (3) the influence of cold stratification at different temperatures, warm stratification preceding cold stratification, light/darkness conditions during the stratification and incubation treatments, seed storage duration and year of seed collection on germination; (4) the effects of temperature and light/darkness conditions, as well as GA_3 on dormancy break and embryo growth; and (5) the influence of seed storage duration on embryo growth.

Materials and methods

Plant material

N. longispathus is a bulbous geophyte, 40–150 cm high when bearing fruit, with one or two $40–60 \times 0.6–1$ cm leaves and flowering stems with one flower (sometimes two) in the apex, with a long spathe (6–10 cm). Flowering occurs from late February until late April, and fruit maturation and seed dispersal take place during the first half of June. The species is

self-compatible, but in the absence of pollinators (*Andrena bicolor*, Andrenidae) flowers hardly ever set seeds. Thus, *N. longispathus* has a mixed mating system, producing both outcrossed and selfed seeds, but few selfed offspring survive to maturity because of high inbreeding depression (Barret *et al.*, 2004; Medrano and Herrera, 2008).

For this study, seeds were collected from the population in Valdeazores (Sierra de Cazorla, Jaen, 30S WG1499, 1330 m above sea level), formed by about 2000 flowering ramets. This population is located on the banks of streams and in permanently waterlogged meadows, on deep marly limestone soils, colonized by herbaceous perennial communities dominated by *Pteridium aquilinum*, *Scirpus holoschoenus*, *Holcus lanatus*, *Cirsium flavispina*, *Schoenus nigricans*, *Aquilegia vulgaris* and *Carex flacca*.

On 15 June 2003, about 550 intact capsules (approximately 22,000 seeds); on 18 June 2004, about 125 intact capsules (approximately 5000 seeds); and on 17 June 2006, about 250 intact capsules (approximately 10,000 seeds) were collected. Seeds were desiccated at room temperature in the laboratory (21–22°C, relative humidity 50–60%) until 1 July in each collection year. At that time, the first tests were started, considering seeds to be aged 0 months. Seeds not used in early tests were kept in paper envelopes at room temperature until they were tested.

Outdoor experiments

Phenology of embryo growth

On 1 July 2003, 50 seeds mixed with sand were placed in each of 12 fine-mesh polyester cloth bags. Bags were buried to a depth of 5 cm in a pot filled with a mixture of peat and sand (3:1 v/v) and placed in an unheated metal-framed shadehouse on the experimental campus of the Technological School of Agronomy in Albacete (150 km from Sierra de Cazorla). This pot was watered to field capacity once a week from 1 October to 31 May and twice a month for the rest of the year, except on winter days when the soil was frozen, trying to mimic the humidity conditions in the natural habitat. Temperatures in the shadehouse were recorded continuously by a thermograph in a weather station, and mean monthly maximum and minimum temperatures were calculated based on daily values. The bags were exhumed each month, starting on 1 August 2003, and the contents sieved (1 mm) to separate the seeds from the sand. Each time, 25 non-germinated seeds were recovered, and embryos were excised immediately and measured. The critical embryo length (i.e. 3.80 mm, see subsection Embryo growth in light, below) was assumed for seeds that germinated within the bag while buried.

Phenology of seedling emergence

On 1 July 2003, three replicates of 200 seeds each were sown in plastic seedtrays (40 × 30 × 5 cm) with drainage holes, in a substrate of sterile peat:sand (3:1), burying the seeds to a depth of 3–4 mm. The seedtrays were placed in the unheated shadehouse described above and subjected to the watering frequency indicated in the previous section. The seedtrays were examined at 7-day intervals until 1 April 2007, and emergent seedlings were counted and removed.

Dormancy break in buried seeds

On 1 July 2003, 200 seeds mixed with sand were placed in each of 12 fine-mesh polyester cloth bags. Bags were buried at a depth of 5 cm in a pot filled with peat and sand and placed in the unheated shadehouse, with a watering programme as described above, and then exhumed every month, starting on 1 August 2003.

Exhumed seeds were classified into one of four categories: (1) seeds germinated within the bag; (2) non-dormant viable seeds, which germinated after 30 d of incubation at 15/4°C in darkness once they were exhumed; (3) dormant viable seeds with a healthy embryo, which remained ungerminated after the previous test; (4) non-viable seeds, decomposed or with an unhealthy appearance, plus seeds ungerminated with an unhealthy embryo.

Laboratory experiments

Effects of stratification and light:dark, seed storage duration and year of collection on germination

Seeds were subjected to different stratification treatments: (1) 4 months of cold, moist stratification (5°C, 9/5°C), or (2) 2 months of warm, moist stratification (normally 1 month at 20/7°C followed by 1 month at 15/4°C), followed by 2 months of cold stratification (5°C). Each treatment was given in both light [12 h cool white fluorescent light, 25 μmol m^{-2} s^{-1} (1.350 lux) each day] and in continuous darkness, which was achieved by wrapping Petri dishes in a double layer of aluminium foil. After the stratification treatments, germination tests were carried out in light and dark at a constant temperature of 5°C and at alternating (12/12 h) temperature regimes of 15/4, 20/7, 25/10, 28/14 and 32/18°C; the light phase was programmed to coincide with the higher temperature, and the dark phase with the lower temperature.

A 100-seed lot was assigned to each treatment and control, distributed into four 25-seed replicates. Each replicate was placed in a 9-cm-diameter Petri dish on a double layer of filter paper moistened with distilled water and sealed with Parafilm, to avoid water loss.

Seeds given stratification treatments were incubated at the test temperatures for 30 d, seeds incubated

in light were checked for germination every 3–4 d. Seeds incubated in darkness were checked only after the end of the test. Ungerminated seeds were checked for viability on the basis of the embryo appearance, paying special attention to the colour and turgidity. Percentages of germination were calculated from viable seeds. The control seeds were incubated in both light and darkness for 5 months, to compare their results with those obtained with seeds stratified for 4 months and incubated for 1 month at different temperatures. Germination was checked every month and dishes incubated in darkness were handled under a green safe lamp (Luna *et al.*, 2004).

The fluctuating temperatures used in this study simulated mean maximum and minimum monthly temperatures during the growing season at medium altitudes on Sierra de Cazorla, the natural habitat of the species and the source region of seeds used in the study. Thus, 15/4°C corresponds to November and March, 20/7°C to October and April, 25/10°C to September and May, 28/14°C to August and June and 32/14°C to July. The 5°C treatment simulates the mean temperature recorded during winter months: December, January and February.

Collection year 2003

On 1 July 2003 fresh seeds (storage duration = 0 months) were tested for germination (control). Four replicates of 25 seeds each were incubated both in light and in darkness at 15/4, 20/7, 25/10, 28/14 and 32/18°C for 5 months. At monthly intervals, germinated seeds were counted and removed. On 1 March 2004 (seed storage duration = 8 months), the same control test described above was repeated. In addition, on 1 July 1 2003, 1300 seeds were placed in each of two 16-cm Petri dishes on two layers of filter paper moistened with 10 cm^3 of distilled water. Dishes were sealed with Parafilm and cold stratified (5°C), one dish in darkness and the other in light. The hydration status of seeds in dishes was checked monthly; seeds stratified in darkness were handled under a green safe lamp. At the end of the stratification period (4 months), ungerminated seeds with a healthy appearance were tested for germination. This cold stratification test was repeated in the same conditions on 1 March 2004 (seed storage duration = 8 months). On 1 July 2003, 1300 seeds were placed in each of two 16-cm Petri dishes and given warm followed by a cold stratification; one dish in light and one in darkness. Dish and seed handling, as well as temperatures and conditions in germination tests were the same as those described above, varying only in the sequence of temperatures in the stratification: 20/7°C for 1 month, 15/4°C for 1 month and 5°C for 2 months (Treatment A). To evaluate the influence of seed storage duration on germination ability, the test with seeds exposed to stratification Treatment A was repeated with seeds stored for 8 and 20 months.

Collection year 2004

To analyse the influence of the year of seed collection on the germination ability, seeds collected in 2004 were subjected to the stratification treatment that produced the highest germination percentages for seeds collected the previous year. On 1 March 2005 (seed storage duration = 8 months) seeds were subjected to Treatment A. The results were compared to those obtained with seeds collected in 2003 and dry stored for 8 months and also subjected to Treatment A.

Collection year 2006

After confirming that Treatment A was the most effective in breaking dormancy of seeds collected in 2003, we decided to investigate the efficiency of other warm + cold stratification treatments in breaking dormancy. In addition, we used another cold stratification temperature, apart from 5°C. These tests were carried out with seeds collected on 17 June 2006. Germination tests were carried out only at temperatures demonstrated to promote high germination percentages in previous tests (i.e. 5, 15/4 and 20/7°C). On 1 July 2006 (seed storage duration = 0 months), 650 seeds were placed in each of two 16-cm Petri dishes on two layers of filter paper moistened with 6 cm^3 of distilled water, assigning one dish to cold stratification (9/5°C) in light and the other one in darkness for 4 months. After cold stratification, ungerminated seeds were incubated. In addition, on 1 July 2006 a control test was initiated under the same conditions as those applied to seeds from 2003, but only at 15/4 and 20/7°C. On 1 July 2006, 650 seeds were placed in each of four 16-cm Petri dishes as described above. Dishes were subjected to a warm + cold stratification. Two dishes were subjected for 1 month to each of the temperatures in the following sequence: 25/10, 20/7, 5 and 5°C (Treatment B), one in light and the other in darkness. Similarly, the other two dishes were subjected for 1 month to each temperature in the following sequence: 28/14, 25/10, 5 and 5°C (Treatment C), one in light and the other in darkness. After both stratification treatments, ungerminated seeds were incubated. To gain more information about the influence of seed storage duration on germination ability, tests with seeds subjected to the stratification Treatments B and C were repeated on 1 March 2007 (seed storage duration = 8 months).

Effects of temperature, light/dark and seed storage duration on embryo growth

On 1 July 2003, 25 freshly matured seeds were placed on two sheets of filter paper moistened with distilled water in a 9-cm Petri dish. After seeds had imbibed at room temperature for 24 h, embryos were excised

with a razor blade and their length measured using a dissecting microscope equipped with a micrometer.

Embryo growth in light

On 1 July 2003 (seed storage duration = 0 months), 25 healthy-looking seeds were placed in each of 20 9-cm Petri dishes, and five dishes in each were placed at 5, 15/4, 25/10 and 28/14°C. After 1, 2, 3, 4 and 5 months (1 month = 30 d), the embryos in one dish at each temperature were excised and measured. Mean length and standard error were calculated for each sample of 25 embryos. In addition, on 1 July 2003, 350 seeds were placed in a 9-cm Petri dish, which was exposed for 1 month to each temperature regime in the following sequence: 20/7, 15/4, 5 and 5°C (Treatment A). Each month, embryos were excised from 25 seeds and measured, calculating the mean length and the standard error. After 4 months, when Treatment A ended, 25 ungerminated seeds with a healthy appearance were placed in each of four 9-cm Petri dishes and incubated at 5, 15/4, 25/10 and 28/14°C for 30 d (Treatment D). At the end of this treatment, mean length and the standard error were calculated for each sample of 25 embryos. In germinated seeds, embryo size was assumed to be that corresponding to the critical embryo length. To determine critical embryo length, seeds subjected to Treatment A and not used in Treatment D (approximately 150 seeds) were incubated at 15/4°C in darkness for 30 d. At the end of this period, embryos in seeds with a split seed coat, but no radicle protrusion, were excised and measured, and the mean length ± standard error of these embryos (critical embryo length, *sensu* Vandelook *et al.*, 2007; Vandelook and Van Asche, 2008a) was 3.80 ± 0.06 mm, $n = 25$, with a range of 3.2–4.3 mm. The ratio of embryo length to seed length (critical E:S ratio) was also measured. It was 0.90 ± 0.01, with a range of 0.82–0.95, where 0.82 is the minimal E:S ratio registered in seeds at the time of germination (threshold E:S ratio).

On 1 July 2003, 100 seeds were placed on moist paper in a 9-cm Petri dish and stratified at 5°C for 4 months, after which 25 ungerminated seeds with a healthy appearance were placed in each of three 9-cm Petri dishes and one dish each was incubated at 15/4, 25/10 and 28/14°C for 30 d (Treatment E). Subsequently, mean length and standard error were calculated for each sample of 25 embryos. In addition, on 1 July 2003, 100 seeds were placed in a 9-cm Petri dish, which was incubated at 28/14°C for 4 months, after which 25 seeds with a healthy appearance were placed in each of three 9-cm Petri dishes and one dish each was incubated at 5, 15/4 and 25/10°C for 30 d (Treatment F). At the end of that period, mean length and standard error were determined for each sample of 25 embryos.

Germination tests in 2004 (seed storage duration = 8 months) showed that germination percentages increased with seed storage duration; consequently, we asked whether seed storage duration can also induce an increase in embryo growth rate. Thus, on 1 March 2005 (seed storage duration = 20 months) embryo growth was measured again, repeating those treatments that had resulted previously in high embryo growth in freshly matured seeds: 15/4°C for 5 months, Treatments A and D (although in Treatment D, after Treatment A, seed transfer was only to 15/4°C). Also, different temperatures were tested for the warm stratification that needs to precede cold stratification for embryo growth, using seeds collected on 17 June 2006. On 1 July 2006, 150 seeds were placed in a 9-cm Petri dish, and exposed for 1 month to each temperature in the following sequence: 25/10, 20/7, 5 and 5°C (Treatment B). In another dish with 150 seeds, the monthly sequence of temperatures was: 28/14, 25/10, 5 and 5°C (Treatment C). Each month a sample of 25 seeds with a healthy appearance was removed and their embryos were excised and measured. After 4 months, 25 seeds with a healthy appearance subjected to Treatment B and another 25 seeds to Treatment C were incubated for 30 d at 15/4°C. At the end of this period, mean length and standard error were calculated for each sample of 25 embryos, assuming that the embryo length in seeds germinated during the experiment corresponded to the critical embryo length.

Embryo growth in darkness

To analyse embryo growth at different temperatures and at different seed storage durations, the same tests described in light conditions (above) were carried out in the dark, with the exception of the test determining the critical embryo length. Seed manipulations were done while using a green safe lamp (Luna *et al.*, 2004).

Effects of GA_3 on dormancy break and embryo growth

On 1 July 2003, 25 seeds were placed on two sheets of filter paper moistened with a solution of 1000 mg/l of gibberellic acid (GA_3) and distilled water in each of 20 9-cm Petri dishes. Ten dishes were placed at 25/10°C and ten at 5°C; in each chamber, five dishes were in light and five in darkness. After 1, 2, 3, 4 and 5 months, a dish from light and one from dark at each temperature were removed and embryos excised and measured. Mean length and standard error were calculated for each sample of 25 embryos; data were compared with those of embryos from seeds incubated in distilled water at the same temperatures for the same period of time. We used the thermoperiod of 25/10°C because it was considered far enough from the conditions required for cold stratification

and similar to those used in previous studies (Baskin *et al.*, 2000; Walck and Hidayati, 2004). The temperature of 5°C was chosen to test whether gibberellic acid (GA_3) could replace the effect of a warm stratification.

Statistical analysis

The effects of incubation at different temperatures, duration of incubation and time of seed storage on embryo growth were analysed by a multifactor analysis of variance (ANOVA). Seed germinability was evaluated by the final cumulative germination percentage, which was compared among treatments by multifactor ANOVAs. In the comparison of the final germination percentage after Treatment A, the effects of four factors were analysed: temperature (six levels), light (two levels), seed storage duration (three levels) and light conditions during stratification (two levels). In the study of influence of the other warm + cold stratification treatments on seed germinability, the effect of five factors were analysed: temperature (three levels), stratification treatments (two levels), seed storage duration (two levels), light condition during incubation (two levels) and light condition during stratification (two levels). When significant main effects existed, differences were detected by a multiple comparison Tukey test. Prior to analysis, normality (Cochran test) and homocedasticity (David test) of data were checked. Values of the final cumulative germination percentage were squared-root arcsine transformed. Statgraphics Plus 5.1 (Statistical Graphics Corporation, Inc., Rockwille, Maryland, USA) software was used for statistical analysis.

Results

Outdoor experiments

Phenology of embryo growth and seedling emergence

In buried seeds, embryo growth was continuous from 1 September 2003 to 1 May 2004, although the highest growth rates occurred during October, December and March. On 1 March 2004, embryo length was 3.02 ± 0.11 mm, 16% of the seeds had germinated

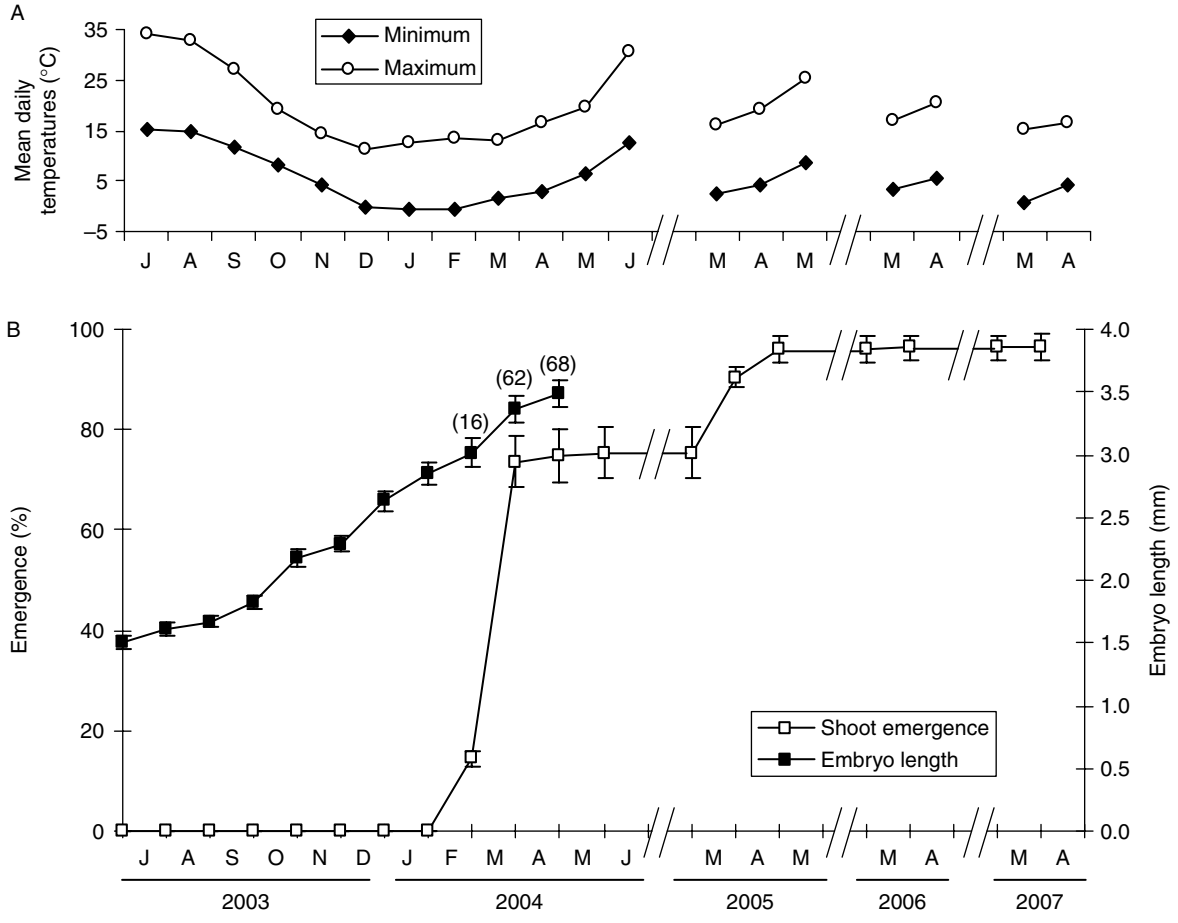

Figure 1. (A) Mean daily minimum and maximum air temperatures and (B) phenology of embryo growth and shoot emergence from seeds sown in July 2003. Numbers between parentheses next to embryo length indicate radicle emergence (%) if > 0.

within the bags and 44% of the embryos had reached the threshold E:S ratio. On 1 April 2004 embryo length was 3.37 ± 0.11 mm, 62% of the seeds had germinated within the bags and 72% of the embryos had reached the threshold E:S ratio. In March 2004, the mean maximum and minimum temperatures were 13 and 2°C, respectively. On 1 May 2004 embryo length reached 3.49 ± 0.10 mm and 68% of the seeds had germinated within the bags (Fig. 1). The next month, only an amorphous mass of seeds with radicles emerged or decomposed was exhumed. Between 1 July 2003 and 1 February 2004, no emergent seedling was recorded in the plastic seedtrays. Seedling emergence started on 1 March 2004 (percentage similar to that of seeds with emerged radicles within exhumed bags) and the highest values were recorded during March 2004; the cumulative emergence at the end of that month was 73.7 ± 4%. In spring 2004, the last seedling emergence was recorded on 8 May, at which time cumulative emergence was 75.3 ± 4.1%. No seedlings emerged between 8 May 2004 and 1 March 2005, but at the end of spring 2005 the cumulative emergence was 96 ± 2%. The last emergence was recorded on 10 April 2007, but the cumulative emergence (96.5 ± 2%) hardly increased during the last 2 years (Fig. 1).

Dormancy break in buried seeds

All seeds exhumed from 1 August 2003 to 1 December 2003 were viable and dormant (Fig. 2). On 1 February 2004, 17.5% of the seeds were viable and non-dormant. On 1 March 2004, 17% of the exhumed seeds had emergent radicles and the percentage had increased to 64% on 1 April 2004 and to 72% on 1 May 2004. From 1 March nearly all seeds in the bags had emerged or were viable non-dormant. From 1 June 2004 only an amorphous mass of decomposed seeds was exhumed (Fig. 2).

Laboratory experiments

Effects of stratification, light:dark, seed storage duration and year of collection on germination

Collection year 2003

In the control tests with freshly matured seeds, germination was null in all cases. In 8-month-old seeds incubated in darkness at 15/4°C, germination was 6 ± 2.2%, being null at the other temperatures and light conditions (data not shown). In freshly matured seeds, the cold stratification treatment at 5°C for 4 months did not promote germination, and no seed germinated when seeds were transferred to the incubation temperatures. In 8-month-old seeds cold stratified at 5°C for 4 months, some seeds germinated when they were transferred to 15/4°C, but the maximum was 5 ± 0.9%, corresponding to seeds stratified and incubated in darkness (data not shown). Stratification Treatment A promoted germination; thus, 0-month-old seeds germinated to 39 ± 3.8% at 15/4°C in darkness. After the stratification in darkness, 20-month-old seeds germinated to 76 ± 4.2%. Maximum germination percentages were achieved at 5 and 15/4°C in darkness, regardless of light conditions during the stratification period, reaching the highest value (86.8 ± 2.9%) at 5°C in darkness after stratification in darkness. Germination percentages increased significantly ($P < 0.001$) with seed storage duration of 20 months evaluated in this study (Fig. 3, Table 1). Seeds incubated in darkness had significantly higher germination percentages than those incubated in light.

Collection year 2004

In 8-month-old seeds subjected to stratification Treatment A, germination percentages were significantly higher ($P = 0.0165$) than those obtained in seeds

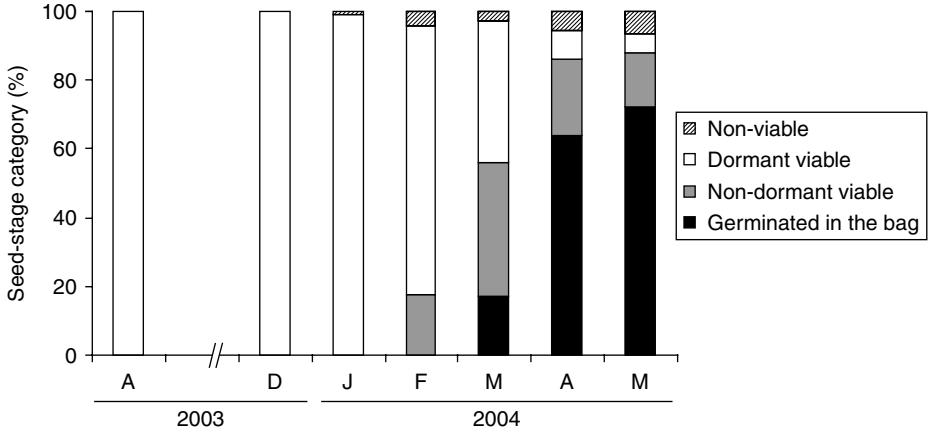

Figure 2. Changes in the percentage of dormant, non-dormant, non-viable and germinated seeds of *N. longispathus* buried on 1 July 2003 and exhumed monthly over a period of 10 months.

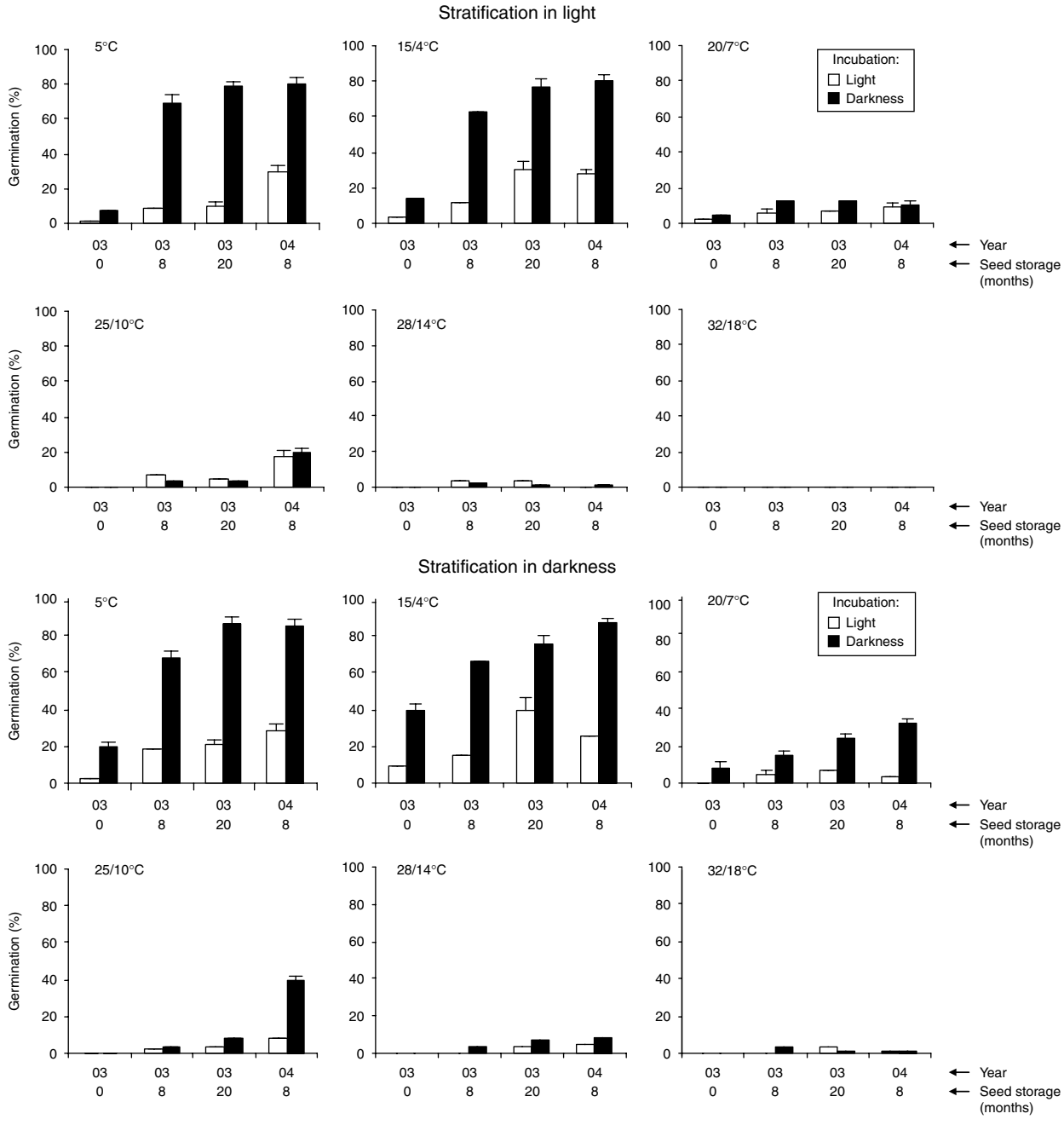

Figure 3. Effect of incubation conditions (light:dark and temperature), time of seed storage (0, 8 and 20 months) and year of seed collection on final germination (mean ± SE, if SE > 2%) of *N. longispathus* seeds. Seeds were stratified for 1 month at each temperature of the sequence 20/7°C + 15/4°C + 5°C + 5°C, and then transferred to temperatures in the figure for 30 d.

similarly aged and treated but collected during the year 2003 (Fig. 3).

Collection year 2006

In the control test with freshly matured seeds, germination was null. The cold stratification treatments at 9/5°C for 4 months hardly promoted germination, and the highest germination (5%) was for seeds stratified in darkness and incubated at 15/4°C in darkness. However, during the cold stratification in darkness 8% of the seeds germinated (data not shown). The warm + cold stratification treatments evaluated in this cohort of seeds promoted germination, and both Treatment B (25/10 + 20/7 + 5 + 5°C) and Treatment C (28/14 + 25/10 + 5 + 5°C) were effective in breaking dormancy and resulted in germination at 15/4°C (Fig. 4). In

Table 1. Main effects on germination of incubation temperature, light conditions during stratification and incubation, and seed storage duration in multifactor analysis of variance of *N. longispathus* collected in 2003. The table shows degrees of freedom (df), *F*-ratio values and categories of factors where germination differences were significant. Different lowercase letters denote significant differences ($P < 0.05$) between factor levels. Residual degrees of freedom: 287

Factor	df	*F*	*P*	Categories
Incubation temperature (°C)	5	108.16	< 0.001	$32/18^a$, $28/14^a$, $25/10^a$, $20/7^b$, 5^c, $15/4^c$
Light conditions (stratification)	1	7.30	0.0073	Light < Darkness
Light conditions (incubation)	1	99.88	< 0.001	Light < Darkness
Seed storage duration (months)	2	68.23	< 0.001	0^a, 8^b, 20^c

freshly matured seeds, the highest germination was 36.3 ± 4.6% (at 15/4°C in darkness after Treatment B in darkness), and in 8-month-old seeds germination increased to 53 ± 1.7%. In addition, germination percentages were significantly higher in seeds incubated in darkness than in seeds incubated with light. Stratification in darkness was more effective in promoting seed germination than stratification in light (Fig. 4, Table 2).

Although Treatments B and C promoted germination (6%, data not shown) in seeds incubated at 5 and 20/7°C, they were less effective than Treatment A (> 80%).

Effects of temperature, light:dark and seed storage duration on embryo growth

Embryo growth in light

Mean length of embryos from freshly matured seeds was 1.50 ± 0.05 mm. The embryo length in seeds incubated at 5, 15/4, 25/10 and 28/14°C for 5 months ranged between 1.98 and 2.42 mm (Table 3A). In no case did they reach the critical embryo length (i.e. embryo size required for seed germination) of 3.80 ± 0.06 mm, nor the threshold E:S ratio of 0.82 from which some seeds can germinate. The threshold E:S ratio was not reached after Treatments E and F, so germination was null (Table 3A). In contrast, after Treatment A which was followed by incubation at 15/4°C (Treatment D) embryo length reached 2.81 ± 0.12 mm, 4% of the seeds germinated and 28% reached the threshold E:S ratio. In 20-month-old seeds subjected to the same temperature sequence, embryo length was significantly higher (2.99 ± 0.12 mm) and 28% germinated (Table 3A).

Embryos in seeds subjected to Treatments B and C for 4 months and then incubated during the fifth month at 15/4°C ranged between 2.34 and 2.49 mm in length but germination was null (Table 4).

Embryo growth in darkness

Embryo growth and germination in darkness showed the same pattern as in light, but values were higher. In freshly matured seeds incubated for 5 months at 5, 25/10 and 28/14°C, the embryo hardly grew. At 15/4°C, however, embryo length was 2.85 ± 0.08 mm and 16% of the embryos reached the threshold E:S ratio (Table 3B). After Treatment A and later incubation at 15/4°C (Treatment D), embryo length was 3.24 ± 0.11 mm, 36% of the seeds had germinated and 56% of the embryos had reached the threshold E:S ratio. In 20-month-old seeds subjected to the same sequence of temperatures, embryo growth was significantly higher (3.78 ± 0.02 mm), 92% of the seeds had germinated and 100% of the embryos had reached the threshold E:S ratio. Moreover, in

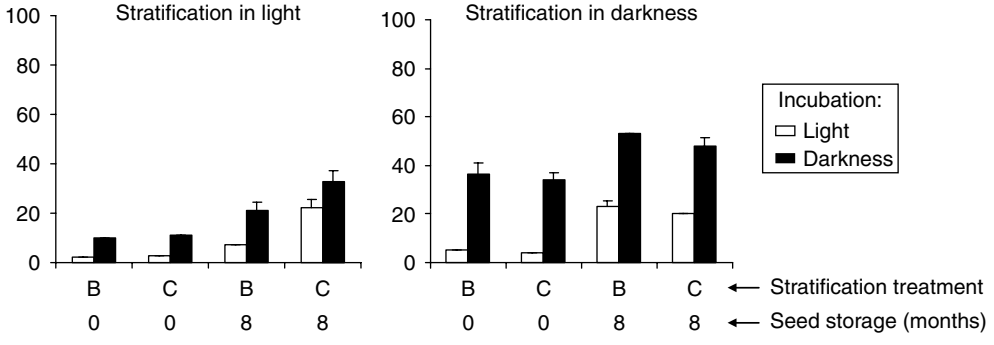

Figure 4. Influence of light:dark, stratification (Treatments B and C) and duration of seed storage on cumulative germination percentages (mean + SE, if SE > 2%) of *N. longispathus* seeds. Incubation at 15/4°C for 30 d. Year of seed collection: 2006. Treatment B: 1 month at 25/10°C, followed by 1 month at 20/7°C, and two additional months at 5°C. Treatment C: 1 month at 28/14°C, followed by 1 month at 25/10°C and two additional months at 5°C.

Table 2. Main effects on germination of light conditions during stratification or incubation, stratification treatment and seed storage duration in multifactor analysis of variance of *N. longispathus* collected in 2006. The table shows degrees of freedom (df), *F*-ratio values and categories of factors where germination differences were significant. Residual degrees of freedom: 59

Factor	df	*F*	*P*	Categories
Light conditions (stratification)	1	43.71	<0.001	Light $<$ Darkness
Light conditions (incubation)	1	107.36	<0.001	Light $<$ Darkness
Stratification treatment	1	1.79	0.1856	
Seed storage duration (months)	1	70.41	<0.001	$0 < 8$

20-month-old seeds incubated at 15/4°C for 5 months, embryo length was significantly higher than that reached by freshly matured seeds from the fourth month of incubation (Table 3B).

In seeds subjected to Treatments B and C for 4 months and incubated during the fifth month at 15/4°C, embryo length ranged between 3.17 and 3.36 mm, and germination percentages ranged between 32 and 36% (Table 4).

Effects of GA_3 on dormancy break and embryo growth

GA_3 did not stimulate embryo growth. In seeds incubated at 25/10°C for 5 months in a GA_3 solution of 1000 ppm, embryo length was 2.36 ± 0.06 mm in light and 2.27 ± 0.06 mm in darkness. No seeds had germinated and no embryo had reached the threshold E:S ratio. In seeds incubated for 5 months at 5°C in GA_3, the embryo length was 2.02 ± 0.04 mm in light and 2.15 ± 0.09 mm in darkness, although germination was 4% in darkness (data not shown).

Discussion

N. longispathus seeds have embryos that are differentiated but underdeveloped (length = 1.50 ± 0.05 mm). Also, embryos required more than 90 d in the most favourable temperature and light conditions (15/4°C in darkness) to reach the critical embryo length (i.e. 3.80 ± 0.06 mm). Thus, it can be concluded that they have morphophysiological dormancy (MPD). To identify the specific level of MPD, it is necessary to determine the environmental conditions that are required to overcome both physiological and morphological dormancy. Seeds with simple levels of MPD require high temperatures ($\geq$15°C) for embryo growth, and those with complex levels of MPD need low temperatures (0–10°C) (Baskin and Baskin, 1998; Walck and Hidayati, 2004). Because embryo growth in *N. longispathus* seeds does not occur at high temperatures (25/10, 28/14°C), nor at 15/4°C or 25/10°C after being stratified at 5°C (Treatment E) or at 28/14°C (Treatment F) (Table 3), the existence of any simple level of MPD can be rejected. Thus, seeds must have one of the three levels of complex MPD. The fact that embryo growth is very low (from 1.50 to 2.05 mm) during 5 months of cold stratification at 5°C (Table 3) leads us to discard the existence of intermediate and deep complex MPDs in *N. longispathus* seeds. Indeed, our study provides robust evidence of non-deep complex MPD for seeds of this taxon. First, maximum values of embryo growth after 120 d of incubation were recorded at 15/4°C (combination of warm + cold stratification) and after the sequence of warm + cold stratification at 20/7, 15/4, 5 and 5°C (Treatment A, Table 3). Secondly, maximum values of embryo growth after 150 d of incubation were recorded after the sequences 20/7, 15/4, 5, 5 and 15/4°C (Treatment D, Table 3); 25/10, 20/7, 5, 5, and 15/4°C; and 28/14, 25/10, 5, 5 and 15/4°C (Table 4). This is the first study reporting non-deep complex MPD in *Narcissus*.

Baskin and Baskin (1998) have shown that in some seeds with non-deep complex MPD, GA_3 can partly substitute for warm stratification but not for cold stratification. In *N. longispathus* seeds, GA_3 was not effective in substituting for warm stratification, since only 4% of the seeds germinated when incubated in darkness at 5°C for 5 months in a GA_3 solution. The highest record of germination promotion by GA_3 in species with this level of dormancy is in *Osmorhiza claytonii*, which did not surpass 24% after 24 weeks of incubation (Baskin and Baskin, 1991).

When fresh seeds of *N. longispathus* were incubated in darkness for 4 months at the temperature sequence in Treatment A (20/7, 15/4, 5 and 5°C), embryos grew from 2.74 to 3.24 mm (increase: 0.50 mm) when transferred to 15/4°C during the fifth month, in contrast to seeds that were only incubated at the latter thermoperiod for the same period (i.e. 5 months), which grew from 2.74 to 2.85 mm (increase: 0.11 mm) during the fifth month (Table 3). A similar record occurred in embryos subjected to warm + cold stratification Treatments B and C and then incubated at 15/4°C during the fifth month (Table 4). So, the exposure to 5°C seems necessary for embryos to finish their growth and reach the critical length. Seeds of *N. longispathus* may be similar to those of *Heracleum sphondylium* in that low (i.e. 2°C) temperatures were required for the breakdown of endosperm storage proteins into soluble amino acids and nitrogenous

Table 3. Influence of temperature, incubation length and duration of seed storage on embryo growth (mean ± SE, $n = 25$ seeds) in *N. longispathus* seeds of collection year 2003. Values followed by different uppercase letters within columns or different lowercase letters within rows are significantly different ($P < 0.05$). The first number within parentheses shows the percentage of germination (radicle emergence) and the second number shows the percentage of seeds with an E:S ratio larger than the threshold E:S ratio (0.82). Treatment A: 20/7°C (1 month) +15/4°C (1 month) +5°C (2 months); length seed storage: 0 and 20 months. Treatment D: after Treatment A, 0-month-old seeds were transferred to 5, 15/4, 25/10 and 28/14°C, and 20-month-old seeds to 15/4°C. Treatment E: 5°C (4 months), then seeds were transferred to 5, 15/4, 25/10 and 28/14°C; length of seed storage: 0 months. Treatment F: 28/14°C (4 months), then seeds were transferred to 5, 15/4, 25/10 and 28/14°C

		Incubation temperatures						
		5°C	15/4°C		25/10°C	28/14°C	Treat. A	
Length seed storage (months)		0	0	20	0	0	0	20
(A) Incubation in light								
Incubation length (months)	1	1.74 ± 0.03^{Aab} (0,0)	1.87 ± 0.04^{Ab} (0,0)	1.89 ± 0.07^{Ab} (0,0)	1.65 ± 0.04^{Aa} (0,0)	1.80 ± 0.04^{Aab} (0,0)	1.79 ± 0.04^{Aab} (0,0)	1.80 ± 0.04^{Aab} (0,0)
	2	1.81 ± 0.04^{Aa} (0,0)	2.01 ± 0.05^{ABabc} (0,0)	2.20 ± 0.07^{Acd} (0,0)	1.91 ± 0.04^{Bab} (0,0)	1.99 ± 0.03^{ABabc} (0,0)	2.09 ± 0.05^{Bbcd} (0,0)	2.25 ± 0.07^{Bd} (0,0)
	3	1.84 ± 0.05^{Aa} (0,0)	2.17 ± 0.03^{ABCbc} (0,0)	2.60 ± 0.08^{Bd} (0,8)	2.07 ± 0.05^{BCab} (0,0)	2.11 ± 0.04^{BCbc} (0,0)	2.13 ± 0.06^{Bbc} (0,0)	2.34 ± 0.08^{Bc} (0,0)
	4	1.94 ± 0.04^{Aa} (0,0)	2.30 ± 0.05^{BCb} (0,0)	2.75 ± 0.10^{BCc} (0,8)	2.12 ± 0.05^{BCab} (0,0)	2.13 ± 0.05^{BCab} (0,0)	2.26 ± 0.08^{Bb} (0,4)	2.70 ± 0.09^{Cc} (0,16)
	5	1.98 ± 0.05^{ABa} (0,0)	2.42 ± 0.09^{Cb} (0,4)	2.86 ± 0.08^{BCc} (4,16)	2.32 ± 0.05^{Cb} (0,0)	2.37 ± 0.05^{Db} (0,0)	–	–
Treat. D		2.88 ± 0.12^{Cb} (0,32)	2.81 ± 0.12^{Db} (4,28)	2.99 ± 0.12^{Cb} (28,32)	2.30 ± 0.09^{Ca} (0,4)	2.29 ± 0.08^{CDa} (0,0)	–	–
Treat. E		1.98 ± 0.05^{ABa} (0,0)	2.04 ± 0.06^{ABa} (0,0)	–	2.00 ± 0.05^{Ba} (0,0)	1.98 ± 0.07^{ABa} (0,0)	–	–
Treat. F		2.23 ± 0.07^{Ba} (0,0)	2.38 ± 0.08^{Ca} (0,0)	–	2.32 ± 0.07^{Ca} (0,0)	2.37 ± 0.05^{Da} (0,0)	–	–
(B) Incubation in darkness								
Incubation length (months)	1	1.64 ± 0.03^{Aa} (0,0)	1.92 ± 0.05^{Ab} (0,0)	1.89 ± 0.05^{Ab} (0,0)	1.86 ± 0.04^{Ab} (0,0)	1.86 ± 0.04^{Ab} (0,0)	1.82 ± 0.05^{Aab} (0,0)	1.84 ± 0.04^{Ab} (0,0)
	2	1.78 ± 0.04^{ABa} (0,0)	2.36 ± 0.07^{BCcd} (0,0)	2.37 ± 0.06^{Bcd} (0,0)	1.95 ± 0.04^{ABab} (0,0)	1.99 ± 0.04^{ABab} (0,0)	2.16 ± 0.05^{Bbc} (0,0)	2.45 ± 0.06^{Bd} (0,0)
	3	1.85 ± 0.05^{ABa} (0,0)	2.49 ± 0.10^{CDbc} (0,12)	2.77 ± 0.08^{Cc} (0,12)	2.10 ± 0.04^{BCa} (0,0)	2.00 ± 0.03^{ABa} (0,0)	2.45 ± 0.07^{Cb} (0,0)	2.52 ± 0.08^{Bbc} (0,4)
	4	1.90 ± 0.04^{ABa} (0,0)	2.74 ± 0.09^{DEb} (0,12)	3.23 ± 0.08^{Dc} (20,52)	2.14 ± 0.04^{BCa} (0,0)	2.12 ± 0.04^{Ba} (0,0)	2.74 ± 0.08^{Db} (0,8)	3.19 ± 0.08^{Cc} (12,56)
	5	2.05 ± 0.08^{Ba} (0,4)	2.85 ± 0.08^{Ec} (0,16)	3.15 ± 0.08^{Dd} (20,52)	2.21 ± 0.06^{CDab} (0,0)	2.35 ± 0.05^{Cb} (0,0)	–	–
Treat. D		3.03 ± 0.12^{Dab} (20,48)	3.24 ± 0.11^{Fb} (36,56)	3.78 ± 0.02^{Ec} (92,100)	3.01 ± 0.08^{Eab} (4,36)	2.82 ± 0.08^{Da} (0,16)	–	–
Treat. E		2.05 ± 0.08^{Ba} (0,0)	2.10 ± 0.04^{ABa} (0,0)	–	2.09 ± 0.03^{ABCa} (0,0)	2.02 ± 0.07^{ABa} (0,0)	–	–
Treat. F		2.61 ± 0.07^{Cbc} (0,0)	2.70 ± 0.05^{CDEc} (0,0)	–	2.45 ± 0.07^{Dab} (0,0)	2.35 ± 0.05^{Ca} (0,0)	–	–

Table 4. Embryo growth (mean ± SE, $n = 25$ seeds) in 0-month-old seeds of *N. longispathus* of collection year 2006, incubated at different temperature/light conditions for 5 months. Treatment B: 25/10°C (1 month) +20/7°C (1 month) +5°C (2 months), then incubation at 15/4°C (1 month). Treatment C: 28/14°C (1 month) +25/10°C (1 month) +5°C (2 months), then incubation at 15/4°C (1 month). Values followed by different uppercase letters within columns or different lowercase letters within rows are significantly different ($P < 0.05$). The first number between parentheses shows the percentage of germination (radicle emergence) and the second number shows the percentage of seeds with an E:S ratio longer than the threshold E:S ratio (0.82)

		Incubation temperature			
		In light		In darkness	
		Treat. B	Treat. C	Treat. B	Treat. C
Incubation length (months)	1	1.96 ± 0.07^{Aa} (0,0)	1.92 ± 0.05^{Aa} (0,0)	1.87 ± 0.04^{Aa} (0,0)	1.98 ± 0.04^{Aa} (0,0)
	2	1.98 ± 0.05^{Aa} (0,0)	1.97 ± 0.05^{Aa} (0,0)	1.90 ± 0.05^{Aa} (0,0)	2.10 ± 0.04^{ABa} (0,0)
	3	1.91 ± 0.04^{Aa} (0,0)	1.88 ± 0.04^{Aa} (0,0)	2.32 ± 0.08^{Bbc} (0,0)	2.34 ± 0.05^{Bbc} (0,0)
	4	2.09 ± 0.05^{Aa} (0,0)	2.10 ± 0.07^{Aa} (0,0)	2.71 ± 0.06^{Cb}(0,4) (0,4)	2.73 ± 0.07^{Cbc} (0,16)
	5	2.34 ± 0.06^{Ba} (0,0)	2.49 ± 0.08^{Ba} (0,12)	3.36 ± 0.09^{Db} (36,72)	3.17 ± 0.11^{Db} (32,68)

compounds required for embryo growth (Stokes, 1953). This hypothesis has been suggested previously for other species whose MPD requires cold stratification, such as *Thaspium pinnatifidum* (Baskin *et al.*, 1992), *Delphinium tricorne* (Baskin and Baskin, 1994), *Anthriscus sylvestris* (Baskin *et al.*, 2000), and *Aconitum napellus* subsp. *castellanum* (Herranz *et al.*, 2010a). In the unheated shadehouse, the embryos grew from 1.67 to 2.18 mm during September and October 2003 (Fig. 1). It is possible that periods of warm stratification occurring during those months may break the non-deep physiological dormancy, while periods of low temperatures (≤10°C) may promote embryo growth, as described in other species with non-deep complex MPD (i.e. *Osmorhiza claytonii*, Baskin and Baskin, 1991). However, the embryo did not elongate to 3 mm until 1 March 2004, when rates of embryo growth and seedling emergence increased. During the period between 1 October 2003 (when seeds started to be constantly moist) and 1 March 2004 (when seedling emergence started) the seeds had received 1432 h of warm stratification (≥15°C) and 2240 h of cold stratification (0.5–10°C), which is equivalent to 8.5 and 13.3 weeks of warm and cold stratification, respectively. The peak of seedling emergence occurred during March 2004, with a percentage similar to that of exhumed seeds with emerged radicles, which discards the possibility of any kind of epicotyl dormancy. Results in this study strongly suggest that in nature (i.e. unheated shadehouse), dormancy break and embryo growth in *N. longispathus* occur during late summer, autumn and winter, and seeds have the ability to germinate in late winter to early spring (March and April), preferably at low (5, 15/4°C) temperatures (Fig. 3), as in other species with this level of MPD: *Osmorhiza longistylis* (Baskin and Baskin, 1984), *O. claytonii* (Baskin and Baskin, 1991), *Erythronium albidum* (Baskin and Baskin, 1985) and *Symphoricarpos orbiculatus* (Hidayati *et al.*, 2001), and in general in seeds with complex levels of MPD (Walck *et al.*, 2002; Vandelook *et al.*, 2007; Herranz *et al.*, 2010b). The ecological consequence of a warm + cold stratification requirement for seed dormancy break and embryo growth is that seeds cannot germinate until late winter, when embryos reach their maximum development, thus avoiding the emergence of seedlings in autumn and their probable mortality due to frost sensibility during the cold winter months.

The increase of germination with seed storage duration found in this study (Fig. 3, Table 1) is related to the increase of embryo growth rate with seed storage duration during the warm + cold stratification period (Treatment A, Table 3). The increase of embryo growth rate with seed storage duration was only found previously in two species: (1) *Narcissus alcaracensis*, a species closely related to *N. longispathus* with intermediate complex MPD (Herranz *et al.*, 2013); and (2) *Merendera montana*, the seeds of which have non-deep complex MPD (Copete *et al.*, 2011b). Although the increase of germination with seed storage duration is frequent in seeds with non-deep physiological dormancy (Baskin and Baskin, 1998; Copete *et al.*, 2005), it is less known in seeds with MPD, having also been cited in two species with intermediate complex MPD: *A. napellus* subsp. *castellanum* (Herranz *et al.*, 2010a) and *Delphinium fissum* subsp. *sordidum* (Herranz *et al.*, 2010b).

The seedling emergence test (Fig. 1) showed that at the end of the first vegetative period 24.7% of the seeds

remained ungerminated in the soil (cumulative seedling emergence $= 75.3 \pm 4.1\%$), with most of them emerging during the second post-sowing spring (cumulative seedling emergence $= 96.0 \pm 2.1\%$). The lack of germination at the end of autumn 2004, even when temperatures were favourable for germination and emergence (15/4, 5°C), could be due to the induction of dormancy by high summer temperatures, just as recorded in species with deep complex MPD such as *Frasera caroliniensis* (Threadgill *et al.*, 1981), or with intermediate complex MPD such as *D. fissum* subsp. *sordidum* (Herranz *et al.*, 2010b). The promotion of seed germination in dark conditions detected in this study also occurs in *D. fissum* subsp. *sordidum* (Herranz *et al.*, 2010b) and *N. alcaracensis* (Herranz *et al.*, 2013), although this is not the general trend in seeds with MPD (Baskin and Baskin, 1994; Hidayati *et al.*, 2000). The ample interannual variability in germination behaviour recorded in the present study (Fig. 3) was previously observed in other species (Beckstead *et al.*, 1996). It is possible that these differences were due to interannual environmental variation (rainfall, temperature) during the seed-ripening phase (Meyer *et al.*, 1989).

The present study provides a guideline for growing plants from seeds to reinforce populations. On 1 September, seeds should be submitted to stratification Treatment A in darkness and subsequently incubated at 15/4°C in darkness for 1 month. In February, seeds with an emergent radicle should be sown in pots. Thus, shoots emerge and seedlings produce a small bulb (2–3 mm in diameter) between March and May. Wet conditions combined with high summer temperatures greatly increase the possibility of bulb decay, so watering must be stopped until November. In the fourth plant cycle, bulbs will have reached 1.5–2.0 cm in diameter around February.

The three Iberian endemic daffodils of the section *Pseudonarcissi* studied up to now have different levels of MPD: *N. alcaracensis*, with intermediate complex MPD, requires only a cold stratification for seed dormancy break (Herranz *et al.*, 2013); *N. longispathus*, with non-deep complex MPD, requires warm + cold stratification to break seed dormancy; and *N. hispanicus*, with deep simple epicotyl MPD, requires a warm stratification for radicle emergence at cool temperatures, and seeds with emerged roots require cold stratification for shoot emergence. In *N. alcaracensis* and *N. longispathus*, the radicle and shoot emerge simultaneously in late winter–early spring. Evolutionary patterns of ecophysiological traits, such as the different levels of seed dormancy, can be studied by comparing species for which phylogenetic relationships have been well resolved (Feder *et al.*, 2000; Vandelook and Van Assche, 2008b). Although phylogeny is not completely solved yet in *Narcissus*, some studies have substantially contributed to disentangling relationships in the group (Graham and Barret, 2004; Medrano and Herrera, 2008; Zonneveld, 2008). From chromosomal affinities and morphological contrasts, Fernandes (1975) established *N. nevadensis* Pugsley as the oldest species in the section *Pseudonarcissi*, and *N. longispathus* and *N. hispanicus* as derived forms resulting from different evolutionary lineages. Such an evolutionary scheme of the group is broadly agreed (Hernández-Bermejo *et al.*, 1999). In *N. nevadensis* embryo growth, radicle and shoot emergence occur during cold stratification, and although GA_3 does not stimulate germination, dry storage of seeds reduces the length of cold stratification required for radicle emergence, so this species also has intermediate complex MPD (unpublished data). If future studies applying molecular techniques confirm the above phylogenetic scheme in the section *Pseudonarcissi*, then intermediate complex MPD, and even deep complex MPD if detected in any species older than *N. nevadensis*, would be the ancestral condition in the group, and both non-deep complex MPD and deep simple epicotyl MPD would be derived. In two unrelated genera (i.e. *Osmorhiza* and *Erythronium*) non-deep complex MPD is also the derived condition (Walck and Hidayati, 2004).

Diverse evolutionary processes with different selection pressures have produced a wide array of ecophysiological traits, such as the different kinds of seed dormancy, observed among species at local, regional and global scales (Baskin and Baskin, 1998; Walck and Hidayati, 2004). In the section *Pseudonarcissi*, intermediate complex MPD (3–4 months of cold stratification) may have led to non-deep complex MPD (2 months of warm +2 months of cold stratification) as a result of adaptation to wetter environments with warmer winters, where a warm stratification is guaranteed during autumn months preceding a period of 2–3 months of cold stratification during winter. In contrast, evolution from intermediate complex MPD to deep simple epicotyl MPD in the group may be explained by adaptation to environments subject to water stress in late spring (i.e. stream edges and rush meadows with seasonal fluctuations in humidity, and shaded rocky crevices). In species with deep simple epicotyl MPD, seedlings have a well-developed root system in early spring when the cotyledons expand, forming a perennating structure necessary for persistence over summer (Kondo *et al.*, 2004). In future studies, it should be determined if other species in the section *Pseudonarcissi* distributed within the Iberian Peninsula and colonizing habitats subjected to summer water stress (i.e. *N. eugeniae*, *N. radinganorum* and *N. bujei*) also have deep simple epicotyl MPD, and, in addition, if any species in northern Spain at higher altitudinal levels in the Pyrenees mountains (i.e. *N. moschatus* and *N. bicolor*) have deep complex MPD.

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References

Aizpuru, I., Ballester, G., Bañares, A., Báscones, J.C., Benito, J.L., Blanca, G., Blanché, C., Blanco, E., Bramwell, D., Bueno, A., *et al*, (2000) Lista Roja de Flora Vascular Española. *Conservación Vegetal* **6**, 11–38.

Bañares, A., Blanca, G., Güemes, J., Moreno, J.C. and Ortiz, S. (Eds) (2003) *Atlas y Libro Rojo de la Flora Vascular Amenazada de España*. Madrid, Dirección General de Conservación de la Naturaleza.

Barret, S.C.H., Cole, W.W. and Herrera, C.M. (2004) Mating patterns and genetic diversity in the wild daffodil *Narcissus longispathus* (Amaryllidaceae). *Heredity* **92**, 459–465.

Baskin, C.C. and Baskin, J.M. (1984) Germination ecophysiology of the woodland herb *Osmorhiza longistylis* (Umbelliferae). *American Journal of Botany* **71**, 687–692.

Baskin, C.C. and Baskin, J.M. (1985) Seed germination ecophysiology of the woodland spring geophytes *Erythronium albidum*. *Botanical Gazette* **146**, 130–136.

Baskin, C.C. and Baskin, J.M. (1991) Nondeep complex morphophysiological dormancy in seeds of *Osmorhiza claytonii* (Apiaceae). *American Journal of Botany* **78**, 588–593.

Baskin, C.C. and Baskin, J.M. (1994) Deep complex morphophysiological dormancy in seeds of the mesic woodland herb *Delphinium tricorne* (Ranunculaceae). *International Journal of Plant Sciences* **155**, 738–743.

Baskin, C.C. and Baskin, J.M. (1998) *Seeds: ecology, biogeography, and evolution of dormancy and germination*. San Diego, California, Academic Press.

Baskin, C.C., Chester, E.W. and Baskin, J.M. (1992) Deep complex morphophysiological dormancy in seeds of *Thaspium pinnatifidum* (Apiaceae). *International Journal of Plant Sciences* **153**, 565–571.

Baskin, C.C., Milberg, P., Andersson, L. and Baskin, J.M. (2000) Deep complex morphophysiological dormancy in seeds of *Anthriscus sylvestris* (Apiaceae). *Flora* **195**, 245–251.

Beckstead, J., Meyer, S.E. and Allen, P.S. (1996) *Bromus tectorum* seed germination: between-population and between-year variation. *Canadian Journal of Botany* **74**, 875–882.

Blanchard, J. (1990) *Narcissus, a guide to wild daffodils*. Woking, UK, Alpine Garden Society.

Cerabolini, B., De Andrés, R., Ceriani, R.M., Pierce, S. and Raimondi, B. (2004) Seed germination and conservation of endangered species from the Italian Alps: *Physoplexis comosa* and *Primula glaucescens*. *Biological Conservation* **117**, 351–356.

Copete, M.A., Herranz, J.M. and Ferrandis, P. (2005) Seed dormancy and germination in threatened Iberian *Coincya* (Brassicaceae) taxa. *Ecoscience* **12**, 257–266.

Copete, E., Herranz, J.M., Ferrandis, P., Baskin, C.C. and Baskin, J.M. (2011a) Physiology, morphology and phenology of seed dormancy-break and germination in the endemic Iberian species *Narcissus hispanicus* (Amaryllidaceae). *Annals of Botany* **107**, 1003–1016.

Copete, E., Herranz, J.M., Copete, M.A., Baskin, J.M. and Baskin, C.C. (2011b) Nondeep complex morphophysiological dormancy in seeds of the Iberian Peninsula endemic geophyte *Merendera montana* (Colchicaceae). *Seed Science Research* **21**, 267–281.

Dorda, E. and Fernández-Casas, J. (1994) Estudios morfológicos en el género *Narcissus* L. Anatomía de hojas y escapo, IV. *Fontquería* **39**, 69–150.

Feder, M.E., Bennett, A.F. and Huey, R.B. (2000) Evolutionary physiology. *Annual Review of Ecology and Systematics* **31**, 315–341.

Fernandes, A. (1975) L'evolution chez le genre *Narcissus* L. *Anales Instituto Botánico de Cavanilles* **32**, 843–872.

Forbis, T.A. and Diggle, P.K. (2001) Subnivean embryo development in the alpine herb *Caltha leptosepala* (Ranunculaceae). *Canadian Journal of Botany* **79**, 635–642.

Giménez-Benavides, L., Escudero, A. and Pérez-García, F. (2005) Seed germination of high mountain Mediterranean species: altitudinal, interpopulation and interannual variability. *Ecological Research* **20**, 433–444.

Graham, S.W. and Barret, S.C.H. (2004) Phylogenetic reconstruction of the evolution of stylar polymorphisms in *Narcissus* (Amaryllidaceae). *American Journal of Botany* **91**, 1007–1021.

Hernández-Bermejo, E., García, M.A., Contreras, P. and Clemente, M. (1999) *Narcissus nevadensis* Pugsley. pp. 195–198 *in* Blanca, G.; Cabezudo, B.; Hernández-Bermejo, E.; Herrera, C.M.; Molero Mesa, J.; Muñoz, J.; Valdés, B. (Eds) *Libro rojo de la flora silvestre amenazada de Andalucía. Especies en peligro de extinción*. Sevilla, Consejería de Medio Ambiente, Junta de Andalucía.

Herranz, J.M., Copete, M.A., Ferrandis, P. and Copete, E. (2010a) Intermediate complex morphophysiological dormancy in the endemic iberic *Aconitum napellus* subsp. *castellanum* (Ranunculaceae). *Seed Science Research* **20**, 109–121.

Herranz, J.M., Ferrandis, P. and Martínez-Duro, E. (2010b) Seed germination ecology of the threatened endemic Iberian *Delphinium fissum* subsp. *sordidum* (Ranunculaceae). *Plant Ecology* **211**, 89–106.

Herranz, J.M., Copete, M.A. and Ferrandis, P. (2013) Environmental regulation of embryo growth, dormancy break and germination of *Narcissus alcaracensis* (Amaryllidaceae), a threatened daffodil endemic iberic. *American Midland Naturalist* **169**, 147–167.

Herrera, C.M., Hernández-Bermejo, E., Luque, P. and Benavente, A. (1999) *Narcissus longispathus* Pugsley. pp. 191–194 *in* Blanca, G.; Cabezudo, B.; Hernández-Bermejo, E.; Herrera, C.M.; Molero Mesa, J.; Muñoz, J.; Valdés, B. (Eds) *Libro Rojo de la flora silvestre amenazada de Andalucía. Especies en peligro de extinción*. Sevilla, Consejería de Medio Ambiente, Junta de Andalucía.

Hidayati, S.N., Baskin, J.M. and Baskin, C.C. (2000) Dormancy-breaking and germination requirements of seeds of four *Lonicera* species (Caprifoliaceae) with underdeveloped embryos. *Seed Science Research* **10**, 459–469.

Hidayati, S.N., Baskin, J.M. and Baskin, C.C. (2001) Dormancy-breaking and germination requirements for seeds of *Symphoricarpos orbiculatus* (Caprifoliaceae). *American Journal of Botany* **88**, 1444–1451.

Kondo, T., Miura, T., Okubo, N., Shimada, M., Baskin, C.C. and Baskin, J.M. (2004) Ecophysiology of deep simple epicotyl morphophysiological dormancy in seeds of *Gagea lutea* (Liliaceae). *Seed Science Research* **14**, 371–378.

Luna, B., Pérez, B., Fernández-González, F. and Moreno, J.M. (2004) Sensitivity to green safelight of 12 Mediterranean species. *Seed Science and Technology* **32**, 113–117.

Mathew, B. (2002) Classification of the genus *Narcissus*. pp. 30–52 *in* Hanks, G.R. (Ed.) *Narcissus and daffodil: the genus Narcissus*. New York, Taylor and Francis.

Medail, F. and Quezel, P. (1997) Hot-spots analysis for conservation of plant biodiversity in the Mediterranean basin. *Annals Missouri Botanical Garden* **84**, 112–127.

Medrano, M. and Herrera, C.M. (2008) Geographical structuring of genetic diversity across the whole distribution range of *Narcissus longispathus*, a habitat-specialist, Mediterranean narrow endemic. *Annals of Botany* **102**, 183–194.

Meyer, S.E., McArthur, E.D. and Jorgensen, G.L. (1989) Variation in germination response to temperature in rubber rabbitbrush (*Chrysothamnus nauseosus*: Asteraceae) and its ecological implications. *American Journal of Botany* **76**, 981–991.

Moreno, J.C. (coord.) (2008) *Lista Roja 2008 de la Flora Vascular Española*. Madrid, Dirección General del Medio Natural y Política Forestal, Ministerio de Medio Ambiente y Medio Rural y Marino.

Moreno, J.C. and Sainz, H. (1992) *Atlas corológico de las monocotiledóneas endémicas de la Península Ibérica y Baleares*. Madrid, Colección Técnica, ICONA.

Navarro, L. and Guitián, J. (2003) Seed germination and seedling survival of two threatened endemic species of the northwest Iberian Peninsula. *Biological Conservation* **109**, 313–320.

Nikolaeva, M.G. (1977) Factors controlling the seed dormancy pattern. pp. 51–74 *in* Khan, A.A. (Ed.) *The physiology and biochemistry of seed dormancy and germination*. Amsterdam, North-Holland.

Phartyal, S.S., Kondo, T., Baskin, J.M. and Baskin, C.C. (2009) Temperature requirements differ for the two stages of seed dormancy break in *Aegopodium podagraria* (Apiaceae), a species with deep complex morphophysiological dormancy. *American Journal of Botany* **96**, 1086–1095.

Ríos-Ruiz, S., Rivera-Nunez, D., Alcaraz-Ariza, F. and Obon-de-Castro, C. (1999) Three new species of *Narcissus* L. subgenus *ajax* Spach (Amaryllidaceae), restricted to the meadows and forests of south-eastern Spain. *Botanical Journal of the Linnean Society* **131**, 153–165.

Scholten, M., Donahue, J., Shaw, N.L. and Serpe, M.D. (2009) Environmental regulation of dormancy loss in seeds of *Lomatium dissectum* (Apiaceae). *Annals of Botany* **103**, 1091–1101.

Stokes, P. (1953) The stimulation of growth by low temperature in embryos of *Heracleum sphondylium* L. *Journal of Experimental Botany* **4**, 222–234.

Threadgill, P.F., Baskin, J.M. and Baskin, C.C. (1981) Dormancy in seeds of *Frasera caroliniensis*. *American Journal of Botany* **68**, 80–86.

Valle, F., Gómez, F., Mota, J.F. and Díaz de la Guardia, C. (1989) *Parque Natural de Cazorla, Segura y las Villas. Guía botánico-ecológica*. Alcorcón, Madrid, Ed. Rueda.

Vandelook, F. and Van Assche, J.A. (2008a) Temperature requirements for seed germination and seedling development determine timing of seedling emergence of three monocotyledonous temperate forest spring geophytes. *Annals of Botany* **102**, 865–875.

Vandelook, F. and Van Assche, J.A. (2008b) Deep complex morphophysiological dormancy in *Sanicula europaea* (Apiaceae) fits a recurring pattern of dormancy types in genera with an Arcto-Tertiary distribution. *Botany* **86**, 1370–1377.

Vandelook, F., Bolle, N. and Van Assche, J.A. (2007) Seed dormancy and germination of the European *Chaerophyllum temulum* (Apiaceae), a member of a trans-atlantic genus. *Annals of Botany* **100**, 233–239.

Walck, J.L. and Hidayati, S.N. (2004) Germination ecophysiology of the western North American species *Osmorhiza depauperata* (Apiaceae): implications of preadaptation and phylogenetic niche conservation in seed dormancy evolution. *Seed Science Research* **14**, 387–394.

Walck, J.L., Hidayati, S.N. and Okagami, N. (2002) Seed germination ecophysiology of the Asian species *Osmorhiza aristata* (Apiaceae): comparison with its North American congeners and implications for evolution of types of dormancy. *American Journal of Botany* **89**, 829–835.

Zonneveld, B.J.M. (2008) The systematic value of nuclear DNA content for all species of *Narcissus* L. (Amaryllidaceae). *Plant Systematics and Evolution* **275**, 109–132.