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Non-deep simple morphophysiological dormancy in seeds of *Viburnum lantana* (Caprifoliaceae), a new dormancy level in the genus *Viburnum*

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Abstract

Seed germination requirements of *Viburnum lantana* were investigated by experiments both in the laboratory and outdoors. Embryo length, radicle emergence and shoot emergence were analysed to determine the level of morphophysiological dormancy (MPD) of seeds. Mean embryo length in fresh seeds was 1.30 mm, and required growth to at least 2.51 mm to germinate. The critical embryo length was 4.1 mm. In the laboratory, the embryo reached 3 mm length after 20 weeks of warm-temperature incubation (20/7 or 25/10°C), which in fact represents a combination of warm + cold stratification. In seeds subjected to cold stratification (1.5 or 5°C) for 24 weeks, embryos hardly grew. Gibberellic acid stimulated embryo growth and germination. In the outdoor phenology test, the embryos grew from 1.30 mm, i.e. fresh seeds sown in September, to 2.98 mm at the end of the following March. In the 'move-along' test (laboratory), starting with temperatures of warm stratification [i.e. 25/10°C (4 weeks) → 20/7°C (4 weeks) → 15/4°C (4 weeks) → 5°C (12 weeks) → 15/4°C (4 weeks)], and in the outdoor phenology study on seeds exposed to a similar temperature sequence, radicle emergence percentages reached 73% after 28 and 35 weeks, respectively. *V. lantana* does not exhibit a delay between root and shoot emergence, dismissing any kind of epicotyl dormancy. Seeds of *V. lantana* have non-deep simple MPD, a level not detected previously in the genus *Viburnum*, with the physiological dormancy component overcome by a combination of warm and cold stratification, preferably in that order.

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Introduction

The genus *Viburnum* (Caprifoliaceae) comprises around 210 species distributed in temperate and subtropical regions in Europe, North Africa, Asia and America (Winkworth and Donoghue, 2005; Ruiz-Téllez and Devesa, 2007). It has long been recognized that seeds of *Viburnum* may require long periods of time (sometimes up to 18 months) to complete germination (Barton, 1958). Piotto and di Noi (2003) stressed the difficulty of eliminating the deep dormancy in this genus. At dispersal, seeds of *Viburnum* have an underdeveloped embryo which, in addition, has a physiological inhibiting mechanism of germination, i.e. morphophysiological dormancy (MPD) (Baskin and Baskin, 1998; Baskin *et al.*, 2009). That is, the embryo must grow for the radicle to emerge from the seed, but a physiological block must also be overcome before, during and/or after elongation of the embryo. There are nine levels of MPD, based on temperature requirements for embryo growth, breaking of physiological dormancy (PD) and emergence of the radicle and shoot, and response of seeds to gibberellic acid (GA_3) (Baskin *et al.*, 2008).

Most of the *Viburnum* species in which germination ecology has been studied have deep simple epicotyl MPD: *V. dentatum, V. dilatatum, V. lentago, V. prunifolium, V. rufidulum* and *V. pubescens* (Giersbach, 1937; Baskin *et al.*, 2008); *V. acerifolium* (Giersbach, 1937; Hidayati *et al.*, 2005), *V. betulifolium* and *V. parvifolium* (Chien *et al.* 2011a), and *V. opulus* (Walck *et al.*, 2012). This level of MPD requires warm stratification to break PD of the radicle, and cold stratification to break PD of the shoot (epicotyl). Thus, the radicle emerges in

autumn and the shoot in spring (Baskin and Baskin, 1998). Recently, a new level of MPD, called non-deep simple epicotyl MPD, was described in seeds of *V. odoratissimum* (Baskin *et al.*, 2008), and also detected in seeds of *V. formosanum* (Baskin *et al.*, 2009). At this level, a delay between root and shoot emergence also exists, but cold stratification is not required to break PD of the epicotyl. Furthermore, from the description of dormancy in seeds of *V. tinus* provided by Karlsson *et al.* (2005), who found that high percentages of roots and shoots emerged from seeds kept continuously at 20/10°C, and Giersbach's (1937) results on *V. nudum* and *V. scabrellum* germination patterns, with shoot emergence delayed for 1–1.5 months in seeds with emerged radicles incubated at 21°C, Baskin *et al.* (2008) concluded that seeds of these species also have non-deep simple epicotyl MPD. Although all *Viburnum* species studied up to now have 'epicotyl dormancy', Baskin *et al.* (2009) suggested both the possibility of absence in some species and the presence of new dormancy levels. As a contribution to a better understanding of the diversity of dormancy in seeds of the large and geographically widespread genus *Viburnum*, we studied the germination ecology of *V. lantana*, in order to test 'epicotyl dormancy' in seeds, and the level of MPD.

Since *V. lantana* has a wide geographical range in Eurasia, being adapted to live at high altitude [up to 2100 m above sea level (a.s.l.)] under extreme continental inland environments in the Iberian Peninsula (Ruiz de la Torre, 2006; Ruiz-Téllez and Devesa, 2007), we hypothesized that a cold stratification may be required to break the PD in the embryo. Embryo growth would occur either (a) during cold stratification (winter), if a complex level of MPD exists, or (b) during warm stratification (spring), after exposure to cold stratification, in which case seeds would have a simple form of MPD. Thus, the general objective of our research was to determine the level of MPD in seeds of *V. lantana*. Accordingly, we analysed: (1) the influence of different temperature and light conditions on embryo growth; (2) the effect of GA_3 on breaking dormancy and embryo growth; (3) the effect of different stratification and incubation conditions on seed germination; and (4) the phenology of embryo growth, break of dormancy, and shoot and seedling emergence.

Knowledge of the temperature conditions promoting germination in *V. lantana* is crucial to develop *ex situ* plant-production programmes to reinforce natural populations (Herrero and Villar-Salvador, 2013). In Andalucía (Southern Spain), *V. lantana* was catalogued as threatened (Herrera *et al.*, 2000), and in Castilla-La Mancha (Central Spain) it is a key component in the Habitat of Special Protection 'sub-Mediterranean deciduous thorny shrublands' (Martín-Herrero *et al.*, 2003).

Materials and methods

Plant material and source of seeds

Ripe, dark blue to blackish fruits of *V. lantana* were collected on 16 August 2008 (*c.* 3000 fruits), on 18 August 2009 (*c.* 10,000 fruits) and on 19 August 2010 (*c.* 3000 fruits), from a population growing in a deciduous thorny shrubland at 1010 m a.s.l. in Corduente (Alto Tajo, Guadalajara province, Central Spain, 30TWL8420). The dispersal unit is a drupe, and except for 1000 fruits collected in 2009, the exocarp and fresh mesocarp (the pulp) were removed, leaving the endocarp around the true seed. Hereafter, the germination unit, consisting of the endocarp plus the true seed, is called a seed. Seeds were washed with water and allowed to air dry at laboratory temperatures [22–24°C, 60–65% relative humidity (RH)] for 12–13 d before studies were initiated.

Laboratory experiments

General conditions for embryo growth and germination experiments

Experiments were conducted in chambers with controlled temperature and light regimes (Ibercex model F-4, Madrid, Spain) equipped with a digital temperature and light control system [±0.1°C, cool white fluorescent light, 25 $\mu mol\, m^{-2}\, s^{-1}$ (1350 lux)]. Seeds were tested for embryo growth and radicle emergence in a 12 h daily photoperiod (hereafter light) and in continuous darkness (hereafter darkness), which was achieved by wrapping Petri dishes in a double layer of aluminium foil, at constant temperatures of 1.5 and 5°C, and at 12/12 h daily fluctuating temperature regimes of 15/4, 20/7, 25/10 and 28/14°C. In the 12/12 h alternating temperature treatments, the higher temperature coincided with the light phase, and the lower temperature with darkness. Seeds were incubated in 9-cm-diameter Petri dishes, on two layers of filter paper moistened with distilled water. Dishes were sealed with Parafilm to minimize the loss of water.

The alternating temperature regimes simulated mean maximum and mean minimum monthly temperatures, characterizing the annual climate cycle in the seed-source region (the mountainous Iberian System in the central Iberian Peninsula): 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 June, July and August. The 5°C treatment simulated the mean temperature recorded during winter months: December, January and February (Elias and Ruiz, 1981). The other low temperature (1.5°C) was chosen because it is within the effective temperature range for cold stratification, and it was

used to simulate the coldest condition in winter in the MPD studies (Walck *et al.*, 1999; Walck and Hidayati, 2004).

Percentage germination (radicles emerged ≥ 0.5 mm, clearly visible) was computed based on the number of apparently viable seeds. Non-germinated seeds were checked for viability on the basis of embryo appearance, paying special attention to the colour and turgidity. Seeds were considered as viable if the embryo showed a white colour and resistance to slight pressure with tweezers. These indicators of seed viability agreed closely with the results obtained when using a tetrazolium test.

Effect of temperature and illumination on embryo growth

The aim of this section was to determine the optimal temperature for embryo growth. First, we measured the mean length of embryos in freshly matured seeds. Thus, on 1 September 2008, 25 seeds were placed on two sheets of filter paper moistened with distilled water in a 9-cm Petri dish at room temperature for 24 h. Embryos from imbibed seeds were excised with a razor blade. Their lengths were measured using a dissecting microscope equipped with a micrometer. Also on 1 September 2008, six dishes with 30 seeds each were placed in light and in dark at 1.5, 5, 20/7, 25/10 and 28/14°C. After 4, 8, 12, 16, 20 and 24 weeks, embryos of 25 seeds from each incubation condition were measured. Mean length and standard error were calculated for each sample of 25 embryos. Further, on 1 September 2008, a move-along experiment (Baskin and Baskin, 2004) was initiated in which seeds were moved from low to high temperatures (increasing series) and from high to low temperatures (decreasing series), to simulate the temperatures to which the seeds are exposed in natural conditions. The temporal sequence of temperatures in each series was as follows:

(a) Increasing series:
5°C (12 weeks) → 15/4°C (4 weeks) → 20/7°C (4 weeks) → 25/10°C (4 weeks).
(b) Decreasing series:
25/10°C (4 weeks) → 20/7°C (4 weeks) → 15/4°C (4 weeks) → 5°C (12 weeks).

Each series was conducted in light and in darkness. Therefore, 24 batches of 30 seeds each were prepared, assigning six batches to each series × light treatment. After 4, 8, 12, 16, 20 and 24 weeks, 25 embryos per batch were excised from healthy seeds and measured, calculating mean length and standard error for each embryo sample. Embryos from seeds from which the radicle had emerged during the experiment were recorded as having a critical embryo length (i.e. assuming it to be the value for every seed that had germinated previously in the treatment studied; Hidayati *et al.*, 2005; Vandelook *et al.*, 2009). That parameter corresponded to the mean embryo length of 40 seeds with split coats, but before radicles emerged (Phartyal *et al.*, 2009; Vandelook *et al.*, 2009). The E:S ratio is the quotient between embryo (E) and endosperm (S) lengths. The critical E:S ratio is the E:S ratio for seeds measured in the calculation of the critical embryo length. Thus, both critical length and critical E:S ratio are mean ($n = 40$) values. The minimum value in the range of those critical parameters corresponds to the minimal value required to germinate, thus being a reliable indicator that the morphological-dormancy component has been surpassed (Copete *et al.*, 2011a, b).

Effect of temperature and light conditions during stratification and incubation on germination

The goal of this section was to evaluate the effect of stratification conditions similar to those in the natural habitat on seed germination. For this purpose, seeds were exposed to the temperature sequences described above: increasing (a) and decreasing (b) series. On 1 September 2009 (seed age = 0 months), 1300 seeds were placed in each of four 16-cm Petri dishes on two sheets of filer paper moistened with distilled water and wrapped with Parafilm. Two dishes were stratified at the increasing series (a) and two dishes at the decreasing series (b), one dish being assigned to light and the other to darkness in each series. The stratification period was 24 weeks. Checks of hydration status and handling of seeds stratified in darkness were done under a green light (500–600 nm $\approx 0.5\,\mu mol\,m^{-2}\,s^{-1}$) (Vandelook *et al.*, 2007). After 24 weeks of stratification, non-germinated seeds (*c*. 35% germinated during the move-along test at increasing temperatures) were incubated for 4 weeks at 5, 15/4, 20/7 and 25/10°C, both in light and in darkness, using four 25-seed replicates for each incubation temperature × light condition. In addition, on 1 September 2009, we started a control test, incubating four replicates of 25 seeds for 28 weeks at each of the eight incubation conditions described above. We checked seed germination once a week, except for seeds stratified and incubated in darkness, which were checked at the end of the incubation period, in order to minimize the risk of exposing seeds to light.

Effect of gibberellic acid on embryo growth and germination

On 1 September 2008, 25 seeds were placed on two sheets of filter paper moistened with a solution of $1000\,mg\,l^{-1}$ of GA_3 in distilled water in each of 12 Petri dishes sealed with Parafilm (Herranz *et al.*, 2010). Six replicates were incubated at 25/10°C, assigning three to light and three to darkness. The test was also carried out at 20/7°C. After 4, 8 and 12 weeks, one replicate was recovered from each temperature and

light condition and 25 embryos were measured and the germination percentage recorded. As controls, we used the seeds incubated at 20/7 and 25/10°C on filter paper moistened with distilled water in the test 'Effect of temperature and illumination on embryo growth' (see above).

Outdoor experiments

Phenology of embryo growth, dormancy break and radicle emergence

On 1 September 2009 we started an experiment with ripe fresh seeds (seed age = 0 months), distributed into 10 batches of 100 seeds each. Seeds were mixed with sterilized sand, put inside nylon bags and buried at depth 6 cm in pots with drainage holes. Pots were placed in a shadehouse without a temperature-control system, located 200 km from the collection site at an altitude of 686 m a.s.l. in Albacete (Castilla-La Mancha, Spain). To simulate humidity conditions of the soil in the natural habitat, the water control system was programmed to water once a week, but it was reduced to twice a month in July and August to simulate the summer drought that is common in the Mediterranean area. In addition, we did not water when the substrate was frozen during the winter.

Air temperature in the shadehouse was recorded continuously by a data logger, assessing monthly averages of maximum and minimum temperatures. A nylon bag was exhumed monthly, starting 1 month after the initial burial. We recorded percentages of seeds with emerged radicles and the mean embryo length, using a sample of 25 healthy seeds. In radicle-emerged seeds embryo length was recorded as the critical embryo length for germination.

Non-germinated exhumed seeds with a healthy appearance, but not used to measure embryo length, were incubated for 4 weeks at 20/7°C in darkness. After seed incubation, we were able to calculate the following seed-status percentages: (1) seeds whose radicle emerged within the bag; (2) viable non-dormant seeds (i.e. those germinating at incubation phase 20/7°C); (3) viable dormant seeds (i.e. those that did not germinate at 20/7°C, but which had healthy embryos); and (4) non-viable seeds (i.e. those having a rotten appearance or showing a dead embryo when excised).

On 1 December 2010, we repeated this experiment, but using seeds collected in August 2010. The goal of this test was to check if exposure of seeds to the temperatures of September, October and November was essential for embryo growth.

Phenology of seedling emergence

On 1 September 2009, 200 seeds (without pulp) collected in August 2009, were sown at a depth of 5 mm in each of three trays (20 cm × 30 cm × 8 cm) with drainage holes, which were filled with a cultivation medium composed of sterilized peat and sand (2:1 v/v). Also, on 1 September 2009, 200 fruits (seeds with pulp) were sown in each of three trays in the same conditions as those described for seeds (above). On 1 December 2010, 200 seeds collected in August 2010 were sown in each of three trays, in the same conditions described above. Seed and fruit trays were placed in the non-heated shadehouse, and were watered as described in the previous section.

Trays were inspected every week, and seedlings with expanded cotyledons (criterion for shoot emergence) were counted and removed. Emergence counts continued until July 2011 in the case of the test started in September 2009, and until June 2012 for the test started in December 2010.

Statistical analysis

Germination data were summarized as cumulative percentages. We calculated means and standard errors of both germination data ($n = 4$) and embryo growth data ($n = 25$). Using a multifactorial analysis of variance (ANOVA), we analysed: (1) the photoperiod effect; (2) the temperature effect during the stratification; and (3) the temperature effect during incubation, both on embryo growth and on germination percentage. In all cases, the factors responsible for the main effects were detected by a multiple comparison Tukey test (significance level = 0.05); significant interactions were studied, contrasting confidence intervals. Previously, the normality (Cochran test) and the homoscedasticity (David test) of data were checked.

Percentages were transformed by arcsine square root to adjust their distribution to normality. Graphic representations of these percentages are shown without transformation.

Results

Laboratory experiments

Effect of temperature and illumination on embryo growth

Mean embryo length in fresh seeds was 1.3 mm, and mean seed length was 6.38 mm. Critical embryo length for germination was 4.1 mm (E:S critical ratio: 0.64). The minimum length of embryo found in a germinated seed was 2.51 mm (threshold at which a seed could germinate) and the value of the minimum E:S ratio was equal to 0.39.

In fresh seeds of *V. lantana* incubated during 24 weeks at alternating temperature regimes (20/7, 25/10 and 28/14°C, both in light and in darkness), embryo length at the end of the test was higher

than the minimum E:S ratio for germination. In light conditions, the embryo reached 3.7 mm at 20/7°C (Figs 1 and 2), 80% of embryos reached the minimum E:S ratio, and 46% of seeds germinated. In contrast, the embryos of seeds subjected to low constant temperatures (5°C and 1.5°C) did not grow during the test period (24 weeks) under either light condition (Fig. 2). 'Move-along' tests confirmed that embryo growth occurred in thermoperiods with higher temperatures, although higher germination (up to 37% in light conditions, and 33% in darkness conditions, at 24 weeks) and a higher percentage of embryos reaching the minimum E:S ratio were noted in the sequence of increasing thermoperiods than in the decreasing sequence ($P < 0.05$) (Fig. 3).

Effect of temperature and light conditions during stratification and incubation on germination

The sequence of increasing temperatures (a) promoted a significantly higher ($P < 0.05$) germination response than sequence (b) in seeds incubated at 15/4°C in darkness conditions (Table 1). The germination of seeds subjected to the treatment of decreasing stratification (b) was higher at 15/4°C than at the other temperatures. The response of seeds incubated continuously at the four temperature regimes increased with a rise in temperature and was highest at 20/7°C.

Effects of gibberellic acid on embryo growth and germination

GA_3 stimulated the rate (speed) of embryo growth in seeds incubated at 20/7°C and 25/10°C (Fig. 2), and significantly ($P < 0.05$) increased the germination percentage. When treated with GA_3 in light and in dark conditions for 12 weeks, 35% of seeds germinated at 20/7°C in the light, and 40% at 25/10°C in the light. In contrast, the final germination percentage obtained in the control treatment was zero (data not shown in figures).

Outdoor experiments

Phenology of embryo growth, dormancy break and radicle emergence

In the test carried out on 1 September 2009, embryos grew from 1.3 to 1.7 mm during September and October. During this period, the maximum and minimum mean temperatures were 25°C and 9°C, respectively (Fig. 4). Between 1 November 2009 and 1 March 2010, coinciding with the natural cold period, embryos hardly grew. In contrast, from 1 to 31 March 2010, embryo length increased from 1.7 to 2.98 mm, above the minimum threshold size of 2.51 mm; in addition, 36% of seeds reached the minimum E:S ratio. In that month, the mean maximum and minimum temperatures were 21°C and 6°C, respectively. During April and May, embryo growth continued, reaching the critical size of 4.1 mm at the end of May. All seeds exhumed between 1 October 2009 and 1 February 2010 were dormant (Fig. 5). In early March 2010, 36% of seeds had broken dormancy, and in early April, 38% of seeds had emerged radicles, and an additional seedlot fraction (21%) was non-dormant. Percentages of seeds with emerged radicles increased up to 62, 83 and 93% in early May, June and July, respectively (Figs 4 and 5).

Phenology of seedling emergence

At the end of April 2010, 39.6% of seedlings had already emerged, with little delay (2–3 weeks) with respect to radicle emergence. Emergence continued during May, June and July, reaching 81.6% at the end of this period (Fig. 4). In the phenological experiment started on 1 September 2009 with seeds including pulp (fruits), only 41.5% of seedlings emerged in early November 2010, with a further increase in cumulative seedling emergence during the following spring, which reached up to 62% on 30 April 2011. Also, there was a delay in the seedling emergence from

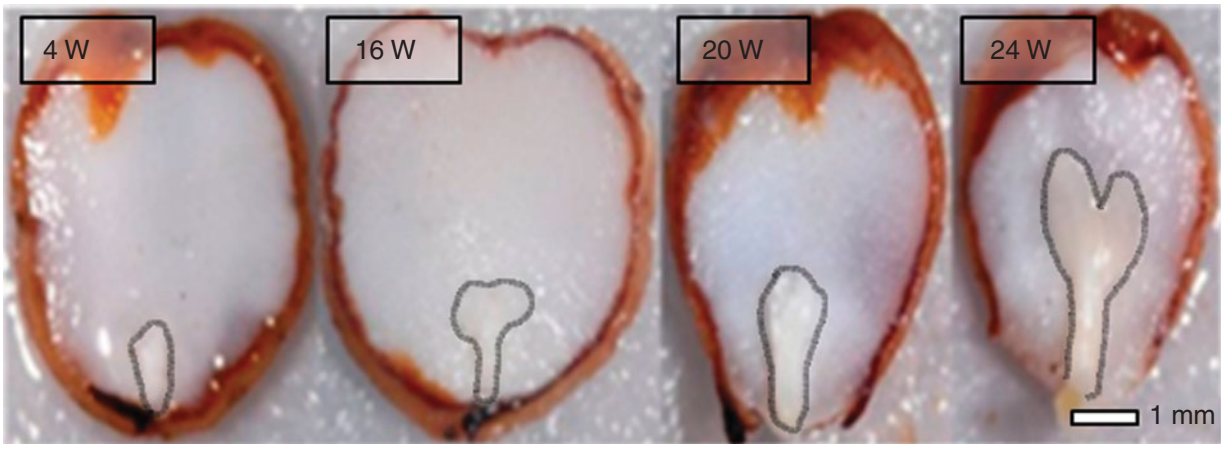

Figure 1. Embryo growth in *Viburnum lantana.* Embryo sizes correspond to the mean growth during incubation at 20/7°C in light conditions after 4, 16, 20 and 24 weeks. Embryos are outlined.

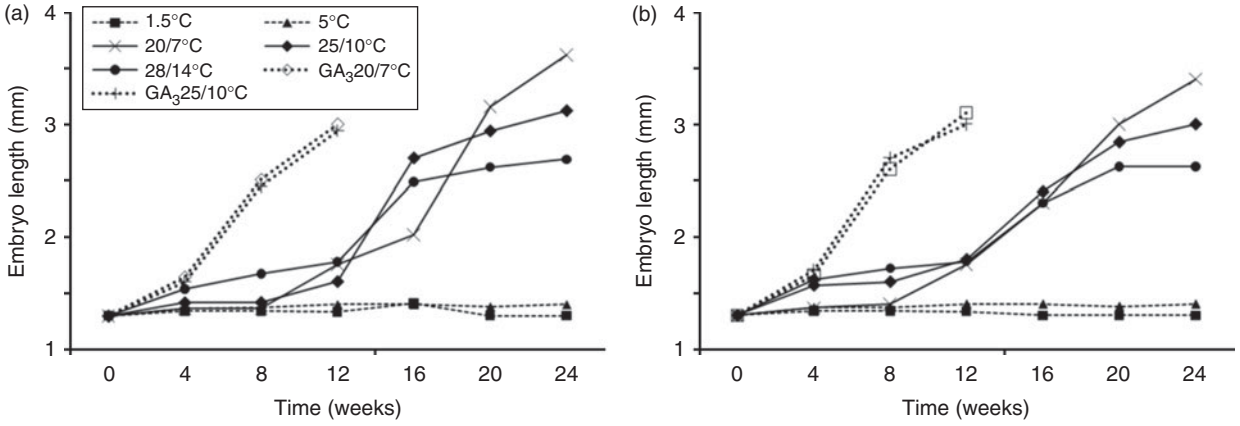

Figure 2. Embryo growth (mm) in freshly matured seeds of *Viburnum lantana* (mean ± SE; $n = 25$) incubated at 1.5°C, 5°C, 20/7°C, 25/10°C or 28/14°C for 24 weeks, or incubated at 20/7°C or 25/10°C with GA_3 for 12 weeks. Incubations were carried out both in light (a) and darkness (b).

fruits, in relation to those without pulp, and in early May 2010 only the 3% of seedlings had emerged, compared to 55% of the seeds without pulp (Fig. 4).

In the phenological experiment started on 1 December 2010, little embryo growth occurred during December, January and February. However, embryo length increased from 1.3 to 2.8 mm between 1 March and 30 April 2011, when maximum and minimum mean temperatures were 16°C and 4°C, respectively. During May and June 2011, embryo growth continued up to 3.5 mm, but it stopped during July, August and September, so the critical size was not reached. Radicle emergence started in May, but during the following months until 1 October 2011 only 40% of radicles and 25.5% of seedlings emerged. The cumulative seedling emergence did not increase between 1 October 2011 and 1 February 2012. From that time to 1 June 2012 emergence increased to 70% (Fig. 4).

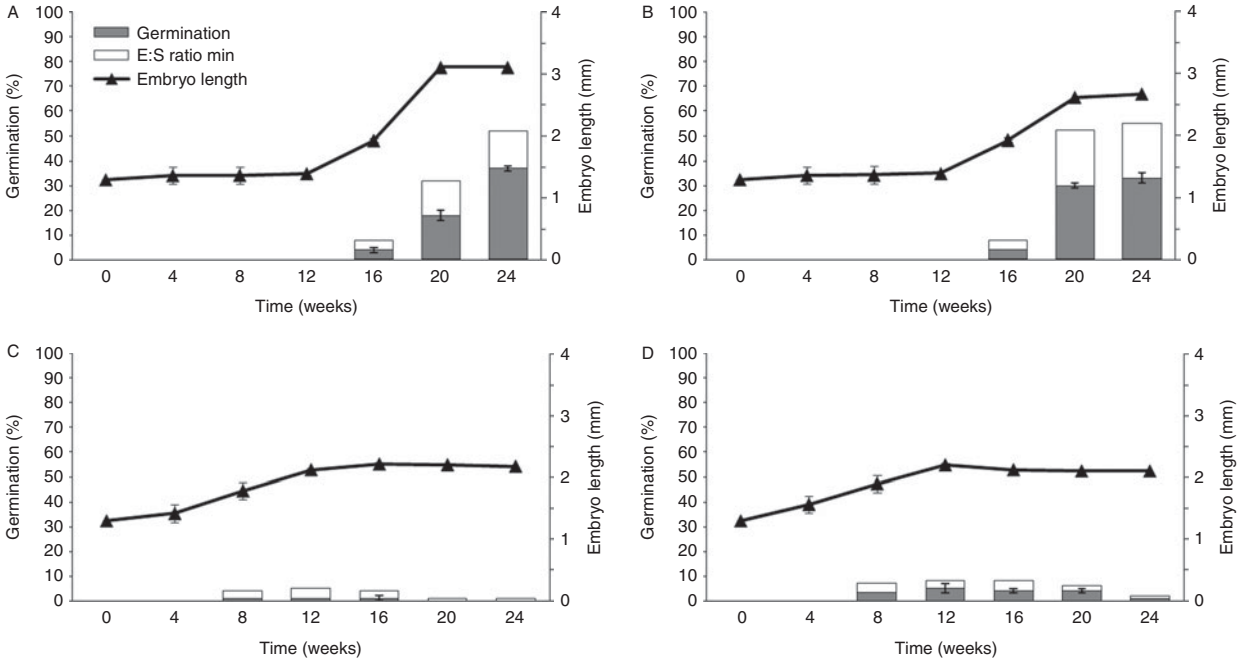

Figure 3. Embryo growth (mm) in fresh seeds of *Viburnum lantana* (mean ± SE; $n = 25$) subjected to (A, B) increasing stratification treatment [5°C (12 weeks) → 15/4°C (4 weeks) → 20/7°C (4 weeks) → 25/10°C (4 weeks)] and (C, D) decreasing stratification treatments [25/10°C (4 weeks) → 20/7°C (4 weeks) → 15/4°C (4 weeks) → 5°C (12 weeks)] in the light (A, C) and dark (B, D). The shaded part of the histograms shows the percentage of germinated seeds (mean ± SE). The white part shows the percentage of seeds with an E:S ratio higher than the minimum E:S ratio (0.39) above which germination is possible.

Table 1. Germination percentages of *Viburnum lantana* fresh seeds (mean ± SE, $n = 4$) incubated in light and dark at 5, 15/4, 20/7 and 25/10°C for 4 weeks following 24 weeks of incubation during which seeds were subjected to: (a) increasing stratification treatment [5°C (12 weeks) → 15/4°C (4 weeks) → 20/7°C (4 weeks) → 25/10°C (4 weeks)], and (b) decreasing stratification treatment [25/10°C (4 weeks) → 20/7°C (4 weeks) → 15/4°C (4 weeks) → 5°C (12 weeks)], in light and in darkness. Capital letters in columns and lower-case letters in rows show significant differences ($\alpha = 0.05$). Control: test duration was 28 weeks

	Incubation							
	5°C		15/4°C		20/7°C		25/10°C	
Stratification	Light	Dark	Light	Dark	Light	Dark	Light	Dark
a, Light	0^{Aa}	0^{Aa}	66 ± 2^{BCd}	90 ± 5^{Ce}	32 ± 3^{Bc}	50 ± 4^{Bd}	6 ± 1^{Ab}	26 ± 1^{Bbc}
a, Dark	4 ± 1^{Ba}	8 ± 1^{Ba}	80 ± 2^{Cde}	95 ± 2^{Ce}	50 ± 2^{Cd}	56 ± 1^{BCd}	20 ± 1^{Cb}	30 ± 3^{BCc}
b, Light	0^{Aa}	0^{Aa}	62 ± 2^{Bef}	70 ± 3^{Bf}	15 ± 1^{Ac}	33 ± 2^{Ad}	9 ± 2^{ABb}	11 ± 1^{Abc}
b, Dark	0^{Aa}	0^{Aa}	59 ± 4^{Bd}	73 ± 2^{Be}	30 ± 1^{Bc}	33 ± 3^{Ac}	13 ± 3^{Bb}	30 ± 2^{BCc}
Control	0^{Aa}	0^{Aa}	4 ± 1^{Aa}	2 ± 1^{Aa}	88 ± 4^{Dd}	85 ± 1^{Dd}	63 ± 3^{Dc}	40 ± 2^{Cb}

Discussion

In the test carried out in the laboratory, the highest embryo growth in *V. lantana* seeds occurred at 20/7°C, either when seeds were exposed to this temperature regime continuously for 24 weeks or during the 4-week period at this regime in the 'move-along' experiment with increasing temperatures. In contrast, embryos of seeds subjected to cold stratification (1.5°C or 5°C) for 24 weeks hardly grew, and none reached the minimum E:S ratio for germination (Figs 2 and 3). In the phenology experiment (Fig. 4), the highest embryo growth occurred in March and April for seeds sown in September or December. Seedling emergence was higher for seeds sown in September. These seeds were exposed to moderately warm temperatures during autumn and then to cold stratification. Furthermore, seedling emergence occurred immediately after radicle emergence, ruling out any kind of epicotyl dormancy in this species, a novel trait in the genus *Viburnum* (Baskin *et al.*, 2009).

Since embryo growth in *V. lantana* seeds occurs at warm temperatures (20/7°C, 25/10°C and 28/14°C), and is negligible at cold stratification, these seeds have one of the six simple levels of MPD, discarding the three levels of complex MPD. The levels of deep simple epicotyl, non-deep simple epicotyl, and deep simple double MPD, could also be discarded due to the lack of delay (2–3 weeks) in shoot emergence in relation to radicle emergence. Additionally, deep simple and intermediate simple MPD are discarded because once embryo growth occurs, a cold stratification period is not required to promote radicle emergence (Baskin and Baskin, 1998, 2004). Thus, seeds of *V. lantana* have non-deep simple MPD. This MPD level is also confirmed by the GA_3-mediated stimulation of embryo growth and germination in seeds incubated at 20/7°C or 25/10°C, and by the rapid radicle emergence in seeds with developed embryos. The presence of non-deep simple MPD in *V. lantana* is a novelty in the genus.

In *Viburnum* species with deep simple epicotyl MPD cited in the introduction, embryo growth and radicle emergence are completed when seeds are exposed to a sequence of temperatures typically recorded in late summer–early autumn, which is not the case for *V. lantana* (Fig. 4). In *V. lantana*, embryo growth commences at 25/10°C and continues when seeds are moved to 20/7°C, as also recorded in *V. tinus*. Furthermore, seeds of both species germinated ≥ 87% when incubated continuously at moderately warm temperatures: 20/7°C for *V. lantana* and 20/10°C for *V. tinus*. This may be because these alternating temperatures include warm and cold stratifications, being suitable for all parts of the germination process: dormancy break, embryo growth and radicle emergence (Karlsson *et al.*, 2005). However, when seeds of *V. tinus* were exposed to the temperature sequence of summer–autumn–winter, radicles emerged at 15/5°C and even during the first weeks at 5°C (Karlsson *et al.*, 2005), a period when radicle emergence in *V. lantana* was zero (Fig. 4). Shoots of *V. tinus* emerged 13–15 weeks after root protrusion, compared to 2–3 weeks in *V. lantana*. In radicle-emerged seeds of *V. tinus*, shoots also emerged after 6 weeks at 25/15°C (Karlsson *et al.*, 2005), leading Baskin *et al.* (2008) to suggest that *V. tinus* may have non-deep simple epicotyl MPD.

In some species with non-deep simple MPD, the physiological dormancy (PD) part of MPD is overcome by a warm stratification, e.g. as in *Chaerophyllum tainturieri* (Baskin and Baskin, 1990), *Corydalis flavula* (Baskin and Baskin, 1994) and *Papaver rhoeas* (Baskin *et al.*, 2002). However, other species require a cold stratification (1.5°C or 5°C) to break PD, after which high (≥15°C) temperatures are required for embryo growth, i.e. PD is broken during winter and MD (embryo growth) occurs when temperatures increase in spring, as recorded in *Thalictrum mirabile*

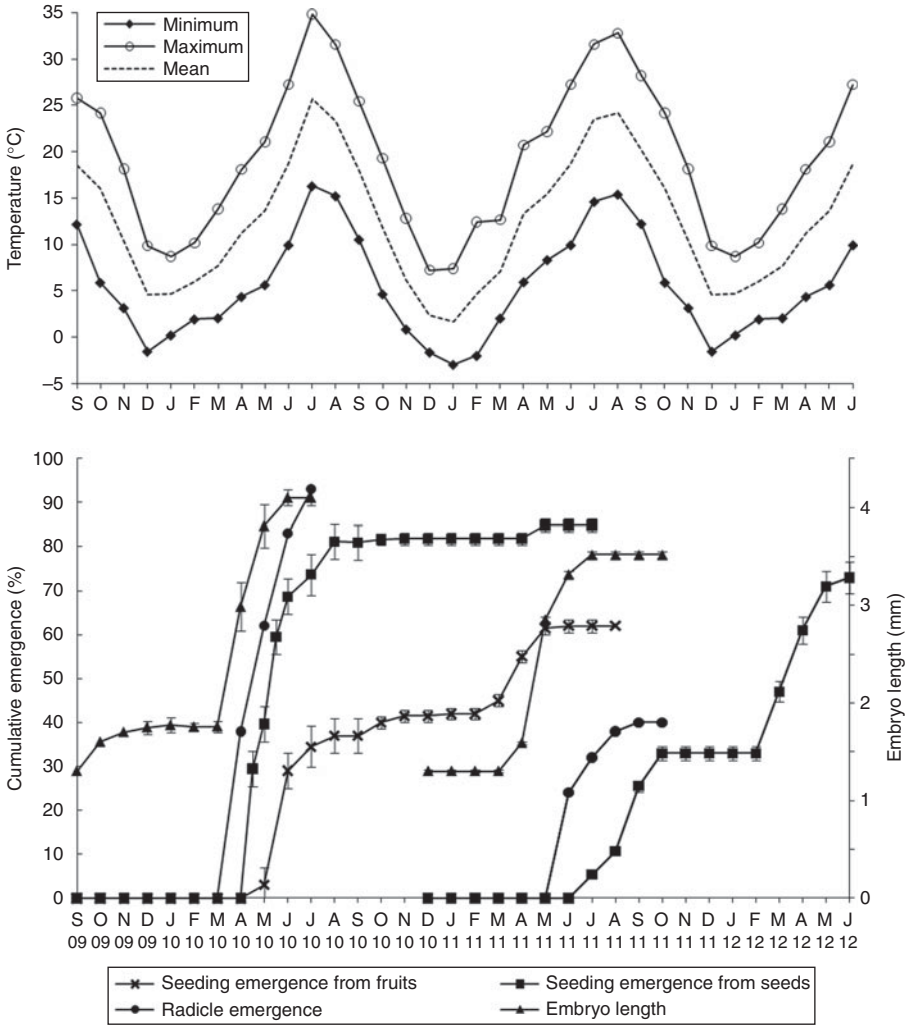

Figure 4. Phenology of embryo growth (mean ± SE, if SE > 0.1; $n = 25$), germination and seedling emergence (mean ± SE; $n = 3$) in *Viburnum lantana* seeds. Experiments were started in September 2009 and December 2010 (see Materials and methods). The upper graph shows changes in temperature (mean of maximum, mean of minimum and mean monthly temperatures) recorded in the shadehouse during the experiment.

(Walck *et al.*, 1999) *Chamaelirium luteum* (Baskin *et al.*, 2001) and *Selinum carvifolia* (Vandelook *et al.*, 2007).

In *V. lantana*, the overcoming of PD prior to embryo growth is promoted by a combination of warm and cold stratification (Figs 3 and 4). Although its seeds germinated up to 88% after a period of 28 weeks at 20/7°C in light conditions (Table 1), this period may be considered as equivalent to 14 weeks of warm stratification and 14 additional weeks of cold stratification (Baskin *et al.*, 2001). If *V. lantana* seeds only required a warm stratification to overcome the PD part of MPD, the highest percentage of embryo growth in the phenological experiment started on 1 September 2009 would have occurred during the next 3 months. During this period the mean maximum temperature was 23°C, and seeds would have received warm stratification (≥15°C) for 1.5 months. This is, in fact, what we recorded in *Lonicera etrusca* (Santiago *et al.*, 2013), another member of the Caprifoliaceae with fruits dispersed in the second half of August, whose seeds have non-deep simple MPD requiring a warm stratification to overcome the PD. If *V. lantana* seeds only needed cold stratification to break PD, most of the embryo growth in the phenological experiment started on 1 December 2010 should have occurred during the following March, as recorded in *Thalictrum mirabile* (Walck *et al.*, 1999) and *S. carvifolia* (Vandelook *et al.*, 2007). However, in *V. lantana* embryo length grew very little (i.e. from 1.3 to 1.6 mm).

Under laboratory conditions, the highest percentages of germination (95%) were obtained in the 'move-along' experiment with increasing stratification temperatures (5°C → 15/4°C → 20/7°C → 25/10°C) and subsequent seed incubation at 15/4°C (Table 1). However, this sequence does not occur completely under natural conditions, where 25/10°C must be

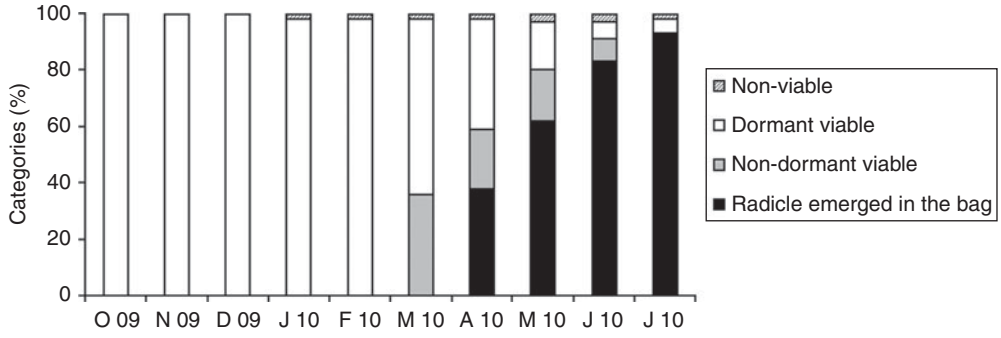

Figure 5. Stage of *Viburnum lantana* seeds exhumed monthly from 1 October 2009. Seed classes were: radicle emerged in the bag, non-dormant viable, dormant viable and non-viable.

followed by a higher temperature than 15/4°C. For this reason, in the phenological experiment started on 1 December 2010 (Fig. 4) a notable increment of the embryo length (from 1.30 to 3.32 mm) occurred during the following months of March, April and May. However, during June, with maximum and minimum mean temperatures of 27°C and 10°C, respectively, the embryo only grew up to 3.51 mm, failing to reach the critical size (4.1 mm). So during the following months (until 1 October 2011) radicle (40%) and seedling (25.5%) emergence were lower than those of the phenological experiment started on 1 September 2009 (93 and 81.6%, respectively). In the latter, seeds were exposed to a natural temperature sequence similar to the sequence in the 'move-along' experiment with decreasing temperature stratification. So, under natural conditions, higher germination values occur when the moderately warm temperatures associated with autumn months precede the cold winter temperatures. Under these conditions, seeds overcame dormancy in early March, 6 months from the beginning of the experiment (Figs 4 and 5). Bezdeckova *et al.* (2009) also recommended 3 months at 20°C followed by 2 months at 4°C to promote germination of *V. lantana*, although these authors did not measure embryo growth during the process. In a test started in September, Adams (1927) recorded 57% of seedlings emerging 10 months later, when seeds of *V. lantana* were sown in boxes placed out-of-doors, but only 17% in boxes placed in a greenhouse, concluding that germination 'is greatly accelerated by exposure for a time to low temperature'.

Our results confirm the initial hypothesis about the need for cold stratification to break physiological dormancy in *V. lantana* seeds. Although embryo growth occurred at moderately warm temperatures, it was only significant after exposure to cold (Figs 3 and 4). Moreover, for seeds incubated permanently at 20/7°C under light conditions (Fig. 2), the change in the curve of the slope of embryo growth took place after 16 weeks, when seeds had been exposed to a treatment which may be considered equivalent to 8 weeks of cold stratification at 7°C (Baskin *et al.*, 2001; Copete *et al.*, 2011b). On the other hand, in the phenological experiment started on 1 December 2010 a considerable increment in seedling emergence occurred during spring 2012, after the second period of cold stratification.

Although in seeds with non-deep simple MPD, the need for a combination of warm and cold stratification to break PD is a little known phenomenon, there are some precedents. Thus, *Ilex maximowicziana* (Chien *et al.*, 2011b) requires a long period (16 weeks) of exposure to moderately warm temperatures (20/10°C or 25/10°C) to obtain 80% germination. However, after a cold stratification at 5°C for 4 weeks, this period is shortened to 8 weeks. This fact shows that although cold stratification is not essential for germination, it is beneficial. Something similar happens in *S. carifolia* (Vandelook *et al.*, 2007) and *Torilis japonica* (Vandelook *et al.*, 2008). In both species, PD is broken by moist chilling (5°C). However, higher germination percentages are achieved at 20/10°C in light when seeds are submitted to 4 weeks of warm stratification (23°C) followed by 12 weeks of cold stratification (5°C) than when seeds are exposed only to cold stratification for 16 weeks.

At the study site, most *V. lantana* seeds are dispersed actively during the second half of August by frugivorous birds that eat their fruits and excrete the seeds in their droppings (personal observation), confirming other records on the natural habitat of this species in the Iberian Peninsula (Ruiz-Téllez and Devesa, 2007). Seeds, which are incorporated into the superficial soil horizons in early September, are exposed to cycles of warm, cold and again warm stratifications described in this work, so up to 80% of seeds may give rise to seedlings which emerge in the following late spring (Fig. 4). In contrast, in fruits (seeds with pulp) which fall directly to the ground after ripening, seedling emergence would be delayed considerably and reduced to less than a half (Fig. 4). The pulp surrounding the seeds complicates germination as a result of a waterproofing effect, the high

osmotic pressure due to dissolved sugars, or the presence of germination inhibitors (Mayer and Poljakoff-Mayber, 1989). Additionally, in seeds dispersed in autumn and with pulp until early December, seedling emergence is delayed notably, occurring mostly during June, July and August of the following year (Fig. 4). This fact may substantially reduce seedling survival probability, due to summer water stress, in those localities of the natural habitat with a more pronounced Mediterranean climatic influence.

The results of this study strongly suggest that most seeds of a cohort require dispersal by frugivorous birds to produce emergent seedlings in the next vegetative period. This fact emphasizes the influence of symbiotic and co-evolutionary relationships between frugivorous birds and shrub species in the habitat of *V. lantana*, which provide berries and drupes during late summer, autumn and early winter (Herrera, 1982; Martín-Herrero *et al.*, 2003). A previous study on several species of *Lonicera* genus cohabiting with *V. lantana* (Santiago *et al.*, 2013) also confirmed the importance of dispersal by frugivorous birds on germination. *V. lantana* seedlings emerging during April and May (up to 69% in the phenological experiment started in September 2009) rely on a period of 6 months to grow and develop before the arrival of winter, providing an opportunity for them to acquire some resistance to cold. Summer storms characterizing the Euro-Siberian region, as well as the Mediterranean mountains above 1300 m a.s.l., result in environmental and soil moisture conditions in the natural habitat which promote the survival of new seedlings (Martín-Herrero *et al.*, 2003; Ruiz de la Torre, 2006). In *V. lantana*, the main ecological consequence of needing a combination of warm and cold stratification to overcome the physiological dormancy component is that germination is concentrated in spring, offering young plants a long frost-free period in which to grow and develop before the outset of winter.

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Conflicts of interest

None.

References

Adams, J. (1927) The germination of the seeds of some plants with fleshy fruits. *American Journal of Botany* **14**, 415–428.

Barton, L.V. (1958) Germination and seedling production of species of *Viburnum*. *Proceedings of the Plant Propagators Society* **8**, 126–134.

Baskin, C.C. and Baskin, J.M. (1990) Germination ecophysiology of seeds of the winter annual *Chaerophyllum tainturieri*: a new type of morphophysiological dormancy. *Journal of Ecology* **78**, 993–1004.

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

Baskin, C.C. and Baskin, J.M. (2004) Determining dormancy-breaking and germination requirements from the fewest seeds. pp. 162–179 *in* Guerrant, E.O.; Havens, K.; Maunder, M. (Eds) *Ex situ plant conservation. Supporting species survival in the wild*. Washington, Island Press.

Baskin, C.C., Baskin, J.M. and Chester, E.W. (2001) Morphophysiological dormancy in seeds of *Chamaelirium luteum*, a long-lived dioecious lily. *Journal of the Torrey Botanical Society* **128**, 7–15.

Baskin, C.C., Milberg, P., Andersson, L. and Baskin, J.M. (2002) Non-deep simple morphophysiological dormancy in seeds of the facultative winter annual *Papaver rhoeas*. *Weed Research* **42**, 194–202.

Baskin, C.C., Chien, C.T., Chen, S.Y. and Baskin, J.M. (2008) Germination of *Viburnum odoratissimum* seeds: a new level of morphophysiological dormancy. *Seed Science Research* **18**, 179–184.

Baskin, C.C., Chen, S.Y., Chien, C.T. and Baskin, J.M. (2009) Overview of seed dormancy in *Viburnum* (*Caprifoliaceae*). *Propagation of Ornamental Plants* **9**, 115–121.

Baskin, J.M. and Baskin, C.C. (1994) Nondeep simple morphophysiological dormancy in seeds of the mesic woodland winter annual *Corydalis flavula* (Fumariaceae). *Bulletin of the Torrey Botanical Club* **121**, 40–46.

Bezdeckova, L., Reznickova, J. and Prochazkova, Z. (2009) Germination of stratified seeds and emergence of non-stratified seeds and fruits of *Viburnum lantana, Euonymus europaeus* and *Staphylea pinnata*. *Zprávy Lesnickeho Výzkumu* **54**, 275–285.

Chien, C.T., Chen, S.Y., Tsai, C.C., Baskin, J.M., Baskin, C.C. and Kuo-Huang, L.L. (2011a) Deep simple epicotyl morphophysiological dormancy in seeds of two *Viburnum* species, with special reference to shoot growth and development inside the seed. *Annals of Botany* **108**, 13–22.

Chien, C.T., Chen, S.Y., Chien, T.Y., Baskin, J.M. and Baskin, C.C. (2011b) Nondeep simple morphophysiological dormancy in seeds of *Ilex maximowicziana* from northern (subtropical) and southern (tropical) Taiwan. *Ecological Restoration* **26**, 163–171.

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

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

Elias, F. and Ruiz, L. (1981) *Estudio agroclimatico de la región de Castilla-La Mancha*. Toledo, Consejería de Agricultura, Junta de Comunidades de Castilla-La Mancha.

Giersbach, J. (1937) Germination and seedling production of species of *Viburnum*. *Contributions from Boyce Thompson Institute* **9**, 79–90.

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

Herrera, C.M. (1982) Seasonal variation in the quality of fruits and diffuse coevolution between plants and avian dispersers. *Ecology* **63**, 773–785.

Herrera, C.M., Manzaneda, A., Benavente, A., Luque, P. and Jordano, P. (2000) *Viburnum lantana L.* pp. 366–370 *in* Blanca, G.; Cabezudo, B.; Hernández-Bermejo, J.E.; Herrera, C.M.; Muñoz, J.; Valdés, B. (Eds) *Libro rojo de la flora silvestre amenazada de Andalucía. Tomo II. Especies vulnerables*. Sevilla, Spain, Junta de Andalucía.

Herrero, N. and Villar-Salvador, P. (2013) *Viburnum lantana* L.,*V. opulus* L., *Viburnum tinus* L. pp. 598–612 *in* Peman, J.; Navarro, R.; Nicolás, J.L.; Prada, M.A.; Serrada, R. (Eds) *Producción y manejo de semillas y plantas forestales*. Madrid, Organismo Autónomo Parques Nacionales.

Hidayati, S.N., Baskin, J.M. and Baskin, C.C. (2005) Epicotyl dormancy in *Viburnum acerifolium* (*Caprifoliaceae*). *The American Midland Naturalist* **153**, 232–244.

Karlsson, L.M., Hidayati, S.N., Walck, J.L. and Milberg, P. (2005) Complex combination of seed dormancy and seedling development determine emergence of *Viburnum tinus* (*Caprifoliaceae*). *Annals of Botany* **95**, 323–330.

Martín-Herrero, J., Cirujano, S., Moreno, M., Peris, J.B. and Stubing, G. (2003) *La vegetación protegida en Castilla-La Mancha*. Toledo, Junta de Comunidades de Castilla-La Mancha.

Mayer, A.M. and Poljakoff-Mayber, A. (1989) *The germination of seeds*. Oxford, Pergamon Press.

Phartyal, S.S., Kondo, T., Hoshino, Y., Baskin, C.C. and Baskin, J.M. (2009) Morphological dormancy in seeds of the autumn-germinating shrub *Lonicera caerulea* var. *emphyllocalyx* (*Caprifoliaceae*). *Plant Species Biology* **24**, 20–26.

Piotto, B. and Di Noi, A. (2003) *Seed propagation of Mediterranean trees and shrubs (Manuali e linee guida)*. Rome, Italy, Agency for the Protection of the Environment and for Technical Services (APAT).

Ruiz de la Torre, J. (2006) *Flora Mayor*. Madrid, Organismo Autónomo Parques Nacionales, Dirección General para la Biodiversidad.

Ruiz-Téllez, T. and Devesa, J.A. (2007) *Lonicerae*. pp. 168–187 *in* Castroviejo, S.; Benedí, C.; Rico, E.; Güemes, J.; Herrero, A. (Eds) *Flora Iberica: plantas vasculares de la Península Ibérica e Islas Baleares*, Vol. 15. Madrid, Spain, Consejo Superior de Investigaciones Científicas, Real Jardín Botánico.

Santiago, A., Herranz, J.M., Copete, E. and Ferrandis, P. (2013) Species-specific environmental requirements to break seed dormancy: implications for selection of regeneration niches in three *Lonicera* (*Caprifoliaceae*) species. *Botany* **91**, 225–233.

Vandelook, F., Bolle, N. and Van Assche, J.A. (2007) Multiple environmental signals required for embryo growth and germination of seeds of *Selinum carvifolia* (L.) L. and *Angelica sylvestris* L. (Apiaceae). *Seed Science Research* **17**, 283–291.

Vandelook, F., Bolle, N. and Van Assche, J.A. (2008) Seasonal dormancy cycles in the biennial *Torilis japonica* (*Apiaceae*), a species with morphophysiological dormancy. *Seed Science Research* **18**, 161–171.

Vandelook, F., Bolle, N. and Van Assche, J.A. (2009) Morphological and physiological dormancy in seeds of *Aegopodium podagraria* (*Apiaceae*) broken successively during cold stratification. *Seed Science Research* **19**, 115–123.

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

Walck, J.L., Baskin, C.C. and Baskin, J.M. (1999) Seeds of *Thalictrum mirabile* (*Ranunculaceae*) require cold stratification for loss of nondeep simple morphophysiological dormancy. *Canadian Journal of Botany* **77**, 1769–1776.

Walck, J.L., Karlsson, L.M., Milberg, P., Hidayati, S.N. and Kondo, T. (2012) Seed germination and seedling development ecology in world-wide populations of a circumboreal Tertiary relict. *AoB plants 2012*, pls007.

Winkworth, R.C. and Donoghue, M.J. (2005) *Viburnum* phylogeny based on combined molecular data: implications for taxonomy and biogeography. *American Journal of Botany* **92**, 653–666.