Seed germination requirements of the rare *Heloscadium repens* (aka *Apium repens*) determine persistence of seeds in the soil seed bank

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Short title as running head: Germination and dormancy breaking of *Heloscadium repens*

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One-sentence summary of the key message of the paper : Due to a strict light requirement for germination, *Heloscadium repens* seeds can remain in the soil for years after primary dormancy is lost during the first year of burial.

Abbreviations: TY: Ter Yde, VRO: De Vroente, VRY: Vrijbroekpark (names of populations)

Abstract:

- The rare and threatened *Heliosciadium repens* grows in moist grasslands and has a particular life cycle. Plants reproduce clonally, although the ramets tend to be rather short-lived, and sexually with seeds that can form a persistent soil seed bank. The germination requirements of *Helosciadium repens* seeds was thoroughly investigated, yielding important knowledge for habitat management and conservation.
- We studied the soil seed bank in three populations, carried out germination experiments and embryo growth measurements with fresh seeds in laboratory, greenhouse and outdoor conditions, and investigated the effects of storage and burial of seeds.
- Our results show that *H. repens* forms a long term persistent (> 6 years) soil seed bank with a very pronounced primary dormancy, but without secondary dormancy or dormancy cycles. Seeds can germinate throughout the growing season when temperatures are sufficiently high. Embryo growth and seed germination are triggered by light or, to a lesser extent, daily temperature fluctuations.
- Seeds of *H. repens* seem to have developed a rather unique germination syndrome with several strategies to remain ungerminated in the soil, until optimal conditions for seedling establishment as well as survival are present. It is obvious that sexual reproduction and seed bank formation are crucial for the long-term survival of the populations.

Introduction

Temporary wetland species growing in temperate climates, not only have to deal with seasonality in temperature for cueing seedling emergence, but also regular flooding. Species growing in wet habitats are characterized by typical adaptations to these specific challenges. Seed germination can be triggered, for example, by changes in daily temperature fluctuations that indicate the water level is decreasing (Thompson and Grime, 1983), sometimes in combination with a light requirement that provides information about depth of burial in the soil or competition from standing vegetation (Grime et al., 1981). Wetland species also typically have a short- or long-term soil seed bank that buffers the population against unpredictability of the environment (van der Valk, 1981).

The rare *Helosciadium repens* (Jacq.) W.D.J.Koch (Apiaceae) is a small creeping clonal plant, formerly known as *Apium repens* (Jacq.) Lag (Ronse et al., 2010). The species occurs mainly in western and southern Europe and has isolated populations in North Africa and the Canary Islands, according to McDonald and Lambrick (2006). It is a rare plant of particular interest, since it is listed in the national Red Lists of all the countries where it occurs (Schnittler and Günther, 1999), as well as in annexes II and IV of the European Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora of the European Community (EC Habitats Directive). It is assumed that the rareness of *H. repens* could be related to its rather peculiar ecology (Burmeier and Jensen, 2008). Although it is widely considered a perennial species, it seems that the ramets (individual stolons) are usually not very long-lived (4 months on average, as obtained by measurements of more than 11000 ramets in four populations, A. Ronse, pers. obs.) and that they are not very competitive (Burmeier and Jensen, 2009). The plants typically grow in moist grasslands, and seemingly require regular disturbance (grazing, mowing, wave action) while benefiting from winter flooding events (Burmeier and

Jensen, 2009). As such, it seems that besides clonal reproduction, successful sexual reproduction through seeds is important for population persistence.

The requirements for seed germination and dormancy breaking of *H. repens* have only been studied to a limited extent. Szwab et al. (2001), described germination tests with seeds from North-French populations. Burmeier and Jensen (2008) reported the existence of a persistent soil seed bank, which was already hinted at by field observations of recovery of an old Belgian population after 50 years of absence (Ronse 2004). It was also found that seeds responded positively to light, daily fluctuating temperatures and cold stratification, and that once dormancy had been relieved, seeds germinated over a wide temperature range (Burmeier and Jensen 2008). However, crucial information about the requirements for dormancy breaking and persistence in the soil seed bank is missing and is the subject of this study.

Seeds of Apiaceae typically have underdeveloped embryos at dispersal, meaning that, after dispersal and prior to germination, the embryo grows inside the seed while consuming the endosperm (Stokes, 1953). Growth of the embryo is very often associated with dormancy breaking that is induced by specific temperature and moisture conditions. Most Apiaceae have seeds with primary physiological dormancy that is broken by either low (0-10°C) or high (>10°C) temperature conditions (Baskin et al., 2014). Once these first layers of dormancy have been overcome, germination can be triggered by exposing seeds to proper light and/or (fluctuating) temperature conditions. Although many Apiaceae have seeds that seem rather short lived in the soil seed bank (e.g. Baskin et al., 2000; Vandelook et al., 2007; Vandelook et al., 2008), especially annual and biennial Apiaceae are known to have seeds that can remain dormant in the soil for up to 10 years (Baskin et al., 2004; Baker et al., 2005).

Here, we provide the first detailed study of the requirements for germination and dormancy breaking in *H. repens* and relate it to persistence in the soil seed bank. More specifically, we investigated (i) the density of the soil seed banks of the natural populations, as well as their germination fate, (ii) the light and temperature requirements for germination, embryo growth and dormancy breaking in controlled conditions, and (iii) seed survival and potential dormancy cycling by burial of seeds in the soil.

Material and methods

All experiments were performed with seeds obtained either directly or indirectly from the three largest then-known Belgian populations of *Helosciadium repens*: Vrijbroek (abbreviated VRY) in Mechelen (Antwerp), De Vroente (VRO) in Herk-de-Stad (Limburg) and Ter Yde (TY) in Koksijde (West-Vlaanderen). More information about these populations can be found in Ronse and Vanhecke (2004).

Regeneration from the soil seed bank

To test whether *H. repens* builds up a persistent soil seed bank, seeds were extracted using soil cores from the natural soil seed bank. Soil samples were randomly extracted with an Edelman auger in an area of about 100 m^2 in areas where *H. repens* was growing. Each sample consisted of six soil cores with an inner diameter of 5 cm and depth of 15 cm. The samples were concentrated according to the method described by Ter Heerdt et al. (1996). Seeds of *H. repens* measure between 1.0 and 1.7 mm (Szwab et al., 2001; Reduron, 2008), therefore we chose a coarse sieve of 3.15 mm mesh width and a finer sieve of 0.8 mm mesh width to sieve the soil to remove both coarse and fine soil material, roots and vegetative parts. The obtained soil fraction was then spread in a thin layer of about 1 to 2 mm in trays of 30 cm x 30 cm, on top of

157 a layer of sterilized potting soil topped with a 4 mm thick layer of sterilized coarse Rhine Sand.
158 The trays were placed in a non-heated greenhouse and were kept moist. We placed them
159 randomly in a block, mixed them with control trays of only potting soil and Rhine sand. We
160 counted all seedlings and removed them carefully immediately after identification, so that they
161 would not hamper other seedlings.
162
163 Two separate seed bank trials were performed and for both trials we computed the seed density
164 in the soil seed bank per compound sample (with six subsamples) as the number of seeds/m^2 by
165 using the formula $6\pi*r^2$ for the surface area with r the radius of the soil cores; this yields a
166 surface area of 0.011781 m^2 per sample. The average seed density per population was then
167 computed.
168
169 In the first trial, three compound soil samples were taken at the end of May 2007, both in the
170 VRY population as well as in the VRO population. Soil samples were only taken in areas where
171 *H. repens* was then growing or where it was known to have been growing, except for the second
172 sample in VRO , which was taken in part of the meadow where no plants of the species have
173 been observed. Samples were divided into an upper layer (from 0 to 6 cm depth) and a lower
174 layer (from 6 to 15 cm). The upper layers of the soil cores were pooled (total volume of 0.083
175 l), as well as the lower layers (0.166 l). The samples were concentrated according to the method
176 described by Ter Heerdt et al. (1996). The emergence and mortality of seedlings was recorded
177 twice a week during the first month, weekly during the second month, and monthly later on;
178 the experiment was continued until no more seeds had germinated over the course of 6 months
179 and lasted for two years. At the end of the experiment, we sieved the topsoil again, and checked
180 for remaining seeds under a stereo microscope. Seeds were considered viable if they were
181 visually intact and firm when pinched with tweezers.

As the first trial had shown the presence of seeds in the upper soil layers, a more thorough sampling was done, according to the size of the populations. For this second trial, respectively 16, 22 and 11 samples were extracted at 0 to 10 cm depth in the VRO, VRY and TY populations in December 2012. In this experiment, we first counted the seeds in the concentrated soil fraction with the microscope; the counted seeds were removed before testing the germination. The remaining soil fraction was then used for germination in trays, to test whether it still contained seeds that had not been counted. This started on 8 April 2013, and we monitored the emergence of seedlings of *H. repens* for a period of three years. At the end of the trial, we summed up the counted seeds with the germinated seeds, to obtain the total number of seeds per sample.

In further seed germination trials, seeds were used that had been harvested from plants grown in the plant collections of Meise Botanic Garden. These plants were seedlings obtained from the soil seed bank of the VRY population (see above). About 50 plants were grown outdoors in Meise Botanic Garden and open pollinated. The seeds used for the trials were air-dried and cleaned; they were stored in paper bags under ambient laboratory conditions (18–25°C, 40–60% relative humidity) until the start of the experiments, unless otherwise stated.

Effects of a short postharvest storage period on seed germination in greenhouse conditions

A preliminary germination test was performed in greenhouse conditions to test for the effect of short storage (< 1 month) on seed germination. Prior to sowing, seeds had been stored in ambient laboratory conditions (~ 20°C; 40-60% RH) during variable periods of time. In total 400 seeds were sown, of which 200 seeds were 26 days old on average (harvested 21 - 31 days previously), 50 seeds were 14 days old, and 150 seeds were 4 days old. Seeds were placed in

plastic pots (15 cm diameter), on top of sterilized commercial potting soil, and covered with a layer of approximately 1 mm soil. Each pot contained ten seeds. The pots were placed in a non-heated greenhouse and watered regularly. The exact temperature conditions in the greenhouse are not known, but the minimum temperature was set at 6°C to avoid frost damage. During sunny days in spring and summer, shade screens covered the greenhouse to avoid temperature peaks. This experiment started in November 2014, and the germination was recorded weekly for 11 months, more than three months after the last germination took place. After 3 months in the greenhouse, as nearly no germination had taken place by then, half of the pots were placed outside to expose them to a cold treatment as it was winter then. All pots were returned to the greenhouse afterwards.

Seed germination and embryo growth under controlled conditions

Temperature and light requirements for dormancy breaking and seed germination were examined by placing seeds on 1% agar (10 g/l) in 9 mm diameter plastic Petri dishes in incubators (LMS Cooled Incubators A 280) at different light and temperature regimes. At the end of each experiment, seeds were pinched with tweezers to check whether they were still firm and viable. Only firm seeds were considered in the analyses.

To determine whether primary dormancy was present, freshly harvested seeds were placed in combinations of three different temperature regimes and two light regimes. Four replicates of 20 seeds per dish were placed at constant 16°C or 20°C, or in daily fluctuating temperatures of 23°C/9°C (12h/12h). Seeds were exposed to a 12h photoperiod (PAR = 36 $\mu mol.m^{-2}.s^{-1}$, with light provided by Philips TLD 80 cool white, fluorescent tubes), coinciding with the high temperature part in case of daily fluctuating temperature regime, or in darkness by wrapping the dishes in aluminum foil. After 26 weeks (about 6 months), the seeds that were incubated in

the dark were also transferred to a 12/12h photoperiod, as they did not show any germination at all in the dark. This experiment ran for 76 weeks. To quantify and compare germination speed between treatments, we calculated 1/T30, as the reciprocal of time (days) required for 30% of the seeds to germinate (Soltani et al. 2015). Although, T50 is more commonly used to quantify germination, we opted to use T30 as 50% germination was not reached in all treatments. Germination speed was calculated for the period starting when seeds were incubated in light.

Effects of pretreatments on the dormancy status of seeds were tested with seeds that had first been dry stored in ambient conditions (c. 20°C; 40-60%RH) for 15 months, or cold stratified at 5°C in the dark for two months. After pretreatment, seed germination was tested at two temperature regimes, constant 16°C and daily fluctuating 23/9°C, with four replicates of 20 seeds per treatment. Germination was recorded for 26 weeks in both light and darkness as described above for each temperature condition.

An additional seed experiment was performed to test the interactive effect of light, daily fluctuating temperatures and KNO_3 on seed germination of *H. repens*. These three factors are known to act as gap-detection signal and to promote germination when environmental conditions are suitable for seedling establishment. In October 2023, we tested 160 seeds of *H. repens* that had been collected in VRY and that had been stored at -20°C and 15% RH for 10.5 years, prior to testing. Seeds were placed on agar in Petri-dishes and incubated in temperature control incubators for 6 weeks. Eight different treatments were applied with two replicates of 10 seeds per treatment. Seeds were incubated in light (8h/16h) or in darkness (wrapped in aluminum foil), at constant 20°C or daily alternating temperatures of 25°C and 10°C (8h/16h) and on agar prepared with distilled water or on agar containing a 101 mg/L KNO_3 solution.

Germinated seeds were counted and discarded weekly. Only intact seeds at the end of the experiment were considered in the analyses.

Embryo length measurements were performed with seeds that had been collected in VRY in August 2013 and that had been preserved at -20°C and 15% relative humidity until one week before the start of the measurements. We measured embryo length and seed length in seeds that had been (i) imbibed in water for one day at 20°C in darkness, (ii) incubated at 20°C in darkness for 28 days and (iii) imbibed at 20°C in light for 28 days. Finally, we also measured the embryo length and seed length in seeds that had just germinated (radicle length between 1 and 3 mm). For each condition 10 replicate seeds were measured. Seed length was measured as the length of the embryo plus endosperm, thus excluding the seed coat. For germinated seeds, only the length of the embryo that was still inside the seed was measured. Finally, the embryo to seed length ratio was calculated as a measure for embryo size.

Seed burial experiment

In a final experiment, the potential longevity of seeds in the soil was tested as well as putative changes in the dormancy status of seeds while in the soil. Freshly harvested seeds were buried in small mesh bags in the Botanic Garden. Each of 48 bags was filled with 20 seeds mixed with 10g of river sand ('Rhine sand') and buried about 5 cm deep. At regular intervals, two bags were dug out and tested for germination. At the start of the experiment (26 September 2016), 40 fresh seeds were tested as a control. Initially the germination tests were performed every 2 months, and after 28 months the intervals were increased to every 6 months or even 2 years for the final bag. The final bags were exhumed after 77 months (c. 6.5 years). For germination, the seeds were retrieved from the bags and were placed on agar in Petri dishes at 23/9°C (12h/12h) with a 12h photoperiod, which had proved to be the optimal regime for germination in earlier

trials. A cut test was performed after each germination trial to distinguish dormant seeds from dead seeds. After 55 months, an extra bag of seeds was exhumed to measure the embryo length of 10 intact seeds.

To quantify seasonal temperature variations that may affect seed germination and dormancy, mean monthly average air temperature values were obtained from the Royal Meteorological Institute in Uccle, Belgium (KMI, 2023).

Statistical analyses

Statistical differences in seed germination percentages were analyzed using logistic regression with logit link function, while differences in mean time to germination and embryo length were analyzed using analyses of variance. Tukey multiple comparisons tests were used to test for significant differences between different categories within a given variable. All these analyses were performed in R (version 3.6.3) using the GLM function in the standard stats package (version 3.6.3) and the multcomp package version 1.4-13 (Hothorn et al., 2008). Statistical differences in germination speed (1/T30) were analysed using analyses of variance in the R stats package (version 3.6.3). 1/T30 was log10 transformed prior to analysis to meet assumptions of normality. Non-transformed means and SE are shown in the graphs.

Results

Regeneration from the soil seed bank

In the first soil seed bank experiment, there was ample germination from the seed bank in the VRY population (table S1), where in total 384 seeds germinated from three soil samples. Seedling emergence started after 18 days, and kept on emerging during more than two years, until week 107 (Figure 1). After 5 months, 80% of all germination had taken place and that had

306 increased to 90% after one year. The final seedling emergence from the topsoil layers was
307 significantly higher than that from the deeper layers (Paired samples T-test t = 8.785; P = 0.013).
308 This translates to a mean soil seed density of 25880 seeds/m^2 in the upper layers and 8375
309 seeds/m^2 in the lower soil layers. In the VRO population no germination of *H. repens* was
310 recorded, except for two seedlings in the deeper layer of one sample (Table S1). At the end of
311 the experiment, a few non-germinated seeds were counted in some samples, but only from the
312 VRY population.
313
314 In the second soil seed bank trial, seedling emergence again started on day 18 and went on until
315 18 months later, in October 2014. Seedling emergence mainly took place within the three first
316 months of the experiment, and we recorded nearly no germination in the coldest months, from
317 December to February. In the VRY population on average 55 seeds per soil sample (4696
318 seeds/m^2) were found, with only one sample without seeds (Table S2). In VRO a much lower
319 average of three seeds per sample (260 seeds/m^2) was found, with individual values ranging
320 from 0 to 8 seeds per sample, equivalent to 0 to 1783 seeds/m^2. In TY rather similar seed
321 densities were found: the average number of seeds per sample also amounted to three, with an
322 average soil seed density of 208 seeds/m^2, and a maximum of 16 seeds per sample (1358
323 seeds/m^2).
324
325 The counting of the seeds under the microscope missed out nearly half of the seeds that were
326 present in the soil, as the small brown seeds are difficult to detect in the soil. Nearly as many
327 seeds germinated as the number of seeds that had previously been counted and removed from
328 the soil samples (Table S2). In total, 646 seeds were counted and 571 germinated in the samples
329 from VRY, 20 were counted and 29 germinated from VRO, and we counted 21 seeds versus 6
330 germinated in TY.

Effects of postharvest short storage on seed germination in greenhouse conditions

No significant interaction (GLM: $P > 0.05$) for seed germination percentage was observed between seed storage periods and incubation conditions (inside or outside greenhouse). Pots that remained in the greenhouse did show significantly higher germination (GLM: $z = -3.453$; $P < 0.001$) as compared to pots that were placed outdoors after three months (Fig. 2). No significant differences (GLM: $P > 0.05$) were found in the germination percentage for seeds of a different age. A decrease in seed germination percentage with increasing seed storage duration was observed for pots placed outdoors, while for pots that remained in the greenhouse highest germination was observed for seeds that had been stored for 14 days.

Seed germination and embryo growth under controlled conditions

Fresh seeds of *H. repens* germinated over an extended period at all temperature conditions, but only when exposed to light (Fig. 3). In the light, germination started already after one week and increased up to 65% after 26 weeks at 20°C. Seeds that were incubated in light continued to germinate at a slow pace until all seeds had germinated at 23/9°C after 76 weeks. After 76 weeks, germination at 16°C was significantly lower as compared to 20°C (GLM post-hoc Tukey: $P < 0.001$) and 23/9°C (GLM post-hoc Tukey: $P < 0.001$).

No seeds had germinated after 26 weeks of incubation in darkness, however, seed germination started rapidly after transfer to light (Fig. 3). No significant interaction effect for 1/T30 was observed between temperature and whether seeds were incubated first in light ($F_{2,17} = 1.110$; $P = 0.35$; Fig 4). A significantly faster germination was observed for seeds that were incubated in darkness first ($F_{1,17} = 392.2$; $P < 0.001$). Germination speed was significantly different at different temperature conditions ($F_{2,17} = 0.01$), although differences were small (Fig 4). After

70 weeks of incubation no significant difference in germination was observed for seeds that were initially incubated in light or in darkness (GLM: $P = 0.298$).

Seeds germinated to significant higher percentages after pre-treatment (GLM: $P < 0.001$), at 23/9°C as compared to 16°C ($P < 0.001$) and in the light ($P < 0.001$; Fig. 5), following 26 weeks of incubation. Significant differences in final germination percentages were observed between all pretreatment conditions (post-hoc Tukey: $P < 0.001$), with highest germination observed after cold-treatment and lowest germination without pre-treatment (Fig. 5). Germination in darkness was lower than 10% in all conditions, and only took place at 23/9°C after pretreatment of the seeds.

All three gap-detection signals (light, daily fluctuating temperatures and nitrates) significantly stimulated germination (Fig. 6), with highly significant effects of light (GLM; $P < 0.001$) and daily fluctuating temperatures (GLM; $P < 0.001$) and a significant effect of KNO_3 (GLM: $P = 0.02$). The interaction between daily temperature and light was the only interaction term that was significant in the model (GLM: $P = 0.049$).

Seeds of *H. repens* had a mean initial embryo length of 0.44 ± 0.02 mm (mean ± SE) and a seed to embryo length ratio of 0.49 ± 0.02. Embryo to seed length ratio had not increased significantly during a 4-week incubation at 20°C in dark conditions (Fig. 7). About half of the seeds placed at 20°C in the light had germinated during the 4-week incubation period and seeds were therefore only used to measure embryo length in the germinated seeds. In germinated seeds, the embryo to seed length ratio was significantly higher ($P < 0.05$) as compared to the initial ratio and as compared to seeds that had been incubated in darkness or that were buried (Fig. 7).

Seed burial experiment

Seeds exhumed from the soil germinated consistently to more than 95% germination when incubated at 23/9°C in the light (Fig. 8). Only in one bag retrieved after 22 months of burial, germination was about 80%. Until about 30 months of burial, seed viability was almost consistently higher than 90%. Irn the bags exhumed, after 34, 40, 48 months and 55 months of burial, quite large variation existed in viability between the bags exhumed, resulting in a very large variation. Fresh seeds germinated on average after close to 250 days (more than 8 months), but germination speed increased rapidly after increased periods of burial. Seeds buried for 10 months germinated on average within 2 weeks and this remained constant for up to four years of burial. Seeds buried for > 4 years germinated within one week after being exhumed and incubated. The embryo to seed length ratio in ungerminated seeds after 4.5 years of burial was not significantly different from that of the initial condition (Fig. 7).

Discussion

The presence of a persistent soil seed bank is a crucial aspect of *H. repens* regeneration and population survival. The production of small seeds that can survive for many years in the soil is quite unusual for clonally reproducing perennial species or for perennial Umbellifer species (Thompson et al., 1993). Moreover, the mechanism they evolved to prevent seeds from germinating in the soil is quite different from that of other wetland species. We argue that these seed characteristics are related to the occurrence of *H. repens* in wet and disturbed sites and to its peculiar ecology and habit.

Our experiments have shown that there is an active seed bank of *H. repens* in the three investigated Belgian populations, and that nearly all the germinated seeds were located in the

406 upper 6 cm of the soil. A higher regeneration from the upper soil layer (0-5 cm) as compared
407 to the lower layer (5-10 cm) was also reported in a population in northern Germany (Burmeier
408 and Jensen, 2008). These observations suggest that either the seeds are unable to penetrate
409 deeper soil layers, or the seeds can only survive in the soil for limited periods of time. Our seed
410 burial experiment also indicates that viability remained above 90 % during up to 2.5 years of
411 burial in the soil, and then became much more variable. In bags dug up after 4.5 years for
412 example, at least 90% of the seeds germinated in one bag while very few viable seeds remained
413 in the other bag, suggesting there might have been an infection or another detrimental event in
414 one of the bags. It seems therefore that it is likely that seeds of *H. repens* can survive for decades
415 in the soil. Long term seed survival in *H. repens* is corroborated by field observations from
416 populations that were recovered after being absent for decades. One example concerns a
417 historical Belgian population along an old cattle drinking pool in the coastal dunes
418 (Houtsaegherduinen, St Idesbald, De Panne, prov. West-Vlaanderen). This population
419 reappeared after 44 years, within one year after removal of a thick sand layer, that was blown
420 in by the wind during all these years (Ronse, 2004).
421
422 The average soil seed density varied considerably among populations, ranging from 208 in TY,
423 260 in VRO, to 4696 seeds/m^2 in VRY. The value of 530 seeds/m^2 calculated for the entire
424 sampled area in one population in northern Germany (Burmeier and Jensen, 2008) lies in the
425 same range. It would be logical that the soil seedbank density is related to the actual or ancient
426 plant density of the populations. This seems indeed to be the case for our data, as in 2003
427 average densities of between 7 and 217 ramets/m^2 were counted in VRY, against densities
428 between 2 and 30 ramets/m^2 in the two other populations (Ronse and Vanhecke, 2004).
429

Seeds of *H. repens* have evolved several strategies to remain ungerminated in the soil seed bank until conditions for seedling establishment and survival are optimal. Firstly, seeds of *H. repens* show considerable primary dormancy at dispersal, although there is a large variation in dormancy between individual seeds. Some of the most dormant freshly harvested seeds germinated only after 70 weeks of incubation, while others germinated within 3 weeks. In the seed burial experiment, primary dormancy loss was evident by an increase in germination rate, with seeds that were buried for 10 months germinating to a 100% within 3 weeks and seeds buried for > 4 years germinating even within one week. The latter observation suggests that dormancy is lost more rapidly in the soil as compared to laboratory conditions. Primary dormancy prevents seed germination immediately after dispersal and is considered a strategy to avoid germination close to the mother plant or immediately after dispersal at the end of the growing season (Hilhorst, 1995; Schütz, 1997), while increasing the chances to become incorporated in the soil seed bank (Venable and Brown, 1988). Since seeds of *H. repens* are dispersed in late summer, seed germination in autumn would be detrimental because of the high risk of flooding and frost damage, which agrees with the general observation of primary dormancy in wetland species (Jensen, 2004).

A second mechanism that inhibits seed germination consists of the inability of *H. repens* seeds to germinate at low winter temperatures. Germination tests at a wide range of temperature would be required to determine the base temperature for germination of *H. repens*. However, some of our experiments suggest that this base temperature for germination may be situated between 5°C to 10°C. Both cold stratification and dry storage significantly reduced the dormancy level in *H. repens*, as was confirmed in earlier studies (Szwab et al., 2001; Burmeier and Jensen, 2008). In contrast to the observation of a wide temperature range for germination by Burmeier and Jensen (2008), seeds of *H. repens* in our study did not germinate at lower

455 temperatures. Very little germination was observed in pots placed outdoors in the winter period
456 as compared to pots in the greenhouse, and germination at 16°C was significantly lower than at
457 20°C. Similarly, seedling emergence in the soil seed bank experiments stopped when
458 temperatures dropped in winter. Hence, a mechanism exists that prevents seeds of *H. repens*
459 from germination during the cold season.
460
461 A third mechanism that prevents germination in the soil, is revealed by our experiments
462 showing that light is important to induce germination of *H. repens*. The presence of this
463 mechanism is not surprising, considering the small seed size and the formation of a persistent
464 soil seed bank. Our own observations on the field support the finding that germination of *H.*
465 *repens* mainly occurs when seeds are exposed to light, as germination was mostly observed on
466 locations with open vegetation or with bare soil, mostly where the vegetation had been removed
467 by grazer trampling (A. Ronse, pers. obs.). Burmeier and Jensen (2008) also found higher
468 germination in light, although they did observe high germination percentages in darkness in
469 certain conditions, which is in stark contrast to our results. Besides a strong light requirement
470 for germination, our results also showed that daily fluctuating temperatures stimulate
471 germination of *H. repens*, especially after a stratification period. Similar findings of increased
472 germination at a fluctuating temperature regime are also mentioned in other studies (Burmeier
473 and Jensen, 2008; Szwab et al., 2001). We also observed a limited stimulating effect of addition
474 of nitrate, which has also been shown to trigger germination in response to vegetation gaps in
475 other species (Pons, 1989). Nonetheless, the requirement for light is a much more obligatory
476 germination signal as compared to daily temperature fluctuations and nitrate. This is quite
477 unusual for wetland species (Thompson and Grime, 1983), but could be related to the small
478 seed size of *H. repens*. Light does not penetrate deeply in the soil (Woolley and Stoller 1978)
479 and as such light is a better signal, as compared to daily fluctuating temperatures, to prevent

small seeds to run out of reserves because of germination too deep in the soil (Van Assche and Vanlerberghe, 1989). Whether the response to light and fluctuating temperatures should be regarded as a dormancy breaking mechanism or a mechanism inducing germination has been, and perhaps still is, a matter of debate (Thompson and Ooi, 2010; Finch-Savage and Footitt, 2012).

Finally, germination is also prevented because seeds of *H. repens* have an underdeveloped embryo at dispersal and showed significant embryo growth prior to germination. Post-dispersal embryo growth is typical for Apiaceae species and has been referred to as morphological dormancy (Baskin and Baskin, 2014). The initial embryo to seed size ratio was towards the higher end of the spectrum in Apiaceae (Vandelook et al., 2012). Nonetheless, a significant increase in embryo length was observed prior to germination. The presence of light is required to trigger embryo growth, which preceded germination, as no significant embryo growth was observed in seeds incubated in darkness or during burial in the soil. Although we did not measure embryo growth at low temperatures, given the positive response of embryo growth to light, it can be assumed that embryo growth starts once primary physiological dormancy is broken and germination is induced. Embryo growth rate is very likely quite high and can be completed in a matter of one to two weeks, as this is the shortest period we observed between exposure to light and seed germination following completion of embryo growth. A similar high embryo growth rate has been observed in other Apiaceae species where embryo growth is triggered by light (Vandelook et al., 2007b, Vandelook et al., 2008).

Perhaps the most surprising observation is that, although seeds of *H. repens* can remain viable in the soil for at least 6.5 years, and probably much longer, they do not show any dormancy cycling or induction of secondary dormancy. Since seeds can survive without any embryo

505 growth for years, they are very likely in a state of reduced metabolism, even though primary
506 physiological dormancy has already been overcome. Light or fluctuating temperatures seem to
507 trigger the seeds out of this state, by inducing embryo growth, eventually culminating into
508 germination. Dormancy cycles are very common in wetland species with a persistent seed bank
509 (Schütz, 2000), as well as in Umbellifers with long-lived seeds (Roberts and Boddrell, 1985;
510 Baskin et al., 1999; Vandelook et al., 2008). Dormancy cycles ensure accurate seasonal
511 germination timing in successive years and prevent germination in periods that are temporarily
512 suitable for germination, but not for seedling establishment. This may be because the conditions
513 that are suitable for germination concur with those for seedling establishment. Indeed, as a low
514 growing species, *H. repens* needs open and low vegetation not only as a seedling, but also
515 afterwards to subsist. Populations rapidly disappear when the vegetation becomes too high,
516 potentially contributing to its rareness (A. Ronse, pers. obs.).
517
518 To conclude, seeds of *H. repens* seem to have a rather unique dormancy and germination
519 syndrome, with unusual features for a perennial and clonally reproducing Umbellifer as well as
520 for a wetland species. The seeds form a persistent soil seed bank with a very pronounced
521 primary dormancy, but without secondary dormancy or dormancy cycles. Seeds germinate
522 throughout the growing season when temperatures are sufficiently high and in response to light,
523 and to a lesser extent daily temperature fluctuations. Our study has provided a deeper
524 understanding of the germination requirements, and therefore also of the ecology of *H. repens*.
525 Considering the rareness and decline of *H. repens* populations, such knowledge is critically
526 important when considering habitat management as well as potential reintroduction efforts. It
527 shows that the sexual reproduction and the formation of a soil seed bank has a crucial role for
528 the long term persistence of the species, given the rather low competitiveness of the individual
529 plants.

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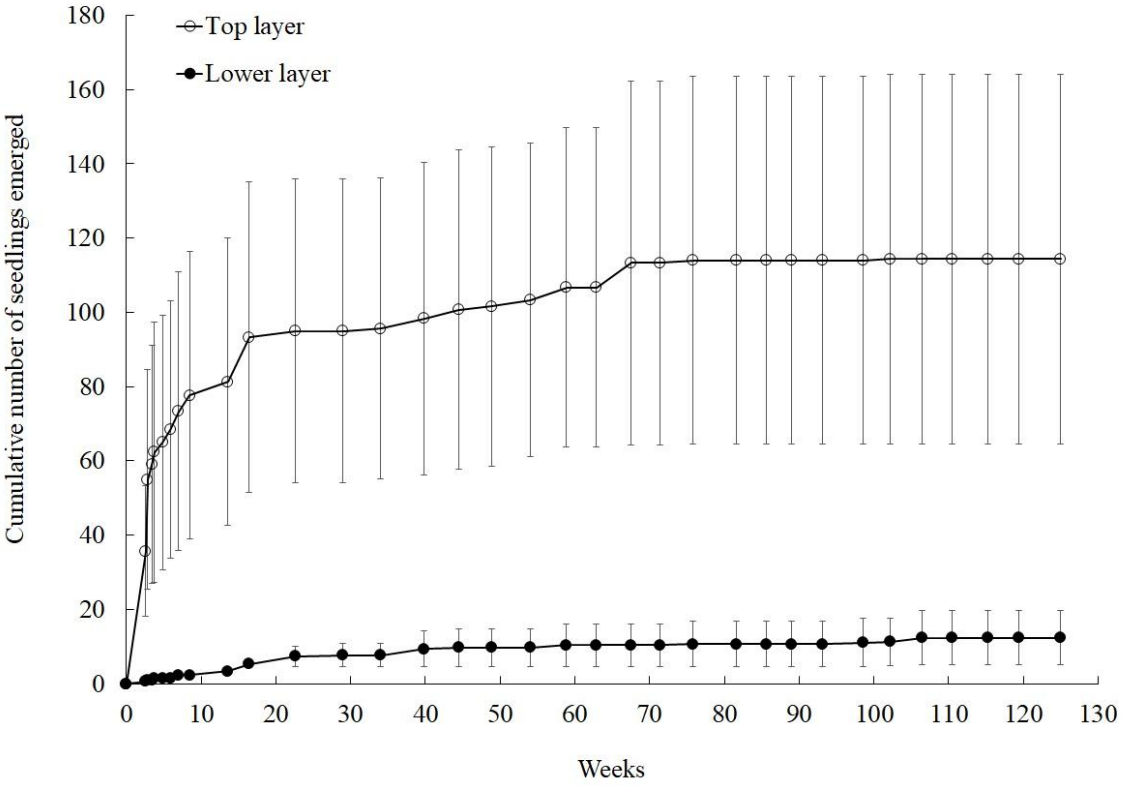
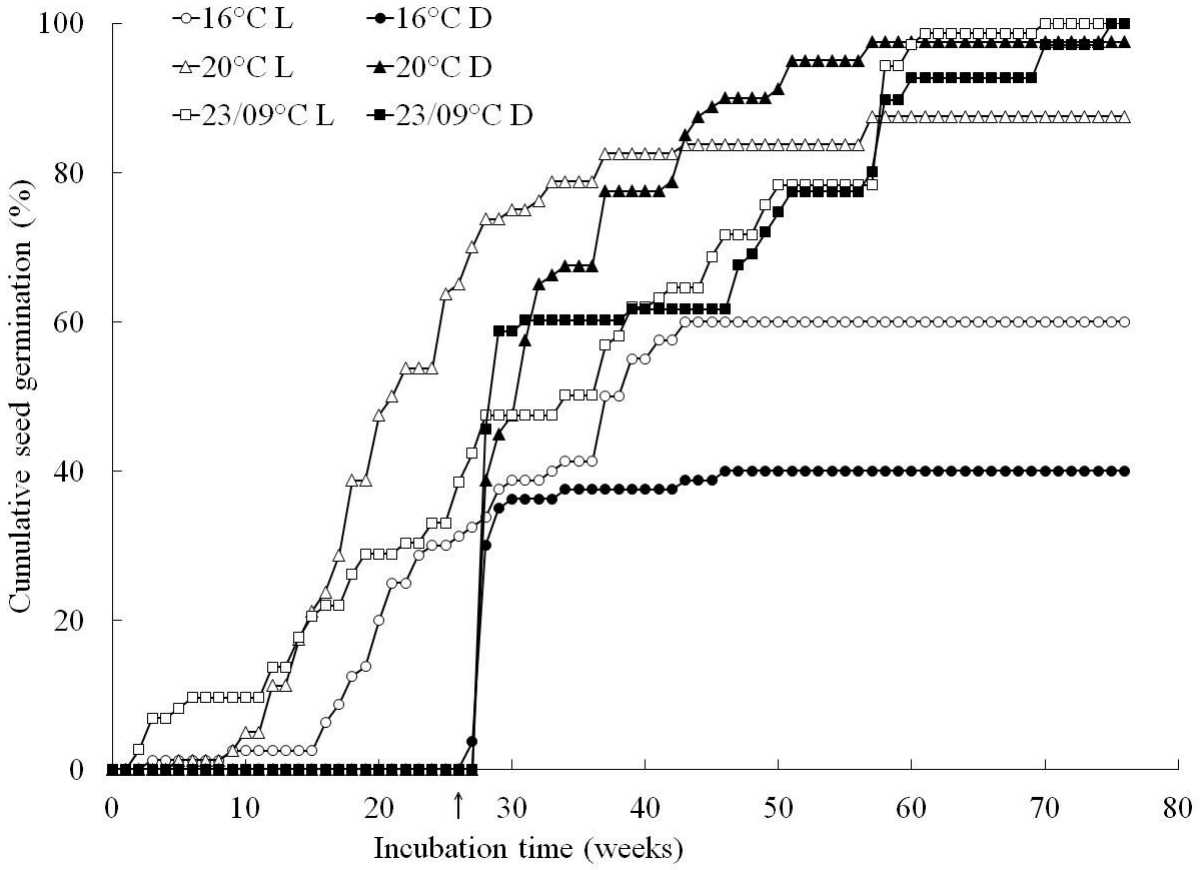
643 Figure legends



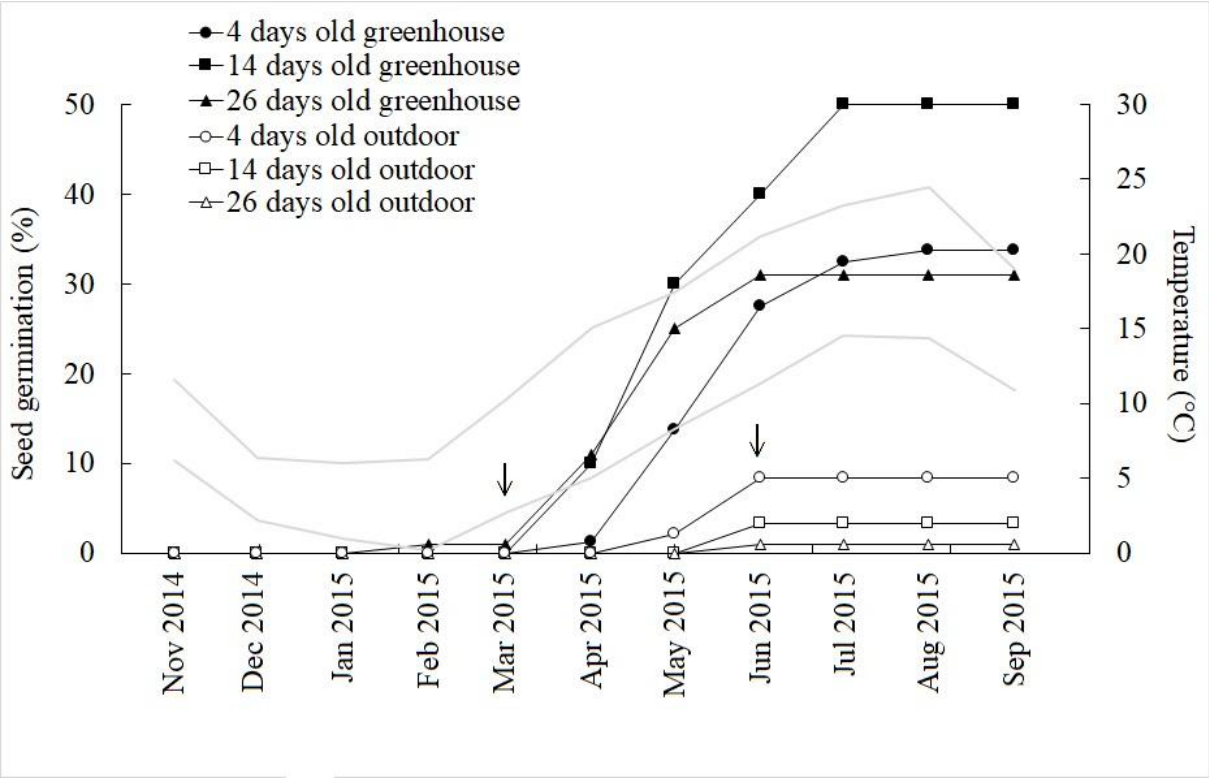
644

645 Figure 1: Mean cumulative germination of seeds from the soil seed bank from Vrijbroekpark
646 (VRY) over a 125-week period in a non-heated greenhouse.
647 Top layer: upper soil layer (0 – 6 cm); Lower layer: deeper soil layer (6 – 15 cm). Errors bars
648 are ± 1SE.



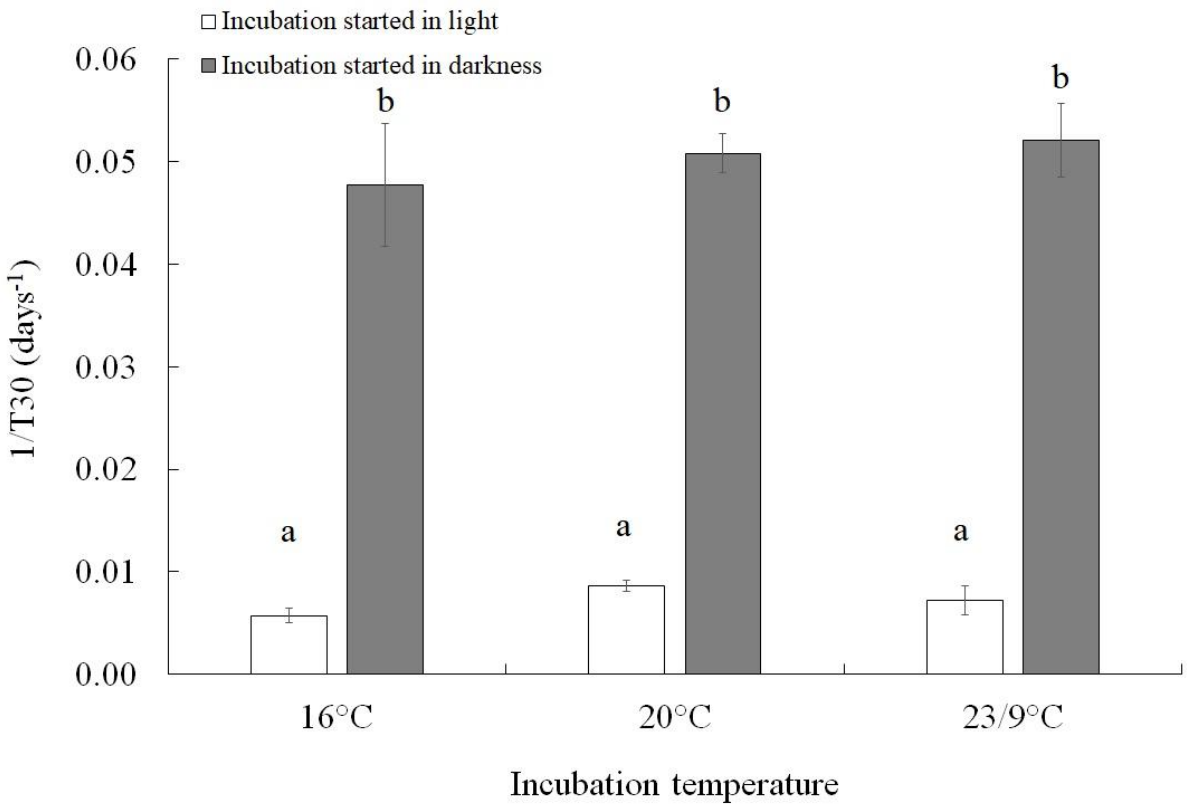
649

650 Figure 2: Cumulative germination percentage of *Helosciadium repens* seeds after 4, 14 and 26
651 days of storage at room temperature conditions. Seeds were sown in pots that were either kept
652 in the greenhouse during later winter (closed symbols) or placed outside (open symbols) after
653 three months in the greenhouse. First arrow indicates when pots were moved outdoor and
654 second arrow indicates when pots were put back in the greenhouse. Gray lines represent mean
655 monthly maximum and minimum air temperature in Uccle, Belgium (KMI, 2023) close to the
656 experimental site.



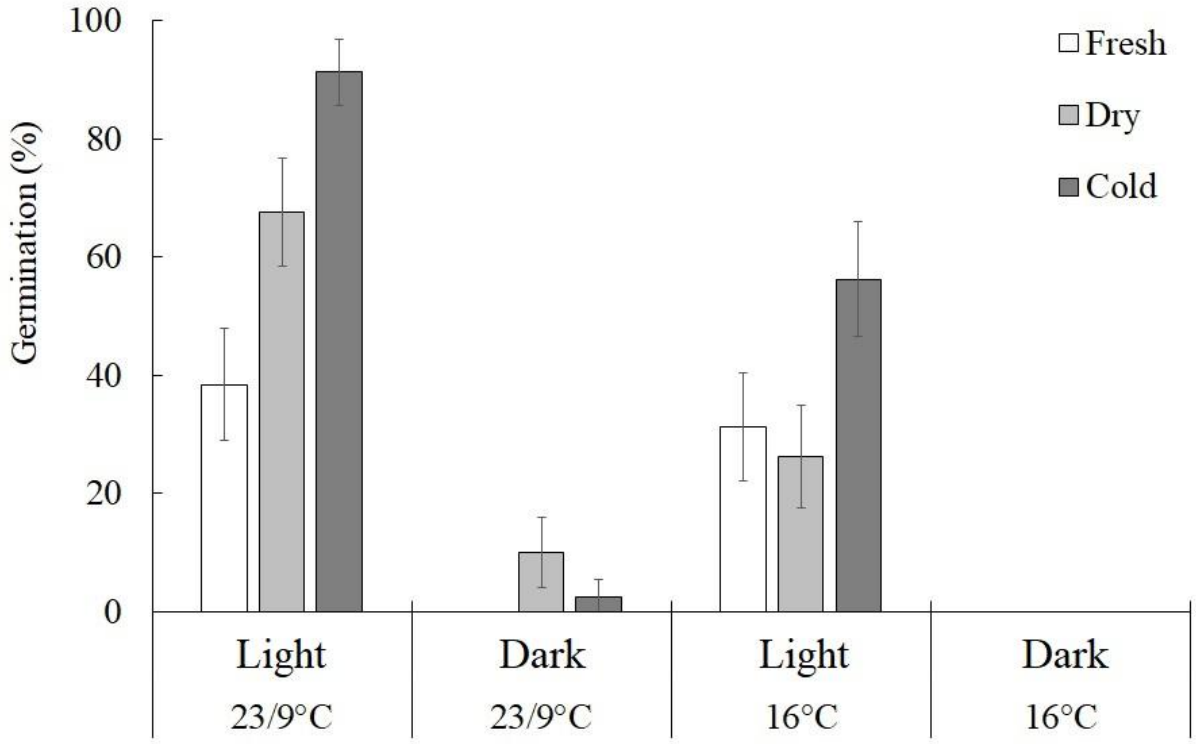
657

658 Figure 3: Cumulative seed germination of fresh seeds at different temperature and light
659 conditions. The arrow indicates transfer from dark to light conditions after 26 weeks. n = 4 for
660 all conditions, maximum SE for all treatments throughout the experiment varied between 7.4
661 (16°C dark) and 13.6 (20°C dark).



662

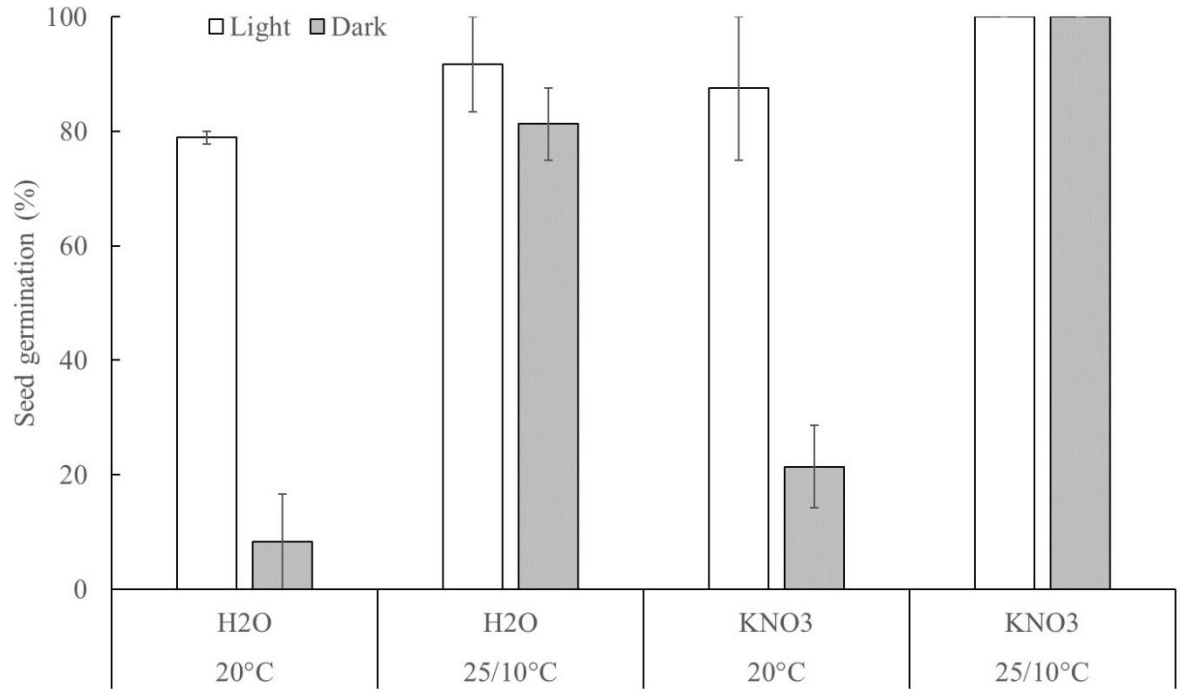
663 Figure 4: Germination speed expressed as 1/T30 ($days^{-1}$) for seeds incubated in light for 50
664 weeks at three different temperature conditions. Seeds were either immediately incubated in
665 light (white bars) or had been incubated in darkness for 26 weeks first (dark bars). Error bars
666 are ± 1 SE. Treatments with different letters above the bars are significantly different (Tukey
667 HSD $\alpha = 0.05$).



668

669 Figure 5: Seed germination percentage after 26 weeks of incubation in light (L) or darkness
670 (D) at constant 16°C or daily fluctuating 23/9°C following a 15-month dry storage (Dry), a
671 two month cold stratification (cold) or no pre-treatment (Fresh). Error bars are 95% Wald C.I.

672



673

674 Figure 6: Mean final seed germination percentages (± 1 SE) of *Heliosciadium repens* seeds
675 incubated in light or darkness, at 20°C or 25/10°C and in water or a 101 mg/L KNO_3 solution
676 for 6 weeks.

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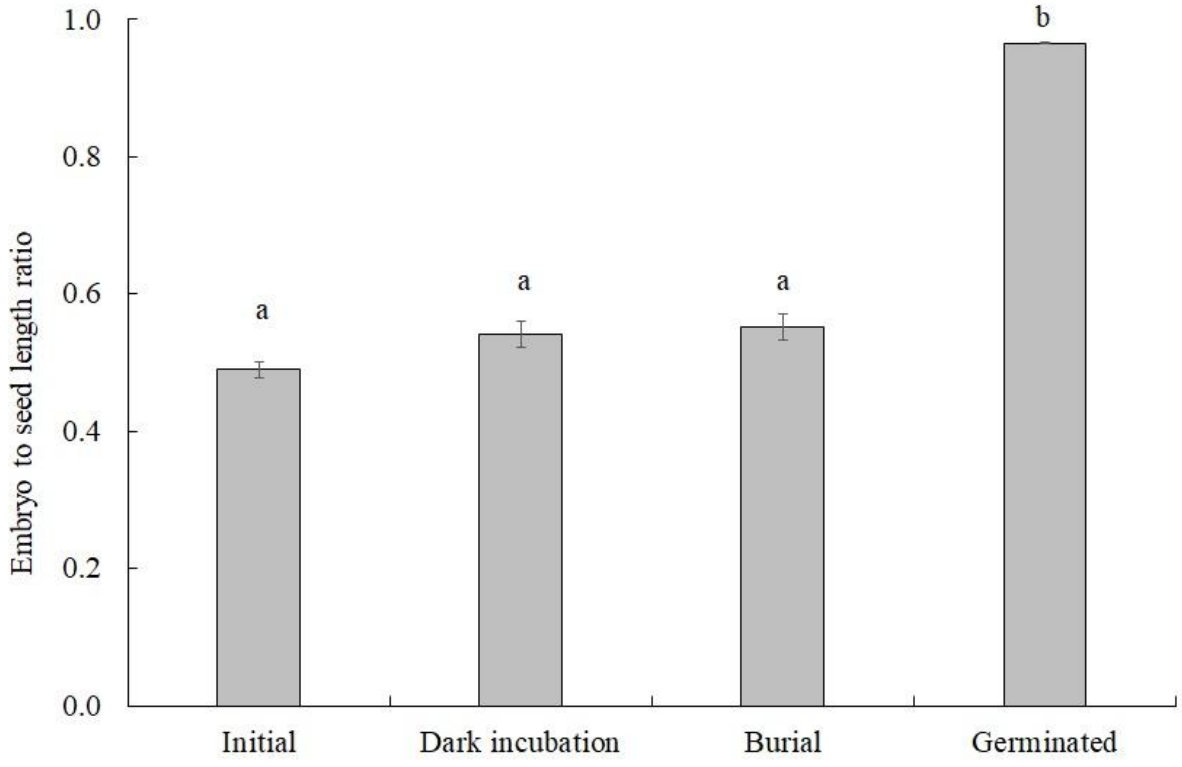
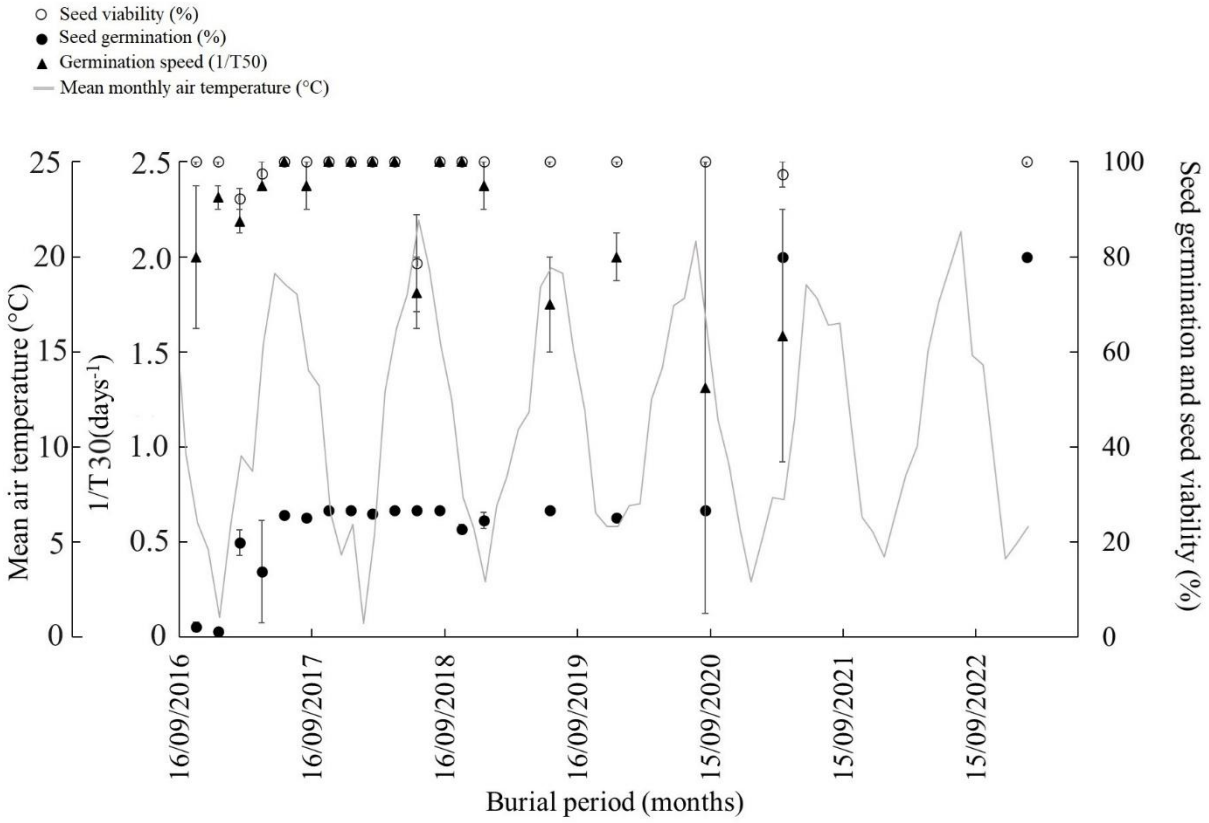


678

679 Figure 7: Mean embryo to seed length ratio (± 1 SE) of *Helosciadium repens* seeds without
680 pretreatment (initial), after 4 wks incubation in darkness at 20°C, after 55 months of burial
681 and at the moment of germination. Treatments with a different letter have a significantly
682 different embryo to seed length ratio (Post Hoc Tukey, $\alpha < 0.05$).



683

684 Figure 8: Seed germination percentage (full triangles), seed viability percentage (empty
685 circles) and germination speed expressed as 1/T30 (full circles) of *Helosciadium repens* seeds
686 at 23/9°C in light after different periods of burial in the soil. Error bars ± 1 SE. Gray line
687 indicates mean monthly air temperature in Uccle, Belgium (KMI, 2023) close to the
688 experimental site.

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