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Review Article

Germination responses in *Callitriche truncata* Gussone

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Abstract. This study investigated the germination responses of seeds of *Callitriche truncata*, an obligate hydrophyte that colonises temporary ponds in the Mediterranean, when subjected to different depths of burial and to varying patterns of initial flooding, and to examine the effect of flooding date on the growth and reproduction effort of this plant. All investigations were carried out at two different seed densities in order to investigate whether this factor would exert any effect on germination success and on accumulation of biomass. Seeds germinated from the 'no burial' treatment and from burial under 1 cm of sterile sediment with the rates of germination success declining rapidly with depth of burial. No germination was recorded from seeds buried deeper than 1 cm. The density of seeds per pot did not influence the results significantly. There was no significant difference in germination success of seeds subjected to 'Autumn flooding' and 'Winter flooding' treatments or across seed densities. Plants grown during the 'Winter flooding' treatment produced less total biomass and a lower proportion of reproductive biomass at the end of the experiment than seeds grown during the 'Autumn flooding' treatment. Although characterised by lower reproductive success, later flooding still permitted completion of life cycles and restocking of the seed bank. These findings are generally consistent with the results of previous studies in other temporary waters of the Mediterranean.

Keywords *Callitriche truncata*, Maltese Islands, germination, burial, biomass, flooding

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1 Introduction

The Southern Water Starwort, *Callitriche truncata* Gussone, is a hydrophyte or amphiphyte (Lansdown 2006) that colonises freshwater habitats in the Mediterranean and Western Europe. It is a frequent colonist of ephemeral freshwater habitats that alternate an aquatic phase with a terrestrial phase (Grillas et al. 2004). In the Maltese islands, this species colonises temporary freshwater rockpools that are inundated by rainfall at the start of the wet season, in September/October, and that subsequently experience one or more hydroperiods before undergoing desiccation at the start of the dry season in March/April (Lanfranco 2004). Nonetheless, in the Maltese Islands, *C.truncata* has only been observed in deeper pools characterised by a single, uninterrupted hydroperiod (Lanfranco and Sammut, in prep.). The life cycle of this species in such pools starts with germination shortly following the first flooding of the pools, growth and production of seeds during the aquatic phase, and survival in the seed stage on or in the bottom sediment of the pool throughout the dry season. Seeds are not actively dispersed but are deposited on the surface of the bottom sediment in the immediate vicinity of the parent plant and may subsequently undergo burial in the sediment (Camilleri 2012). The cyclic nature of the habitat, alternating a dry phase with an aquatic phase, imposes an annual life cycle on the species, necessitating re-establishment of populations every autumn. As such, the germination success of the seeds in the seed bank at the start of the wet season is a proximal initial determinant of the establishment of this species in the pool macrophyte community, implying that response to environmental cues for germination is a key factor in the persistence of this plant across years. Previous studies

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have indicated that germination responses of *C.truncata* seeds from temporary marshes in the Camargue are sensitive to depth of burial (Bonis et al. 1994) and seed density (Bonis et al. 1996) whilst the date of initial flooding has been found to lead to higher biomass and seed production of macrophytes in the temporary marshes of the Camargue (Grillas et al. 1998).

This study aimed to investigate the germination responses of seeds of *Callitriche truncata* when subjected to different depths of burial and to varying patterns of initial flooding, and to examine the effect of flooding date on the growth and reproduction effort of this plant. A negative relationship between density of seeds and rate of germination of *C.truncata* was observed by Bonis et al (1996). As such, all investigations in this study were carried out at two different seed densities in order to test whether this factor would exert any effect on germination success and on accumulation of biomass in local samples.

The following hypotheses were addressed:

Hypothesis 1: Germination success is reduced with greater depth of seed burial

Previous work, including Bonis et al. (1994) and Jurik et al. (1994) has shown that germination of seeds and emergence of seedlings from wetland seed banks is known to depend on the depth of burial of seeds in the sediment. Bonis et al. (1994) showed that most seeds, including those of *Callitriche truncata*, that germinated from seed banks of temporary marshes, did so from the seed reserve in the top 2 cm of sediment. Jurik et al. (1994) indicated that overlying sediment of depth as low as 0.25 cm significantly reduced the number of species and the total number of individuals recruited from wetland seed banks. It is hypothesised that, in accordance with the findings of Bonis et al. (1994), germination of *Callitriche truncata* seeds in freshwater rockpool seed banks of the Maltese Islands is sensitive to depth of seed burial, with higher germination success expected from seeds at the surface of the sediment layer.

Hypothesis 2: Germination success is higher at the start of the wet season

Later emergence from the seed bank would be expected to reduce the probability of macrophytes surviving to reproduction, since germination in periods closer to the end of the wet-season may not provide sufficient time for completion of the life-cycle. It is hypothesised that the germination success of *Callitriche truncata* seeds is higher in autumn, at the start of the wet-season, than it would be in winter.

Hypothesis 3: Production of biomass and reproductive success are dependent on flooding date

Early flooding has been found to increase the biomass and seed production of macrophytes in the temporary marshes of the Camargue (Grillas et al. 1998). It is hypothesised that a similar pattern occurs in *Callitriche truncata* populations in Maltese pools, where earlier flooding dates would lead to the formation of more vegetative and reproductive biomass than that produced in response to later flooding.

2 Material and methods

All seeds used in this study were collected from a single rock pool, situated at San Pawl tat-Targa, Malta, which was known to be colonised by *C.truncata*. Samples of dry sediment, which were assumed to contain recent seeds deposited at the end of the previous wet season, were collected from the surface of the sediment layer of the basin in August 2011. The sediment was stored in paper bags and subsequently stored in closed containers, in dark and dry conditions before undergoing sorting and analysis. Isolation of seeds from the sediment was started in mid-September 2011. During this process, the sediment was sieved under low-intensity jets of water through different mesh-sizes using the method described by Grillas et al. (1993) and Greenwood et al. (2005). The seeds and other debris collected by each sieve were filtered, and the residue left to air dry for 24 hours. The residue was subsequently observed through a stereomicroscope and seeds of *C. truncata* were removed using fine forceps. The seeds were subsequently stored in closed containers, in dark and dry conditions until required for the germination experiments.

The number of seeds recovered from the sediment samples was small compared to that utilised in studies with comparable objectives, such as Bonis et al. (1993). This was a consequence of the restricted volume of the sediment layer (and hence, of the seed bank) of the rockpools in the area of study. As such, the range of experimental treatments and number of replicates was constrained by the small number of seeds available. In all treatments, the criterion for germination was the emergence of the first plant structure (radicle or shoot) above the sediment layer. Use of this criterion therefore classified seeds that would have germinated but whose structures did not actually emerge above the sediment surface as 'ungerminated'. This limitation was considered unavoidable. During the course of the experiments, the pots were checked for germinated seeds at intervals ranging from three days to nine days, well within the monitoring ranges suggested by Baskin et al. (2001). Germlings were not removed from the pots so as to simulate natural intraspecific competitive effects.

Experiment 1: Effect of depth of burial on germination of seeds

The effect of depth of burial on germination responses was investigated using opaque, circular plastic containers (henceforth referred to as 'pots') of diameter 6.5 cm and height 8.3 cm in which a thin, level bed of sterile sediment was placed. Ten or twenty seeds, depending on the treatment, were placed in each pot using fine tweezers and distributed on the sediment surface along a uniform grid. The seeds were subsequently buried under different depths of sterile sediment. Five different treatments were used: no burial, burial under 1 cm, 2 cm, 3 cm and 4 cm of sterile sediment. The upper limit of burial was based on the conclusions of Bonis et al. (1994), who, investigating a number of species from temporary marshes, indicated that only Charophyte oospores germinated from burial under more than 4 cm of sediment. Each treatment was repeated with ten seeds per pot and with twenty seeds per pot in order to investigate any possible effects of density-dependence on germination rates. Four replicates were prepared for each treatment and these were arranged in a grid pattern in a small ventilated greenhouse and completely filled with water obtained from a domestic reverse-osmosis apparatus. Filling of the pots was carried out at the beginning of October 2011 and these were subsequently examined regularly for a period of five months during which water levels were replenished when necessary. The positions of the pots were rotated weekly in order to eliminate any effects that may have been attributable to the position of specific pots. Germination rates were calculated using Maguire's equation (M) as described by Ranal et al. (2006) and as applied by Greenwood et al. (2005):

$$M = \sum \frac{n_i}{t_i}$$

where n_i is the cumulative number of seeds that would have germinated at time t_i (days).

The duration of this experiment is much longer than the two-week to four-week duration for germination experiments suggested by Baskin et al. (2001). The long duration of this experiment was intended to mimic the natural situation, where seeds in rockpools are inundated for several months. As such, the length of this experiment would have detected any 'late' germination that may have occurred.

Experiment 2: Effect of flooding date on germination success

The effect of different flooding dates on germination success were investigated using pots identical to the ones utilised for the 'burial' experiment described previously, with a number of uniformly-distributed seeds being placed on the surface of a level bed of sterile sediment of approximate thickness 2 *cm* in each pot. Two treatments were designed, each simulating a different flooding period:

(1): 'Autumn flooding'. Pots were inundated on 15th October 2011 in order to simulate flooding in the warmer part of the wet season. The experiment proceeded for 61 days.

(2): 'Winter flooding'. Pots were inundated on 15th December 2011 in order to simulate a late wet-season. The experiment proceeded for 63 days.

Each treatment was repeated with ten seeds per pot and twenty seeds per pot with four replicates being prepared for each treatment. Flooded pots were maintained in a small ventilated greenhouse, exposed to diurnal cycles of ambient light and temperatures and their positions rotated weekly in order to eliminate any effects that may have been a consequence of the position of specific pots. The pots were examined regularly until the end of the experiments and the number of germinated seedlings noted.

Experiment 3: Effect of flooding date on accumulation of biomass

The effect of flooding date on accumulation of biomass was investigated by subjecting seeds to two treatments simulating different dates of flooding followed by a continuous hydroperiod and subsequent desiccation. This experiment was carried out using plastic pots measuring 17 cm × 9.5 cm × 7 cm (depth) with a number of seeds (either 25 or 50, depending on the treatment) being placed on the surface of a level bed of sterile sediment of approximate thickness 2 cm in each pot. The same treatments and number of replicates described for Experiment 2 were used. The 'Autumn flooding' treatment was started at the end of October 2011 and the 'Winter flooding' treatment at the end of December 2011. The 'Autumn flooding' and 'Winter flooding' treatments proceeded for 135 and 73 days respectively, ending in mid-March 2012. During this period, the pots were examined regularly and flowers and fruits were noted and counted and left on the parent plants. At the end of the experiment, the plants in each pot were harvested, rinsed carefully to remove any sediment, and dried for three hours at 45°C − 65°C. The total dry weight of plant material in each pot, including belowground biomass, was subsequently measured. An estimate for reproductive effort was calculated by comparing the biomass of fruit (representing reproductive structures) with biomass of vegetative material. Seven randomly-selected plants from each pot utilised for this purpose. The fruits were detached and weighed separately and fruit biomass was expressed as a proportion of total biomass.

3 Analysis of data

Proportion data was subject to an arcsine square-root transformation in order to normalise the distribution of the data (Legendre et al. 2012). Student's t-test was used when comparison of two datasets (after controlling for normality and equal variance) was required. The Shapiro-Wilk test was used to test for normality of the data whilst an Equal Variance test was utilised to compare variances in the groups being tested. When the assumptions of normality or equal variance were not satisfied, the groups were compared using a Mann-Whitney Rank Sum Test. Comparisons involving more than two groups were carried out using a One-way ANOVA , Two-Way ANOVA or a Kruskal-Wallis One Way Analysis of Variance on Ranks after controlling for distribution and variance. All tests were carried out using SigmaPlot for Windows version 11.0 (Systat Software 2008).

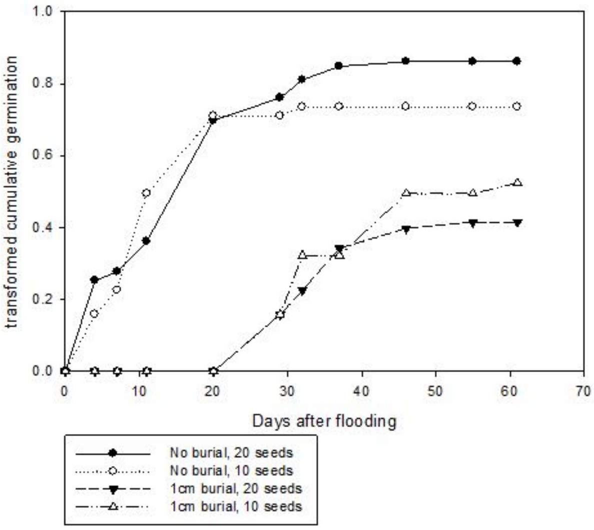

Figure 1: Cumulative germination success of seeds placed on the sediment surface and seeds buried under 1 *cm* of sterile sediment at two different seed densities (20 seeds per pot and 10 seeds per pot). Proportions of germinated seeds were arcsine square-root transformed.

4 Results

Effect of depth of burial on germination success

The effects of the depth of burial on seed germination are summarised in, Figure 1, and in Table 1. Seeds that were buried under 2 *cm*, 3 *cm* and 4 cm of sediment did not germinate and the results of these treatments were therefore omitted from subsequent analyses. Cumulative germination at the end of the experiment was highest in seeds that were not subject to burial (58% ± 6% with 20 seeds per pot; 45% ± 24% with 10 seeds per pot) and declined considerably in seeds that were buried under 1 cm of sediment (16% ± 14% with 20 seeds per pot; 25% ± 17% with 10 seeds per pot). The difference in cumulative germination between the seeds placed on the surface and those buried under 1 cm of sediment was tested using Student's t-test and was statistically-significant ($t = 3.738$; $P = 0.002$; $\pi = 0.931$). Seeds buried under 1 cm of sediment also germinated much later than seeds that had not been buried (Figure 1), although that may partly be a consequence of the time required for emergence of the shoot from deeper layers of sediment. First and last germination events in unburied seeds were noted 5.8 ± 3.5 days and 37.3 ± 6.9 days after flooding respectively whilst the analogous events in buried seeds were recorded 32.7 ± 4.0 days and 45.5 ± 13.1 days after flooding respectively. Peak germination rates were generally attained between $20-46$ days after initial flooding of the pots in all treatments. The interaction of seed density and depth of burial was examined using a Two-Way ANOVA. The results obtained indicated that the interaction between depth of burial and seed density was not significant in its effect on rates of germination ($F = 1.645$; $P = 0.224$; $\pi = 0.109$).

Table 1. Rates of germination success; mean number of days after flooding on which first germination events were recorded and mean number of days after flooding on which last germination events were recorded. Results are given for each treatment (different depths of burial of seeds) and for different seed densities per pot (20 seeds per pot and 10 seeds per pot). No germination was recorded from seeds buried under 2 cm, 3 cm and 4 cm of sediment.

	Germination success		First germination		Last germination	
Depth of burial	20 seeds	10 seeds	20 seeds	10 seeds	20 seeds	10 seeds
0 *cm*	58% ± 6%	45% ± 24%	5.8 ± 3.5	9.3 ± 3.5	37.3 ± 6.9	23.0 ± 6.0
1 *cm*	16% ± 14%	25% ± 17%	32.7 ± 4.0	38.5 ± 15.1	38.7 ± 14.2	45.5 ± 13.1
2 *cm*	0%	0%	-	-	-	-
2 *cm*	0%	0%	-	-	-	-
2 *cm*	0%	0%	-	-	-	-

Effect of flooding date on germination responses

The effects of early-onset and late-onset flooding on germination responses are summarised in Figure 2 and in Table 2. Mean germination rates ranged from 70% ± 8% of seeds in the 'Autumn Flooding' treatment to 55% ± 38% of seeds in the 'Winter Flooding' treatment. A One-Way ANOVA did not indicate any overall significant difference in germination rates ($F = 0.309; P = 0.819; \pi = 0.05$) across flooding treatments ($t = 0.533; P = 0.602; \pi = 0.05$) and across density of seeds per pot ($t = -0.425; P = 0.677; \pi = 0.05$). First germination in all treatments was recorded within 5.3 ± 6.7 to 10.0 ± 6.0 days of flooding whilst latest germination was recorded between 13.3 ± 9.6 to 56.0 ± 0.0 days after flooding. Peak germination rates were attained within 3 to 15 days after initial flooding (Figure 2) with no consistent difference in germination rate being noted across flooding treatments and across different seed densities. A Two-Way ANOVA indicated that differences in germination success across seed densities per pot were not statistically significant (F = 0.4405; P = 0.537). No significant interaction between seed density and flooding date, in relation to germination success, was detected (F = 0.351; P = 0.565).

Table 2. Rates of germination success; mean number of days after flooding on which first germination events were recorded and mean number of days after flooding on which last germination events were recorded. Results are given for each treatment (different flooding dates) and for different seed densities per pot (20 seeds per pot and 10 seeds per pot).

	Germination success		First germination		Last germination	
Treatment	20 seeds	10 seeds	20 seeds	10 seeds	20 seeds	10 seeds
'Autumn'	$56\% \pm 18\%$	$70\% \pm 8\%$	9.5 ± 2.9	10.0 ± 6.0	33.0 ± 12.7	27.0 ± 9.2
'Winter'	$59\% \pm 24\%$	$55\% \pm 38\%$	10.0 ± 3.5	5.3 ± 6.7	56.0 ± 0.0	13.3 ± 9.6

Effect of flooding date on accumulation of biomass

The effects of flooding date on accumulation of biomass and reproductive biomass are summarised in Figure 3, Figure 4, and in Table 3. Plants that germinated during the 'Autumn Flooding' treatment accumulated greater total biomass (after standardisation to account for the initial number of seeds per pot) at the end of the experiment than plants that germinated during the 'Winter Flooding' treatment with ranges of standardised total biomass of $0.94 \pm 0.58\,g$ to $1.16 \pm 0.40\,g$ per pot for the 'Autumn Flooding' treatment and from $0.13 \pm 0.05\,g$ to $0.14 \pm 0.08g$ per pot in the 'Winter Flooding' treatment. The difference in production of biomass between the two flooding treatments was statistically significant ($T = 100.00; P < 0.001$). The plants in the 'Winter Flooding' treatment also produced significantly less reproductive biomass than those in the 'Autumn Flooding' treatment ($t = 3.378; P = 0.005; \pi = 0.864$). Seed density did not exert significant effects on production of biomass in either treatment ($t = -1.314; P = 0.237; \pi = 0.108$ for 'Autumn Flooding' and $t = -0.318; P = 0.761; \pi = 0.05$ for 'Winter Flooding'). A Two-Way ANOVA did not detect any significant interaction between flooding date and seed density in relation to accumulation of reproductive biomass ($F = 0.272; P = 0.612$).

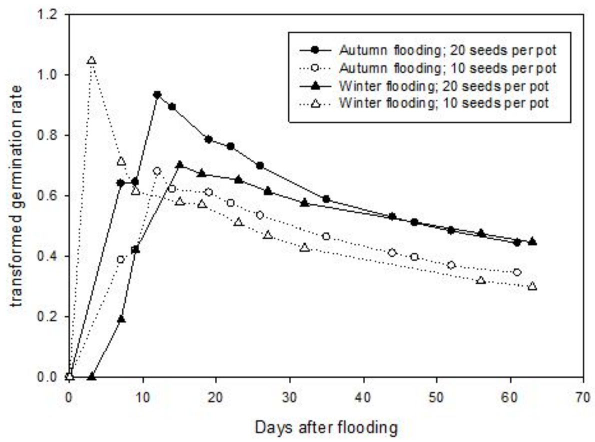

Figure 2: Germination rate over time for seeds subjected to 'Autumn flooding' and 'Winter flooding' treatments at two different seed densities (20 seeds per pot and 10 seeds per pot). Germination rates were arcsine square-root transformed.

5 Discussion

Effect of burial on germination success

Germination success was highest in seeds situated on the surface of the sediment and declined sharply with depth, with no germination recorded from seeds buried under 2 cm of sediment or more. These results corresponded with those of other studies (Galinato et al. 1986; Bonis et al. 1994; Jurik et al. 1994, Rhazi et al. 2007), all of which indicated that burial was associated with a sharp decrease in germination capacity of seeds. Germination success for seeds of *C.truncata* placed on the surface of the sediment was comparable to that recorded for seeds of the same species from the surface layers of the Cerisière marsh by Bonis et al. (1994). These authors recorded a germination rate of 57.1% when these seeds were placed on the surface of a layer of sterile sediment, and no germination for buried seeds.

In a natural setting, seeds are deposited on the surface of the sediment layer and may subsequently percolate into deeper layers of sediment following transport by wind during the dry season (Espinar et al. 2007). Burial of seeds would attenuate the influence of environmental cues, including light (Bonis et al. 1994) and temperature fluctuations (Thompson et al. 1983), which would be important for the germination process. Inhibition of germination of buried seeds is adaptive for relatively small-seeded species such as *Callitriche truncata* since these are constrained by limited endosperm reserves that may not be sufficient to enable seedlings emerging from buried seeds to reach the surface of the sediment and start photosynthesising. Buried seeds may act as perennial repositories, giving rise to a 'storage effect' (Chesson

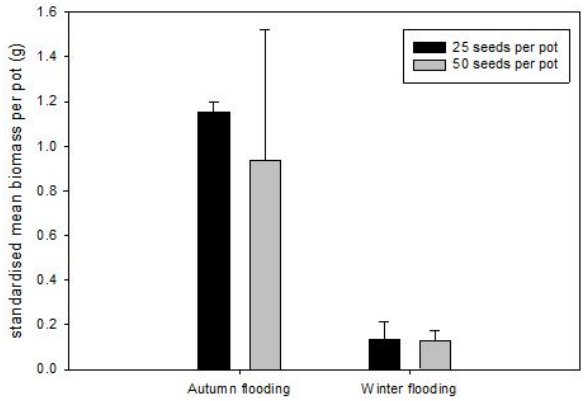

Figure 3: Standardised mean total biomass, expressed in grams per pot, produced by plants germinating under two different flooding treatments: 'Autumn flooding' and 'Winter flooding'. Each treatment was repeated with two different seed densities: 50 seeds and 25 seeds per pot. Biomass measurements were standardised to account for the number of seeds per pot in order to facilitate comparisons.

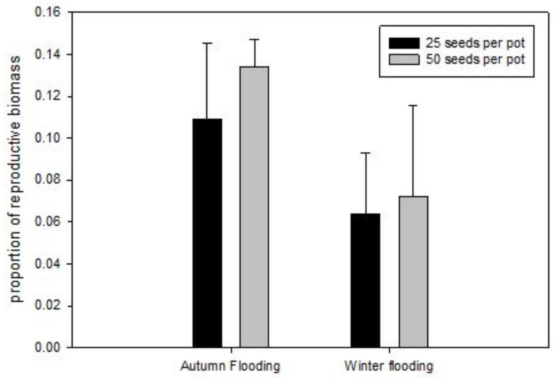

Figure 4: Proportion of reproductive biomass relative to total biomass produced for plants germinating under two different flooding treatments: 'Autumn flooding' and 'Winter flooding'. Each treatment was repeated with two different seed densities: 50 seeds and 25 seeds per pot.

1985) by magnifying the effect of 'favourable' years and diluting the effect of 'unfavourable' years, ensuring the survival and re-establishment of the plant throughout several years.

Effect of flooding date

Rates of germination success of seeds subjected to the 'Autumn flooding' and 'Winter flooding' treatments were comparable and not significantly different. Nonetheless, plants that emerged in winter accumulated significantly less total biomass and reproductive biomass than plants that emerged in autumn. This decrease in total biomass as a consequence of later flooding is analogous to that reported by Grillas et al. (1998) for marshland plants in the Camargue, where a three-week delay in flooding led to a reduction in biomass produced at the end of the growing season. This reduction in production of biomass was attributed to a decrease in the time available for plant growth before the onset of environmental conditions (colder temperatures and shorter day length) that limited photosynthesis and consequently limited growth (Grillas et al. 1998). The results obtained in Experiment 3 of this study suggest that delayed flooding in a natural setting, as may occur during a wet season in which the hydroperiod is fragmented, would limit plant growth but would still permit successful completion of life cycles and sufficient fruit production for restocking of the seed bank. Such compressed life cycles are characteristic of macrophytes of temporary ponds in general.

Density-dependent effects

The density of seeds in each pot did not exert significant effects on any of the experimental results. This is in contrast to the results of Bonis et al (1996), in which higher densities of seeds were associated with a statistically-significant reduction in germination rates. In a natural setting, seeds are deposited directly on the sediment surface in the vicinity of the parent plant and such localised patches may be characterised by relatively high densities of seeds. The results obtained do not suggest that clustering of seeds leads to any density-dependent inhibition of germination, a physiological response that would be adaptive as it would maximise replenishment of the seed bank.

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