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Seed germination of mudflat species responds differently to prior exposure to hypoxic (flooded) environments

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Abstract

Mudflats are exposed for short periods after flood water drawdown. They support fast-growing annual herbs with a ruderal strategy. To optimize their recruitment success, seeds of mudflat species germinate better under fluctuating temperatures, full illumination and aerobic environments that indicate the presence of optimal (non-flooded) conditions for plant growth and development. Here, we hypothesize that prior exposure of mudflat seeds to hypoxic (flooded) environment interferes with the germination process and results in more vigorous germination once aerobic conditions are regained. To test this hypothesis, seeds of five mudflat species were incubated in both aerobic and hypoxic environments at four (14/6, 22/14, 22/22 and 30/22°C) temperature regimes, reflecting different (seasonal) conditions when drawdowns may occur. All species responded positively to four temperature regimes; however, moderate 22/14 and 22/22°C temperatures were optimum for high percentages and rates (speed) of seed germination. Since seeds of four species germinated exclusively under aerobic conditions, they were moved from hypoxic to aerobic conditions. Prior exposure of seeds to hypoxic environment facilitated high percentages, rates and synchronization of germination of *Limosella aquatica*, *Peplis portula* and *Samolus valerandi* seeds compared to incubation under strict aerobic conditions. However, prior exposure to hypoxic environment induced secondary dormancy in non-dormant seeds of *Hypericum humifusum* but broke dormancy in *Lythrum hyssopifolia* seeds that otherwise required cold stratification to overcome physiological dormancy. All species that have a narrow ecological niche (strictly occurring in mudflat habitats) showed positive responses to prior exposure to hypoxic environments. In contrast, *H. humifusum* that has a wide ecological niche (from mudflats to moist sandy grasslands) showed a negative response. We conclude that the hypoxic environment may strongly affect seed germination behaviour once the aerobic environment is regained. The most striking effect is the acceleration of the germination process and, therefore, life cycle supporting the survival in an ephemeral habitat.

Introduction

Available information indicates that seeds of several wetland species can tolerate flooding and remain viable even after long periods of submergence (Parolin et al., 2003; Lucas et al., 2012; de Melo et al., 2015; Poschlod and Rosbakh, 2018). Some of them can germinate or even form seedlings while they are submerged under water (de Oliveira Wittmann et al., 2007; Ferreira et al., 2007; Phartyal et al., 2018; Valdez et al., 2019), whereas others may not tolerate flooding at all (de Melo et al., 2015; Valdez et al., 2019). However, to our knowledge, there is very little information available (Phartyal et al., 2020; Rosbakh et al., 2020) on how seed germination of wetland species such as mudflat species that remain viable under long flooded (hypoxic) conditions respond to non-flooding (aerobic) conditions immediately after water drawdown.

Regenerative traits like seed dormancy, germination timing and seedling establishment (Poschlod et al., 2013; Saatkamp et al., 2019; Phartyal et al., 2020; Rosbakh et al., 2020) are extremely sensitive to abiotic factors, including temperature, light, moisture and aerobic/hypoxic conditions, that represent the major bottleneck to species recruitment (Fenner and Thompson, 2005). A slight mismatch between regenerative traits and abiotic factors may negatively affect the synchronization of seed germination with the most favourable conditions not only for seedling development but also for juvenile plant recruitment (Saatkamp et al., 2019). Thus, the present study investigated how does prior exposure to flooding impacts the seed germination process of wetland (mudflat) species.

In temperate regions, the wetland habitats along the margin of rivers, streams, backwaters and oxbow lakes are often flooded during the winter season and the period of water drawdown coincides with the growing summer season (Voigtlander and Poppe, 1989). These recently exposed nutrient-rich muddy sediments support quick growth and dominance of annual herbs (Webb et al., 1988; Baskin et al., 1993, 2004; Phartyal et al., 2020). This kind of mudflat habitats are temporal, unpredictable and unstable in nature, often existing only for a few weeks at every part of the hydroperiod gradient, provided they are drained, open and free of litter. In some extreme cases, they may remain under flooded (hypoxic) conditions for decades or even centuries (Poschlod and Rosbakh, 2018). This unpredictable environment exposes seeds of mudflat species to a quick transition from hypoxic to aerobic conditions and alters their regeneration pattern either by promoting or inhibiting the seed germination process (Baskin et al., 1994; Böckelmann et al., 2017; Phartyal et al., 2020; Rosbakh et al., 2020).

There is contrasting evidence on how flooding affects plant regeneration in wetlands. On the one hand, flooding has been shown to facilitate seed dormancy break and enhance germination in seeds of *Cyperus erythrorhizos*, *C. flavicomus* (Baskin et al., 1993), *Rotala ramosior* (Baskin et al., 2002a), *Schoenoplectus purshianus* (Baskin et al., 2000a), *Scirpus juncoides*, *S. mucronatus*, *S. smithii* and *S. wallichii* (Watanabe and Miyahara, 1989). On the other hand, flooding was shown to induce secondary dormancy and suppressed germination in seeds of *Bidens tripartita* (Benvenuti and Macchia, 1997), *Echinochloa crus-galli* (Honěk and Martinkova, 1992; Holguín et al., 2020), *Lobelia dortmanna* (Farmer and Spence, 1987) and *Vallisneria americana* (Jarvis and Moore, 2008). As for mudflat plants, the seeds of many species have been demonstrated to come out of dormancy in a higher percentage under flooded (hypoxic) than under non-flooded (aerobic) conditions (Baskin et al., 2002b). In temperate regions, where mudflat habitats are flooded during the unfavourable (winter) season and remain non-flooded during the favourable (summer) season, dormancy will be broken while seeds are still flooded. However, in seeds of the summer annual *Leucospora multifidi*, dormancy release is initiated in late autumn during non-flooded conditions and continues even during winter flooding. The non-dormant seeds germinated in summer but, if seeds were flooded prior to the onset of dormancy release, flooding prevented dormancy break in seeds of this species (Baskin et al., 1994). Therefore, this adaptation can result in much higher germination percentages and rates and a more synchronized germination when the water recedes (Baskin and Baskin, 2014). Another adaptation of seeds of mudflat species is that high summer temperatures do not cause flooded seeds to re-enter dormancy (Baskin et al., 1993). Thus, regardless of when waters recede during the growing season (from early spring to early autumn), seeds remain non-dormant and can germinate (Baskin and Baskin, 2014) if exposed to an appropriate temperature regime.

Considering the quick transition from flooding to non-flooding, we hypothesized that there should be a close integration of physiological responses of germinating seeds with a change in oxygen and temperature regimes. We were specifically interested to know whether or not hypoxic conditions interfere with the induction/release of seed dormancy in mudflat species exposed to different temperature regimes. Flooding and non-flooding conditions at an appropriate temperature regime (season) are supposed to play a crucial role in triggering germination at a time most suitable for seedling growth or to induce dormancy to avoid an unfavourable time for seedling survival. For example, seeds of the winter annual grass (*Alopecurus carolinianus*) flooded in autumn did not enter secondary dormancy during exposure to low winter temperatures in Kentucky (USA), but those buried in non-flooded (moist) soil and exposed to the same temperatures did so. Thus, it is indicated that hypoxic conditions prevented the induction of dormancy in non-dormant seeds (Baskin et al., 2000b). Taking all these factors into account, in the present study, we specifically asked the following questions: (1) Does prior exposure of seeds of mudflat species to hypoxic conditions help them germinate in high percentages, rates (speed), and in a more synchronized manner when shifted to aerobic conditions? (2) Does exposure of dormant seeds to hypoxic conditions substitute for requirements of a cold stratification treatment to break dormancy, if any? (3) Does prior exposure of non-dormant seeds to hypoxic conditions induce secondary dormancy or suppress germination?

Material and methods

Study species

We selected five mudflat species: *Hypericum humifusum* L. (Hypericaceae); *Limosella aquatica* L. (Scrophulariaceae); *Lythrum hyssopifolia* L. (Lythraceae); *Peplis portula* L. (Lythraceae) and *Samolus valerandi* L. (Primulaceae), typically inhibiting mudflat habitats of Central Europe. *H. humifusum* primarily belongs to moist sandy grasslands/trampled communities along mudflats, *L. aquatica*, *L. hyssopifolia* and *P. portula* occur only in mudflats, whereas *S. valerandi* mainly occurs in salt marshes along with mudflat habitats (Oberdorfer, 2001). In our previous research, seeds of *L. hyssopifolia*, *P. portula* and *S. valerandi* germinated moderately well in both aerobic and hypoxic conditions irrespective of temperature fluctuation with full illumination and were categorized as 'flood-resistant mudflat species', whereas the seeds of *L. aquatica* germinated well only under fully illuminated aerobic conditions at fluctuating temperatures and were categorized as 'true mudflat species'. In contrast, the seeds of *H. humifusum* had a high germination percentage and rate only under aerobic conditions at both constant and fluctuating temperatures with and without light and were categorized as 'facultative mudflat species' (Phartyal et al., 2020).

Fully ripened fruits of these species were harvested in several natural populations from randomly chosen individuals (>50 individuals per species) growing at a step-away distance from each other and thoroughly mixed. After collection, seeds were separated from the fruits by hand, air-dried for several days, cleaned and kept dry in a cold room at 4°C until the beginning of the germination experiments (Baskin and Baskin, 2014). Seeds of all study species were non-dormant except for *L. hyssopifolia* that possess physiological dormancy and require cold moist stratification to overcome dormancy (Phartyal et al., 2020).

Seed germination experiments

To determine optimum conditions for high percentages, rates (speed) and synchronization of germination, seeds of each species were incubated under different combinations of temperature and oxygen. In each incubation treatment, five replicates of 20 seeds were placed on two layers of moist filter paper in a Petri dish. All the experiments were conducted in germination chambers (RUMED GmbH, Laatzen, Germany) at one constant (22/22°C) and three diurnal fluctuating temperatures (14/6, 22/14 and 30/22°C – representing climate of cool spring, mild and warm summer seasons in temperate mudflat habitats) in 14/10 h of the light/dark regime.

To simulate hypoxic conditions, Petri dishes supplied with vents to provide consistent gas exchange were placed in a

Table 1. Outline of the seed incubation treatments

Treatment abbreviations	Treatment details (arrow '→' indicates seeds moved from hypoxic to aerobic condition at the same temperature regime after 6 weeks of incubation)
22/22 Aerobic	Constant temperature (22/22°C) + Aerobic condition
22/22 Hypoxia	Constant temperature (22/22°C) + Hypoxic condition → Aerobic condition
30/22 Aerobic	Fluctuating temperature (30/22°C) + Aerobic condition
30/22 Hypoxia	Fluctuating temperature (30/22°C) + Hypoxic condition → Aerobic condition
22/14 Aerobic	Fluctuating temperature (22/14°C) + Aerobic condition
22/14 Hypoxia	Fluctuating temperature (22/14°C) + Hypoxic condition → Aerobic condition
14/10 Aerobic	Fluctuating temperature (14/10°C) + Aerobic condition
14/10 Hypoxia	Fluctuating temperature (14/10°C) + Hypoxic condition → Aerobic condition

desiccator and their air volume was substituted with pure nitrogen. We repeated this procedure each time when germination was scored. Dishes to which this treatment was not applied are referred to as aerobic. The number of seeds germinated was scored every third day for 6 weeks. Germination was defined as the protrusion of a radicle through the seed coat. As highlighted above, mudflat habitats can experience flooding to non-flooding conditions in a very short period of time, which exposes seeds a rapid transition from hypoxic to aerobic conditions during the germination process. Since seeds of none of the study species germinated under strict hypoxic conditions they were, therefore, shifted to aerobic conditions at a similar temperature and light/dark conditions for another 6 weeks (see Table 1 for treatment details), to determine how prior exposure to hypoxic conditions affects seed germination.

Statistical analysis

Seed germination percentages, mean germination time (MGT) and germination synchronization index (Z) were calculated using *GerminaR* package for R (Lozano-Isla et al., 2019). A lower value of MGT reflects faster rates (speed) of seed germination in a particular incubation treatment. In the case of the synchronization index, a Z-value close to one indicates that germination of all seeds occurs at the same time (more synchronized germination), while a Z-value close to zero indicates that seed germination of at least two seeds occurred at a different time (less synchronized germination). To infer statistically significant differences among the incubation treatments, Fisher's exact test was used. Seed germination percentages at any given treatments were displayed using mean values and binomial confidence intervals. All statistical analyses were carried out using R 3.4.3 (R core development team, 2020).

Results

None of the seeds of any study species germinated under strict hypoxic conditions at any temperature regimes, except those of *L. hyssopifolia*, within 6 weeks of incubation. Only 5% seeds of *L. hyssopifolia* germinated under strict hypoxic conditions at the warm (30/22°C) temperature regime. However, prior exposure to hypoxic conditions significantly ($P < 0.05$) stimulated seed germination percentage, MGT and germination synchrony of three study species (*L. aquatica*, *P. portula* and *S. valerandi*) at almost all temperature regimes when seeds were moved to aerobic conditions (Figs 1–3).

On the contrary, germination percentage, MGT and germination synchrony of *H. humifusum* seeds were significantly ($P < 0.05$) suppressed by prior exposure to hypoxic conditions at most of the tested temperature except at the cool temperature (14/6°C) regime (Figs 1–3). The seeds germinated equally well (>90%) in both strict aerobic conditions and when moved from hypoxic to aerobic conditions at the cool temperature regime.

In contrast to seeds of the other four study species, those of *L. hyssopifolia* either failed to germinate or had a low (0–37) germination percentage when incubated only under strict aerobic conditions. Whereas a prior exposure to hypoxic conditions significantly ($P < 0.05$) stimulated seed germination (58–93%), MGT and germination synchrony at all test temperature regimes (Figs 1–3).

Prior exposure to hypoxic conditions induced dormancy in non-dormant seeds of *H. humifusum* except at low cool (14/6°C) temperature, whereas it acted as a dormancy-breaking treatment for dormant seeds of *L. hyssopifolia*.

Overall, majority of the germination parameters (germination percentage, MGT and synchrony index) performed significantly ($P < 0.05$) well at moderately warm fluctuating (22/14°C) temperatures followed by constant (22/22°C) temperatures in comparison to warm (33/22°C) and cool (14/6°C) temperature regimes, which were found to be comparatively sub-optimal for seed germination in most of the study species (Figs 1–3).

Discussion

Seed germination of none of the study species displayed any positive response only to strict hypoxic (except for *L. hyssopifolia*) conditions but generally displayed a positive response to strict aerobic conditions. This confirms our previous findings that the majority of mudflat species need aerobic conditions to germinate (Phartyal et al., 2020; Rosbakh et al., 2020). However, the overall trends indicate that prior exposure of seeds to hypoxic conditions stimulates germination in the majority of our study species, thus confirming our hypothesis (Table 2).

Non-dormant seeds of *H. humifusum* germinated in a moderately broad range of environments including dark except under hypoxic conditions (Phartyal et al., 2020). Seeds of this species had a higher percentage, faster rate and more synchronized germination only in aerobic conditions in all tested temperature regimes. However, seeds responded poorly when moved from

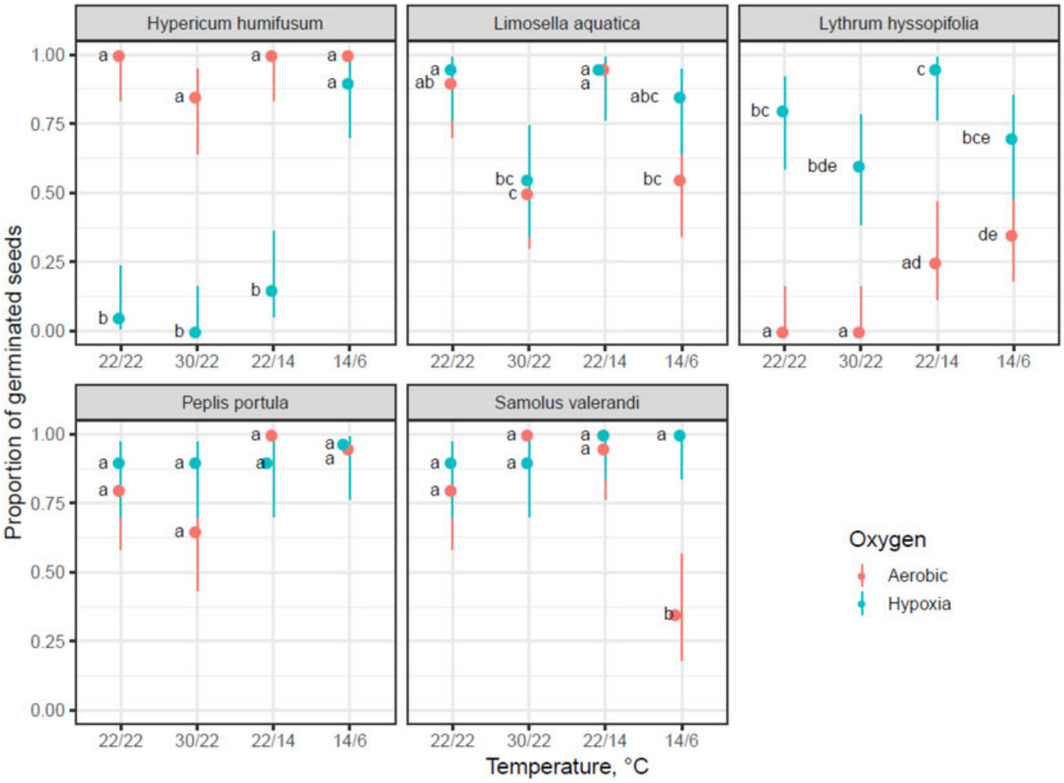

Fig. 1. Bar plots illustrating median and quartiles of seed germination percentage of mudflat species incubated under a wide range of environmental conditions. Letters represent subsets with significant ($P < 0.005$) differences.

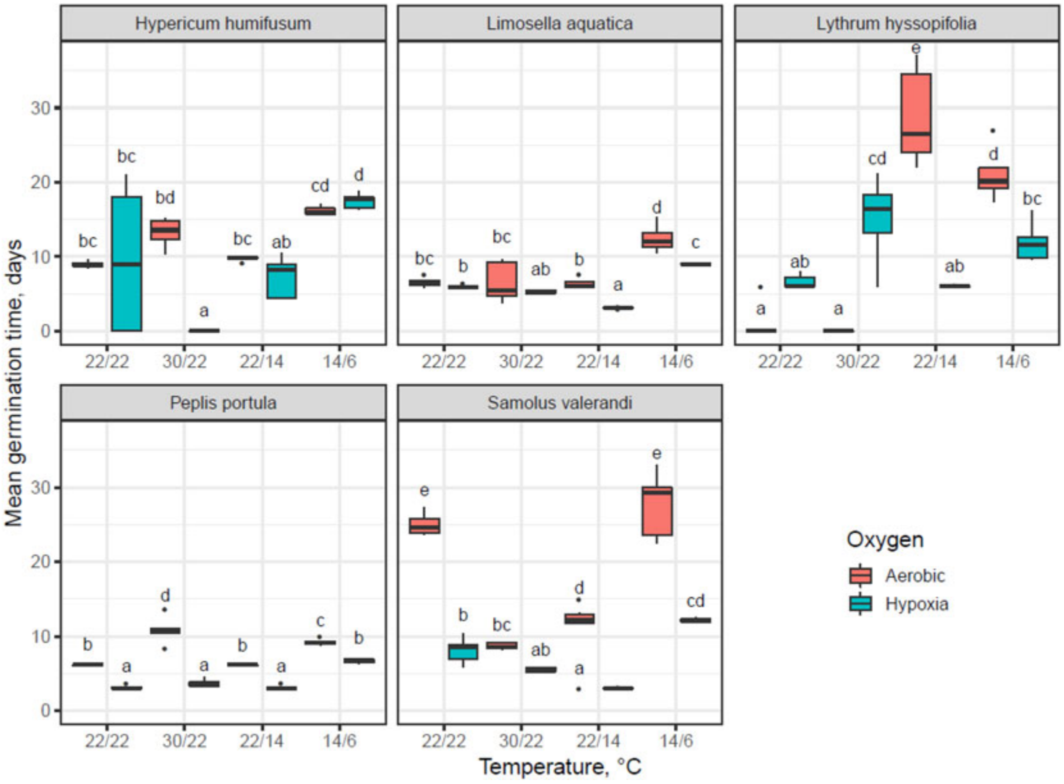

Fig. 2. Box plots illustrating median, quartiles and outliers (o) of MGT of mudflat species incubated under a wide range of environmental conditions. Letters represent subsets with significant ($P < 0.05$) differences.

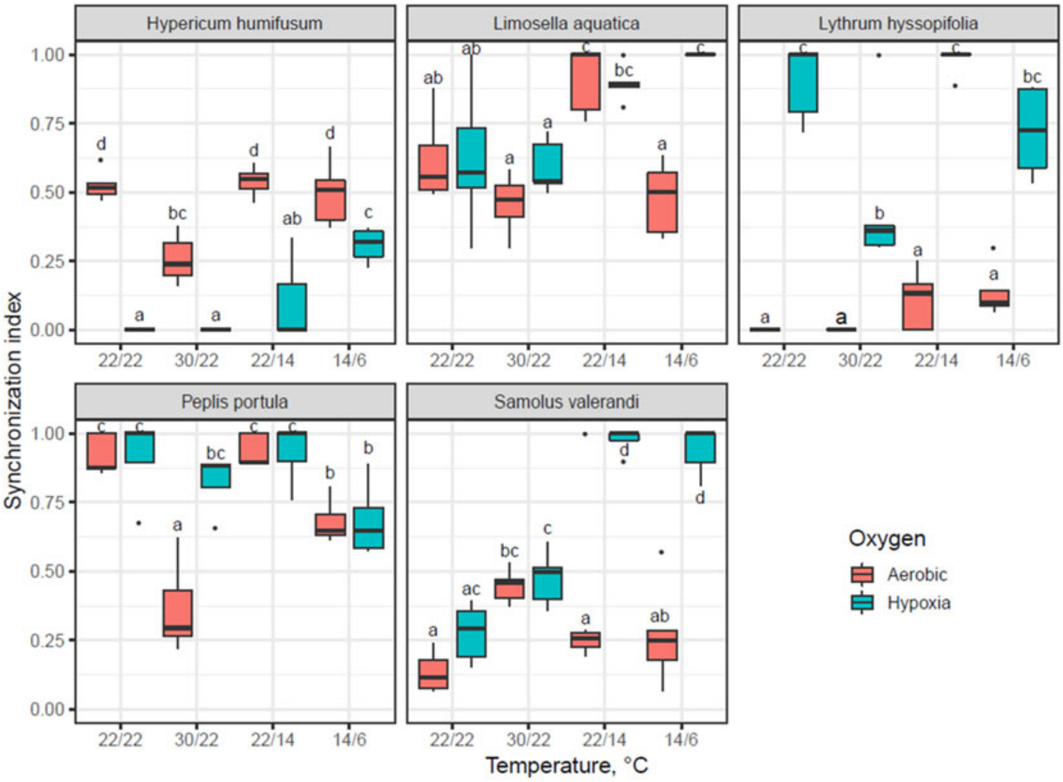

Fig. 3. Box plots illustrating median, quartiles and outliers (o) of germination synchronization index of mudflat species incubated under a wide range of environmental conditions. Letters represent subsets with significant ($P < 0.05$) differences.

Table 2. A summary of seed response to prior exposure to hypoxic environments. Values in column 3 and 4 represent the overall mean of four incubation temperature regimes

		Seed incubation environments			
Species	Germination parameters	Aerobic environment	Hypoxic → Aerobic environment	Response	Remarks
H. humifusum	Germination (%)	96	27	Negative	Induce secondary dormancy and suppress seed germination process
	MGT (days)	12.1	13.6		
	Synchrony Index	0.50	0.20		
L. aquatica	Germination (%)	73	83	Positive	Stimulate seed germination process
	MGT (days)	8.0	5.8		
	Synchrony Index	0.62	0.78		
L. hyssopifolia	Germination (%)	15	75	Positive	Help to release physiological dormancy without cold moist stratification
	MGT (days)	18.6	9.9		
	Synchrony Index	0.12	0.78		
P. portula	Germination (%)	85	90	Positive	Stimulate seed germination process
	MGT (days)	8.1	4.2		
	Synchrony Index	0.73	0.84		
S. valerandi	Germination (%)	77	96	Positive	Stimulate seed germination process
	MGT (days)	18.6	7.2		
	Synchrony Index	0.27	0.67		

hypoxic to aerobic conditions at warmer temperatures (22/14, 22/22 and 30/22°C) but not a low temperature (14/6°C). Thus, prior exposure of seeds to hypoxic conditions induced secondary dormancy except at low temperature, suitable for cold stratification. This also confirms earlier reports that flooding (low oxygen level) may induce secondary dormancy in seeds of several wetland species (Farmer and Spence, 1987; Honěk and Martinkova, 1992; Benvenuti and Macchia, 1997; Nishihiro et al., 2004a,b; Jarvis and Moore, 2008; Holguín et al., 2020). Secondary dormancy may be eventually broken by cold stratification. This is an unusual behaviour of a species of an ephemeral habitat such as mudflats that might be explained by the main occurrence of *H. humifusum* in moist sandy grasslands or trampled sites with a comparatively low probability of flooding (Oberdorfer, 2001). Thus, our results confirm the classification of this species by Phartyal et al. (2020) as a 'facultative mudflat species' that demonstrated a broad germination niche width (Phartyal et al., 2020) as the species occurs not only in the mudflats.

Physiologically dormant seeds of *L. hyssopifolia* germinated over a broad range of environmental conditions, including hypoxic conditions after cold stratification (Phartyal et al., 2020). However, seeds of this species either failed to germinate or germinated poorly under aerobic conditions at all tested temperatures. However, if seeds were exposed to hypoxic conditions then moved to aerobic conditions, they had a higher percentage, rate and synchronization of germination even without a cold stratification treatment. A high proportion of seeds of this species also germinated under hypoxic conditions at low temperature (4°C) during cold stratification (S. Phartyal, personal observation). Thus, a high percentage of seeds of several mudflat species, including *L. hyssopifolia*, classified as a 'flooded mudflat species' by Phartyal et al. (2020) come out of dormancy after they have experienced flooded (hypoxic) conditions (Watanabe and Miyahara, 1989; Baskin et al., 1993, 2000a, 2002a,b). This finding is in agreement with the results from previous studies that lack of oxygen can sometimes break dormancy even in terrestrial species, especially if their seeds require cold stratification to overcome dormancy (Come et al., 1991).

In contrast to the response of *H. humifusum* and *L. hyssopifolia* seeds to hypoxic conditions, non-dormant seeds of *L. aquatica* germinated only in a narrow range of environments of light, aerobic and fluctuating temperatures (Phartyal et al., 2020). Seeds had a higher percentage, rate and synchronization of germination when moved from hypoxic to aerobic conditions than if they were exposed directly to aerobic conditions. These results justify the classification of *L. aquatica* as 'true mudflat species' (Phartyal et al., 2020) and its phytosociological classification into 'Nano-Cyperetea' (Oberdorfer, 2001). A similar effect of prior exposure to hypoxic conditions was demonstrated in seeds of *P. portula* and *S. valerandi*. This reveals that a flooding pretreatment may help seeds to germinate as fast as possible immediately after water drawdown, which would allow seedlings to take full advantage of the short growing period in an unpredictable and unstable environment, which is the case of mudflat habitats. Prior exposure of seeds to flooding events was also reported to promote germination in other wetland species like *Sphaeranthus indicus* (Shetty, 1967) and *Panicum laxum* (Cole, 1977). In a recent study on dormant seeds of *Echinochloa crus-galli*, Peralta Ogorek et al. (2019) reported that hypoxic conditions weaken primary dormancy as well as hindered induction of secondary dormancy at warm temperatures. They also reported that dormancy-breaking signals are overridden during hypoxic conditions, which help to prevent seed germination when submerged in water.

The majority of mudflat species produce non-dormant seeds and show a narrow germination niche width (Phartyal et al., 2020) as compared to the germination niche of other wetlands species (Rosbakh et al., 2020). Thus, low seedling mortality and optimize establishment may be promoted if seed germination is only triggered by high oxygen concentrations that are typical for exposed soils or sediment surfaces after water drawdown (Coops and van der Velde, 1995). We conclude that in view of the very short favourable growing period for mudflat species the promotive effect of flooding (hypoxic condition) on percentage, speed and synchronization of germination when the floods ends is adaptive in the ephemeral mudflat habitat. This is another crucial role hypoxic environment play, apart from seeds to persist in submerged sediments (Poschlod and Rosbakh, 2018), to control germination traits of mudflat species.

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