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Dose–response of germinating *Rudbeckia mollis* (Asteraceae) seeds exposed to various thermal scenarios

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Abstract

Temperature is a key environmental signal regulating germination. A thorough understanding of how seed populations respond to various temperatures can inform end-users regarding effective establishment strategies and forms the basis for questions related to a taxon's thermo-biology. Although abundant information exists regarding germination responses of economically important crops to several temperature scenarios, much less is known concerning the seed biology of wild germplasm. To address this, we examined the germination response of non-dormant *Rudbeckia mollis* seeds to various doses of constant or simulated seasonal diel temperatures. Germination response was sigmoidal. Seeds of *R. mollis* were capable of germinating within a few days to high percentages (>95%) at relatively cool constant (15–25°C) or 12-hour alternating (22/11–33/24°C) temperatures, with optimum temperatures for germination occurring at 25°C or 29/19°C. Germination was inhibited as temperatures increased to 30°C or 33/24°C with early and late germinating phenotypes displaying differential responses at these temperatures. No germination occurred at 35°C. Results are discussed in terms of seedling establishment of *R. mollis* outside its natural range and implications of climate change on germination.

Keywords: germination rate, heat stress, optimum temperature, phenotype, *Rudbeckia mollis*, t_{50}, thermoinhibition

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Introduction

The germination process, delimited by imbibition and emergence of the embryonic axis through its covering structures, represents a fundamental shift in life-history stage and is a key component in the production process for many taxa of economic importance. Temperature is one abiotic signal that significantly impacts the germination process and related descriptive parameters, maintenance of seed viability and alleviation or continuation of seed dormancy. For example, temperature fluctuations have been shown to alter rates of imbibition (Baskin *et al.*, 2007) and drive metabolic or biophysical reactions associated with germination or, alternatively, viability loss (Walters, 1998; Corbineau *et al.*, 2002; Bazin *et al.*, 2011). From an eco-physiological perspective, seeds must be capable of responding to temperature fluctuations in order to change their dormancy and germination status (J.M. Baskin and C.C. Baskin, 2004). Typically the reciprocal of the mean or median time for a population of seeds to complete germination (i.e. germination rate) is used to describe germination kinetics. However, germination within a seed population will be temporally distributed such that subpopulations of seeds completing germination at different times can be identified; with temperature being a main determinant of the distribution for each subpopulation (Garcia-Huidobro *et al.*, 1982; Covell *et al.*, 1986).

Therefore, while water is essential for commencement of germination, temperature is considered one of the most important physical factors regulating germination (Probert, 2000; J.M. Baskin and C.C. Baskin, 2004). Constant or alternating temperature ranges that regulate germination are taxa specific and may be correlated with local climate and geography (Covell *et al.*, 1986; Probert, 2000; Fenner and Thompson, 2005). Although ambient temperatures are predicted to increase as a result of global climate change (IPCC, 2007), scarce evidence has been

presented regarding the implications of such alterations to seed ecology and recruitment dynamics outside sub-arctic regions (Walck *et al.*, 2011).

Temperature requirements for efficient germination, field establishment and maintenance of viability are well known for many economically important crops. Moreover, thermal methods such as stratification, which promote germination, are widely recognized for crop and ornamental taxa. Alternatively, a significant gap occurs in knowledge regarding the seed biology of wild germplasm (C.C. Baskin and J.M. Baskin, 2004). Nevertheless, interest in the use of native wildflower seeds for a variety of applications, such as ecological restoration, slope stabilization, wildflower gardening and roadside beautification, is gaining acceptance globally (Milstein, 2005). The wildflower seed industry represents new opportunities for countries in Asia, South America and Africa that are expanding horticultural production (Milstein, 2005) and possibly restoration activities. The genetic implications of using seeds of domesticated wildflowers or seeds from non-local sources for restoration and conservation purposes are discussed elsewhere (Lesica and Allendorf, 1999; Mijnsbrugge *et al.*, 2010). The wildflower seed industry in the south-eastern USA is considered to be an emerging market with potential for expansion. Producers in this region utilize wild collected seeds for which practically no seed biology information has been reported (Kauth and Pérez, 2011).

Regardless of production region, stakeholders in the wildflower seed industry will have to improve production efficiency to remain profitable and provide high-quality seeds or finished plants that will be demanded as opportunities arise for expansion (Milstein, 2005). Likewise, returns on investments for ecological restoration and conservation projects can be improved when practitioners have information, such as germination timing, that augment their ability to measure outcomes and monitor benchmarks (Hong *et al.*, 1998; Menges, 2008). Knowledge of seed responses to various temperature regimes can thereby inform end-users in terms of effective propagation, production scheduling and field establishment conditions. This new understanding also forms the basis for further germination enhancements through application of seed technologies such as priming. Moreover, understanding germination responses at various temperatures can facilitate development of predictive germination models and studies related to germination ecology, ecotypic differentiation, and the potential effects of different climate change scenarios on population biology and commercial production.

Our ultimate goal is to improve understanding of wildflower seed biology and the application of this new knowledge to the problem of global climate change. In this manuscript we analyse trends in the germination response of *Rudbeckia mollis* Elliott (Asteraceae, Heliantheae) seeds exposed to constant and simulated seasonal temperatures. We selected *R. mollis* because it is a wildflower currently in demand but of limited availability due primarily to a paucity of seed biology information. *R. mollis* occurs in Alabama, Georgia and South Carolina and on exposed xeric sandhills and open hammocks in north Florida (Wunderlin and Hansen, 2004). The Köppen–Geiger climate classification for this region is Cfa, which is characterized as temperate, with hot summers and no dry season (Peel *et al.*, 2007). *R. mollis* is an herbaceous annual reaching 1 m in height. In Florida, flowering takes place from June to July and fruit shedding occurs in late September. Inflorescences consist of showy, yellow, ligulate ray florets, while the disk is flattened with brown or purplish florets. Aside from its horticultural potential, *R. mollis* could be used in natural area restoration seed mixes. This taxon also produces anti-carcinogenic ambrosanolides (Herz and Kumar, 1981).

Materials and methods

Seed material

Achenes, referred to hereafter as seeds, of *R. mollis* were harvested by hand during late September 2009 from natural stands occurring in Suwannee County, Florida, USA. Mature seeds were easily detached from dry inflorescences. Debris, weed seeds, and unfilled or immature seeds were removed by passing the entire lot through an air-density separator (STS-WM2-SV, SeedTech Systems Inc., Wilton, California, USA). Cleaned seeds were stored at approximately 24°C and 25–35% relative humidity (RH) for approximately 4 weeks until experimentation began.

Germination tests

Seeds were surface sown on 10 mm of screened and washed quartz sand (Sakrete of North America, LLC, Charlotte, North Carolina, USA) contained within 20 × 90 mm Petri dishes. Sand was pre-moistened with 23 ml of distilled water prior to sowing, and distilled water was added as necessary. After sowing, seeds were exposed to constant temperatures of 15, 20, 25, 30 or 35°C, or one of four simulated seasonal diel temperatures: 22/11, 27/15, 29/19 or 33/24°C. Constant temperatures were achieved by using a thermo-gradient table (Model 5010.00, Seed Processing Holland BV, Enkhuizen, The Netherlands). Simulated seasonal temperatures were derived from average monthly maxima and minima collected by the Southeast Regional Climate Center over a 30-year period at

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sites across Florida and represent temperatures experienced during the winter (22/11°C), late autumn or early spring (27/15°C), early autumn or late spring (29/19°C), and summer (33/24°C). Alternating temperatures fluctuated every 12 h and were programmed into one of four germination chambers (I-30-VL, Percival Scientific, Inc., Perry, Iowa, USA). All seeds were exposed to a 12-h daily photoperiod provided by cool white fluorescent lamps. Illumination coincided with higher temperatures in the alternating regimes. Photosynthetic photon flux density at seed level was $52 \pm 5\,\mu mol\,m^{-2}\,s^{-1}$ (mean ± SD) on the thermo-gradient table and $57 \pm 7\,\mu mol\,m^{-2}\,s^{-1}$ within the chambers. All treatments consisted of four 100-seed replicates that were randomly assigned to each temperature. This experiment utilized a randomized complete block design with dishes blocked for potential temperature and light differences.

Viability testing

Seed viability was examined using the tetrazolium (TZ) staining technique (Peters, 2000) prior to germination tests and for any non-germinated seeds remaining at the conclusion of a germination test. The pre-germination viability test was conducted on a sample of four 100-seed replicates. Briefly, seeds were randomly assigned to one of four beakers containing 5 ml of 0.1% TZ solution (pH 7) and all were incubated in the dark at 35°C for 24 h. Seeds were bisected longitudinally and staining patterns were examined under 15–20× magnification. Post-germination viability (V) was calculated as $V = \text{FGP} + S_p$, where FGP equals final germination percentage and S_p equals percentage of remaining seeds that display a positive staining pattern.

Computation of germination parameters

Germination counts were performed daily for 34 d. A seed was considered germinated upon emergence of the radicle and cotyledons. We recognize that by including post-radicle emergence events, such as emergence of cotyledons, a more conservative approach to measuring the completion of germination has been applied. Seedlings were removed from replicates after germination. Trends in germination were analysed by non-linear curve-fitting. The progress of cumulative germination over the 34 d experimental period was fitted to the sigmoid model $f = a{\cdot}(1 + \exp^{(-(x - X_0)/b)})^{-1}$; where a, b and X_0 are the parameters. For each temperature treatment, the time to 25, 50 and 75% of final germination was calculated from visual inspection of cumulative germination graphs and the fitted equations. Germination uniformity (U) was calculated as $U = t_{75} - t_{25}$.

Data analysis

Curve-fitting and regression analysis were carried out in SigmaPlot (v 10.0., Systat Software Inc., San José, California, USA). SigmaPlot uses the Marquardt–Levenberg algorithm in an iterative process until differences between residual sums of squares no longer decrease significantly, thus giving the best fit. Additionally, more complex mathematical models containing excessive parameters were excluded when parameter dependencies were equal to 1.0 (i.e. over-parameterization). Normality and constant variance were tested using the Kolmogrov–Smirnov and Spearman Rank Correlation tests, respectively, with $P = 0.05$. The Durbin–Watson statistic was used to test independence of residuals with acceptable deviation from 2.0 set at 0.5. Germination data were transformed via the $\text{asin}\sqrt{Y}$ function as necessary; however, non-transformed data are presented.

Results

Seed viability and final germination

Initial seed lot viability was 96.0 ± 0.0% (mean ± SE). Viability remained above 96% for seeds exposed to relatively low or moderate constant and alternating temperatures but began declining quadratically as constant ($R^2 = 0.8749$; $P < 0.001$) and alternating ($R^2 = 0.9136$; $P < 0.001$) temperatures increased (Fig. 1A, B). Alternatively, the relationship between constant ($R^2 = 0.9875$; $P < 0.001$) or alternating ($R^2 = 0.9972$; $P < 0.001$) temperature and FGP was sigmoid (Fig. 1A, B), such that germination remained above 95% between 15 and 25°C, and 22/11 and 29/19°C, followed by a rapid decline at elevated temperatures. Notably, seeds exposed to a constant 35°C did not germinate. Seeds exposed to simulated summer temperatures (33/24°C) maintained some, albeit significantly reduced, germination ability (FGP ≈ 33%). Viability was approximately nine- and three-fold greater than FGP at the highest constant and alternating temperatures, respectively (Fig. 1A, B). The threshold for germination decline was more abrupt at alternating than at constant temperatures, with calculated $°C_{50}$ values for constant and alternating temperatures of 31°C and 28°C, respectively (Fig. 1A, B). However, the majority of seedlings produced after day 15 at 30°C appeared abnormal (i.e. pale green or yellow). An average alternating temperature of 28°C corresponds to an average maximum and minimum fluctuation of 33/23°C when constrained to the simulated seasonal temperatures used here.

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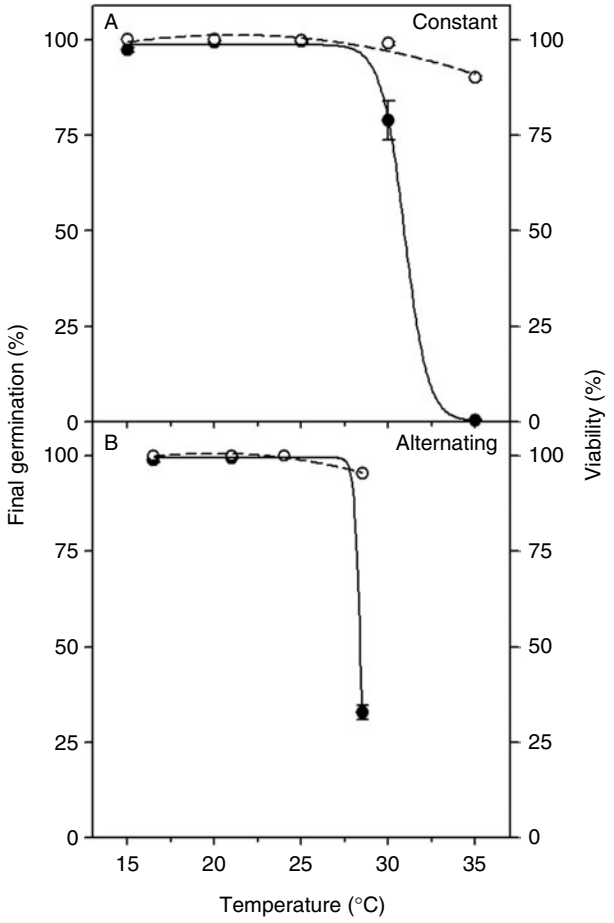

Figure 1. Germination response curves for seeds of *Rudbeckia mollis* exposed to constant and alternating temperatures for 34 d. Final germination percentage (●) and viability (○) of seeds exposed to (A) constant or (B) alternating temperatures. Viability data are from post-germination tetrazolium tests. The viability response at constant ($R^2 = 0.8749$; $P < 0.001$) and alternating ($R^2 = 0.9136$; $P < 0.001$) temperatures was described best by the quadratic function: $f = Y_0 + ax + bx^2$. Final germination percentage for constant ($R^2 = 0.9875$; $P < 0.001$) and alternating ($R^2 = 0.9972$; $P < 0.001$) temperatures was best described by the three-parameter sigmoid function: $f = a{\cdot}(1+ \exp^{(-(x-X_0)/b)})^{-1}$. Alternating temperatures simulate average daily maxima and minima for the winter (22/11°C), early spring or late autumn (27/15°C), early autumn or late spring (29/19°C), or summer (33/24°C) throughout Florida. Final germination percentage and viability were regressed against the average maximum and minimum temperatures for each season. Error bars = ±SE.

Cumulative germination

The trend in cumulative germination was sigmoid (R^2 range: 0.9031–0.9987; $P < 0.001$) for all temperatures where germination occurred (Fig. 2A, B). Lag time, defined as the time required for germination to commence after sowing, ranged from about 3–10 d and 4–6 d for constant and alternating temperatures, respectively. Lag time was inversely proportional to germination temperature. Germination was relatively rapid when seeds were exposed to intermediate constant temperatures or temperatures that simulated conditions experienced during the autumn, winter and spring. However, seeds at 25°C or 29/19°C (i.e. early autumn or late spring) germinated faster than seeds at any other constant or alternating temperature (Fig. 2A, B; Table 1). The number of days required to reach maximum FGP for seeds exposed to constant temperatures ranged between about 20 and 33 d; whereas maximum FGP was achieved between about 22 and 31 d for seeds exposed to alternating

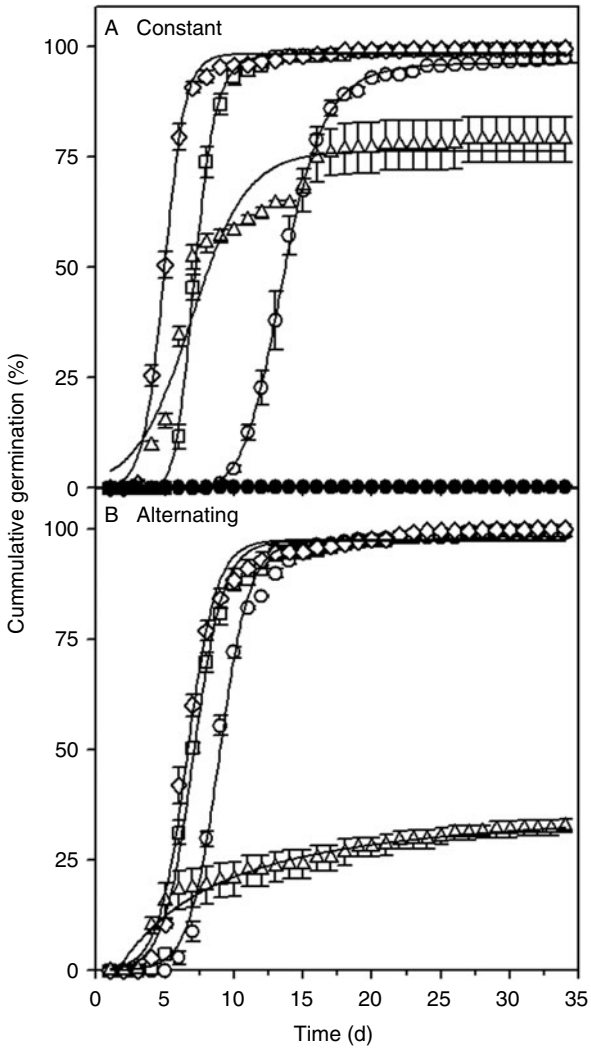

Figure 2. Germination progress curves of *Rudbeckia mollis* seeds exposed to (A) constant or (B) alternating temperatures. For (A): 15 (○), 20 (□), 25 (◇), 30 (Δ) and 35°C (●). For (B): 22/11 (○), 27/15 (□), 29/19 (◇) and 33/24°C (Δ). Cumulative germination was regressed against the average maximum and minimum temperatures for each season. Cumulative germination was best described (R^2 range: 0.9031–0.9987; $P < 0.001$) by the three-parameter sigmoid function: $f = a{\cdot}(1 + \exp^{(-(x - X_0)/b)})^{-1}$. Error bars = ±SE.

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Table 1. Germination parameters of *Rudbeckia mollis* seeds exposed to constant or seasonally simulated alternating germination temperatures

Temperature (°C)	Time to germination (d)			Germination rate ($1 \cdot t_{50}^{-1}$)	Germination uniformity ($t_{75} - t_{25}$)
	t_{25}	t_{50}	t_{75}		
Constant					
15	12.2	13.8	15.5	0.0725	3.3
20	6.5	7.3	8.1	0.1370	1.6
25	4.1	5.0	5.8	0.2000	1.7
30[a]	5.5	8.2	15.2	0.1220	9.7
35	nd	nd	nd	nd	nd
Alternating					
22/11	7.8	9.0	10.3	0.1111	2.5
27/15	5.9	7.1	8.4	0.1408	2.5
29/19	5.5	6.7	7.9	0.1493	2.4
33/24[b]	3.4	7.9	12.9	0.1266	9.5

$t_{25,50,75}$ = time in days required for seed population to reach 25, 50 or 75% of final germination percentage (FGP). Germination uniformity was calculated as the difference between t_{75} and t_{25}. Alternating temperatures simulate average daily maxima and minima for the winter (22/11°C), early spring or late autumn (27/15°C), early autumn or late spring (29/19°C) or summer (33/24°C) throughout Florida. nd = values not determined due to lack of germination.
[a] FGP ≈ 79%.
[b] FGP ≈ 33%.

temperatures (Fig. 2A, B). Germination was about two times more uniform at 20 or 25°C than at 15°C. Alternatively, the differences in germination uniformity for seeds exposed to simulated autumn, winter and spring temperatures were negligible (Table 1).

Seeds exposed to 30°C or 33/24°C displayed erratic patterns in germination response compared to seeds incubated at cooler constant or alternating temperatures, respectively. For example, seeds held at 30°C consisted of two populations. In the first population germination was not inhibited at this elevated temperature and seeds completed germination in about 6 d. Germination was inhibited in the second population and some seeds continued to germinate slowly over the next 28 d (FGP ≈ 78% cf. viability Fig. 1, Fig. 2A). Similarly, a small fraction of seeds (18%) exposed to simulated summer temperatures (33/24°C) germinated rapidly, while the majority of remaining seeds did not germinate but remained viable (Figs 1B and 2B). The time required to reach 25, 50 and 75% germination for seeds at 30°C, or 50 and 75% germination for seeds at 33/24°C, was 1.3–2.6 times greater than the time required to reach these proportions for seeds held at 25°C or 29/19°C, suggesting that germination rate peaked at the latter temperatures (Table 1). Moreover, germination was about 3.0–6.0 and 4.0 times less uniform at 30°C and 33/24°C, respectively, than at cooler temperatures (Table 1).

Discussion

Plants have evolved mechanisms to temporally distribute germination, thus allowing seed populations to respond to favourable and unfavourable environmental heterogeneity. Therefore, the ability of seeds to act as effective thermal sensors after separation from the parent plant is fundamental to ultimate seedling fate (Probert, 2000; Donohue, 2002; Donohue *et al.*, 2005; Fenner and Thompson, 2005). The temperature requirements for seed germination of most economically important taxa are well known, which facilitates their commercial propagation and production on a global scale. Despite the ubiquity of wildflowers in most ecosystems of the world, the benefits they confer in such ecosystems and potential advantages provided to humans, the seed biology of nearly all native wildflowers remains unstudied. Here, we investigated the dose–response of *R. mollis* seeds to various germination temperatures to improve understanding of wildflower seed biology. Moreover, this investigation is necessary to gain insight on how subpopulations of seeds respond physiologically to thermal signals, and to form the basis for further seed-related studies on the ecology and physiology of plants under changing climatic conditions.

The rapidity and uniformity with which seeds of *R. mollis* achieved high germination percentages over a broad range of temperatures suggest that seeds were non-dormant when sown. It is possible, however, that seeds may have been dormant at shedding and that the relative humidity and temperature combination used for storing these seeds facilitated dormancy alleviation through the dry afterripening process. Bazin *et al.* (2011) have shown that physiological dormancy in sunflower (*Helianthus annus* L., Asteraceae, Heliantheae) seeds is alleviated in 2–5 weeks when seeds afterripen at 15–25°C and 32–33.5%

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RH. *R. mollis* seeds used in this study were stored under similar conditions. Based on the relatively short amount of time required to alleviate dormancy under conditions that can facilitate dry afterripening in this tribe (Heliantheae) and the completeness of germination for *R. mollis* seeds over a wide range of temperatures after such exposure, we hypothesize that if dormancy occurs in freshly shed seeds of *R. mollis* then it is non-deep physiological dormancy (*sensu* J.M. Baskin and C.C. Baskin, 2004).

The high-temperature inhibition of germination (Figs 1 and 2; Table 1) for non-dormant seeds of *R. mollis* suggests that outdoor production or field establishment during the warmest times of the year within regions of similar climate (i.e. Köppen–Geiger climate classification = Cfa) may be limited. Fundamental mechanisms proposed for the thermal inhibition of germination at elevated temperatures include damage to membrane systems at $T > T_{opt}$ (Hendricks and Taylorson, 1979) and shifts in base water potential to higher values as T surpasses T_{opt}, thereby limiting germination under moist conditions (Alvarado and Bradford, 2002; Watt *et al.*, 2011). Additionally, expression of thermal inhibition in seeds of some members of Asteraceae is consistent with elevated levels of endogenous abscisic acid (ABA) and decreased biosynthesis of gibberellic acid (GA) and ethylene (Taylor *et al.*, 2005; Argyris *et al.*, 2008). Analysis of germination percentage, rate and uniformity indicate that 25°C is the optimum constant temperature for *R. mollis* germination. Similarly, conditions experienced during early autumn or late spring (29/19°C) represent the optimum alternating germination temperatures.

The appearance of abnormal seedlings for late germinating phenotypes at 30°C, onset of viability loss at 35°C or under simulated summer temperatures (33/24°C), and decreases in germination rate suggest that *R. mollis* seeds exposed to these temperatures may have begun accumulating ageing-associated damage (Corbineau *et al.*, 2002). However, viability loss was mitigated at alternating temperatures. This may be part of a stress response mechanism in *R. mollis* that allows a proportion of non-dormant seeds to remain viable for some period of time yet avoid germination when seasonal temperatures are limiting (Probert, 2000; Fenner and Thompson, 2005). For example, Watt *et al.* (2011) conclude that thermal inhibition can be a bet-hedging strategy, while Taylor *et al.* (2005) suggest that thermal inhibition, similar to secondary dormancy, can be alleviated and re-imposed several times. Nevertheless, viability loss is predicted to continue in a taxon-specific manner for seeds in the soil, particularly those exposed to supra-optimal temperatures (Roberts, 1973; Priestly, 1986; Walters, 1998).

It is interesting to note that a portion of early germinating *R. mollis* phenotypes were selected by simulated summer temperatures to develop into seedlings during the experimental period (Fig. 2B). We speculate that sustained increases in seasonal temperature maxima and minima due to a warming climate (IPCC, 2007) would increase selection pressure on seed populations of *R. mollis.* Selective pressure could be expressed as comparative flattening of cumulative germination curves with perhaps an associated shift to the right along the time axis. This type of response may be different from that of taxa occurring in colder regions, wherein it is anticipated that climate warming may be beneficial for regeneration by seeds (Fenner and Thompson, 2005). A flattening of cumulative germination curves and shift in germination timing as temperatures increase has been shown for other taxa (Washitani and Takenaka, 1986; Orozco-Segovia *et al.*, 1996; Alvarado and Bradford, 2002). Disruption of germination patterns resulting in altered life-history traits or lower genetic variation in seedling populations can be maladaptive (Baskin and Baskin, 2001; Donohue, 2002; Donohue *et al.*, 2005; Fenner and Thompson, 2005).

Conclusions

Non-dormant seeds of *R. mollis* are equally capable of achieving high and rapid germination at relatively cool constant or alternating temperatures. Elevated constant (>31°C) or alternating temperatures (mean >28°C) will limit germination and may facilitate ageing-associated damage. Attempts to establish seedlings should occur when average soil temperatures are between 20 and 25°C. Subpopulations of *R. mollis* seeds respond to stressful thermal environments differently. Therefore, marginal increases in germination temperature, which correspond to forecasted climate change scenarios (IPCC, 2007), should be investigated further to determine possible effects on germination kinetics and recruitment dynamics.

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