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The germination niches of grassland species targeted for restoration: effects of seed pre-treatments

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Abstract

Restoration of semi-natural grassland communities involves a combination of (1) sward disturbance to create a temporal window for establishment, and (2) target species introduction, the latter usually by seed sowing. With great regularity, particular species establish only poorly. More reliable establishment could improve outcome of restoration projects and increase cost-effectiveness. We investigated the abiotic germination niche of ten poorly establishing calcareous grassland species by simultaneously exploring the effects of moisture and light availability and temperature fluctuation on percentage germination and speed of germination. We also investigated the effects of three different pre-treatments used to enhance seed germination – cold-stratification, osmotic priming and priming in combination with gibberellic acid (GA_3) – and how these affected abiotic germination niches. Species varied markedly in width of abiotic germination niche, ranging from *Carex flacca* with very strict abiotic requirements, to several species reliably germinating across the whole range of abiotic conditions. Our results suggest pronounced differences between species in gap requirements for establishment. Germination was improved in most species by at least one pre-treatment. Evidence for positive effects of adding GA_3 to seed priming solutions was limited. In several species, pre-treated seeds germinated under a wider range of abiotic conditions than untreated seeds. Improved knowledge of species-specific germination niches and the effects of seed pre-treatments may help to improve species establishment by sowing, and to identify species for which sowing at a later stage of restoration or introduction as small plants may represent a more viable strategy.

Introduction

European calcareous grasslands support a diverse flora and fauna and have high conservation and cultural values (Hillier *et al.*, 1990; WallisDeVries *et al.*, 2002). Once widespread, they have declined massively due to agricultural intensification and abandonment, both on the European mainland (WallisDeVries *et al.*, 2002) and in the UK (Braithwaite *et al.*, 2006). Remaining fragments are often small and isolated from each other, resulting in an increased risk to the persistence of many of the more specialist species (Fischer and Stöcklin, 1997; Bruun, 2000). Furthermore, the effects of past habitat fragmentation on grassland biodiversity may not yet have become fully manifest (Helm *et al.*, 2006). Both diversification of degraded sites and creation of additional semi-natural grassland are urgently required to help avert further species loss (Walker *et al.*, 2004), and these actions are included in conservation initiatives such as the UK Biodiversity Action Plan (UK Biodiversity Group, 1998).

After extended periods of agricultural intensification or abandonment, desirable target species are often no longer present in the seed bank (Davies and Waite, 1998; Bossuyt *et al.*, 2006; Fagan *et al.*, 2010). Natural re-colonization via seed rain or seed transfer by grazing livestock is possible if source habitats are nearby (Barbaro *et al.*, 2001; Fagan *et al.*, 2008). However, this process is often slow and unreliable even under favourable conditions, and frequently leads to floristically impoverished communities (Stampfli and Zeiter, 1999). Thus, active introduction is usually required (Pywell *et al.*, 2002, 2007).

There are various methods for species introduction. Planting usually works well in terms of plant survival (Wells *et al.*, 1989; Davies *et al.*, 1999; Wallin *et al.*, 2009) but is both labour- and cost-intensive. It is most often

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applied on small scales to diversify existing species-poor vegetation (Manchester *et al.*, 1999). If species-rich hay donor sites are available, spreading of hay from such sites can work well for hay meadow communities (Patzelt *et al.*, 2001; Hölzel and Otte, 2003). The method tends to work less well for communities predominantly managed by grazing (Edwards *et al.*, 2007). Because of the limitations of these alternative approaches, the most common method of introduction in grassland restoration is by sowing of seeds purchased from wildflower seed companies or collected by hand (Walker *et al.*, 2004; Hedberg and Kotowski, 2010). However, commercial wildflower seeds are often expensive; e.g. 1 kg of *Succisa pratensis* seeds typically costs GBP 410 (= EUR 475) (Emorsgate Seeds, 2010). Gathering of seeds from local sites, even when possible, has high labour costs and often only collects small quantities of seeds.

Even when seeds are available, efforts to re-establish target species of infertile communities often fail (Hodgson, 1989; Pywell *et al.*, 2003). For most species, successful regeneration from seeds depends on availability of suitable establishment microsites during the species' germination season (Bakker, 1989; Schütz, 2000; Kahmen and Poschlod, 2008). For example, germination of species from unimproved grassland is usually higher in swards that are only moderately dense (Fenner, 1978). Accordingly, when diversifying agriculturally improved swards, it is common practice to carry out pre-sowing disturbance to reduce or eliminate competition by the existing sward (Isselstein *et al.*, 2002; Hofmann and Isselstein, 2004; Pywell *et al.*, 2007). At fertile sites, gaps created for sowing are often short-lived, as the existing sward regenerates rapidly (Pywell *et al.*, 2007) or large numbers of seedlings of undesirable species emerge from the soil seed-bank (Galatowitsch, 2008). For these reasons, success and cost-effectiveness of restoration crucially depend on rapid and reliable seedling emergence (Pywell *et al.*, 2003).

Many species of unimproved grassland show primary seed dormancy, i.e. their seeds are initially unable to germinate after being shed. They lose their dormancy gradually, turning from being absolutely dormant (i.e. unable to germinate at all) to being conditionally dormant (i.e. able to germinate under an initially narrow but gradually widening set of environmental conditions), and finally to being non-dormant (i.e. able to germinate under the widest possible range of environmental conditions) (Baskin and Baskin, 1998). Seed dormancy may also vary within populations, resulting in intermittent germination of many species of unimproved grassland (Olff *et al.*, 1994). In contrast, species of agriculturally improved grassland often lack dormancy and can germinate soon after being shed (Olff *et al.*, 1994).

Germination of non-dormant seeds is still contingent upon suitable environmental conditions, most importantly temperature, light and moisture (Schütz, 2000). Light availability and quality and diurnal temperature fluctuations commonly act as cues indicating gaps in the sward (Silvertown, 1980; Thompson and Grime, 1983; see also Bullock, 2000), and uptake of moisture enables the seeds to commence germination. As the seedling is usually the life stage most sensitive to unfavourable environmental conditions, responses to such cues can optimize timing of germination to minimize mortality (Angevine and Chabot, 1979). Similarly, intermittent germination at the population level may spread these risks in time (Cavers *et al.*, 2000). While such adaptive germination behaviour aids persistence of plant species in their natural habitats, it may hinder species introductions during restoration, as the window of opportunity for establishment associated with pre-sowing disturbance is usually short-lived.

A wide range of pre-treatments is available to ensure rapid and complete germination and to help overcome seed dormancy (Halmer, 2004). Such pre-treatments may hold great potential to improve establishment success in ecological restoration. They include seed priming techniques that allow seeds to partially hydrate and commence germination metabolism, while at the same time avoiding radicle emergence. Other pre-treatments are designed to overcome specific types of seed dormancy. For example, cold–moist stratification, i.e. exposure of imbibed seeds to cold temperatures for several weeks to months, can serve to overcome physiological seed dormancy. This is considered the most common type of seed dormancy among temperate grassland species and under natural conditions ensures germination in spring (Washitani and Masuda, 1990; Olff *et al.*, 1994). Depending on the exact subtype of physiological dormancy operating in a species, this may be substituted by soaking of seeds in gibberellic acid solution. Results can be further improved by combining several pre-treatments (Halmer, 2004). Seed pre-treatments often widen the window of environmental conditions that will stimulate germination, and so may also affect establishment success in restoration projects.

The present study has three main objectives: (1) to investigate the abiotic germination niches of grassland restoration target species which were previously identified as difficult to establish in restoration projects; (2) to investigate the potential of various seed pre-treatments to achieve quicker and more complete germination; and (3) to investigate how pre-treatments may affect species-specific abiotic germination niches. Improved knowledge of these aspects may help increase the establishment success of such species in future restoration projects.

Materials and methods

Test species

Ten species were included in our study (Table 1). All species occur in calcareous grassland (Rodwell, 1992) and are adapted to environmental stress associated with this habitat (Table 1). However, meta-analyses of restoration project outcomes have indicated them to be difficult to establish at grassland restoration sites (Pywell *et al.*, 2003; R.F. Pywell, unpublished data). The only exception was *Thymus pulegioides*, a species less regularly used in UK restoration projects, but closely related to *Thymus polytrichus*, another species included in our study that establishes poorly in restoration. *Bromopsis erecta* performs better in restoration than the other species studied, but is still a relatively poorly establishing grass (Pywell *et al.*, 2003). All seeds used in this study were collected in summer/autumn 2007, air-dried at room temperature and stored in darkness at 4°C until used for this study. Some afterripening may have occurred during storage. However, the storage regime was chosen for its similarity to storage regimes used in restoration projects (e.g. Gustafson *et al.*, 2004; Reckinger *et al.*, 2010). Our results thus indicate what controls germination of our target species in the context of sowing for restoration.

Experimental design

From January 2009, germination experiments were carried out using untreated seeds as well as seeds subjected to either of three different pre-treatments: (1) for cold-stratification, seeds were stored in a refrigerator at 4°C for 5 weeks, between two layers of nylon cloth buried in a tray filled with moist sand;

(2) osmotic seed priming was carried out by soaking seeds in a solution of polyethylene glycol (PEG)-6000 with a nominal osmotic potential of -1.0 MPa, for which the weight ratios of PEG-6000 and distilled water were calculated with the computer program SPMM (Michel and Radcliffe, 1995); and (3) osmotic priming was also applied in combination with a seed pre-soaking in gibberellic acid (GA$_3$). In agricultural and horticultural species, the latter can further increase benefits associated with priming (Finch-Savage, 1991; Rogis *et al.*, 2004). To this end, we dissolved GA$_3$ in the PEG solution at a concentration of 1000 mg l^{-1}. For priming, seeds were placed into 90-mm Petri dishes, with 200 seeds per dish placed on a double layer of filter paper (VWR, No. 413) soaked with 12 ml of the respective solution. The resulting solution volume:air-dry filter paper ratio exceeded the critical value of 12 ml g^{-1} above which concentration due to exclusion of PEG from filter paper is minimized (Hardegree and Emmerich, 1990). Dishes were sealed with Parafilm 'M' (Pechiney Plastic Packaging Company, Chicago, Illinois, USA). Priming was carried out in darkness for 14 d at 16°C, using the incubator (MIR-253; Sanyo Electric Co., Ltd., Osaka, Japan) subsequently also used in the germination study. The selected water potential and duration are known to work well across a wide range of grassland species (Adegbuyi *et al.*, 1981; Tallowin *et al.*, 1994). At the end of the 5-week cold-stratification or 14-d priming periods, seeds were thoroughly rinsed in distilled water and blotted dry, and then sown into 55-mm Petri dishes containing two layers of filter paper (VWR, No. 413). We only used seeds that appeared full and undamaged, but carried out no formal assessment of seed viability. Seeds of the three smallest-seeded species (Table 1) were assessed using a stereomicroscope. Each replicate consisted of 20 seeds or, for the large-seeded *B. erecta*, 15 seeds.

Table 1. List of test species used in this study. Nomenclature follows Stace (1997). Seed weights from Liu *et al.* (2008), CSR strategy types according to Grime *et al.* (2007): C, competitive; S, stress-tolerant; R, ruderal. Information on primary seed dormancy types from various literature sources: ND, non-dormant; PD, physiologically dormant; PY, physically dormant; MPD, morpho-physiologically dormant

Species	Family	Seed weight (mg)	CSR type	Seed dormancy type	Source of dormancy information
Bromopsis erecta (Huds.) Fourr.	Poaceae	4.63	SC/CSR	ND	Grime *et al.* (1981)
Campanula glomerata L.	Campanulaceae	0.13	–	PD	Hitchmough *et al.* (2008)
Carex flacca Schreb.	Cyperaceae	0.87	S	PD	Taylor (1956)
Filipendula vulgaris Moench	Rosaceae	0.81	S/CSR	ND	Partzsch (2008)
Helianthemum nummularium (L.) Mill.	Cistaceae	1.15	S	PY	Thanos *et al.* (1992)
Pimpinella saxifraga L.	Apiaceae	1.26	SR/CSR	MPD	Flemion and Henrickson (1949); Grime *et al.* (1981)
Stachys officinalis (L.) Trevis.	Lamiaceae	1.01	S/CSR	PD[1] or ND[2]	[1]Kotorová and Lepš (1999); [2]Grime *et al.* (1981)
Succisa pratensis Moench	Dipsacaceae	1.30	S/CSR	PD[1] or ND[2]	[1]Maas (1989); [2]Grime *et al.* (1981)
Thymus polytrichus Borbás	Lamiaceae	0.13	S	ND	Pigott (1955); Grime *et al.* (1981)
Thymus pulegioides L.	Lamiaceae	0.15	–	ND	Pigott (1955)

In a fully factorial design, the various seed pre-treatments were combined with a range of abiotic regimes involving manipulation of temperature, moisture and light conditions. Seeds were exposed either to a constant (16°C) or a diurnally fluctuating (12 h at 22°C and 12 h at 10°C) temperature regime which corresponds to a typical spring soil surface regime at one of our restoration sites (M. Wagner, unpublished data). Moisture was manipulated by moistening the filter paper with 4.5 ml of either distilled water for a 'high water potential' treatment or a solution of PEG-6000 for a 'low water potential' treatment of nominally -0.6 MPa designed to generate moderate drought stress while still providing enough moisture to allow germination (e.g. Evans and Etherington, 1990). Again, the solution volume:air-dry filter paper ratio exceeded the critical value of $12 \, \text{ml g}^{-1}$ (Hardegree and Emmerich, 1990). For the untreated control, cold-stratification and $PEG + GA_3$ seed priming pre-treatments, we combined these temperature and moisture regimes with three different light regimes. These were: (1) a full-light treatment with Petri dishes placed on to the top incubator shelf, lit from above by a 15 W fluorescent lamp for $12 \, \text{h d}^{-1}$, coinciding with the warmer temperature in the fluctuating temperature regime; (2) a shade treatment with Petri dishes placed on to the second shelf from the top, and dishes shaded by 'full-light' dishes and a layer of green high-density polyethylene (HDPE) shade netting (65–70% shading) attached to the underside of the top shelf; and (3) a dark treatment with Petri dishes wrapped in aluminium foil and placed in a sealed cardboard box. During measurements with a Cropscan MSR16R sensor (Cropscan Inc., Rochester, Minnesota, USA) the green HDPE netting indeed reduced light levels across much of the visible spectrum by *c.* 70%. The effect was stronger for red wavelengths around 660 nm (*c.* 77% reduction), but about average for far-red wavelengths around 730 nm (*c.* 71% reduction), and shading thus reduced the red/far-red ratio of light in similar ways, but weaker, as a green leaf canopy would have done. Because of lack of time and space, germination following priming without added GA_3 was tested only under full light.

Each treatment combination was replicated three times per species. Because of limited incubator space, we tested different pre-treatments in sequence. Tests with untreated seeds were started on 21 January 2009, tests with osmotically primed seeds (PEG only and $PEG + GA_3$) on 4 February 2009, and tests with cold-stratified seeds on 18 February 2009. The three replicate dishes of the full-light and shade treatments were stacked. To minimize solute concentration effects, dishes were sealed with Parafilm 'M' after initial moistening and between counts. In addition, in the full-light and shade treatments, one-third of the solution was replaced at weekly intervals during counts.

Germinated seeds in these treatments were counted and removed after 2, 5, 7, 9, 12, 14 d and then at 7-d intervals until day 42, by which time germination had ceased in most species. At each count, the topmost Petri dish of a stack was moved to the bottom, to ensure that each replicate received approximately equal amounts of light. To avoid disruption of the treatment, seed germination in darkness was recorded only once, after 42 d. Substantial germination of *Carex flacca* occurred beyond the 42-d period, and therefore monitoring of this species was continued for a further 28 d in the light and shade treatments, i.e. for 10 weeks in total. For *B. erecta*, we only tested germination of untreated seeds, as substantial germination had occurred in this species during the cold-stratification and priming pre-treatments.

Data analysis

Statistical analyses were carried out using R, Version 2.10.0 (R Development Core Team, 2009). For each species we determined general linear models that best described the dependence of final germination after 6 weeks on pre-treatments as well as abiotic conditions and their interactions with each other. To accommodate for the fact that priming without added GA_3 was only assessed in the full-light regime, we ran two separate analyses per species. The seed pre-treatment analysis compared all four pre-treatment levels across the various moisture and temperature regimes at full light. The light regime analysis compared control, cold-stratification and $PEG + GA_3$ pre-treatments only, but did so across the full range of light regimes. While the latter explored the widest possible range of abiotic regimes, the former, via inclusion of both priming methods (PEG only and $PEG + GA_3$) enabled assessment of whether effects of the $PEG + GA_3$ treatment may have been caused mainly by the hydration effect of priming, the hormonal effect of GA_3, or a combination.

Analysis of final percentage germination was based on arcsine-transformed data. For both the seed pre-treatment and light regime analyses, we started with fully factorial maximal models including all main factors as fixed factors and all possible interactions. We then removed non-significant model terms, one at a time, beginning with the highest-order terms, and re-ran the analysis. Model simplification was stopped when all of the lower-order terms had appeared in at least one significant higher-order interaction (Crawley, 2005).

To characterize speed of germination in the full-light and shade regimes, we calculated t_{50} values, specifying time to 50% of final germination after 6 weeks in each Petri dish. For *C. flacca*, calculation was based on final germination after 10 weeks.

Characterization of germination speed based on seeds that germinate per experimental unit, rather than on all viable seeds, is commonly used in seed ecological studies (e.g. Grime *et al.*, 1981; Gibson-Roy *et al.*, 2007).The alternative of basing calculations on 50% of all viable seeds would have required germination in different treatments to regularly exceed 50% of viable seeds, which was obviously not the case in our study. Where necessary, we interpolated between consecutive count dates. To assess treatment effects on t_{50} we used the same two types of analyses as for final germination, with three differences. First, data were Box–Cox transformed prior to analysis. Box–Cox exponents were estimated using the procedure in R's MASS library. Second, as no information was available on temporal patterns of dark germination, no such data were included in the light regime analyses. Third, while General Linear Modelling (GLM) analyses were straightforward for *Filipendula vulgaris*, *Helianthemum nummularium*, *T. pulegioides* and *T. polytrichus*, all of which showed reasonable germination across the whole range of abiotic conditions, this was not the case for the remaining species. *Campanula glomerata*, *C. flacca*, *Stachys officinalis*, and *S. pratensis* germinated very poorly in the 'low water availability' treatments, and *C. flacca* also in the constant-temperature treatments (see Results), rendering precise estimates of t_{50} impossible. Therefore, the respective subsets of treatments were not included in data analyses of t_{50} for these species. By excluding complete subsets of treatments, a crossed and balanced design for analysis was maintained, and model simplification was carried out as for analyses of final germination. Due to very low final germination throughout, no t_{50} values were calculated for *Pimpinella saxifraga*.

Results

In different species, abiotic factors and seed pre-treatments tended to affect germination differently, the sole exception being soil moisture, low levels of which generally resulted in both lower final germination and reduced speed of germination (t_{50}). Thus, results are reported separately for each species, with species characterized by similar germination responses being grouped.

Bromopsis erecta

Irrespective of temperature or moisture conditions, untreated seeds of *B. erecta* germinated to nearly 100% in full light and shade and to more than 80% in darkness. Substantial germination also occurred during seed priming and cold-stratification. It thus appears highly unlikely that low germination rates

contribute to the comparatively poor performance of *B. erecta* in restoration, and so results for this species are not presented in more detail.

Thymus polytrichus and Thymus pulegioides

Germination of untreated *T. polytrichus* and *T. pulegioides* seeds reached 75–100% in all shading and full-light treatment combinations (Fig. 1A, B), and accordingly, there was little room for further stimulation of germination in response to seed pre-treatments. Moreover, *T. polytrichus* showed reduced seed germination in response to cold-stratification, and this reduction was particularly pronounced at sub-optimal moisture levels (Fig. 1A), which was also reflected in a significant interaction between pre-treatment and moisture in the seed pre-treatment analysis (Table 2). In darkness, germination of untreated seeds was sporadic (Fig. 1A, B). However, in both *Thymus* species, seed priming with PEG + GA$_3$ substituted for the light requirement for germination (Fig. 1), and this is reflected by significant two-way and three-way interactions in the light regime analyses for both species (Table 3).

Germination speed of untreated seeds was largely unaffected both by shading and by temperature fluctuation in both *T. polytrichus* and *T. pulegioides* (Fig. 2A, B). In the former, priming with PEG + GA$_3$, but not without added GA$_3$, appeared to mitigate the slowing down of germination in response to drought stress (Fig. 2A). At the same time, seeds of this species germinated more slowly after cold-stratification, particularly when moisture availability was reduced (Fig. 2A). These findings were also reflected in a significant interaction between pre-treatment and moisture level in both the seed pre-treatment analysis (Table 4) and in the light regime analysis (Table 5). In *T. pulegioides*, priming with PEG + GA$_3$ accelerated germination more in shaded conditions than at full light, as indicated by a significant interaction between pre-treatment and light in the light regime analysis (Table 5).

Filipendula vulgaris and Helianthemum nummularium

Irrespective of seed pre-treatment, final germination in both species tended to reach 30–50% across the various abiotic treatment combinations, but was lower in *F. vulgaris* for both untreated seeds and cold-stratified seeds in various treatment combinations involving darkness (Fig. 1C). Raised levels of dark germination in *F. vulgaris* in response to PEG + GA$_3$ priming were reflected by a significant interaction between pre-treatment and light regime in the light regime analysis (Table 3). At the same time, at full
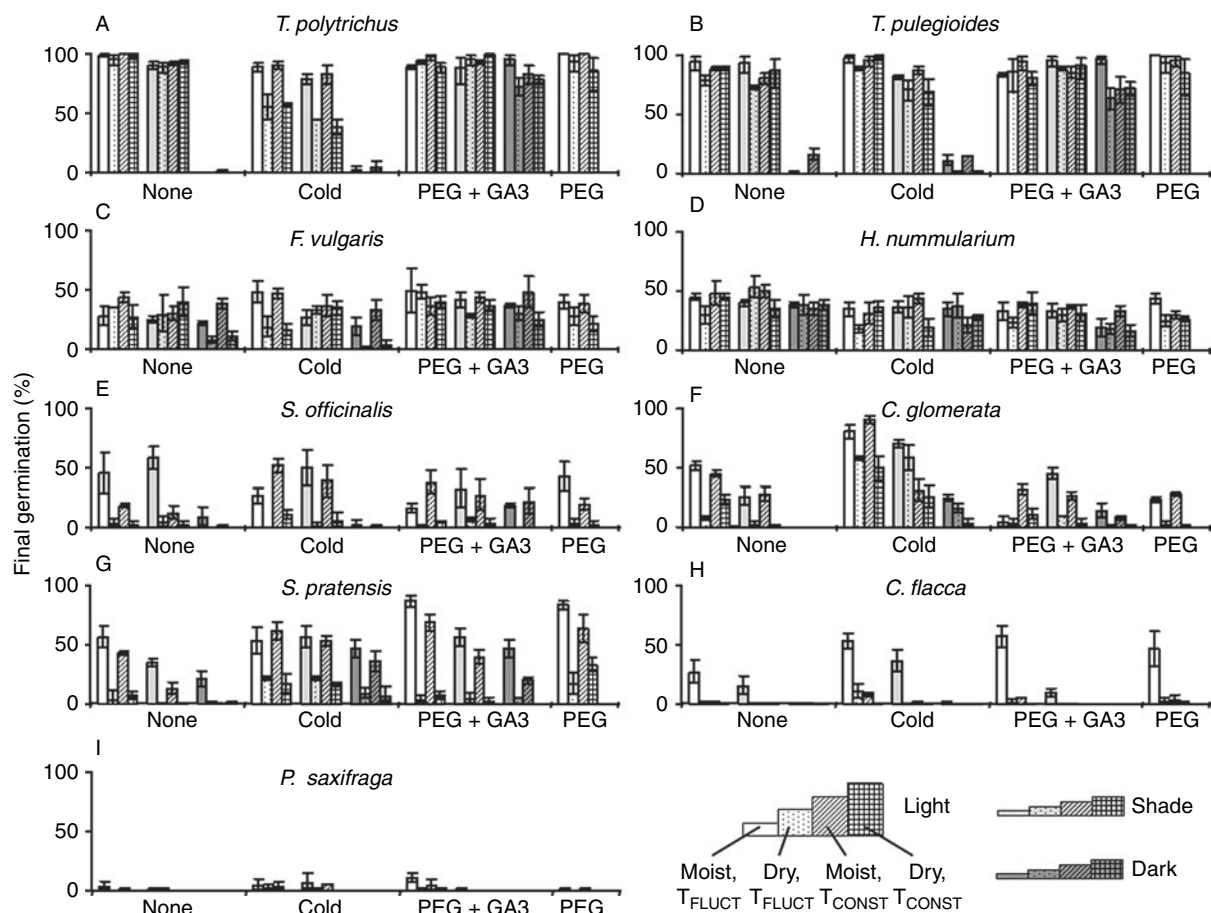
Figure 1. Percentage final germination of nine species of calcareous grassland under 12 different abiotic regimes, combining three light regimes with two different moisture levels and two temperature regimes, constant versus fluctuating. Results are shown for untreated seeds, cold-stratified seeds, seeds primed osmotically with a PEG solution containing GA_3, and seeds primed osmotically with a PEG solution not containing GA_3. Back-transformed means and standard errors are shown.

light, cold-stratification appeared to have induced an increased susceptibility to drought stress in *F. vulgaris* (Fig. 1C), reflected by a significant interaction between seed pre-treatment and moisture level (Table 2).

In *H. nummularium*, germination was not reduced by darkness. However, irrespective of abiotic conditions, final germination in this species was slightly – but nevertheless significantly – reduced both by cold-stratification and seed priming (Tables 2 and 3; Fig. 1D).

As expressed by lower t_{50} values, germination of *F. vulgaris* was accelerated by seed priming, irrespective of whether GA_3 was added to the PEG solution (Fig. 2C; see also Tables 4 and 5). At least in the full-light regime, priming both with and without added GA_3 also accelerated germination in *H. nummularium* (Fig. 2D, Table 4). Priming also mitigated the slowing down of germination in response to drought stress that was observed in untreated seeds (Fig. 2D), as underlined by a significant interaction between pre-treatment and moisture level in both analyses of t_{50} data (Tables 4 and 5). At the same time,

cold-stratification mitigated the deceleration of germination that was observed in untreated seeds in response to shading (Fig. 2D), and this was also reflected by a significant interaction between pre-treatment and light in the light regime analysis (Table 5).

Stachys officinalis

Germination of untreated seeds of *S. officinalis* was strongly reduced by constant temperature, by water potential reduction and by darkness, but not by shading (Fig. 1E). As indicated by significant interaction effects between pre-treatment and temperature regime (Tables 2 and 3), both cold-stratification as well as $PEG + GA_3$ priming substituted for the temperature fluctuation requirement (Fig. 1E). In addition, $PEG + GA_3$ priming promoted dark germination at the unreduced water potential, and this was reflected in the light regime analysis by a significant three-way interaction between pre-treatment, light and moisture (Table 3).

Table 2. Summary of seed pre-treatment GLM analyses of final germination percentages after 6 weeks, based on results in the full-light regime for all four pre-treatment levels. Rows show F values and term significance levels (NS, non-significant and maintained in the final model because of significant higher-order interaction; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). 'Excluded' indicates non-significant terms not included in the final model

Source	d.f.	C. glomerata	C. flacca	F. vulgaris	H. nummularium	P. saxifraga	S. officinalis	S. pratensis	T. polytrichus	T. pulegioides
Intercept	1	1216.7***	201.1***	937.2***	1724.4***	37.8***	342.0***	825.4***	3996.4***	1904.5***
Pre-treatment (P)	3	99.6***	3.8*	2.1NS	3.6*	3.6*	0.6NS	6.0**	20.8***	Excluded
Moisture (M)	1	101.5***	91.1***	10.1**	7.5**	15.3***	111.1***	168.2***	29.1***	4.4*
Temperature (T)	1	7.9**	77.7***	Excluded	4.1NS	Excluded	1.4NS	0.2NS	Excluded	Excluded
P × M	3	3.7*	Excluded	3.4*	Excluded	Excluded	Excluded	4.5**	3.9*	Excluded
P × T	3	3.8*	Excluded	Excluded	Excluded	Excluded	8.4***	Excluded	Excluded	Excluded
M × T	1	1.5NS	26.3***	Excluded	7.7**	Excluded	Excluded	5.5*	Excluded	Excluded
P × M × T	3	3.5*	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded
d.f. corrected model	15	6	7	6	4	8	9	7	1
d.f. error	32	41	40	41	43	39	38	40	46

Whereas temperature fluctuation enhanced overall germination of *S. officinalis*, it did not affect germination speed as expressed by t_{50} (Fig. 2E). Seed priming accelerated germination, irrespective of temperature regime or shading (Fig. 2E), as reflected by significant pre-treatment main effects in both analyses, combined with non-significant pre-treatment interactions with both light and temperature (Tables 4 and 5).

Campanula glomerata and *Succisa pratensis*

In both *C. glomerata* and *S. pratensis*, a combination of full light, temperature fluctuation, and unreduced water potential was most conducive to high final germination. However, even this optimal combination of abiotic factors resulted in a final germination of untreated seeds only slightly higher than 50% in both species (Fig. 1F, G). In both species, constant temperature only slightly reduced final germination, whereas both shading and reduced water potential resulted in a much stronger reduction (Fig. 1F, G).

In both species, at least one pre-treatment resulted in a marked increase in germination. In *C. glomerata*, final germination reached about 90% in the most optimal abiotic treatment in response to cold-stratification (Fig. 1F). Cold-stratification in this species also induced dark germination in a proportion of seeds, and also reduced the moisture requirement for germination, in particular in the shaded and dark treatments (Fig. 1F). This was also reflected in a highly significant interaction between pre-treatment, light regime and moisture regime in the light regime analysis (Table 3). Priming of *C. glomerata* seeds resulted in slightly lower final germination in the full-light regime; however, PEG + GA_3 priming appeared to slightly enhance germination in the shade and dark treatments (Fig. 1F).

Final germination in *S. pratensis* was strongly enhanced by PEG + GA_3 priming, but this was only observed at the unreduced water potential (Fig. 1G). By contrast, cold-stratification enhanced germination at both water potentials, although at the unreduced water potential, the effect was small in comparison to the effect of PEG + GA_3 priming (Fig. 1G). Both cold-stratification and PEG + GA_3 priming promoted dark germination in *S. pratensis* (Fig. 1G). The described pre-treatment and abiotic responses are also reflected by the highly significant interactions between pre-treatment and moisture in the seed pre-treatment analysis (Table 2), and between pre-treatment, light and moisture in the light regime analysis (Table 3).

In *C. glomerata*, both cold-stratification and PEG + GA_3 priming markedly reduced t_{50}, i.e. they accelerated seed germination (Fig. 2F), and this was also reflected by significant pre-treatment main and interaction effects (Tables 4 and 5).

Table 3. Summary of light regime GLM analyses of final germination percentages after 6 weeks, based on results for three different light regimes in combination with three different pre-treatment levels, not including the 'PEG only' option. See Table 2 for notation

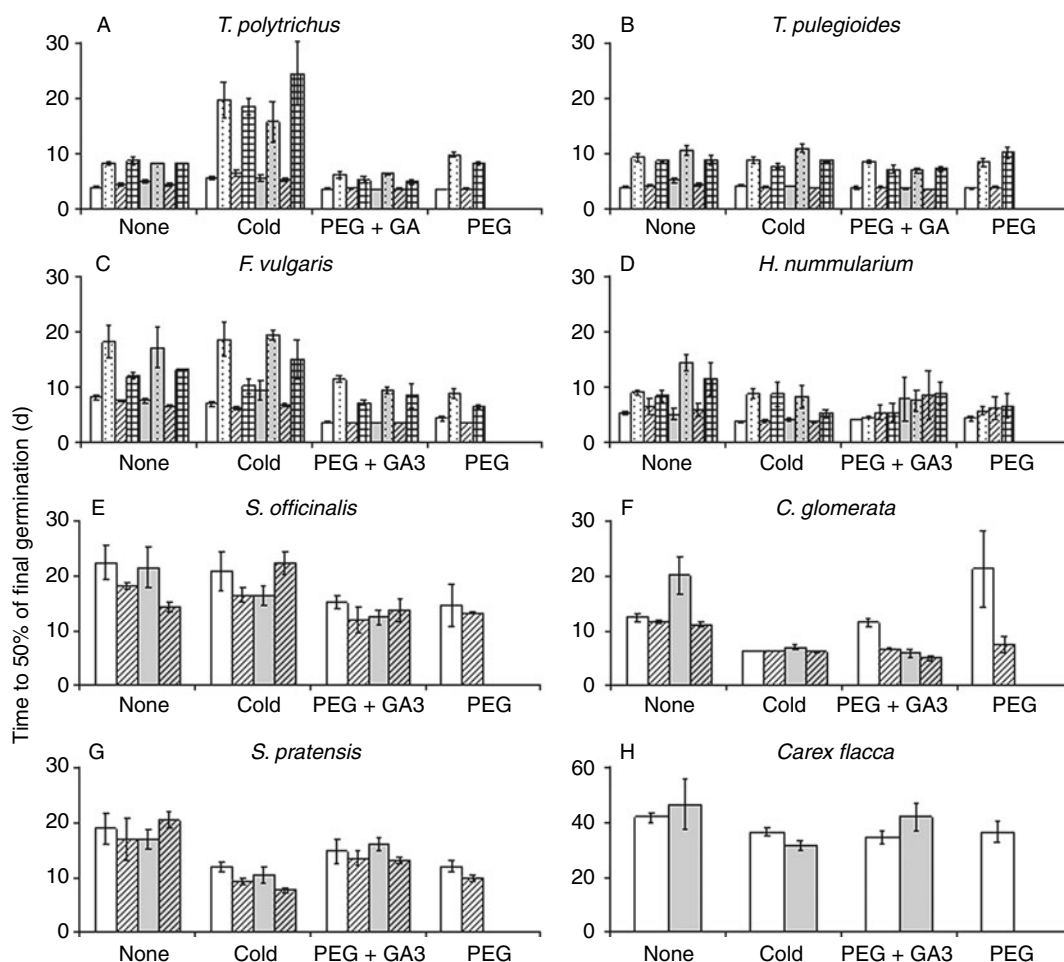| Source	d.f.	C. glomerata	C. flacca	F. vulgaris	H. nummularium	P. saxifraga	S. officinalis	S. pratensis	T. polytrichus	T. pulegioides
Intercept	1	1634.9***	237.4***	1838.1***	3150.7***	51.8***	395.7***	1493.0***	6323.0***	3389.8***
Pre-treatment (P)	2	118.8***	10.0***	12.6***	13.0*	3.7*	0.4^{NS}	51.0***	199.8***	41.6***
Light (L)	2	178.4***	81.0***	16.6***	4.6*	16.7***	35.4***	42.9***	500.7***	222.2***
Moisture (M)	1	134.2***	148.2***	24.2***	4.8*	19.1***	150.2***	402.5***	32.8***	19.6***
Temperature (T)	1	12.9***	124.6***	Excluded	Excluded	5.7*	0.2^{NS}	18.6***	Excluded	Excluded
P × L	4	20.9***	3.6**	2.5*	Excluded	3.5*	2.4^{NS}	3.3*	94.3***	38.7***
P × M	2	1.7^{NS}	3.1^{NS}	3.6*	Excluded	Excluded	0.3^{NS}	11.2***	16.3***	Excluded
P × T	2	14.1***	Excluded	Excluded	Excluded	Excluded	8.0***	2.0^{NS}	Excluded	Excluded
L × M	2	7.3**	38.6***	8.9***	0.7^{NS}	6.0**	4.4*	9.1***	1.6^{NS}	Excluded
L × T	2	16.4***	32.8***	Excluded	3.6*	Excluded	4.6*	4.0*	Excluded	Excluded
M × T	1	Excluded	62.8***	Excluded	0.2^{NS}	Excluded	4.4*	10.1**	Excluded	Excluded
P × L × M	4	6.0***	2.7*	2.6*	Excluded	Excluded	2.7*	3.2*	4.5**	Excluded
P × L × T	4	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded
P × M × T	2	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	4.8*	Excluded	Excluded
L × M × T	2	Excluded	13.2***	Excluded	5.6**	Excluded	Excluded	Excluded	Excluded	Excluded
P × L × M × T	4	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded
d.f. corrected model		22	23	17	13	12	12	25	17	9
d.f. error		85	84	90	94	95	84	82	90	98

Figure 2. Time in days to 50% of final germination of eight species of calcareous grassland under 12 different abiotic regimes, combining two light regimes, full light and shade, with two different moisture levels and two temperature regimes, constant versus fluctuating. Results are shown for untreated seeds, cold-stratified seeds, seeds primed osmotically with a PEG solution containing GA_3, and seeds primed osmotically with a PEG solution not containing GA_3. Untransformed means and standard errors are shown. For the key see Fig. 1.

In *S. pratensis*, all pre-treatments resulted in faster germination, with PEG + GA_3 priming being slightly less effective (Fig. 2G). This was reflected by significant pre-treatment effects on t_{50} in both the seed pre-treatment analysis (Table 4) and the light regime analysis (Table 5). At the same time, the absence of significant interactions between pre-treatment and abiotic factors (Tables 4 and 5) indicated that pre-treatment effects on germination speed in this species occurred independent of abiotic regime.

Table 4. Summary of seed pre-treatment GLM analyses of t_{50}, based on results in the full-light regime for all four pre-treatment levels. See Table 2 for notation

Source	d.f.	C. glomerata	C. flacca	F. vulgaris	H. nummularium	S. officinalis	S. pratensis	T. polytrichus	T. pulegioides
Intercept	1	10 774.3***	478343.0***	21 333.1***	15 688.8***	3455.7***	1277603.1***	89 242.3***	67 404.6***
Pre-treatment (P)	2	10.2***	Excluded	91.8***	5.3**	4.4*	10.5***	82.8***	Excluded
Moisture (M)	1	Not tested	Not tested	391.7***	21.8***	Not tested	Not tested	535.3***	707.3***
Temperature (T)	1	17.3***	Not tested	47.6***	Excluded	Excluded	7.4*	0.1^{NS}	Excluded
P × M	2	Not tested	Not tested	6.2**	4.9**	Not tested	Not tested	7.4***	Excluded
P × T	2	5.7**	Not tested	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded
M × T	1	Not tested	Not tested	14.7***	Excluded	Not tested	Not tested	4.9*	Excluded
P × M × T	2	Not tested	Not tested	Excluded	Excluded	Not tested	Not tested	Excluded	Excluded
d.f. corrected model		7	0	9	7	3	4	9	1
d.f. error		16	11	38	40	20	19	38	46

Table 5. Summary of light regime GLM analyses of t_{50}, based on results for three different light regimes in combination with three different pre-treatment levels, not including the 'PEG only' option. See Table 2 for notation

Source	d.f.	C. glomerata	C. flacca	F. vulgaris	H. nummularium	S. officinalis	S. pratensis	T. polytrichus	T. pulegioides
Intercept	1	43 937.0***	51231669.2***	15 308.1***	16 130.9***	7952.9***	16 067.6***	91 559.9***	41 208.1***
Pre-treatment (P)	2	89.5***	Excluded	103.7***	8.6***	9.1***	28.3***	154.9***	19.9***
Light (L)	2	9.8**	Excluded	0.9[NS]	1.4[NS]	Excluded	Excluded	Excluded	2.0[NS]
Moisture (M)	1	Not tested	Not tested	348.2***	37.2***	Not tested	Not tested	440.6***	963.4***
Temperature (T)	1	21.4***	Not tested	29.1***	Excluded	Excluded	Excluded	Excluded	11.4**
P × L	4	22.8***	Excluded	3.3*	3.4*	Excluded	Excluded	Excluded	5.4**
P × M	2	Not tested	Not tested	4.7*	5.1**	Not tested	Not tested	3.5*	Excluded
P × T	2	2.8[NS]	Not tested	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded
L × M	2	Not tested	Not tested	Excluded	Excluded	Not tested	Not tested	Excluded	Excluded
L × T	2	0.3[NS]	Not tested	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded
M × T	1	Not tested	Not tested	8.4**	Excluded	Not tested	Not tested	Excluded	Excluded
P × L × M	4	Not tested	Not tested	Excluded	Excluded	Not tested	Not tested	Excluded	Excluded
P × L × T	4	4.8*	Not tested	Excluded	Excluded	Excluded	Excluded	Excluded	Excluded
P × M × T	2	Not tested	Not tested	Excluded	Excluded	Not tested	Not tested	Excluded	Excluded
L × M × T	2	Not tested	Not tested	Excluded	Excluded	Not tested	Not tested	Excluded	Excluded
P × L × M × T	4	Not tested	Not tested	Excluded	Excluded	Not tested	Not tested	Excluded	Excluded
d.f. corrected model		11	0	10	8	2	2	5	7
d.f. error		24	17	61	63	33	33	66	64

Carex flacca

Notable germination of untreated *C. flacca* seeds occurred only at the higher of the two water potential levels, and only in the fluctuating temperature regime. Under these optimal conditions, germination after 6 weeks reached 27% in the full-light regime, and 15% in the shade regime (Fig. 1H). After 10 weeks, this had increased to 51% and 23%, respectively (data not shown). Cold-stratification boosted germination both in the full-light and shade optimal abiotic combinations, but PEG seed priming – both with and without added GA_3 – did so only at full light (Fig. 1H). None of the pre-treatments did, however, stimulate germination at the reduced water potential or at the constant temperature, and neither did they induce any dark germination (Fig. 1H). Accordingly, while pre-treatment interacted with light regime in the light regime analysis (Table 5), it showed no other significant interactions with abiotic factors in either of the two analyses (Tables 4 and 5).

A pre-treatment effect on t_{50} of *C. flacca* fell short of significance in the light regime analysis ($P = 0.072$), although results suggest a mean reduction of t_{50} of 6 days in response to cold-stratification (Fig. 2H).

Pimpinella saxifraga

Final germination of *P. saxifraga* was very low, but a slight increase was detected after cold-stratification (Fig. 1I; Table 3).

Discussion

Our investigation of abiotic germination requirements of calcareous grassland species illustrates the mechanisms that underlie spatial and temporal variation in germination niches thought to promote high species richness in this ecosystem (Grubb, 1977; Thompson *et al.*, 1996). Previous research has identified a main axis describing a gradient in germination requirements, ranging from species that germinate indiscriminately soon after primary seed dispersal (e.g. many Poaceae and large-seeded dicots) to more temporally opportunistic species (e.g. many small-seeded dicots; Thompson *et al.*, 1996). While one species (*B. erecta*) among those we tested germinated indiscriminately, the other nine species exhibited large variation in germination responses to treatments, which suggests the existence of further germination niche axes. In the following, we discuss abiotic germination niches of our species, the effects of seed pre-treatments, and the wider implications for ecological restoration.

Abiotic germination niches

Of all tested species, *C. flacca* had the most specific abiotic requirements for germination. Moderate drought stress effectively prevented its germination. Our results also confirmed strong requirements of light (Grime *et al.*, 1981) and of temperature fluctuation (Thompson and Grime, 1983; Schütz and Rave, 1999), suggesting a germination preference for large vegetation gaps. Schütz and Rave (1999) showed that

the requirement for temperature fluctuation is more absolute in *C. flacca* than in many other *Carex* species: In their study, *C. flacca* was one of very few species in which constant high temperature did not substitute for temperature fluctuation. In our study, 5 weeks of cold-stratification promoted germination, but did not widen the range of abiotic conditions conducive to germination. Under field conditions, *C. flacca* is strictly spring-germinating (Thompson *et al.*, 1996), suggesting a more absolute cold requirement than found in our study. However, in our study, germination of untreated *C. flacca* seeds may have been promoted by the extended storage period, as dry storage has been reported as beneficial for germinability of this species (Grime *et al.*, 1981). Time to 50% final germination appeared reduced in response to cold-stratification, but this was not significant, possibly due to comparatively low statistical power.

Due to their cold requirement, when introduced by sowing, the seeds of *C. flacca* require sufficient exposure to cold temperatures, either by sowing in autumn or by cold-stratification prior to sowing in spring. However, while cold-stratification usually promotes germination of *C. flacca* under abiotic conditions conducive to its germination, it did not appear to widen the narrow abiotic germination niche of this species. Even when cold-stratified, seeds have a strong requirement of high light and moisture levels, and of temperature fluctuation, which all have to be adequately met. Therefore, and as *C. flacca* has a very high potential for vegetative spread (Grime *et al.*, 2007), introduction as small plants or vegetative fragments may be a good alternative when restoring populations of this species.

Four species were characterized by a very wide abiotic niche for germination. Irrespective of abiotic regime, untreated seeds of *F. vulgaris* and *H. nummularium* almost always showed 30–50% germination, with slightly lower levels of germination in those treatments that combined low water potential with darkness (Fig. 1C, D). By contrast, untreated seeds of the small-seeded *T. polytrichus* and *T. pulegioides* (Table 1) germinated nearly completely in full-light and shade treatments, but not in darkness. Final germination of all four species appeared uninfluenced by temperature fluctuation, and light requirement was relatively minimal, suggesting no adaptation to germinate only in larger gaps. In *T. polytrichus*, cold-stratification slowed down and reduced percentage germination at reduced water potential. This may be interpreted as a form of conditional dormancy that could increase fitness if dry periods were more frequent or more extended in spring than in autumn.

Cold-stratification did not increase germination in any of the four species, suggesting that they are not specialized to germinate in spring. In agreement with this, Stampfli and Zeiter (2008) found that both *H. nummularium* and *T. pulegioides* germinate throughout the cool season, i.e. from autumn to spring. Similarly, Thompson *et al.* (1996) showed that both *H. nummularium* and *T. polytrichus* emerge in autumn and spring. Finally, Partzsch (2008) found that seeds of *F. vulgaris* germinated readily shortly after field-collection without pre-treatment.

It appears unlikely that lack of germination explains the poor performance of these four species in restoration, although enhancement of germination in those species with incomplete germination might nonetheless boost seedling establishment. In *F. vulgaris*, this was achieved by seed priming. *H. nummularium* is characterized by physical seed dormancy (Table 1; see also Thanos *et al.*, 1992), i.e. by initial seed-coat impermeability for water. While there are different seed pre-treatments available in this situation (Baskin and Baskin, 1998), this also explains why priming did not increase final germination, in spite of accelerating it. Seeds whose coats were already permeable germinated faster, but physically dormant seeds did not benefit. Negative effects of stratification and priming on final germination in *H. nummularium* did not depend on abiotic conditions, i.e. were unlikely to be due to narrowing of the abiotic window permissive to germination. As we did not test the viability of ungerminated seeds at the end of the experiment, we cannot preclude mortality during the pre-treatment phase.

Three species were intermediate in terms of abiotic germination-niche width. *S. pratensis*, *C. glomerata* and *S. officinalis* all germinated comparatively well under shaded conditions (for *S. pratensis* see also Grime *et al.*, 1981), but at the same time had a relatively high moisture requirement. However, whereas the former two did not require temperature fluctuation, this was the case for *S. officinalis* (see Patzelt *et al.*, 2001). All three species thus appear to have a lesser necessity for large gaps than *C. flacca*, although the requirement for temperature fluctuation in *S. officinalis* may suggest otherwise. For *S. pratensis*, our finding of germination under shaded conditions fits with the observation that establishment may even benefit from the presence of a plant canopy (Isselstein *et al.*, 2002).

Cold-stratification promoted germination in *C. glomerata* and *S. pratensis*, implying specialization towards spring germination (for *S. pratensis* see Maas, 1989; Patzelt *et al.*, 2001), but did not generally increase germination of *S. officinalis* which is early summer germinating (Patzelt *et al.*, 2001).

Our results suggest that cold-stratification best improves establishment of *C. glomerata* and *S. pratensis*, and that seed priming can at least accelerate establishment of *S. officinalis*. Even when unaccompanied by an increase in overall germination,

this may increase the ability to compete, e.g. with high densities of weed seedlings that can quickly establish on ex-arable land.

Low levels of *P. saxifraga* germination may, at least in part, have been due to many seeds either lacking a viable embryo, or having an underdeveloped one, not able to germinate prior to maturation in an imbibed state (= morphophysiological dormancy *sensu* Baskin and Baskin, 1998; see also Flemion and Henrickson, 1949), although decline in seed viability during seed storage prior to the study may also have contributed. Cold-stratification may thus only affect seeds with more or less mature embryos, resulting in weak stimulation of germination. This agrees with the observation that *P. saxifraga* mainly emerges in spring (Stampfli and Zeiter, 2008). Morphophysiological dormancy is characteristic for many Apiaceae species, and these species often appear only in the second year after sowing in field experiments (Silvertown and Tremlett, 1989). To break their dormancy, either an extended period of cold-stratification, or of warm-stratification followed by cold-stratification, is suggested (Baskin and Baskin, 1998).

Wider implications for restoration

In calcareous grassland, physical hazards such as drought are a major cause of seedling mortality, and shelter provided by neighbouring plants may, for many species, more than outweigh the potential disadvantage of seedlings having to compete with these plants (Ryser, 1993). However, the balance between facilitative and competitive effects will be different under the more fertile conditions usually encountered at grassland restoration sites (Bruno *et al.*, 2003; Wagner, 2004). Even those species requiring relatively small gaps for regeneration in unimproved grassland may thus depend on pre-sowing disturbance in the denser swards of restoration sites. Interestingly, a gradient in seed and germination attributes quite similar to the one identified by Thompson *et al.* (1996) – from species germinating indiscriminately and rapidly to species with very specific abiotic requirements – has also been identified for species replacement during conversion of productive grassland to unproductive grassland (Olff *et al.*, 1994). This may suggest that immediate germination could be a better adaptation to more productive conditions. Accordingly, species known to establish poorly when introduced in the productive early stages of restoration may do so because their specific requirements for germination and/or seedling establishment are not met at that stage. This would confirm the argument that abiotic features cannot be ignored when dealing with questions of biotic composition in restoration (Hobbs and Norton, 2004). Such effects may be most pronounced in species with very strict abiotic requirements and a very narrow seasonal window for germination, such as *C. flacca* in our study. For such species, sowing at a later stage of restoration may work better (Pywell *et al.*, 2007), although for species with a known capacity for vegetative spread, early introduction by planting might also yield satisfactory results.

Our results suggest that seed pre-treatments can improve rates and overall levels of germination in species characterized by opportunistic germination, enabling them to better exploit the short temporal window of opportunity for establishment associated with pre-sowing disturbance. While we cannot prove this, it seems likely that the stimulation of dark germination after $PEG + GA_3$ priming, observed in several species, was caused by the addition of GA_3. Similar effects have been observed in other species (Finch-Savage, 1991; Finch-Savage *et al.*, 1991). Otherwise, we found little evidence for positive effects on seed germination of adding GA_3 to the priming solution. We would thus not generally recommend such a combination, especially as GA_3, at higher concentrations, can induce development of morphologically abnormal seedlings with reduced fitness (e.g. Rogis *et al.*, 2004). We tested only a limited range of pre-treatments, and variation of factors, such as pre-treatment duration, PEG solution concentration, temperature during the pre-treatment phase or use of other priming methods, may increase pre-treatment efficiency. In some species, dry storage at room temperature may be sufficient to overcome dormancy, as found in previous studies for *C. flacca* (Grime *et al.*, 1981) and for *S. pratensis* (Kotorová and Lepš, 1999).

Widening of the abiotic germination niche by seed pre-treatments may also enhance establishment success. Even when such widening may be accompanied by an increased risk of seedling mortality, e.g. by dessication after relaxation of the moisture requirement for germination, this risk may be well worth taking given that the temporal window of opportunity for seedling establishment associated with pre-sowing disturbance is often short-lived. While it remains to be demonstrated in field experiments that the most promising pre-treatments can indeed increase establishment success under field conditions, it seems likely that, eventually, a deeper knowledge of species-specific germination niches and how these can be modified by seed pre-treatments may help to optimize the success of introduction by sowing in restoration. Further, such knowledge may also help to identify species for which sowing at a later stage or planting may represent a more viable strategy than sowing in the initial stage of restoration.

Acknowledgements

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