

***WHY SOME SPECIES CANNOT COLONIZE RESTORED HABITATS? THE EFFECTS OF SEED AND MICROSITE AVAILABILITY***

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Journal for Nature Conservation, accepted. DOI: 10.1016/j.jnc.2012.12.005

Editor version available at

<http://www.elsevier.com/journals/journal-for-nature-conservation/1617-1381>

## Abstract

Restoration of calcareous grasslands was promoted as a conservation strategy to reduce the risks imposed by habitat loss and fragmentation. Restoration already provided promising results for several taxa, however some specialist species still fail at colonizing restored habitats. We aimed at explaining this lack of colonization success for three calcareous grasslands specialist species in Southern Belgium: *Pulsatilla vulgaris*, *Trifolium montanum* and *Veronica prostrata*. We studied: (i) Germination in control and outdoor conditions (cold, heat, smoke and litter effects); (ii) In-situ seedling emergence patterns (effects of seed addition and germination microsites availability). The three species were able to germinate in Petri dishes in the absence of treatment. Cold enhanced the germination of *V. prostrata*. Fire-related treatments (heat shock and smoke exposure) did not enhance germination and were deleterious to *V. prostrata*. Litter cover improved *P. vulgaris* emergence in outdoor containers, but had a negative effect on *V. prostrata*. In the field, *V. prostrata* did not emerge. *T. montanum* seedlings were observed in the reference grasslands when seeds were added, but not in the restored grasslands. *P. vulgaris* emerged in the reference grasslands, and to a lower degree in the restored grasslands. The combination of seed addition and microsites availability for seed germination resulted in enhanced seedling emergence for *P. vulgaris*. Our results suggest that seed and microsite availability can be limiting factors for site colonization, but the combination of both is likely much more limiting. Lower seedling emergence in restored than in reference grasslands suggests a lower habitat quality in restored grasslands.

Keywords: Belgium; Calcareous grasslands; Fire-related cues; Microsite availability; Restoration; Seed availability.

## Introduction

The last century has shown a decline in natural and semi-natural habitats throughout many parts of Europe. One consequence of habitat fragmentation is a decrease in specialist plant species population size and connectivity, leading to increased risk of extinctions (Leimu et al. 2006). Jongman and Pungetti (2004) proposed habitat restoration as a conservation strategy to reduce the risks imposed by habitat loss and fragmentation. One aim of ecosystem restoration is to create and expand habitat that support species characterizing former plant communities (Society for Ecological Restoration International Science & Policy Working Group 2004).

Species colonization within a site is largely controlled by two factors that limit seedling recruitment, including seed availability within the dispersal area (i.e. the number of seeds reaching the site) and seedling establishment based on microsite availability (i.e. number of microsites suitable for regeneration) (Coulson et al. 2001). These two processes determine if seeds spread over a site and if seeds establish as seedlings once settled on a site. Limited or lack of dispersal is a well-known constraint for semi-natural habitat restoration (Turnbull et al. 2000). Also microsite availability is a vital element in site colonization (Foster 2001; Münzbergová and Herben 2005). Biotic factors, such as inter-specific competition, notably with grass species, or litter effects (mechanical interaction, alteration of light and moisture conditions) can be responsible for the lack of microsite availability, reducing seedling survival. Microsite availability can be improved through application of disturbance regimes, which creates regeneration niches or gaps for germination and establishment through vegetation and/or litter effect removal. Moreover, disturbance may enhance seedling emergence after seed addition (Edwards et al. 2007).

In Belgium, approximately 100 ha of calcareous grasslands have been restored over the last 20 years. All restored sites were pre-forest communities comprised of 40-100 years old forests. Restoration protocols included tree and shrub clearing followed by sheep and goat grazing. As for restoration projects in Europe (Dzwonko and Loster 1998; Barbaro et al. 2001; Baba 2003), restored grasslands in Belgium globally tended to resemble reference habitats despite some differences in vegetation structure and floristic composition (Piqueray et al. 2011a). At the site scale, species richness rapidly recover when new habitat areas are created (Piqueray et al. 2011b). However, some characteristic species may fail at colonizing restored sites, despite the close vicinity of seed sources (Zobel et al. 1996; Piqueray et al. 2011a). Most calcareous grassland species exhibit low spatial and temporal dispersal (seed bank) ability (Kalamees and Zobel 1997; Stampfli and Zeiter 1999; Bisteau and Mahy 2005). In addition, Zobel et al. (2000) showed that some calcareous grassland species were dependant on microsite availability for recruitment. In this type of habitat, the influence of fire on germination and microsite availability is particularly important. The indirect effects of fire can lead to a reduction in competition by vegetation removal or a direct effect on seed germination through the actions of heat and smoke (Thomas et

al. 2003). Fire was formerly used as a traditional management technique to prevent shrub overgrowth (Kahmen et al. 2002). Currently, this practice is not recommended as a management tool, as comparative studies revealed it was not effective at maintaining traditional calcareous grassland communities (Kahmen et al. 2002; Köhler et al. 2005; Piqueray and Mahy 2010). However, at the species level, these same studies revealed that some plants benefit from fire treatments and some specialist species present in calcareous grassland seed banks germinate better after heat shock (e.g. *Thymus pulegioides*, *Globularia bisnagarica*) (Bossuyt and Honnay 2008).

In this study, we evaluated germination, and seed and microsite availability in three calcareous grassland species (*Pulsatilla vulgaris*, *Trifolium montanum* and *Veronica prostrata*) that failed at colonizing restored calcareous grasslands despite the close vicinity of seed sources. Our objective was to determine the factors limiting the spread and establishment of these species by addressing the following questions: (i) do the study species produce viable seed and do the seeds require treatments (cold, heat shock and smoke exposure) to germinate?; (ii) do microsite (presence of litter and/or disturbance) conditions influence seedling emergence?; (iii) does seedling emergence differ between restored and reference calcareous grasslands?; and (iv) can seed addition improve seedling emergence?

## Methodology

### *Study species and seed collection*

Our study evaluated three rare species in Belgium occurring only in calcareous grasslands: *Pulsatilla vulgaris*, *Trifolium montanum* and *Veronica prostrata*. The species are highly threatened and listed in the regional Red Data Book (Saintenoy-Simon et al. 2006). Species morphological descriptions can be found in Tutin et al. (1964-1980) and were therefore not reported here.

*Pulsatilla vulgaris* Mill. (Ranunculaceae) seeds mature from May-June (J. Piqueray, pers. obs.). Achenes are 1 × 3.5-4.5 mm, bearing a ca. 3 cm appendage. The species has however a low potential of wind dispersal (Tackenberg 2003). Achenes average mass is 4.2 mg and they exhibit no dormancy (BIOPOP Database, Poschlod et al. 2003). Seed bank is transient (Thompson et al. 1997).

*Trifolium montanum* L. (Fabaceae) seed maturity occurs in July-September (J. Piqueray, pers. obs.). Seeds are 1.2-1.7 × 0.8-1 mm (Bojnansky and Fargasova 2007), and mass is 0.85 mg in average (Cerabolini et al. 2003). Seeds do not exhibit dormancy and the soil seed bank is mainly transient as few seeds are able to survive more than one year in the soil (Thompson et al. 1997; Tremlová and Münzbergová 2007). It may be dispersed by endozoochory (BIOPOP Database, Poschlod et al. 2003). Seed bank is transient (Thompson et al. 1997).

*Veronica prostrata* L. (Scrophulariaceae) seed maturity occurs in June-July (J. Piqueray, pers. obs.). Seeds are subglobose to obovoid, compressed, dorsal side is slight convex, the ventral with central oval hilum and seam, 1.4-1.6 × 1.2-1.4 mm (Bojnansky and Fargasova 2007). Seeds may disperse by boleochory (BIOPOP Database, Poschlod et al. 2003), but due to the plant small size, it is likely that dispersal ability is low (Vittoz and Engler 2007). Only *V. prostrata* subsp. *scheereri* occurs in Belgium. Matus et al. (2003) found that seed bank was likely short-term persistent.

All three species are mainly associated with species typical of old calcareous grasslands (e.g. *Helianthemum nummularium*, *Koeleria macrantha*, *Brachypodium pinnatum*) (Wells and Barling 1971; Gibson and Brown 1992; Butaye et al. 2005; Piqueray et al. 2007).

Seeds of each species were hand-collected in 2006 for germination in Petri dishes and outdoor container experiments, and for in-situ experiments at maturity in 2007 (Table 1). For *P. vulgaris* and *T. montanum*, the seeds of 10 randomly selected flower heads were collected in each population, each year. For *V. prostrata*, the seeds of 15 randomly selected racemes were collected in each population, each year.

### *Germination in Petri dishes*

We studied germination in Petri dishes for each of the three species under three treatments as follows: (i) cold; (ii) heat; and (iii) smoke. This resulted in eight treatment combinations (control (o), cold (c), heat (h), smoke (s), cold+heat (ch), cold+smoke (cs), smoke+heat (sh), and cold+heat+smoke (csh)). Seeds were subjected to the following treatments before germination: cold treatment for two months at 4°C; heat treatment of 20 min at 60°C in an oven, which corresponds to topsoil conditions during a fire (Auld and Bradstock 1996); and seeds exposed to a smoke treatment by burning *Brachypodium pinnatum* (Poaceae) hay for 20 minutes, with smoke produced using a bee smoker injected into airtight glass bottles containing seeds. Experiment was replicated four times. Following seed treatments for each of the three study species, we established a total of 96 Petri dishes, containing 10 seeds each, placed on double filter paper moistened with distilled water. Petri dishes were randomly placed (the three species mixed) in a climatic chamber for 120 days (18°C constant temperature, 16h photoperiod, 70-80% humidity). We proceeded to dishes randomization and removing of rotten seeds every week. In case of high fungi development, dishes were replaced in order to avoid contamination of remaining seeds.

Data were analyzed independently for each species. Effects of the three treatments on final germination percentages (data for *P. vulgaris* and *V. prostrata* was arcsin-transformed in order to improve homoscedasticity) were tested through three-ways ANOVA. In case of significant effects, pairwise comparisons of treatment combinations were made using Tukey's tests.

## *Emergence and development in containers*

At the end of October 2006, an outdoor container experiment was established on an open homogenous area in Gembloux (Belgium; 50°33'57"N; 4°42'11"E; alt. 153 m; annual mean temperature ca. 9°C, annual rainfall ca. 800mm). Area was settled in an arable field. It was fenced and covered with a geotextile. Container size was 0.6m length × 0.15m width × 0.15m depth. Each container was filled with a bottom layer of calcareous stones (5 L, caliber 40 mm) followed by a commercial compost (pH = 7.5). Twenty seeds were sown in each container. Three treatments were set up: (i) no treatment = "control treatment"; (ii) seeds sown after heat and smoke treatments (see Petri dish experiment for procedure) = "fire treatment"; and (iii) containers covered with 2 cm *B. pinnatum* litter = "litter treatment". Each treatment was repeated four times for each species (total: 36 containers). Containers were randomly placed within area, the three species mixed. In each container, emergence and survival rates were recorded monthly. In November 2007, seedling size for all three species (mean of the two largest perpendicular plant diameters in mm) and number of leaves of *P. vulgaris* was recorded. *B. pinnatum* litter was re-applied at this time. The final number of individuals per container was recorded at the end of the experiment in April 2008 (Total duration of the experiment: 18 months).

The percent final emergence, seedling size at the end of the first growing season and final number of individuals were compared among the three treatments using one-way ANOVAs, followed by Tukey's tests in the case of significant differences. In order to improve homoscedasticity, *P. vulgaris* germination was arcsin-transformed, *P. vulgaris* seedling size, *T. montanum* seedling size and *V. prostrata* final number of individuals were log-transformed, *T. montanum* germination and final number of individuals were sqrt-transformed. Seedling fate (survival or mortality) at the end of the experiment was analyzed among the three treatments using a chi-square test.

## *In situ experiments*

In situ experiments were conducted at five sites. Sites were selected to meet the following conditions: (i) the sites exhibited a large population (hundreds individuals) of one of the study species, called "target species" (each site having one target species, see Table 1); (ii) a restored area occurred in close vicinity to the population; and (iii) the target species did not occur in the restored area. Each site was composed of two parcels, the "reference parcel" where the species occurred and the "restored parcel", where the species was absent. These sites were therefore suitable for studying the absence of re-colonization of restored sites by the target species, despite the close vicinity of a seed source. Under these very limited conditions, we were able to find only one site supporting *V. prostrata* and two sites for *T. montanum* and *P. vulgaris* (Table 1). The site "Montagne-aux-Buis" (target species: *V. prostrata*) is located in the Viroin Valley, the

other sites are located in the Lesse Valley (see Butaye et al. (2005) and Piqueray et al. (2007) for vegetation descriptions). Restored parcels were formerly forest stands that were clear cut at different periods (Table 1). Since 2003, all sites have been managed by grazing using migrating sheep flocks (duration: 2-3 weeks/year, resulting to a grazing intensity of 1-2 sheep/ha\*year; restored parcels are grazed each year, reference parcels every 2 or 3 years depending on site). The experiment was settled at the five selected sites from May to August 2007, at the propagule release time for each species.

**Table 1: Study sites for each of the study species. Time since parcel restoration.**

Name	Localisation	Target species	Time since restoration
Les Grignaux	50°07'N - 5°10'30"E	Pulsatilla vulgaris	3 years
Tienne des Vignes	50°06'N - 5°10'E	Pulsatilla vulgaris	10 years
Lorinchamps	50°06'N - 5°14'E	Trifolium montanum	4 years
Les Pairées	50°06'N - 5°11'E	Trifolium montanum	12 years
Montagne-aux-Buis	50°05'30"N - 4°34'E	Veronica prostrata	9 years

Within each parcel (restored parcel and reference parcel) of each site we established four 2 m x 2 m experimental units. Each unit was divided into four 1 m x 1m subunits, corresponding to: (i) control (no treatment, O); (ii) seed addition (S); (iii) disturbance (D); and (iv) seed addition + disturbance (SD). Seed addition was the manual addition of seeds (25 seeds). Disturbance was litter and above-ground vegetation removing in 25 5cm x 5cm microsites. These treatments were randomly assigned to subunits. At each site, added seeds were collected within the site in order to insure the genetic integrity of local populations. In May 2008, seedlings were counted in each subunit (Experiment duration: 13 months, 10 months and 11 months respectively for *P. vulgaris*, *T. montanum* and *V. prostrata*).

In-situ seedling emergence was analyzed using three-ways ANOVA. A preliminary analysis tested for site effects. In the case of significant site effects (single effect or interaction with treatments or parcel type), further analyses were computed independently for each site. In case of significant effects, pairwise comparisons between treatments were made using Tukey's tests. No transformation permitted to improve homoscedasticity.

# Results

## *Germination in Petri dishes*

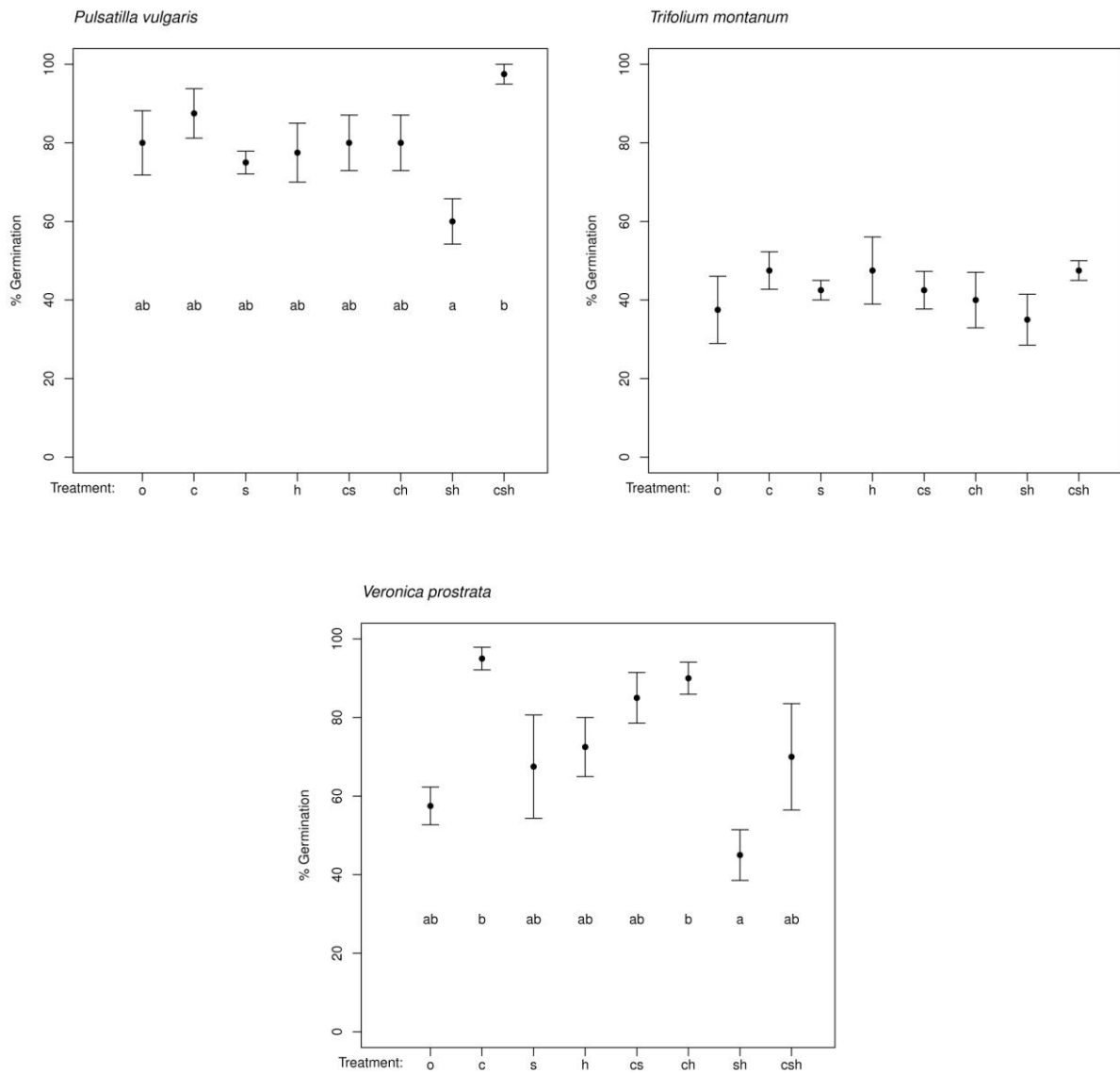


Fig. 1: Petri dish germination of each study species under the eight treatment combinations. Error bars are standard errors. The letters c, h and s correspond respectively to cold, heat and smoke treatments, and their combinations. Different letters under plots indicate significant differences.



Germination initiated following 4, 11 and 18 days for *T. montanum*, *V. prostrata* and *P. vulgaris*, respectively. The control treatment germination percentage for *T. montanum* was 37.5% (SE=8.54%) and was not significantly changed under the treatment conditions (Table 2; Fig. 1). Control *V. prostrata* germination percentage was 57.5% (SE=4.79%). Germination percentage of this species significantly increased with cold treatment ( $P<0.001$ ). Smoke treatment tended to decrease germination percentage ( $p=0.044$ ). However we observed a nearly significant heat\*smoke interaction ( $p=0.098$ ). No clear conclusions can therefore be made about smoke effect. The highest germination percentage was obtained when cold treatment was applied alone (Fig.1). Germination in the absence of treatment was 80% (SE=8.16%) for *P. vulgaris*. ANOVA detected a significant effect of cold treatment ( $p=0.007$ ). This treatment tended to enhanced germination. However significant or nearly significant interactions between effects (e.g.  $p=0.048$  for cold\*smoke,  $p=0.063$  for cold\*smoke\*heat, Table 2) made the interpretation of the results difficult. Tukey's test among treatment combinations revealed only the difference between cold+smoke+heat (csh: 97.5 %) and smoke+heat (sh: 60 %) as significant (Fig. 1).

**Table 2 : ANOVA table of the response of germination in Petri dishes for the three study species. Response to cold, heat and smoke treatment are given as well as their interactions.**

	Pulsatilla vulgaris		Trifolium montanum		Veronica prostrata	
	$F_{[1;24]}$	p	$F_{[1;24]}$	p	$F_{[1;24]}$	p
Cold	8.82	0.007	0.76	0.392	20.01	<0.001
Heat	0.03	0.857	0.08	0.774	1.95	0.176
Smoke	0.04	0.844	0.00	1.000	4.52	0.044
Cold*Heat	2.16	0.155	0.34	0.566	0.71	0.409
Cold*Smoke	4.33	0.048	0.08	0.774	0.94	0.342
Heat*Smoke	1.79	0.193	0.08	0.774	2.96	0.098
Cold*Heat*Smoke	3.80	0.063	3.04	0.094	1.07	0.312

### *Emergence and development in containers*

Container growing experiments revealed different phenological patterns between species (Fig. 2). *T. montanum* and *V. prostrata* emerged both in autumn and spring, but in different proportions. Respectively, for *T. montanum* and *V. prostrata*, we recorded the following emergence percentages: 32% and 11% in the first autumn (2006), 62% and 86% in the first spring (2007), and 6% and 3% in the second autumn (2007). Both species exhibited an emergence peak in March and mortality occurred primarily in the first spring, with a peak in April. *P. vulgaris* emerged entirely in summer between mid-June and mid-August and the highest mortality occurred the following winter.

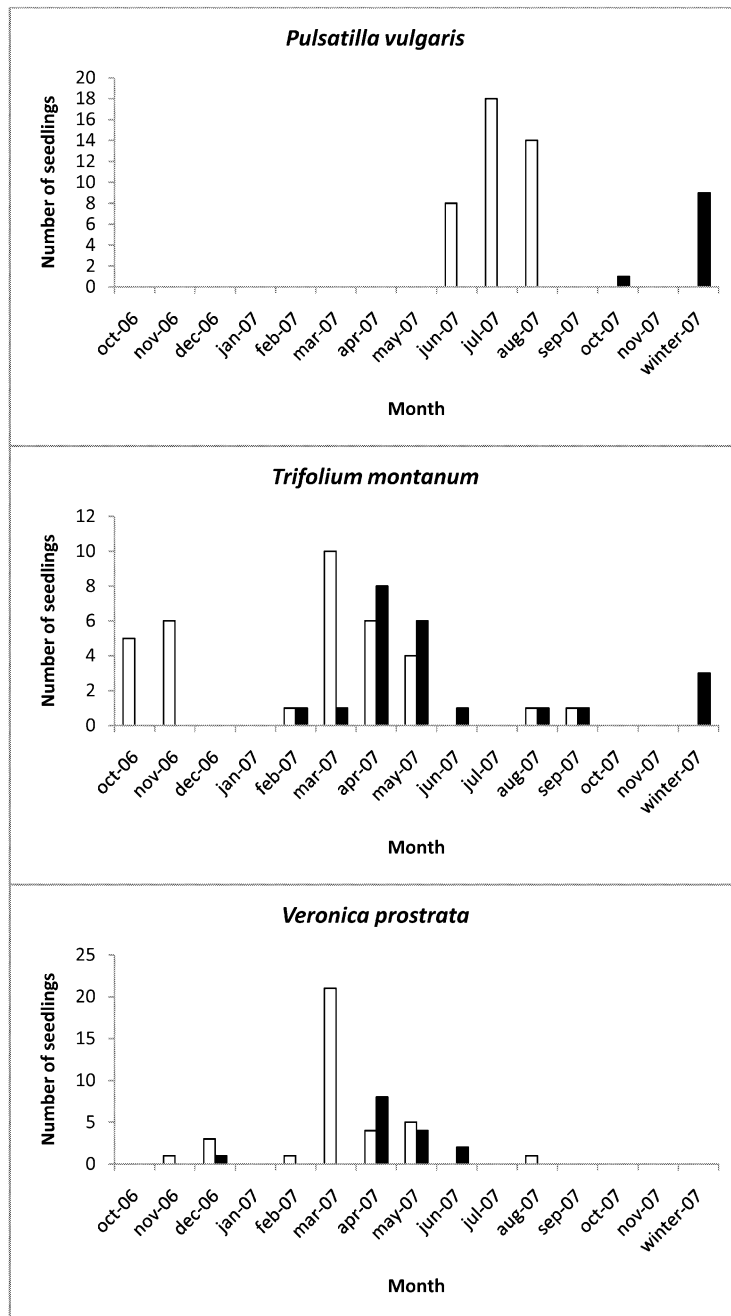


Fig. 2: Emergence (white bars) and mortality (black bars) phenology in outdoor containers for each study species. Species dependant y-scales were used in graphs. Due to low occurrences of emergence/mortality events at this period, the months from December 2007 to April 2008 were pooled under the term "winter-07".

*V. prostrata* emergence and survival percentages were reduced by fire treatment (smoke + heat) relative to the control (emergence: 6% vs. 31%;  $P = 0.014$ ; survival: 0% vs. 64%,  $P = 0.012$ ; Table 3). *Brachypodium* litter reduced *V. prostrata* emergence rate (7% vs. 31% in control,  $P = 0.014$ ; Table 3). However, *Brachypodium* litter resulted in an increased emergence (30% vs. 11% in control,  $P = 0.014$ ), final number of individuals per container (5.25 vs 1.25 in control,  $P = 0.003$ ) and survival (87% vs 44% in control,  $P = 0.038$ ) of *P. vulgaris* (Table 3). Finally, *T. montanum* survival was the lower in control (12% vs. 57% with fire treatment and 60% with litter treatment,  $P = 0.016$ ; Table 3). However, the final number of individuals per container was not significantly different among treatments for this species due to an inverse pattern in germination.

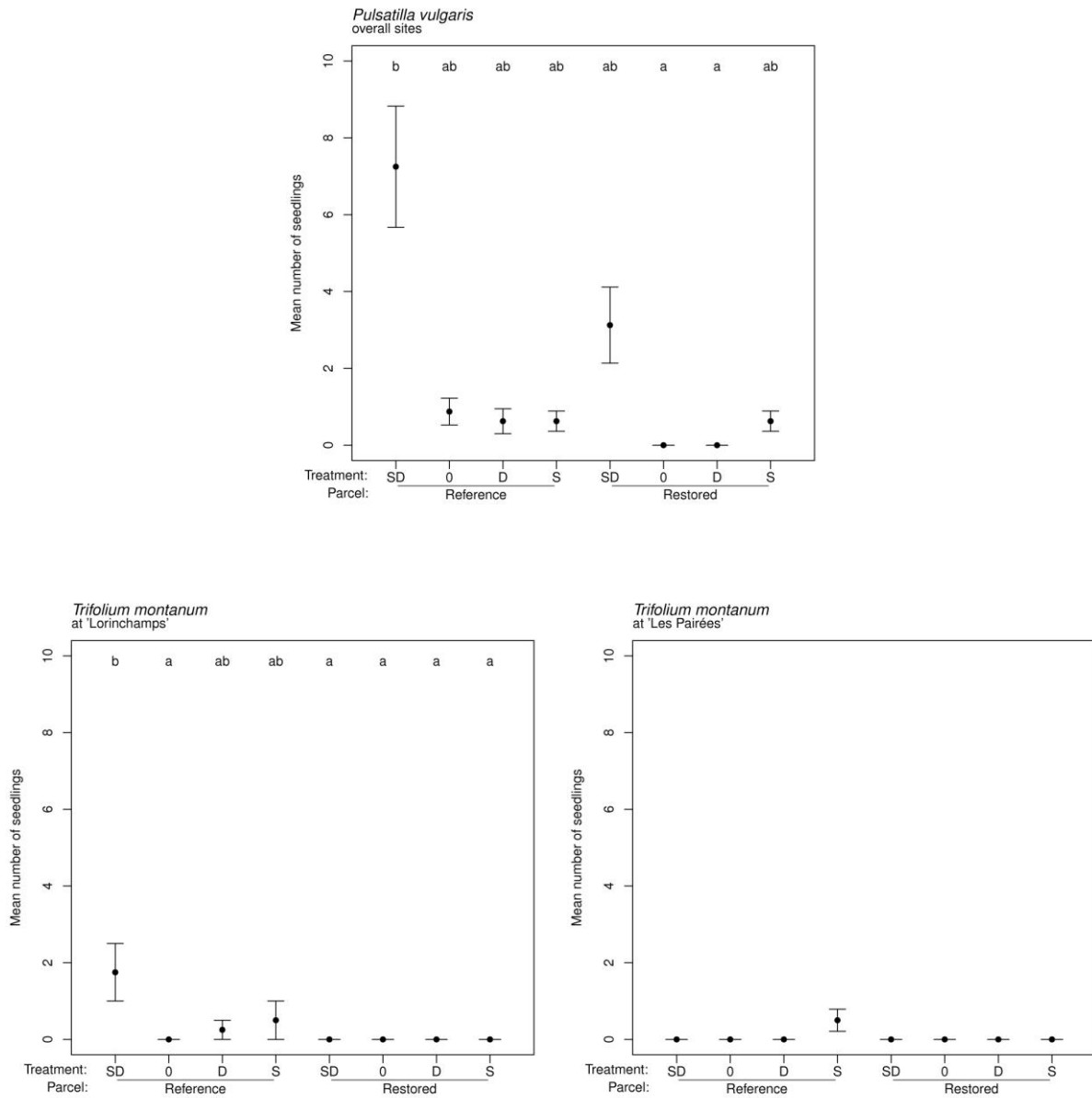
**Table 3: Study species response in outdoor containers under three treatments. Under fire treatment conditions, seeds were submitted to heat (20 min at 60°C) and smoke (20 min of smoke) before sowing. Under litter treatment conditions, containers were covered with *Brachypodium* litter. P indicates the p-value of the performed test. When significant, a pair-wise comparison was computed; letters indicate significant differences. Final number of individuals is given per container.**

	Treatment			test	P
	Control	Fire	Brachypodium		
<i>Pulsatilla vulgaris</i>					
Germination (%)	11 <sup>ab</sup>	9 <sup>b</sup>	30 <sup>a</sup>	$F_{[2;9]}=5.84$	0.024
Survivorship (%)	44	71	87	$\chi^2_{[2]}=6.53$	0.038
Final number of individuals	1.25 <sup>b</sup>	1.00 <sup>b</sup>	5.25 <sup>a</sup>	$F_{[2;9]}=11.70$	0.003
Seedling size (mm <sup>2</sup> )	270	215	577	$F_{[2;7]}=2.88$	0.122
Number of leaves	2.2	1.9	2.8	$F_{[2;7]}=1.97$	0.209
<i>Trifolium montanum</i>					
Germination (%)	21	9	12	$F_{[2;9]}=0.30$	0.750
Survivorship (%)	12	57	60	$\chi^2_{[2]}=8.26$	0.016
Final number of individuals	0.50	1.00	1.50	$F_{[2;9]}=0.46$	0.644
Seedling size (mm <sup>2</sup> )	49	2761	1732	$F_{[2;5]}=1.24$	0.366
<i>Veronica prostrata</i>					
Germination (%)	31 <sup>b</sup>	6 <sup>a</sup>	7 <sup>a</sup>	$F_{[2;9]}=7.06$	0.014
Survivorship (%)	64	0	83	$\chi^2_{[2]}=8.87$	0.012
Final number of individuals	4.00 <sup>a</sup>	0.00 <sup>b</sup>	1.25 <sup>ab</sup>	$F_{[2;9]}=6.82$	0.016
Seedling size (mm <sup>2</sup> )	14413	-	16768	$F_{[1;4]}=0.10$	0.771

### *In situ experiments*

In-situ experiments revealed highly contrasting patterns among species. Germination was not observed under any treatment conditions for *V. prostrata*.

Twelve *T. montanum* seedlings were recorded. A preliminary ANOVA revealed several significant interactions for sites, notably for seed addition and disturbance (results not shown). As a consequence, we analyzed the effects of treatments and parcels in each site separately applying a three-way ANOVA (Table 4). At "Les Pairées", only two seedlings emerged and no significant effect was found. At "Lorinchamps", seedling emergence only occurred in reference parcel, resulting in a significant difference with restored parcel ( $P=0.013$ ). At this site, seed addition increased seedling emergence ( $P=0.043$ ). The higher seedling emergence was observed when disturbance and seed addition were combined (7 seedlings vs. 0, 1 and 2 seedlings, respectively for the control, disturbance alone, and seed addition alone, Fig. 3).



**Fig. 3: In-situ seedling emergence (mean number of seedlings per 1 x 1 m subunit) under the different treatments in the two parcel types for: (a) *Pulsatilla vulgaris* (2 sites pooled), (b) *Trifolium montanum* at "Lorinchamps", and (c) *Trifolium montanum* at "Les Pairées". Error bars are standard errors. The letters D and S are indicated respectively when disturbance and seed addition occurred.**

We observed a total of 105 *P. vulgaris* seedlings. Emergence patterns were similar at "Les Grignaux" and "Tienne des Vignes" (no significant interactions with site effect, result not shown). Significantly lower seedling emergence was recorded in restored than in reference parcels (75 seedlings vs. 30 seedlings,  $P=0.006$ ; Fig. 3). Both seed addition and disturbance had a significant positive effect ( $P<0.001$  in both cases). Moreover, the significant interaction between both

treatments ( $P < 0.001$ ) indicates that seedling emergence was enhanced by a combination of disturbance and seed addition. The significant triple interaction ( $P = 0.030$ ) indicates that this enhancement was more important within reference parcels (Fig. 3, Table 4).

**Table 4: Response of *P. vulgaris* and *T. montanum* to in-situ experiments treatments (ANOVA). The tested effects were parcel type (reference Vs. restored; parcel), disturbance (D) and seed addition (S).**

	<i>Pulsatilla vulgaris</i> overall sites		<i>Trifolium montanum</i> at « Lorinchamps »		<i>Trifolium montanum</i> at « Les Pairées »	
	$F_{[1;56]}$	p	$F_{[1;24]}$	P	$F_{[1;24]}$	P
Parcel	8.25	0.006	7.14	0.013	3.00	0.096
D	20.53	<0.001	2.57	0.122	3.00	0.096
S	26.72	<0.001	4.57	0.043	3.00	0.096
Parcel*D	3.91	0.053	2.57	0.122	3.00	0.096
Parcel*S	1.75	0.192	4.57	0.043	3.00	0.096
D*S	22.91	<0.001	1.14	0.296	3.00	0.096
Parcel* D*S	4.99	0.030	1.14	0.296	3.00	0.096

## Discussion

### *Germination and seedling development*

The three species evaluated in this study generated viable seeds, with a germination percentage varying from 40% to 80% in Petri dishes in the absence of treatment. *T. montanum* germination without treatment was lowest. However, it reached approximately 45% over all treatments, which is consistent with the results of Schleuning and Matthies (2009).

We did not proceed to viability tests of non-germinating seeds in Petri dishes experiment, in order to make distinction between non-viable and dormant seeds. For *P. vulgaris*, the high germinability under control treatment suggests that few seeds were dormant. At the contrary, the nearly 100% germination of *V. prostrata* after cold treatment, compared to the ca. 60% germination in control treatment, suggests that nearly all seed were viable, but some were dormant. Death of seeds due to fire or smoke treatment was however probable in other treatments. Nearly all non-germinating *T. montanum* seeds had rotten during experiment and were not viable anymore.

In outdoor containers, a small number of *V. prostrata* seeds germinated in autumn, the majority germinated in spring. This result could be related to a necessity for a cold period, which was demonstrated to enhance germination in Petri dishes. However, a similar phenological pattern was observed for *T. montanum*, although this species exhibited no significant increase in germinability after cold treatment. Spring germination, observed for *P. vulgaris*, allows a reduction in winter seedling mortality, while seedlings emerging in autumn have a competitive advantage if they survive, which is more likely during a mild winter. Bi-seasonality (observed for *T. montanum* and *V. prostrata*) in seedling emergence can therefore be a bet-hedging strategy to reduce year-to-year variation in recruitment (Masuda and Washitani 1992).

The *T. montanum* and *V. prostrata* mortality pattern (greater in spring than in winter) likely reflects weather conditions than an intrinsic characteristic of the species. Indeed, the 2006-2007 winter was exceptionally mild in Belgium, with mean temperatures 3.5°C higher than usual over the period from December to February, with only a few days below 0°C. However, these mild winter conditions were likely sufficient to meet the cold requirement for *V. prostrata* germination, as mean temperature was similar to these applied during cold treatment in the Petri dishes experiment. In other respects, an exceptional drought occurred in April 2007, with the total absence of rain for this month likely explaining the observed high mortality (MeteoBelgique 2010 for weather conditions).

None of the three species was favoured by fire-related treatments (heat and smoke), regardless of the experimental conditions (Petri dishes or outdoor containers). Even, *V. prostrata* exhibited a decreased germination percentage following fire-related treatments. Indeed heat can be responsible for the death of seeds (Baskin and Baskin 1998).

Response to litter addition in outdoor containers was two-fold and species dependant. On the one hand, *T. montanum* and *V. prostrata* exhibited a decreased germination percentage in containers covered by *B. pinnatum* litter, although this effect was not significant for *T. montanum*. On the other hand, seedling survival was significantly higher for *T. montanum*. This dual effect of litter has already been described in fen grasslands (Rasran et al. 2007). Litter can inhibit recruitment by decreasing light availability for seedlings and mechanically prevent emergence (Xiong and Nilsson 1999). Alternatively, litter can protect seedlings against severe drought or chill, and therefore facilitate emergence. A similar effect was described for moss layer in calcareous grasslands (Jeschke and Kiehl 2008). Inhibition and facilitation may act differently following the plant life stage, and their interplay is likely to strongly influence final emergence (Callaway and Walker 1997). As a whole, litter was favourable for *T. montanum* recruitment and exhibited a negative effect on *V. prostrata* evidenced by decreased germination. Finally, *P. vulgaris* exhibited the best performance in containers covered by litter. This result is congruent with Pfeifer et al. (2002), who found that artificial shadowing increased *P. vulgaris* performance under transplantation conditions.

## *Seed or microsite limitations?*

A lack of seed can be limiting for both recruitment within populations or new site colonization in grassland habitats (Zobel et al. 2000; Donath et al. 2007; Edwards et al. 2007; Rasran et al. 2007). When seed addition enhances seedling emergence in restoration areas, this indicates dispersal limitations for new site colonization (Münzbergová and Herben 2005). We identified such a dispersal limitation for *P. vulgaris* that emerged in restored areas only with the addition of seeds. In reference parcels emergence enhancement observed for *P. vulgaris* and *T. montanum* suggests that recruitment within population is also seed limited. However, little recruitment was observed in reference parcels for both species without seed addition, implying that natural recruitment is not null. This recruitment is likely due to seed rain, given the transient nature of seed bank of both species (Thompson et al. 1997).

*V. prostrata* and *T. montanum* did not emerge in restored areas. As a consequence, no conclusion could be made for these species about limitations for colonization operating in restored sites. Even in reference grasslands no (*V. prostrata*) or little (*T. montanum*) seedlings emerged. It is likely that the low number of added seeds in in-situ experiments (25/m<sup>2</sup>, 400/site) can be part of the cause for the low emergence of this species. Indeed, even in control treatment in container experiment, these species exhibited low final emergence (20% and 2.5% for *V. prostrata* and *T. montanum* respectively). In the field, the additional competition constraint likely reduced emergence and lead to the low final emergence observed, making significant differences unlikely to occur. This could have been avoided by increasing the number of sown seed. However, due to the rarity of the species, we were allowed to collect a limited seed number, which limited the extent of the experiment. This limitation was likely responsible for a lack of power in statistical tests overall the study. It is however worth noting that *T. montanum* emergence in the field was between 1% and 5% based on site. Although these percentages indicate low emergence rates, they are slightly higher than those reported by Schleuning et al. (2009) for the same species and considered sufficient to maintain population size if management practices were suitable.

For *P. vulgaris*, a significant positive effect of seed addition and disturbance combination was shown in both reference and restoration grasslands. In reference grasslands, each treatment alone did not result in more seedlings than the control treatment. Limitations to recruitment are therefore not a dichotomy of seeds vs. microsites, but rather a combination of both effects. It was already observed that disturbance increased the effectiveness of seed addition (Zobel et al. 2000; Hofmann and Isselstein 2004; Poschlod and Biewer 2005; Edwards et al. 2007; Hellström et al. 2009). The same pattern was observed in *T. montanum* at the "Lorinchamps" site, but the effect was not significant. A replication of the experiment, including higher level of seed addition, would therefore be needed to confirm this pattern.



The results observed in outdoor containers are valuable to explain field results. The better a species performed under *Brachypodium* litter in outdoor containers, the better it performed in the field. *P. vulgaris* that showed enhanced performance with litter exhibited the best emergence in the field in contrast to *V. prostrata* that did not emerge in the field. This pattern suggests that the 5 cm x 5 cm microsites used were too small for microsite dependant species, particularly *V. prostrata*. Therefore, it would be worth studying the effect of microsite size and type on *V. prostrata* recruitment and, to a lesser extent, *T. montanum*. Also, global shadowing and crown cover would be interesting to be put in relation with emergence of these species. Germinability may also be partly responsible for the patterns observed in containers and in the field. The better a species germinated in Petri dishes, the better it performed in outdoor containers. *T. montanum* had a relatively low germinability in Petri dishes under control treatment (37.5%). This low germinability, combined to the low number of added seeds in in-situ experiments (25/m<sup>2</sup>, 400/site) can be part of the cause for the low emergence of this species.

### *Implications for conservation*

One of the aims of ecological restoration is to provide new habitat opportunities for characteristic species in order to create new populations and increase population size and connectivity. In our study, while target species produce viable seed that survive under controlled conditions, the three target species failed at colonizing restored areas. Seedling emergence in restored areas could only be achieved after seed addition. These results point out the importance of propagule availability for restoration success. Several authors stated that occurrence of seed sources in the near vicinity of restored sites was an important determinant for this (Bakker et al. 1996; Kiefer and Poschlod 1996; Pärtel et al. 1998; Willems and Bik 1998). Our study demonstrated that it is not always sufficient and that supplementary actions may be needed to completely recover target species pool in restored areas, even when restoration occur at a small distance from old grasslands.

Also, sufficient bare ground availability is important to be achieved. Both *T. montanum* and *V. prostrata* had lower seedling emergence under litter accumulation conditions and *P. vulgaris* emergence in the field was enhanced by combining sowing and disturbance. Piqueray et al. (2011a) reported that current management did not permit to maintain bare ground in restored areas. Further studies are therefore necessary to determine if alternative management practices i.e. type, timing and intensity (different from the current grazing management) can improve microsite availability. Increased disturbance and gap creation is required to reduce litter accumulation, leading to better recruitment, essential in individual species population dynamics. It is especially essential for *V. prostrata* which optimal habitats in Belgium are the most xeric grasslands, characterized by a 15% bare ground cover in average, and a lower vegetation height (Piqueray et al. 2007). In less xeric grasslands, such as our study site, such vegetation characteristic only occur at earlier successional stages obtained under high grazing intensity. Applied disturbance (5 cm x 5 cm microsites) was shown to be insufficient for the recruitment of this species.

Finally, fewer seedlings of *P. vulgaris* were observed in restored areas than in reference grasslands. Restored areas showed the absence of seedling emergence for *T. montanum* and *V. prostrata*. These results suggested that conditions in restored parcels are less suitable for species recruitment (seedling establishment and survival). Piqueray et al. (2011a) found no difference in soil conditions between restored and reference parcels within study sites. They however revealed that restored areas supported an increased frequency of the competitive grasses *B. pinnatum* and *Bromus erectus*. Management to limit the extent of these species may therefore be a prerequisite to target species establishment.

## Acknowledgments

We thank the Public Service of Wallonia for giving us authorization to collect seeds of protected species and to work on protected areas. We also express thanks to both anonymous reviewers for their pertinent comments on the manuscript. This study was supported by the FRS-FNRS (contract FRFC 2.4556.05). J.-P. Bizoux is a postdoctoral researcher of FRS-FNRS.

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