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Germination and survival of endangered *Pulsatilla grandis* (ranunculaceae) after artificial seeding, as affected by various disturbances

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ABSTRACT

Pulsatilla grandis is a rare and endangered species in Central Europe. A field experiment was set up to test the effects of disturbances on its germination and survival rates after artificial seeding. Disturbances were simulated by the following treatments: burning, hoeing, and removal of vegetation. The germination percentage decreased rapidly with time, to only 2% in 2-year-old fruits. The germination rate of fresh fruits was the highest in hoed plots (29.2 ± 12.9%), followed by removed-vegetation plots (21.8 ± 17.3%) and burned plots (1.8 ± 2.2%). No germination was observed in control plots. The survival rate of seedlings was monitored over the next 5 years. In the year following the treatment, all the seedlings survived in the burned plots; in the hoed and in removed-vegetation plots, survival rates were 38.5 ± 2 and 55 ± 27%, respectively. Approximately half of them were alive after 5 years. Grassland vegetation in burned quadrates recovered very quickly, whereas in other disturbed plots the numbers of species and vegetation cover were very limited in the first year, but recovered in the following two years. In-seeding was successful as a short-term solution. As a long-term solution the traditional management procedures (mowing) must include a wider area and create disturbances by opening gaps within the closed grassland sward.

Keywords: conservation, disturbance, dry grasslands, fire, germination rate, hoeing, seed longevity, seeding, vegetation removing

INTRODUCTION

Extensively used species-rich calcareous grasslands form one of the most vulnerable and endangered habitats in Central Europe. Some decades ago these semi-natural grasslands, widespread in Central and Western Europe, were used for livestock grazing and for hay production, but in recent decades they have declined in size and numbers (Wolkinger and Plank, 1981; Willems, 2001). Changes in traditional land use present the most important danger, through both intensification of agriculture and abandonment followed by bush encroachment (Keymer and Leach, 1990). Habitat deterioration, fragmentation, and isolation of populations are among the main factors causing decline of populations and species (Keymer and Leach, 1990; Eriksson and Ehrlén, 2001; Lindborg and Eriksson, 2004). Thus, dry grasslands

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represent spots of high diversity and sometimes form refuges for thermophilous grassland species and their populations (Kaligarič, 1998). Many rare and threatened species are adapted to the following: (a) nutrient-poor conditions, also described as mineral nutrient stress (Grime, 1979), (b) calcareous substrates (rich in calcium carbonate) (Kinzel, 1982), and (c) extensive traditional land use (Kaligarič, 1998). Factors such as mycorrhiza (Zobel et al., 1996) and dispersal of species in time and space (Poschold et al., 1998) play important roles and should be taken into account within revitalization and restoration strategies for dry calcareous grasslands. The maintenance and revitalization of low-intensity grazing or mowing has, therefore, become an important tool of biological conservation throughout Europe (Zobel et al., 1996; Ostermann, 1998; Austrheim et al., 1999; Willems, 2001).

In Slovenia's sub-Mediterranean region, dry calcareous grasslands are still widespread, but in its Central European region extensive dry grasslands remain only in small patches. Those that are still in good condition have survived only in remote areas, where traditional land use is still practised (Kaligarič, 1998). Fragmentation of those dry grassland habitats that are still managed is a major problem for species survival. Several studies have addressed population viability analysis (Amler et al., 1999; Possingham et al., 2001), and practical tools for conservation analysis have been proposed (Settele et al., 1996; Huxel and Hastings, 1999), but they have been subjected to lively discussion in recent literature (e.g., Lindenmayer et al., 2003; Henle et al., 2004).

Following Slovenia's accession to the EU, some dry grassland conservation programs recently have started. However, one of the first revitalization experiments was already initiated 15 years ago by a local community. The main objective was to preserve a flagship species, the Pasque flower *Pulsatilla grandis* Wenderoth, on a popular and frequently visited site in the mountains of Boč (see map, Fig. 1). Historical data for the Boč area revealed how the *P. grandis* population declined in the last 50 years from 1.1 to 0.06 ha (Fig. 2). The eastern (steppe) distribution of this species, which also includes occurrences in Central, Eastern, and Southeastern Europe (Hegi, 1974; Godicl, 1980), shows that during recent decades the number of localities where it is found has fallen by half, to only four, which cover not more than 1.6 ha.

The general aim of the study was to test the possibility of artificial seeding of *Pulsatilla grandis* as a means of enhancing the population sustainability. In our experiment we simulated cutting, which is a basic regional-scale management practice in secondary grasslands, and also various physical disturbances that occur on differing micro-scales and intensities. Such disturbances could be induced by natural gap creation, e.g., by deer or cattle, which took place in the past in the course of the late (autumnal) grazing season. Also fire, as a natural or a human-induced factor in the form of an occasional management tool, was taken into consideration. In other studies that addressed other species, not all the above disturbances were studied in one experiment.

Our specific aims were (1) to test the germination of *Pulsatilla grandis* fruits of various ages, in order to estimate their longevity during storage; (2) to test the effects of physical disturbances, simulated by treatments with fire, vegetation removal, and hoeing, on the germination and survival rates of seedlings; and (3) to study the vegetation recovery on the treated plots during 2 years following the treatments.

It is known that the introduction of seeds or plants is recommended only in special cases such as those involving extremely rare and threatened populations (Oostermeijer et al., 1998); it is recommended that regeneration from available seed banks should be tried first (Bekker et al., 1997). However, in the case of degradation of restored sites, absence of seed bank relicts, and fragmentation and isolation of restored sites, it may be difficult for many species to re-colonize without artificial seeding or even transplantation (Lindborg and Eriksson, 2004). Since the decline of *Pulsatilla grandis* in the Boč area is a typical example of such a situation, and since the initiative has been taken locally, we were highly motivated and encouraged to perform this experiment in order to examine its wider applicability.

MATERIALS AND METHODS

The study site

The study site was located in the Boč mountains (46°16′N, 15°35′E; 650 m asl), at the edge of the Pannonian plain, an area that forms the last ascent of the Alps towards the southeast (Fig. 1). The geological bedrock is comprised mainly of calcareous limestone and dolomite. The soil is a relatively thin (<20 cm) and undeveloped Rendzina, with an A–C soil profile, and neutral to slightly basic chemical reaction.

Meteorological data are available for the nearby city of Celje (Branovec, 2000). The mean annual temperature is 10.3 °C and the mean annual rainfall is 1,285 mm. It is a steeply sloping area, mainly covered by calcareous Illyrian beech forests, with some thermophilous *Ostrya carpinifolia-Fraxinus ornus* forests. Grassland vegetation is developed in the central plain at around 650–680 m asl. Some decades ago the whole grassland

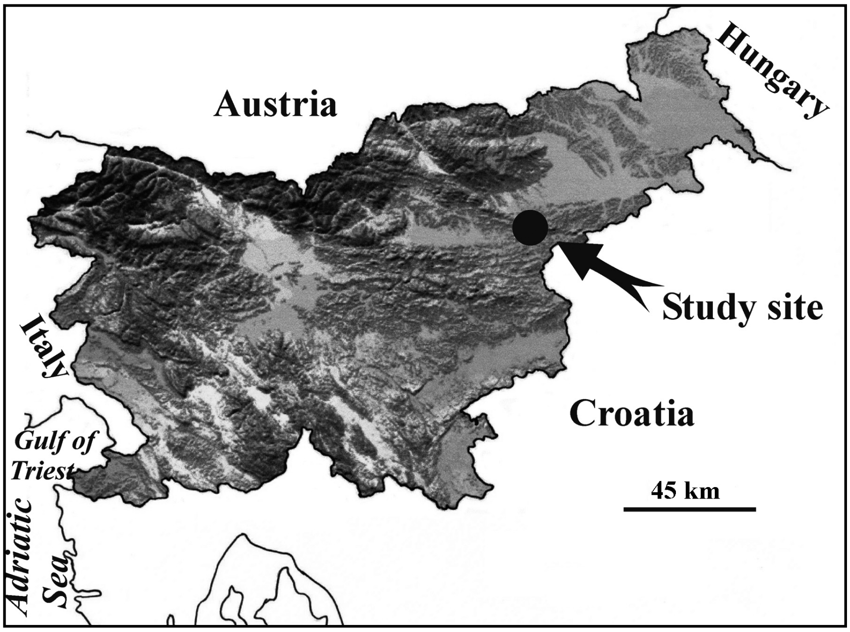

Fig. 1. Map of Slovenia with location of the study site (Mt. Boč).

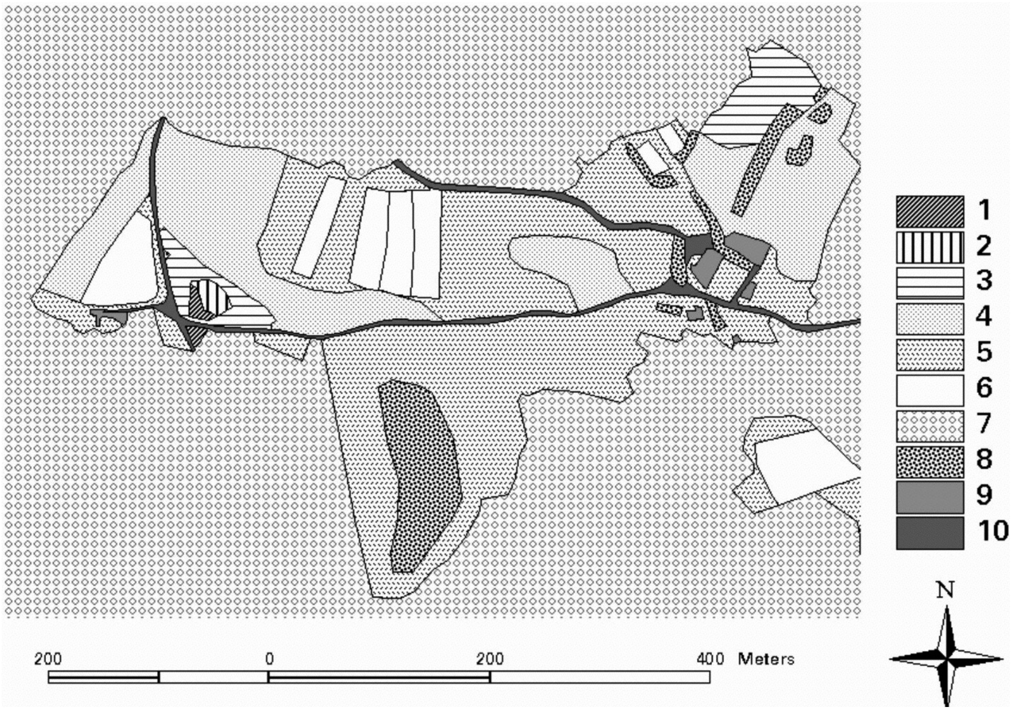

Fig. 2. Historical data about the declining population of *Pulsatilla grandis* on the location of Boč (NE Slovenia). 1—actual population size on 0.06 ha. 2—the location of experimental plots. 1 and 2—population size before 1990 on 0.13 ha. 1, 2, and 3—population size in 1960 and 1970 (before 1980) on 1.1 ha. 4—appropriate habitat for *Pulsatilla grandis* before 1990. Possibly colonized before 1950. Old literature data are not precise. 5—improved grasslands. 6—fields. 7—forest. 8—single trees and hedges. 9—buildings. 10—streets.

area was used as extensive unfertilized meadows, but later a major part was converted into arable land and intensively fertilized meadows. Today the remnants of the extensive, floristically rich grasslands are derelict, with only a small area being protected and fenced because of the presence of *Pulsatilla grandis* (Fig. 2). The grassland vegetation belongs to the association *Scabioso hladnikianae-Caricetum humilis* (nom. sin. *Bromo-Plantaginetum mediae*) (classis *Festuco-Brometea*) (Kaligarič and Škornik, 2002).

Traditional management, extensive mowing, and occasional burning were essential tools in preservation of these grasslands. Autumnal grazing by cattle, and also by deer, which are very common in the area, make disturbances in the form of creation of gaps very likely. The impact of deer must not be neglected with regard to herbivory also.

Production of fruits for experiments

We started with production of *Pulsatilla grandis* fruits in 1990. The fruits were derived from the original population, in light of the fact that alien genotypes could contaminate the receptor population. The use of propagules from neighboring populations was also suggested by Lindborg and Eriksson (2004), and the collection of fruits from the centers of geographic ranges has also been advocated (Fenster and Dudash, 1994).

Thirty *Pulsatilla grandis* fruits were sown separately in (20 × 17)-cm plastic pots filled with local soil, at a depth of 1 cm. The pots were kept in a greenhouse under natural illumination. Fruits were sown in June 1990, and each pot was supplied with tap water at 7-day intervals to moisten the soil. The temperature range was 15–26 °C. The nursery period ran from May 1990 to May 1991, after which the plants were transplanted to a garden. The plants started to flower and produce fruits in 1993. We stored the fruits produced in 1995, 1996, and 1997 for use in a germination experiment in May 1997. They were stored under dry conditions in a greenhouse at a year-round room temperature of 20–24 °C. The fruits for field experiments were collected in May 1998.

Germination percentage in greenhouse

The germination experiments were conducted in May 1997. For germination, *Pulsatilla grandis* fruits produced in 1995, 1996, and 1997 were placed on moist filter paper in glass Petri dishes. To prevent drying out these were then set on zigzag-folded blotting-paper, which was placed in a plastic pot filled with tap water. Fifty fruits were tested in each of five parallel experiments for each year of fruit production, i.e., 50 fruits × 3 years in each experiment. We considered that a fruit had germinated when it had a radicle at least 2 mm long. Germination continued until September 1997, and during the germination period the mean temperature of the greenhouse was 21.8 ± 1.8 °C and the lighting was the natural daylight of the period May to September.

Experimental design and sampling

The experimental disturbances, which included three types of treatments—burning, hoeing, and vegetation removal—were performed in May 1998, before artificial seeding on an area adjacent to the remnant population (Fig. 2). In hoed plots the soil was earthed up with the hoe. In vegetation-removal plots the vegetation and litter were removed, and the surface of the topsoil was disturbed. In the burned plots, a mosaic of bare ground and surviving patches was created.

The sampling scheme was designed in six replicated blocks, each comprising three treatment plots and a control plot, i.e., a total of 24 plots, all measuring 1 × 1 m. In each plot 150 fruits were seeded at an even spacing. Water was added to all plots and hay was spread over the ground to reduce the evaporation, except for the control plots. In order to simulate a traditional management regime on the studied grassland vegetation, all the plots were cut and the cut above-ground biomass was removed every August for the following 5 years, i.e., until 2003.

Seedlings were counted in mid-June 1998, and for the following 5 years living plants were counted in order to determine the survival rates. To monitor the recovery of vegetation after the treatments, vegetation was sampled in all plots at the peak of the vegetation growth season (mid-July) in 1998, 1999, and 2000. Sampling included recording of the species composition and estimation of the cover of each plot. The covers were determined by estimation of the percentage of the total plot area covered by each species. The species were classified as grassland species, scrub species, and forest understorey species, according to their syntaxonomy on the class level. The taxonomic nomenclature follows Martinčič et al. (1999).

Statistical analysis

The impact of fruit age on the germination of *Pulsatilla grandis* in the greenhouse, and the impacts of treatments on the germination and survival of *P. grandis* seedlings, on the flowering and fruit production of *P. grandis* individuals, and on species richness in experimental plots were examined with one-way ANOVA, in which the independent variable was fruit age or treatment. In the statistical analysis the result was considered statistically significant if $p < 0.05$. After ANOVA, the differences between fruit ages or treatments were subjected post-hoc to Tukey's HSD (Honestly Significant

Difference) Test for Multiple Comparisons (Sokal and Rohlf, 1995). All analyses were performed with the computer package STATISTICA (StatSoft, Inc.).

RESULTS

Germination rates

The germination rate of the *Pulsatilla grandis* fresh fruits in the greenhouse was 90 ± 14.1% (mean ± standard deviation, N = 5); that of 1-year-old fruits was already significantly lower, at 66 ± 11.4%, and that of 2-year-old fruits had decreased even more strongly, with only 2 ± 4.5% remaining viable for germination.

Germination rates of the *Pulsatilla grandis* fresh fruits in situ, recorded in June 1998, were highest on hoed plots, at 29.2 ± 12.9% (mean ± standard deviation, N = 6); next were the vegetation-removal plots, with 21.8 ± 17.3%; and in the burned plots the germination rates was significantly lower, at only 1.8% ± 2.2%. In the untreated, control, quadrates no germination was observed.

Survival rates

The survival rates of seedlings are presented in Table 1. In the burned plots all the seedlings that developed from the few germinated seeds survived into the year following the treatment (1999). In the hoed and vegetation-removal plots, the survival rates were significantly lower, with about half or fewer of the seedlings surviving the first year. Approximately half of those that survived the first year were still alive after 5 years. When the absolute numbers of germinated and surviving seedlings are considered, the most favorable treatment for germination of *Pulsatilla grandis* seems to be hoeing, although removal of the vegetation matrix yielded quite similar results.

The plants started to flower in 2001, the third year after seeding (Table 2), and all the plants that flowered produced fruits. The numbers of flowering and fruiting individuals on treated plots were small, and they did not differ significantly between treatments. No new germination was observed in either treated or untreated (control) plots of fruits produced on *Pulsatilla grandis* plants during the experiment. Moreover, no natural colonization was observed.

Vegetation recovery following the treatments

The species composition in the untreated control plots was representative of typical dry grassland vegetation, with some plant species that indicated overgrowing and development of Saum vegetation: *Erica carnea* L., *Geranium sanguineum* L., *Polygonatum odoratum* (Mill.) Druce, *Daphne cneorum* L. Two forest understorey species were already present: *Cyclamen purpurascens* Mill. and *Mercurialis ovata* Sternb. & Hoppe. The rest

Table 1
Survival rates (%) of *Pulsatilla grandis* seedlings in 1999–2003 (mean ± standard deviation, N = 6). Means bearing the same letter are not significantly different at $p < 0.05$

	Treatment			
	Burning	Hoeing	Vegetation removal	Control
1999	100 ± 0[a]	38.5 ± 2[b]	55 ± 27[b]	0 ± 0[c]
2000	100 ± 0[a]	32.4 ± 24[b]	33.9 ± 17.2[b]	0 ± 0[c]
2001	100 ± 0[a]	19.2 ± 7.6[bc]	29.2 ± 25.4[b]	0 ± 0[c]
2002	47.2 ± 45.2[a]	10.3 ± 1.3[ab]	29.2 ± 25.4[ab]	0 ± 0[b]
2003	47.2 ± 45.2[a]	9.8 ± 11[ab]	24.1 ± 27[ab]	0 ± 0[b]

Table 2
Flowering and fruit-producing individuals of *Pulsatilla grandis* (%) (mean ± standard deviation, N = 6) on experimental plots in years 2001, 2002, and 2003

	Treatment			
	Burning	Hoeing	Vegetation removal	Control
2001	25 ± 41.8	18.8 ± 40.1	11.8 ± 18.3	0 ± 0
2002	25 ± 41.8	13.8 ± 19.6	19.6 ± 13.7	0 ± 0
2003	25 ± 41.8	24.3 ± 20.5	21.4 ± 17.3	0 ± 0

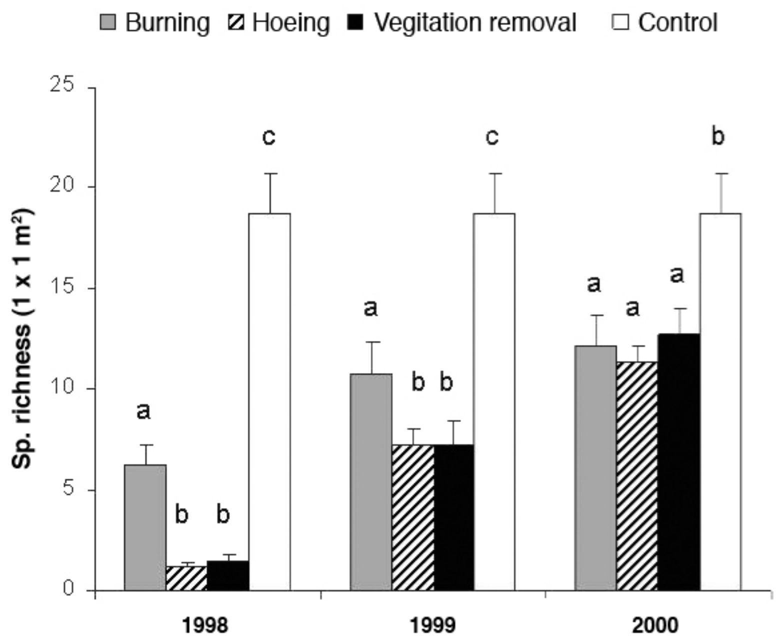

Fig. 3. Species richness (mean $\pm$ standard deviation, N = 6) in experimental plots, which in May 1998 were subjected to burning, hoeing, vegetation removal, and no disturbance (control), recorded in 1998, 1999, and 2000. Means bearing the same letter are not significantly different at $p < 0.05$.

constituted a typical semi-natural calcareous grassland species inventory (not shown).

Species richness in burned plots in 1998 and 1999 was significantly higher than in other disturbed plots (Fig. 3). Vegetation in the burned plots recovered very quickly, and almost no signs of fire were observed in the summer of 1999 (Figs. 3 and 4). In other disturbed plots the numbers of species and the vegetation cover were very limited in the first year, but recovered in the following two years (Figs. 3 and 4). Some of the typical grassland species had stabilized as early as 1 year after the treatments (Fig. 4). No further sampling was done, as no differences between treated and untreated plots could be noticed after that period.

DISCUSSION

Since the re-colonization ability of a species is affected by its longevity in a seed bank (Lindborg and Eriksson, 2004), some preliminary germination tests of *Pulsatila grandis* fruits of different ages were necessary. The results of our greenhouse germination experiments indicated that fruits lose their germination capability relatively quickly and therefore have only minor importance in the persistence of a soil seed bank, although room-stored fruits are not equivalent to those in a natural soil seed bank. Their germination strategy is based on penetration through the litter and vegetation matrix to the soil, and, therefore, they could germinate already in the year of fruit production, without stratification. We could speculate about the relationships between fruit morphology, penetration capability, early fruit production, and the strategy of germination within the same year. If the fruits had longer germination capability, their presence in the soil seed bank could not simply be neglected. Similar observations were reported from Hungarian dry grasslands, where the seed bank of the sensitive grassland specialist *Pulsatilla pratensis* (L.) Miller subsp. *hungarica* Soó disappeared during grass encroachment (Matus et al., 2003). Most of the species that inhabit grasslands are not long-lived in the soil seed bank (Eriksson and Eriksson, 1997). Moreover, only approximately half of the plant species in grasslands contribute seeds to the seed bank (Bakker and Berendse, 2001).

Reduced competition under the favorable abiotic conditions provided by removal of the vegetation matrix, i.e., enhanced availability of light and soil resources, enabled relatively successful germination of *Pulsatilla grandis* fruits in hoed and vegetation-removal plots. Nevertheless we cannot completely exclude possible variation of soil properties among the plots with the various treatments, since the top layer of the soil was disturbed in hoed plots and also, to some extent, in the vegetation-removal ones. After burning, the germination was limited by the rapid recovery of the vegetation. Some species recovered very quickly, e.g., grasses (*Bromus erectus* Huds., *Phleum phleoides* (L.) Karsten, *Festuca rupicola* Heuff.), and species

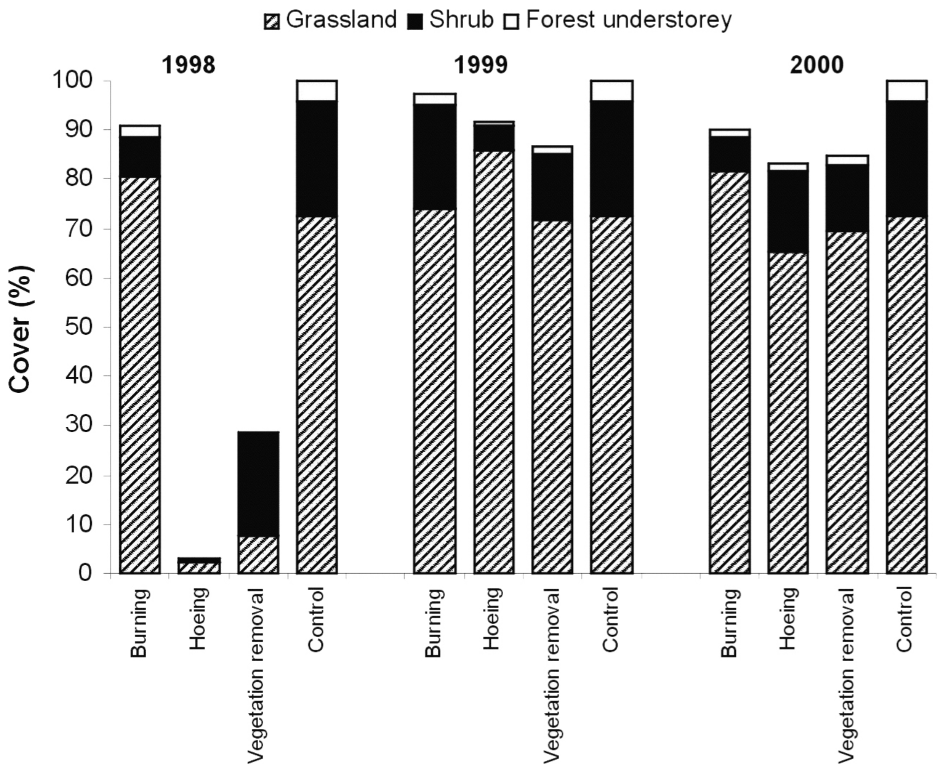

Fig. 4. Vegetation cover (%) in experimental plots, which in May 1998 were subjected to burning, hoeing, vegetation removal, and no disturbance (control), estimated in 1998, 1999, and 2000. Percentages (%) of grassland, scrub, and forest species are also indicated on the bars.

with above-ground (*Thymus pulegioides* L., *Veronica chamaedrys* L.) or below-ground clonality (*Anthericum ramosum* L., *Polygonatum odoratum, Euphorbia verrucosa* L.). In burned plots the roots, the rhizomes, and also the soil seed bank remained practically untouched; therefore, the competition remained unchanged. Similar negative effects were noted in grasslands of *Pulsatilla pratensis* subsp. *hungarica* (Matus et al., 2003). There are also contrary cases, in which fire is used as a tool for reducing the dominance of non-native species (Dyer, 2002), or the sward is too thick for establishment and persistence of typical grassland species (Lindborg and Eriksson, 2004). Vegetation removal was quite favorable for germination, but its application in grassland management is questionable.

The mortality of seedlings during the first winter was rather high; some were frozen in the autumn, and others were probably eaten by herbivores, e.g., deer, or attacked by diseases. Low survival rates in the subsequent years were related to competitive exclusion by dominant grasses, through encroachment of the grassland sward. As to why no germination was observed in untreated (control) quadrates: we saw from the greenhouse experiment that 3-year-old fruits already had very low germination capability, and this is why no new germination was observed after 5 years. In the second year of the experiment fruits were already 3 years old, an age at which a very low germination percentage (2%) was obtained in the greenhouse.

We expected at least minimal natural colonization or natural re-seeding from newly germinated plants, which started to produce fruits in 2001 on treated plots. However, only plants that germinated in the year of treatment (1998) were present until 2003. Within a few years after the treatments the vegetation recovered almost completely, with no gaps in the vegetation cover and no visible difference from the control plots. No disturbances by deer or by grazing, which could create gaps, were observed on either the treated or the untreated plots.

Danielsson (1987) reported that a related species—*Pulsatilla vernalis* (L.) Mill.—could regenerate only in patches where the bottom layer had been disturbed, and Kellner (1993) reached conclusions for the same species, which had 30 times higher density of seedlings in clear-cut areas than in adjacent closed pine stands. Our present results confirm their conclusions, and our experiment showed that a disturbance that opens gaps enables germination of *Pulsatilla grandis*.

Muller (2002) tried to fix *Pulsatilla alba* Rchb., a rare and endangered species in Lorraine, on a triangle, inspired by Grime's primary strategies triangle (Grime, 1977). The species was positioned between a "grazing" and a "no or very low exploitation" type. Grime (1977) recommended a low-pasturing regime for its habitats, and classified *Pulsatilla alba* in the "disturbance-depend rare plants" category, as defined by Pavlovic (1994). We suggest the same classification for its relative, *Pulsatilla grandis*.

We are aware that the substantial decline in population size, which also affects the viability of the plant, could not be resolved just by in-seeding in a small protected area. Apart from the fact that proper management is needed, the most important factor in the decline has been the reduction in the population's growing area from its former size. Maintenance of a viable population on the remaining restricted area by standard management alone, i.e., regular mowing, proved unsuccessful also because of human interference in the form of rooting and picking of individual plants. Nevertheless, even under a proper management regime at the site, and with the survival of individual plants, the reproduction capacity in general would be limited, since only a small and isolated part of the originally bigger population is preserved. Apart from any external factors, the genetic drift and lower population viability are characteristic of small plant populations. Two genetic processes reduce genetic variation when population size decreases: random genetic drift, and inbreeding (Oostermeijer et al., 1998). In the case of *Pulsatilla grandis* in Boč, weak fruit production is probably caused both by genetic "problems" and human activity, i.e., picking flowers. These factors further constrain the natural sustainability of this population. On the basis of the results of our field experiment we suggest a short-term and a long-term solution.

1. A short-term solution would be to maintain the existing population by in-seeding. Until the long-term solution is implemented, the population should be periodically enriched by artificial seeding. In order to achieve this, some kind of disturbance to create gaps in the encroaching vegetation is necessary. In our present study removal of the vegetation was successful, and we consider it the most appropriate. However, the short-term solution represents the kind of management that would be designated as "ex situ" rather, than "in situ". Treatments that closely resemble gardening do not achieve habitat preservation in the long run, but only keep the site attractive as scenery.

2. A long-term solution would be to restore the larger area of grasslands where *Pulsatilla grandis* grew in the past, and to maintain a big enough population by means of proper extensive management. As we concluded from our present field experiment, creating gaps in the vegetation matrix is essential to support natural re-seeding and re-colonization. Therefore, grazing by sheep, or cattle if sheep are not available, is important as a means of ensuring a long-term, light, but permanent disturbance and "gap opening" in the grassland. Since the traditional local way of managing dry grasslands, known as the "mowing-grazing system" is nearly extinct, we strongly recommend its re-introduction. There are parallel examples from Germany, which demonstrate that European cultural landscapes, including secondary dry grasslands, should be restored only when they are integrated into the respective land-use systems (Pfadenhauer, 2001). Preserving the traditional land use of the region will increase the chances of the presence of sustainable populations of *Pulsatilla grandis* in dry calcareous grasslands in the area.

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