DOES SEED MODIFICATION AND NITROGEN ADDITION AFFECT SEED GERMINATION OF *PULSATILLA GRANDIS*?[*]

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Pulsatilla grandis is an endangered species in the Czech Republic and is protected in whole Europe because the number of its populations is declining. One of the possible causes is the deposition of atmospheric nitrogen. In our research, we investigated how nitrogen concentrations and seed appendage removal directly affect the species' seed germination. Seeds were allowed to germinate under laboratory conditions in water solutions of NH_4NO_3 ranging in concentration from 0 to 4239 mg N l^{-1}. They were able to germinate up to the concentration of 848 mg N l^{-1} even when covered with mycelium, which supports the idea that they can tolerate being strongly infected by fungi. We also found a significant positive effect of seed appendage removal on seed germination. Seeds without appendages germinated, on the average, with 11% greater probability, compared to seeds with appendages. We conclude that the germination of *P. grandis* is not directly affected by high N concentrations in rain water, which can range from 10 to 13 mg N l^{-1} near large cities. Surprisingly, low concentrations of N (up to 34 mg N l^{-1}) might even slightly support the seed germination of *P. grandis*. The negative effect of N deposition on seeds is indirect and acts in conjunction with the absence of management at localities.

fungal infection, nitrogen deposition, pasqueflower, Ranunculaceae

INTRODUCTION

In the last decades, populations of dry calcareous grassland species such as *Pulsatilla grandis* have been decreasing throughout Central Europe and therefore have been listed under the Bern convention (D o s t á l o v á , K i r á l y, 2013).

The population decline has many causes such as lack of management, intensification of agriculture and higher N deposition often leading to increased nitrogen content in rainwater and soil (Fišák et al., 2002;

B o b b i n k et al., 2010; D o s t á l o v á , K i r á l y, 2013).

As a consequence, native species of dry calcareous grasslands are replaced by tall, expansive grasses (*Arrhenatherum elatius, Brachypodium pinnatum*), shrubs (*Rosa* spp.), and trees (*Ailanthus altissima, Pinus nigra* or *Robinia pseudoacacia*) (K u b í k o v á et al., 2007; B o b b i n k et al., 2010; R a n d i ć et al., 2013).

Seed germination is a critical stage in the plant life cycle and can be negatively affected by different biotic and abiotic factors. One factor possibly affect-

ing *P. grandis* seeds is higher nitrogen (N) content in the soil, as has been found for the similar species *Pulsatilla pratensis* (B o c h e n k o v á et al., 2015).

Many studies have examined the effects of nitrogen compounds (NH_4^+ and NO_3^- in particular) on seed germination and the subsequent reduction of species richness and deterioration of dry grasslands (e.g. B o b b i n k et al., 1998, 2010; T i p p i n g et al., 2013). The effect of higher nitrogen content on seed germination depends on the species and the range of N concentrations. Seed germination of weedy species, such as *Amaranthus powelli*, *Galium tricornotum*, *Medicago arabica*, *Thlaspi villosa,* and *Atriplex sagitata*, was found to be stimulated by a certain amount of nitrogen (10–500 mg N l^{-1}) and inhibited by higher concentrations (M a n d á k, P y š e k, 2001; C h a u h a n et al., 2006). Even very low N concentrations can have negative effects on some endangered species, such as the orchid *Pseudorchis albida,* whose seed germination is blocked already by concentrations of 2 mg NO_3^- l^{-1} (P o n e r t et al., 2013).

Seed germination in dry grasslands can also be negatively affected indirectly through higher plant cover as a consequence of higher N availability and absence of management with disturbances. Stronger tall grasses compete with native dry grassland species for light, water, and nutrients. The absence of disturbances creating essential 'gaps' for seed germination weakens native species even more (W i l s o n, T i l m a n, 1993; K a l i g a r i č et al., 2006; B o r e r et al., 2014). Such conditions impede the subsequent transition from germinated seeds to young seedlings and juvenile plants, as has been found for the similar species *P. pratensis* and *Pulsatilla vulgaris* (B o c h e n k o v á et al., 2012; P i q u e r a y et al., 2013).

Seeds of *P. grandis* create only transient seed banks (K a l i g a r i č et al., 2006). Freshly collected seeds do not need to undergo stratification and can germinate already in autumn of the same year. Seeds can reach germination rates of up to 29% under *in situ* conditions and 90% in a greenhouse, but their viability decreases fast to only 2% during the first two years after collection (Kaligarič et al., 2006). A similar reduction in germination has been found for *P. vulgaris* (W e l l s, B a r l i n g, 1971; T h o m p s o n et al., 1997) and likely also for *Pulsatilla patens* (K a l l i o v i r t a et al., 2006). Such low seed persistence can be another reason behind the species' disappearance from seed banks and even its local extinction, besides overgrowing of habitats, which can be prevented by suitable management (M a t u s et al., 2003; K a l i g a r i č et al., 2006).

Another factor possibly affecting seed germination is fungal infection, negatively connected with higher N and biomass content. Sporal infection of the embryo can decrease seed viability or even seed death (K i r k p a t r i c k, B a z z a z, 1979; C r i s t, F r i e s e, 1993; G o v i n t h a s a m y, C a v e r s, 1995;

S c h a f e r, K o t a n e n, 2003, 2004). The strength of the infection can be affected by factors such as plant species, moisture, and nutrient availability (S c h a f e r, K o t a n e n, 2004; M o r d e c a i, 2012).

Seeds of all *Pulsatilla* species have a special feathery style (appendage) aiding anemochory or zoochory, and according to W e l l s, B a r l i n g (1971), its other role is to make seeds embed themselves in the soil (W e l l s, B a r l i n g, 1971). Dispersal of *Pulsatilla* seeds by wind over long distances is very rare (T a c k e n b e r g et al., 2003). The feathery style can help seeds reach better germination conditions and avoid predation by hygroscopic drilling, as in *Stipa pulcherrima* and many other dry grassland species (S e n d t k o, 1999). Nevertheless, such seeds can also get stuck on vegetation without reaching the soil and the seed bank, but the significance of the feathery style for the germination of *Pulsatilla* seeds is not completely known (W e l l s, B a r l i n g, 1971). The feathery style was removed in several experiments with different *Pulsatilla* species, but, unfortunately, this modification was usually not evaluated (e.g. K a l a m e e s et al., 2005; Š e d i v á, Ž l e b č í k, 2012). According to the feathery style of *Pulsatilla vulgaris* (W e l l s, B a r l i n g, 1971; W a l k e r, P i n c h e s, 2011) and *P. pratensis* (B o c h e n k o v á et al., 2015) appears to have no decisive effect on seed germination itself and is used only for dispersal. Removal of the feathery style can also affect seed germination, as seeds without appendages provide less biomass for their potential fungal infection (B o c h e n k o v á et al., 2015).

In this study we tested whether increased N content in the soil can affect the seed germination of *P. grandis* and whether the germination is affected by mechanical removal of seed appendages and fungal infection of seeds.

The aims of this study were to (*i*) ascertain the range of N concentrations which allows the germination of *P. grandis* seeds and (*ii*) determine whether the removal of seed appendages affects germination. We also asked (*iii*) whether the species' seed germination under high N concentrations in the soil is negatively affected by higher rates of fungal infection.

MATERIAL AND METHODS

Species description

P. grandis Wenderoth from the family Ranunculaceae is an endangered species in the Czech Republic (D e c r e e N o. 3 9 5 / 1 9 9 2 C o l l.) and is listed in Annex II of the Habitats Directive (C o u n c i l D i r e c t i v e 9 2 / 4 3 / E E C, 1992). The species grows in Central, Southern, and Eastern Europe on calcium-rich dry grasslands, rocky outcrops, and in open thermophilous oak and pine forests (S k a l i c k ý,

Treatment	1(Control)	2	3	4	6	8
mg N l^{-1}	0	6.8	34	170	848	4 239
mg NH_4NO_3 l^{-1}	0	19	97	484	2 422	12 111

1988; D o s t á l o v á , K i r á l y , 2013). It is a perennial, 213- cm (during fruiting 13–40 cm) tall, hairy, self-compatible hemicryptophyte (L i n d e l l , 1998) that flowers from March to May with erect pale purple flowers. Its fruits are 4–5 cm long achenes (hereafter referred to as seeds). According to a recent estimate, there are approximately 200 localities of *P. grandis* in the Czech Republic, altogether with several tens of thousands of individuals (R y b k a , et al. 2004; D o s t á l o v á , K i r á l y , 2013). The largest population occurs at the locality Kamenný vrch in South Moravia (see following subsection).

Seed collection and seed description

Seeds of *P. grandis* were collected in Moravia from different wild plants on 15th May 2009 in the Kamenný vrch Protected Reserve and Site of Community Interest (49°11′2.290″N, 16°33′5.988″E). The locality is famous for having the largest population of the species in the Czech Republic with 60 000 flowering individuals (Ř i h á č e k , 2009). The locality is situated 4 km SW of the centre of Brno at the elevation of 366–384 m a.s.l. The mean annual temperature is 9.4°C, and mean annual precipitation is 505 mm. The study site is located on a southern slope with an inclination of 4–6°. The soil is a typical Cambisol developed on diorite bedrock (M a c k o v č i n et al., 2007).

P. grandis produces achenes consisting of two parts: a 3–5 mm long seed with a small embryo inside and a 3–5 cm long persistent feathery style (appendage). Both parts are covered with silky silver hair. In our experiment we only used ripe and healthy seeds. Seeds without any deformations, that is those not differing in size, shape, or having a short undeveloped feathery style, were considered healthy (W e l l s , B a r l i n g , 1971; B o c h e n k o v á et al., 2015).

Germination experiment

The germination experiment was carried out in a laboratory at the Czech University of Life Sciences Prague in November 2009. The ambient temperature was 22°C, and the seeds were exposed to a 16/8 h light/dark regime in Petri dishes 10 cm in diameter with three layers of filter paper sterilized for 20 min at 120°C in a Labo Autoclave. The test consisted of six different treatments. A total of 3000 fully-developed and healthy seeds (50 per Petri dish) were subjected to five N solution treatments and one control treatment (distilled water only) with five replicates and two variants containing seeds with and without hairy appendages. To test the effect of hairy appendages on germination and fungal infection, the appendages were removed 2 mm from the seed (hereafter referred to as seed modification).

To simulate atmospheric nitrogen deposition, ammonium nitrate (NH_4NO_3, 35% N) diluted with distilled water was used to obtain the required N concentrations (Table 1). The level of dilution was
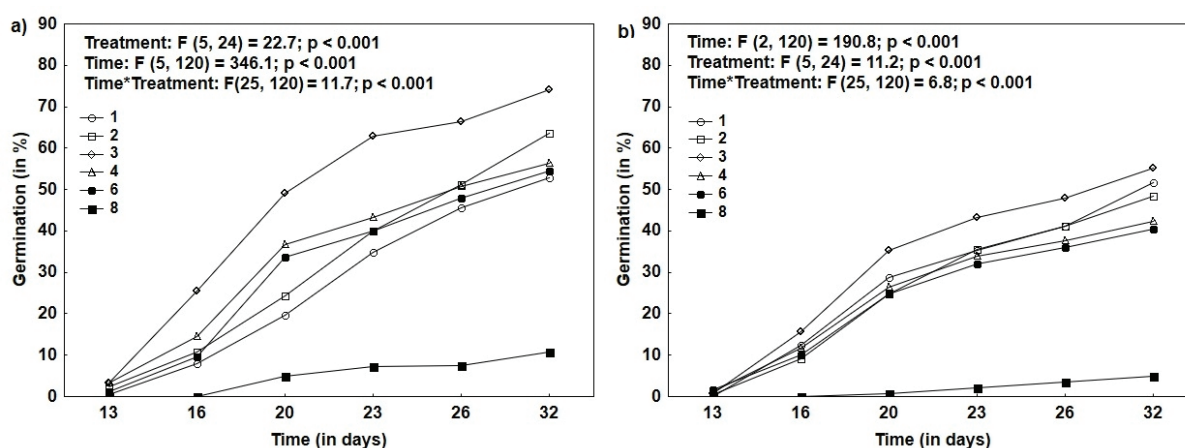

Fig. 1. Mean cumulative germination of (a) modified and (b) unmodified seeds collected on days 13, 16, 20, 23, 26, and 32 of the experiment. Treatment abbreviations are given in Table 1. The F and P values were obtained by repeated measures ANOVA

equal between samples of the same treatment. To each Petri dish, 5 ml of different N solutions were added, according to the treatment, on the first day of the experiment. The final N concentrations used were estimated as in similar experiments (e.g. L u g o , 1955; M a n d á k , P y š e k , 2001; B o c h e n k o v á et al., 2015).

Data collection and analysis

Rates of germination and fungal infection were recorded after 13, 16, 20, 23, 26, and 32 days since the start of the experiment. Seed with visible, at least 1 mm long radicle sprouts were considered germinated. A seed was considered infected if more than half of its surface was covered with fungal mycelium. Infected seeds which were able to germinate were considered as germinated and infected.

The percentage data on germination and fungal infection were arc-sin transformed for the analysis and then transformed back to create graphs. The transformed data were evaluated by repeated measures ANOVA (treatment, time) and by factorial one-way ANOVA (effects of N solution treatment, seed modification, and their interaction) with Tukey's post-hoc test. All analyses were performed in STATISTICA software Version 10.1 (www.statsoft.cz).

RESULTS

Seed germination

Seeds of *P. grandis* started to germinate on the twelfth day, after which both unmodified (with the appendage) and modified (without the appendage) seeds germinated steadily. The mean cumulative germination

rate of modified and unmodified seeds on the last day of the experiment was 52 and 40.4%, respectively, with a statistically significant difference ($P < 0.001$). The course of germination was significantly affected by treatment, time, and the interaction between time and treatment (Fig. 1a, b) for both modified and unmodified seeds, but with differences in the order in which seeds subjected to different treatments germinated.

Modified seeds had by approximately 11% greater average germination rate across all treatments compared to unmodified seeds. Treatment 3 (34 mg N l^{-1}) induced the greatest germination rate: 74% and 55.2% for modified and unmodified seeds, respectively. The lowest germination was reached in the treatment with the highest level of N (10.8% and 4.8% for modified and unmodified seeds, respectively). Modified seeds subjected to the remaining treatments germinated in the following order: treatment 2 (63.6%), treatment 4 (56.4%), treatment 6 (54.4%), and treatment 1 (52.8%). Unmodified seeds germinated as follows: treatment 1 (51.6%), treatment 2 (48.4%), treatment 4 (42.4%), and treatment 6 (40.4%).

Factorial ANOVA revealed a significant effect of treatment ($P < 0.001$), seed modification ($P < 0.001$), and interaction between these factors ($P = 0.008$) on seed germination (Fig. 3a). Tukey's post-hoc test within each group of seed modification then distinguished three groups for modified seeds and two groups for unmodified seeds (Fig. 3a). One-way ANOVA comparing treatments of modified and unmodified seeds detected a significant difference between treatments 2 and 4 (Fig. 3a).

Fungal infection

The mean extent of fungal infection for each modification category did not differ significantly across treatments 1–8 (0–4239 mg N l^{-1}). The results show
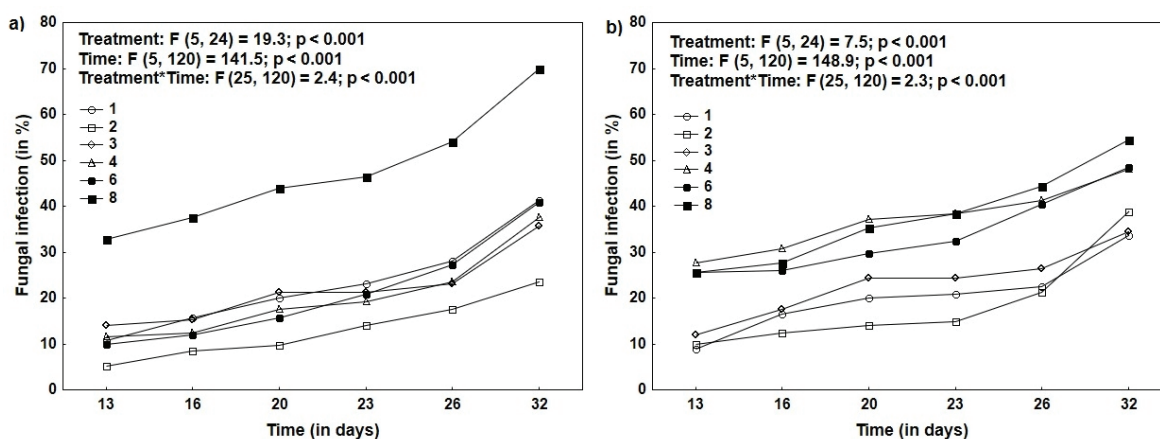

Fig. 2. Mean fungal infection of (a) modified and (b) unmodified seeds collected on days 13, 16, 20, 23, 26, and 32 of the experiment. Treatment abbreviations are given in Table 1. The F and P values were obtained by repeated measures ANOVA

a 41.5% and 43% rate of fungal infection for modified and unmodified seeds, respectively. The extent of fungal infection was significantly affected by treatment, time, and the interaction between time and treatment for both categories of seed modification (Fig. 2a, b).

The course of fungal infection was similar in both modified and unmodified seeds, except treatment 8 for modified seeds, which had by approximately 15% greater fungal infection rate compared to unmodified seeds (compare Fig. 2a, b).

The greatest fungal infection rate was found in the treatment with the highest N concentration (treatment 8; 4239 mg N l^{-1}) for both categories of seed modification, but the infection rate was not significantly different between them ($P = 0.101$). The lowest fungal infection was reached in treatment 2 (23.6%) for modified seeds and in treatment 1 (33.6%) for unmodified seeds (Fig. 2a, b).

Using factorial ANOVA, we compared the rates of fungal infection between modified and unmodified seeds, and found significant effects for treatment ($P < 0.001$) and the interaction between treatment and seed modification ($P = 0.016$), but not for the modification itself ($P = 0.669$, Fig. 3b). Tukey's post-hoc test distinguished two distinct groups for modified and unmodified seeds. One-way ANOVA comparing different treatments applied to modified and unmodified seeds found treatment 2 to differ significantly (Fig. 3b).

DISCUSSION

Seed germination

In our experiment we found *P. grandis* to have a high germinability under a wide range of N con-

centrations, which agrees with our previous study which focused on the similar species *P. pratensis* (B o c h e n k o v á et al., 2015). Seeds were able to germinate under relatively high N concentrations (848 mg N l^{-1}) compared to N concentrations commonly recorded in wet deposition near large cities, ranging only between 10 and 13 mg N l^{-1} (F i š á k et al., 2002). Even though the total amount of N deposition in the study was based on N deposition data only, and despite the fact that the total amount of N which affects the environment is unknown, concentrations greater than 848 mg N l^{-1} do not occur naturally in dry grasslands at present. The critical concentration of nitrogen which blocks the germination of *P. grandis* (2422 mg N l^{-1}) corresponds with results obtained for the related species *P. pratensis* (B o c h e n k o v á et al., 2015). Such high critical N concentrations are more typical for weedy species and ruderal habitats (M a n d á k, P y š e k, 2001). Seeds of dry grassland species exposed to high N concentrations can get damaged by salt plasmolysis, moisture scarcity or direct toxicity (G o w a r i k e r et al., 2009; T a i z et al., 2014). Higher N levels in soil also negatively affect seed banks because they facilitate the growth of tall grasses (B a s t o et al., 2015). Dense vegetation and a thick litter layer block seeds from reaching the soil and creating a seed bank (D o n a t h, E c k s t e i n, 2010; R u p r e c h t, S z a b ó, 2012), which can be crucial for plants that create only transient seed banks, such as *P. grandis* (K a l i g a r i č et al., 2006).

Seed viability of *P. grandis* decreases in the second year after ripening, and the six months of dry storage before our experiment probably had only a slight effect on seed germination. Under greenhouse conditions, one-year-old seeds have a lower germination rate by about 30%, but the decrease in
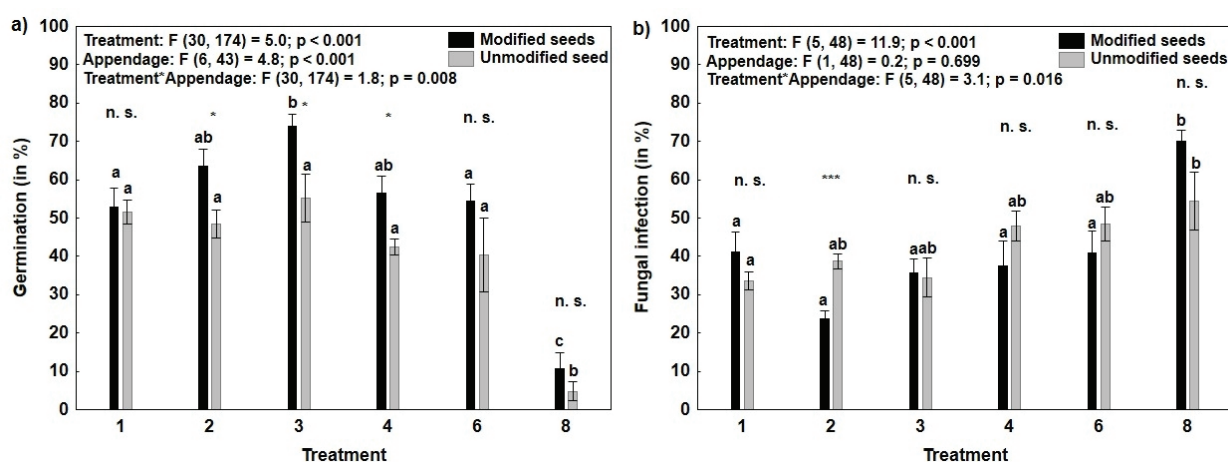

Fig. 3. (a) Mean cumulative germination and (b) mean extent of fungal infection of modified and unmodified seeds at day 32 of the experiment. Treatment descriptions are given in Table 1. Vertical lines represent standard errors of the mean (SE), F and P values were obtained by factorial ANOVA. Using the Tukey's post-hoc test (α = 0.05), treatments with the same letter within the same type of seed modification (calculated separately for black and grey columns) were not significantly different. One-way ANOVA was used to evaluate differences within each treatment (black and grey columns). Differences between black and grey columns were not statistically significant (n.s.) or significant at the probability levels of *P< 0.05 and ***P < 0.001

germination is much stronger after two years of storage (Kaligarič et al., 2006). However, it is interesting that the decrease in seed viability in the first two years is much lower in the similar species *P. pratensis* (B o c h e n k o v á et al., 2015), *Pulsatilla cernua* (S a n g et al., 1996) or *Pulsatilla slavica* (L h o t s k á , M o r a v c o v á , 1989).

Fungal infection

We found no significant difference across treatments 1–8 (0–4239 mg N l^{-1}) for both modification categories, but the extent of fungal infection was significantly affected by treatment, time, and the interaction between time and treatment. Treatment 8 of modified seeds had fungal infection rate greater by about 15%. This could be due to some random deviation because the fungal infection in other treatments had almost the same course. During our experiment, we observed that even seeds completely covered with mycelium were able to germinate under high nitrogen concentrations, as was also observed by M a n d á k , P y š e k , (2001) and C h a u h a n et al. (2006). Healthy and undamaged seeds of *P. grandis* have a hard seed coat which protects seeds against fungal infection. When the seed coat is disrupted, water and spores of fungi can infect the embryo and break physiological dormancy, which can cause seed death or germination at an inappropriate time (B a s k i n et al., 2000).

The intensity of fungal infection depends on the combination of fungi, plant species, temperature, nutrient levels and moisture availability (S c h a f e r , K o t a n e n , 2004; M o r d e c a i , 2012). Certain combinations of these factors can lower seed viability, decrease germinability and alter seedling survivorship (K i r k p a t r i c k , B a z z a z , 1979; C r i s t , F r i e s e , 1993; G o v i n t h a s a m y , C a v e r s , 1995; S c h a f e r , K o t a n e n 2003, 2004). However, it is necessary to emphasize that some fungi can increase seed mortality whereas others are harmless commensals (C r i s t , F r i e s e , 1993).

Seed modification

In our experiment we examined the effect of feathery appendages on seed germination, which is reported for *P. vulgaris* in a review by W e l l s , B a r l i n g (1971). In that paper, modified seeds (without appendages) germinated at a slightly lower rate (about 8%). Modified seeds of *P. pratensis* also exhibited a lower germination rate (B o c h e n k o v á et al., 2015). Our results indicate that seed modification can improve the germination rate of *P. grandis* with about 11%, probably because fungal pathogens have less nutrients to feed on.

Recommendations for *in situ* and *ex situ* management

To support populations of *P. grandis*, we recommend to keep overgrowth biomass (e.g. using fire management, mowing or grazing) at a certain level and to create 'gaps' around maternal plants to increase seed germination and seedling survival. According to K a l i g a r i č et al. (2006), burnt patches provide perfect conditions for the seed germination and seedling survival of *P. grandis*, as fire removes all overgrowth biomass. However, an adequate vegetation cover (e.g. by solitary bushes) can also play an important role as a canopy. This can affect the temperature and humidity and thereby influence seed germination and seedling emergence and survival. Small seedlings can be supported by covering them with fabric or hay during winters with little snow, as is also recommended for the similar and more sensitive species *P. patens* (B j ö r k é n et al., 2015). In our unpublished study, we found the survival rate of *P. grandis* seedlings under greenhouse conditions to be 15% (n = 804) compared to the 1% (*n* = 309) survival rate of *P. pratensis* (Bochenková, unpublished). *P. grandis* seems to be a stronger dry grassland competitor compared to the similar species *P. pratensis*. Under *ex situ* conditions, the germination of *P. grandis* can be supported by careful seed modification and a small amount of added N.

CONCLUSION

We conclude that seeds of *P. grandis* are able to germinate under a wide range of N concentrations (up to 848 mg l^{-1}). Our results also support the hypothesis that *P. grandis* has a seed coat which is more resistant against fungal infection and that appendage removal can improve the seed germination of this *Pulsatilla* species. The results of our experiment do not indicate that increased *in situ* levels of N directly reduce germination and seedling survival of *P. grandis*. Any influence of N on seeds is probably indirect and acts through interspecies competition within the plant community due to the absence of management and lack of 'gaps'. Populations of *P. grandis* grow in dry and nutrient-poor habitats, and fungal infection due to higher nutrient availability is probably not the main cause of their recent decline.

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