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Dactylorhiza maculata, *Platanthera bifolia* and *Listera ovata* survive N application under P limitation

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A B S T R A C T

Increased nutrient availability is generally believed to be harmful for terrestrial orchids. However, no information is available on whether grassland orchids survive decades-long calcium (Ca), nitrogen (N), phosphorus (P) and potassium (K) application. We took advantage of sampling in one of the oldest long-term experiments in the world, the Rengen Grassland Experiment (RGE), which was established in Germany in 1941 on low-productive *Violion caninae* grassland, and we monitored orchids from 2005 to 2009.

Dactylorhiza maculata, *Platanthera bifolia* and *Listera ovata* survived decades-long application of Ca and CaN fertilizer, but not CaN together with P. Cover of *D. maculata* was highest in areas of Ca treatment. The number of flowers per plant of *L. ovata* was significantly higher in the CaN treatment compared with the control indicating that enhanced N availability stimulated its growth. Long-term N application (NH_4NO_3 at an annual rate of 100 kg N ha^{-1}) was not fatal to the studied orchids if not accompanied by P application. It is more likely that enhanced N availability can cause loss of studied orchids only where the availability of P in soil is high enough to enable the growth of productive species that can better compete for light on eutrophic soils.

1. Introduction

Across a wide range of European grasslands, a number of endangered orchid species have suffered dramatic declines. Remaining populations often tend to be small with uncertain long-term viability (Jacquemyn et al., 2005; Kull and Hutchings, 2006). Eutrophication of orchid localities due to fertilizer application, high mineralization rates of organic matter and atmospheric deposition of N compounds are believed to be the main reasons for orchid decline in recent decades (Jacquemyn et al., 2007; Wotavová et al., 2004; Janečková et al., 2006).

Increased nutrient supply is generally assumed to be harmful for orchids, so field experiments have been conducted to investigate effects of N, P and K availability on adult orchids. According to Silvertown et al. (1994), application of inorganic fertilizers returning equivalent amounts of N, P, Mg and 80% of K to that removed in the annual hay crop significantly decreased flowering spike numbers of *Orchis morio* over six years of treatment. In the same experiment, N decreased flowering spike numbers, although the applied annual rates were only 22–88 kg N ha^{-1}. Two applications of P at a rate of 40 kg ha^{-1} (80 kg P ha^{-1} together) over six years greatly reduced flowering spike numbers, apparently for decades. The effect of P on *O. morio* was out of all proportion to its effect on hay yield, suggesting that P application may have been toxic to the orchid. In an experiment by Dijk and Olff (1994), the application of 250 kg N ha^{-1} in the form of NH_4NO_3 and 80 kg P ha^{-1} in the form of NaH_2PO_4 was sufficient to decrease the abundance, total biomass and flowering of *Dactylorhiza majalis* in a *Juncus acutiformis* wet hay meadow in the Netherlands. In a one-season experiment by Mattila and Kuitunen (2000), a single application of organic slow-release N, P and K fertilizer immediately increased relative capsule production in *Platanthera bifolia* plants with small inflorescences but not in plants with large inflorescences, which might indicate that plant response to fertilizer input is related to plant size.

Most information about the effects of N, P and K availability on orchids comes from laboratory and pot experiments. In a pot experiment with natural soil, the growth of *O. morio* was not significantly affected by N application whereas the overall growth of *Dactylorhiza fuchsii*, and especially that of roots, was markedly reduced (McKendrick, 1996). Direct toxic effects of N and P fertilizers on the growth of seedlings of Dutch marsh orchids were recognized in *in vitro* cultures by Dijk and Eck (1995). Only large N

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concentrations had a general negative effect on orchid axenic growth. *Dactylorhiza praetermissa* responded positively to the addition of mineral N whereas *Dactylorhiza incarnata* showed the most negative response. *D. majalis* showed the highest growth in the greatest P concentration of all species, in contrast to the most negative response of *D. praetermissa*. Further, calcicole *D. incarnata* suffered from ammonium N toxicity rather than from nitrate N toxicity (Dijk and Eck, 1995).

To the author's knowledge (Hejcman and Schellberg, 2008), it has never been experimentally investigated whether grassland orchids withstand decades-long N, P and K application under field conditions. Such information is necessary for predicting the response of orchids to recent increases in nutrient availability in many ecosystems. Further, according to Wassen et al. (2005), more endangered species persist under P-limited rather than under N-limited conditions, and therefore enhanced P is more likely to induce species loss than N enrichment. It remains unclear if this conclusion holds for orchids as well.

The only known worldwide, long-term grassland fertilizer experiment where assessments of orchids have been performed is the Rengen Grassland Experiment (RGE), established by Prof. Ernst Klapp in the Eifel Mountains in Germany in 1941 (Schellberg et al., 1999). The RGE is probably the oldest continuously-running and properly-designed grassland fertilizer experiment worldwide. Three orchid species, *Dactylorhiza maculata*, *P. bifolia* and *Listera ovata*, were recorded there in the 1940s, 1990s and again in 2005 (Hejcman et al., 2007). To describe the effect of fertilizer application on these orchids, cover of orchids was monitored from 2005 to 2009. Furthermore, we investigated the effects of long-term Ca and N application on plant height, number of flowers and length of leaves of *L. ovata*.

Fig. 1. Aerial photograph of the Rengen Grassland Experiment (RGE) taken in late June 2005. The RGE was established in 1941 by Prof. Klapp and is probably the oldest continuously-running grassland fertilizer experiment with proper randomization worldwide (photo Michal Hejcman©). "Block IV" is in the foreground, "Block II" in the background. Control plots without any fertilizer application (where the students are working) are to the right of "Block II".

2. Materials and methods

2.1. Study site

The RGE was set up at the Rengen Grassland Research Station of the University of Bonn in the Eifel Mountains (50°13′N, 6°51′E) at 475 m above sea level. At the study site, the mean annual precipitation is 811 mm and mean annual temperature is 6.9 °C (Rengen meteorological station). The soil is a pseudogley, indicated by temporarily wet conditions in the turf layer after rainfall in winter and spring and the slow vertical rise of capillary water in summer. Further soil information is given in Schellberg et al. (1999).

2.2. Experimental design

The experiment is arranged in a completely randomized block design with five fertilizer treatments (treatment B = Ca; C = Ca/N; D = Ca/N/P; E = Ca/N/P/KCl; F = Ca/N/P/K_2SO_4) and the same number of replicates (see Fig. 1 and Table 1 for details). As an unlimed control was originally missing in the RGE, five replicates of a treatment without any fertilizer input (further referred to as control) were added in 1998. The treatments were introduced in the vicinity of the existing plots on an area exhibiting the same soil properties. This area was protected as an unfertilized control, has never been fertilized and has been cut at the same time in which the experimental plots were harvested. The experiment is arranged in two blocks, originally named "Block II" and "Block IV", each including five of the abovementioned treatments. Until now, studies on floristic composition concentrated on Block IV only. All experimental plots, including the control, have been cut twice a year in late June or early July and in mid-October.

Long-term fertilizer application created significantly different plant communities within the near neighborhood (Chytrý et al., 2009). Unfertilized control plots supported oligotrophic *Nardus* grassland (*Violion caninae* alliance) whereas vegetation in the Ca and CaN treatments mostly resembled montane meadow (*Polygono-Trisetion* alliance). *Arrhenatherum elatius* hay meadow (*Arrhenatherion* alliance) developed in the CaNP, CaNPKCl and CaNPK_2SO_4 treatments. Long-term fertilizer application resulted in a high gradient of aboveground biomass production (Hejcman et al., 2010a, Table 1) as well as soil chemical properties (Hejcman et al., 2009, Table 2).

Table 1
Amounts of nutrients (kg ha^{-1}) supplied annually to the treatment plots since 1941 according to Schellberg et al. (1999), classification of vegetation into alliances according to Chytrý et al. (2009) and aboveground dry matter biomass production according to Hejcman et al. (2010a).

Treatment abbrev.	Applied nutrients (kg ha^{-1})	Alliance	Biomass production (t ha^{-1})
Con	Non-fertilized control	*Violion caninae*	2.5
Ca	Ca = 715; Mg = 67	*Polygono-Trisetion*	2.9
CaN	Ca = 752; N = 100; Mg = 67	*Polygono-Trisetion*	4.9
CaNP	Ca = 936; N = 100; P = 35; Mg = 75	*Arrhenatherion*	6.5
CaNPKCl	Ca = 936; N = 100; P = 35; K = 133; Mg = 90	*Arrhenatherion*	8.9
CaNPK_2SO_4	Ca = 936; N = 100; P = 35; K = 133; Mg = 75	*Arrhenatherion*	9.6

Table 2
Results of basic soil chemical analyses of the 0–10 cm layer (mg 100 g^{-1} of soil, according to Hejcman et al., 2009). Numbers represent mean values of five replicates in Block IV.

Treatment	P	K	pH	N (%)	C (%)	C/N	Mg
Control	1.5	4.3	4.9	0.373	4.9	13.1	13.2
Ca	0.6	2.5	6.5	0.350	4.2	12.0	19.9
CaN	0.4	2.3	6.5	0.364	4.4	12.0	19.5
CaNP	31.1	3.2	6.6	0.363	4.3	11.8	19.9
CaNPKCl	22.6	9.4	6.5	0.363	4.4	12.0	20.7
$CaNPK_2SO_4$	22.2	10.5	6.6	0.367	4.5	12.3	20.7

Soil pH was potentiometrically measured in a suspension with 0.01 M $CaCl_2$. Plant-available P and K were extracted by a calcium-acetate–lactate solution and measured colorimetrically and photometrically, respectively. Mg was extracted with 0.01 M $CaCl_2$ and measured with flame atomic absorbance spectrometry (AAS). Total carbon (C) and N were quantified by elemental analyses (Carbo-Erba, Italy).

2.3. Data collection

Cover of *D. maculata*, *P. bifolia* and *L. ovata* was visually estimated directly in percentages in control plots and in "Block IV" in late June from 2005 to 2009. Plant height, number of flowers and length of both leaves of *L. ovata* were measured non-destructively and counted in late June 2009 in the control and in "Block II" and "Block IV" together, since there were not enough plants in "Block IV" in this year. Similar data for the other two species were not collected, as the number of flowering individuals recorded in all treatments was too low.

2.4. Data analysis

Repeated measures ANOVA was used to analyze cover data of investigated species. One-way ANOVA followed by *post-hoc* comparisons with Tukey's test was used to identify significant differences among treatments for plant height, number of flowers and length of both leaves of *L. ovata*.

3. Results

After more than 60 years since the onset of the experiment, the orchid species investigated in this study were recorded throughout five years of vegetation monitoring in the unfertilized control, Ca and CaN treatments, but never in the CaNP, CaNPKCl and $CaNPK_2SO_4$ treatments. Mean cover of *D. maculata* was below 2% in all treatments where they were present (Fig. 2a). Cover of *D. maculata* was significantly affected by treatment, but not by year or treatment*year interaction (Table 3). The highest and relatively stable cover of *D. maculata* was recorded in the Ca treatment in all investigated years. *D. maculata* was recorded in four and three seasons in the control and CaN treatments, respectively.

Mean cover of *P. bifolia* and *L. ovata* was below 1% in the control, Ca and CaN treatments in all seasons. Its cover was neither significantly affected by treatment, nor by year and treatment*year interaction (Table 3). Both species were recorded in the control plot in all five investigated seasons, whereas in the Ca and CaN treatments they were identified only in two and three seasons, respectively.

Although no plants of *L. ovata* were recorded in CaN treatment in "Block IV" in 2009, ten plants were recorded in CaN treatment in "Block II". This enabled us to measure ten plants in the control, Ca and CaN treatments, respectively. Although the effect of treatment on the height of *L. ovata* plants was not significant, there was a clear trend within the data (Table 4). Mean plant height was slightly increased in the CaN treatment compared to control. This trend was clearly reflected by the number of flowers per plant, which

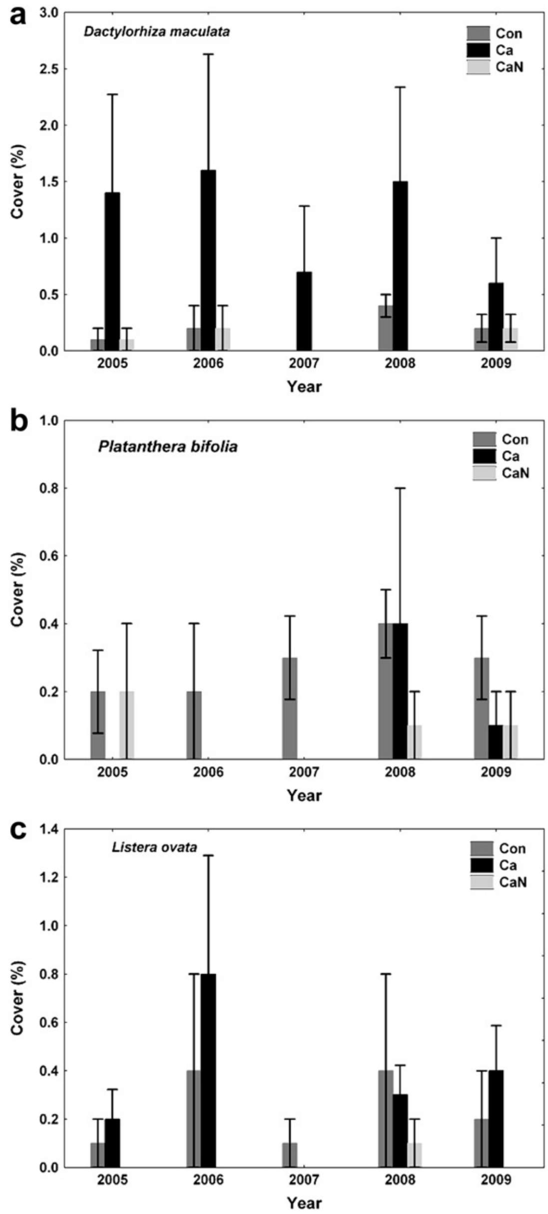

Fig. 2. Effect of unfertilized control (Con), Ca and CaN treatments on cover of orchid species *Dactylorhiza maculata* (a), *Platanthera bifolia* (b) and *Listera ovata* (c). Error bars represent the standard error of the mean (SE).

increased significantly from control to CaN treatment (Table 4). The length of both leaves was slightly increased in the CaN treatment compared to control (Table 4), but this effect was not statistically significant.

Table 3
Results of repeated measures ANOVA of cover data. Degrees of freedom were 5 for treatment, 4 for year and 20 for treatment*year interaction. F – F value, P – obtained probability value, significant result in bold.

Tested variable		Treatment	Year	Treatment*Year
Dactylorhiza maculata	F	8.3	0.5	0.4
	P	**0.001**	0.728	0.942
Platanthera bifolia	F	2.8	1.1	0.5
	P	0.069	0.354	0.883
Listera ovata	F	2.9	1.3	0.6
	P	0.063	0.269	0.811

Table 4
Results of one-way ANOVA (F – F value, P – obtained probability value, significant result in bold) of plant height, number of flowers per plant, length of lower leaf and length of upper leaf of *Listera ovata* measured in 2009. Treatment abbreviations (Ca and CaN) are given in Table 1. Numbers represent averages of ten replicates ± standard error of the mean (SE). Treatments with the same letter are not significantly different.

Tested variable	F	P	Control	Ca	CaN
Plant height (cm)	1.3	0.284	30.1 ± 1.5[a]	32.1 ± 1.9[a]	34 ± 1.6[a]
Number of flowers per plant	8.6	**0.001**	25.6 ± 1.6[a]	31.4 ± 2.1[ab]	37 ± 2.1[b]
Length of lower leaf (cm)	0.2	0.805	5.9 ± 0.2[a]	6.1 ± 0.2[a]	6.1 ± 0.4[a]
Length of upper leaf (cm)	1.5	0.239	6.2 ± 0.2[a]	6.5 ± 0.2[a]	6.8 ± 0.4[a]

4. Discussion

The principal finding of this study is that *D. maculata, P. bifolia* and *L. ovata* survive decades-long Ca or Ca and N application, but not when Ca, N and P are applied together. The main conclusion is that long-term application of N (NH_4NO_3 at an annual rate of 100 kg N ha^{-1}) is not fatal for the orchid species studied here if this is not accompanied with P application on soils with low P availability. This conclusion is especially interesting in the case of *D. maculata*, as this species is characteristic for oligotrophic soils (Ellenberg indicator value for nutrients (EIVN) is 2; Ellenberg et al., 1991) in contrast to the more nutrient-demanding *P. bifolia* (EIVN = indifferent) and *L. ovata* (EIVN = 7). On the other hand, Ca applied together with N decreased the frequency of occurrence of all orchids, as they were recorded in control plots almost in all years, but only in one or three years out of the five investigated in the CaN treatment. This indicates that although long-term application of N did not eliminate the studied orchid species from the sward, there were some negative effects of N application on their vitality. Similarly to CaN treatment, a slightly negative effect of Ca treatment on the occurrence of *P. bifolia* was recorded, but this was not the case for *D. maculata* and *L. ovata*. Cover of *D. maculata* markedly increased in the Ca treatment in comparison with control, indicating no harmful effect of liming on this species. Studied orchids were evidently able to tolerate a wide range of soil pH, ranging from 4.9 in control to 6.5 in the Ca treatment, although they prefer neutral or alkaline soils as indicated by Ellenberg indicator values for pH (7 for all species). The highest cover of *D. maculata* observed in the Ca treatment speaks for its preference for neutral soils.

All the studied orchids are long-living species with underground tubers (*D. maculata* and *P. bifolia*) or rhizomes (*L. ovata*; Kull and Hutchings, 2006). Therefore, their absence in aboveground biomass in a particular year can indicate dormancy rather than extinction. Additionally, we can conclude that the effects of fertilizer application on these orchid species should be evaluated by long-term studies.

We have learned from earlier experimentation in the RGE that long-term application of N increased the extraction of plant-available P from the soil (Hejcman et al., 2010a), and subsequently, plant-available P became extremely rare in the CaN treatment (see Table 2 for plant-available P concentration in the upper 10 cm soil layer). A substantial increase in biomass production that could successfully compete with orchids was inhibited by P limitation in the CaN treatment. This is clearly indicated by soil analysis (Hejcman et al., 2009), the N:P ratio in aboveground biomass of vascular plants (Hejcman et al., 2010c) and by the N:P ratio in bryophyte biomass (Hejcman et al., 2010b). We fully agree with the conclusions made by Janssens et al. (1998), Niinemets and Kull (2005) or by Wassen et al. (2005) that many endangered species, like orchids recorded in the RGE, can persist under P-limited rather than under N-limited conditions. Enhanced N availability is more likely to cause the loss of studied orchids only if P availability exceeds a threshold which is unknown at this time.

Although Silvertown et al. (1994) and Dijk and Olff (1994) described the negative effects of N application on orchids in field experiments, many of these effects were generated by an increase in biomass production of the surrounding vegetation competing for light. Based on the result that 67 years of N application was not enough to eliminate orchid species in the RGE, the question can be raised as to whether the results of pot and field experiments with N application performed only for several years allow for long-term extrapolation. We conclude that as long as N application under P limitation is insufficient to increase biomass production substantially, *D. maculata*, *P. bifolia* and *L. ovata* can survive a long-term increase in N availability. Tolerance of *P. bifolia* and *L. ovata* against increased N availability can probably explain why these orchids are amongst the most common in Central Europe (Brzosko and Wróblewska, 2003; Brzosko et al., 2009).

Measurements on plant performance in the RGE support the adaptability of studied orchids to moderate N levels. An increase in the number of flowers per plant in *L. ovata* clearly demonstrates even a positive effect of N application. This indicates that *L. ovata* has the highest N requirements of all studied orchids. In turn, the negative effects of applied N leading to the disappearance of many grassland orchids as described in observational studies (Jacquemyn et al., 2005; Wotavová et al., 2004; Janečková et al., 2006) can probably be expected on soils with sufficiently high plant-available P content, thus enabling the growth of more productive and competitive species in the sward. This is undoubtedly demonstrated by the fact that the studied orchids were generally missing in NP and NPK treatments in the RGE, which produced more aboveground biomass than the Ca and CaN treatments.

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