Received: 3 May 2024 | Accepted: 3 November 2024

DOI: 10.1111/1365-2435.14710

Functional Ecology | BRITISH ECOLOGICAL SOCIETY

RESEARCH ARTICLE

Nitrate inhibition of germination in Ericaceae relates to seed size and mycoheterotrophy

Tomáš Figura[1,2,3] | Edita Tylová[3] | Yosuke Matsuda[4] | Martina Janoušková[2] | Jan Ponert[2,3,5]

[1]Naturalis Biodiversity Center, Leiden, The Netherlands

[2]Institute of Botany, Czech Academy of Sciences, Průhonice, Czech Republic

[3]Department of Experimental Plant Biology, Faculty of Science, Charles University, Prague, Czech Republic

[4]Graduate School of Bioresources, Mie University, Tsu, Mie, Japan

[5]Prague Botanical Garden, Prague, Czech Republic

Correspondence
Jan Ponert
Email: ponert@natur.cuni.cz

Funding information
Akademie Věd České Republiky, Grant/Award Number: RVO 67985939; Grantová Agentura České Republiky, Grant/Award Number: ThiefHunt GN23-05310O

Handling Editor: Sandra Varga

Abstract

1. Nitrate is a well-known regulator of several processes in plants, including seed germination. It generally induces germination, but its effect is species-specific, and germination of some species is even inhibited. It is not clear what drives these differences.
2. Nevertheless, it is striking that the germination of initially mycoheterotrophic orchids is generally inhibited by nitrate, indicating that this may be an adaptive trait allowing the seeds to detect sites with low nutrient availability, favourable for mycoheterotrophic plants. Whether this unusual negative response to nitrate is a general feature of mycoheterotrophic plant germination remains unknown.
3. We focused on the family Ericaceae, which contains lineages that are either fully or initially mycoheterotrophic together with autotrophic ones, and we tested the germination response of selected species to nitrate.
4. We found high variability in the germination responses even within members of this family. Fully and initially mycoheterotrophic species reacted more negatively to nitrate compared to autotrophic species. However, the species responses to nitrate also strongly correlated with seed dimensions. As a control, we also tested responses to another common nitrogen form, ammonium ion, but these showed little or no correlations with the tested predictors indicating that the observed germination responses are specific to nitrate.
5. Two factors may be responsible for the negative response of mycoheterotrophic species of both Ericaceae and orchids to nitrate: seed size and the mode of carbon acquisition (mycoheterotrophy); nevertheless, both factors are closely linked. Thus, mycoheterotrophic plants in general, which nearly all produce tiny dust seeds, are likely to be negatively affected by nitrate.

KEYWORDS
ammonium, carbon acquisition mode, Ericaceae, mycoheterotrophy, nitrate, nitrogen, seed anatomy

1 | INTRODUCTION

Nitrogen is one of the most important nutrients for plants usually taken up from the soil. Most of the soil nitrogen is bound in soil organic matter, which is not directly accessible to plants. The activity of soil microorganisms and inputs from external sources produces readily accessible forms of soil nitrogen. These include nitrate, ammonium and some small organic molecules (Farzadfar et al., 2021). Nitrate is regarded to be the most important form of nitrogen for plants growing in aerobic soils (Glass, 2009; Maathuis, 2009). However, nitrogen is also a key nutrient for other organisms what creates a strong competition for nitrate between different soil organisms (Kuzyakov & Xu, 2013). Thus, plants must possess efficient mechanisms for its uptake and sensing. To date, two nitrate receptors have been identified: NRT1.1 nitrate transporter (Ho et al., 2009) and the transcription factor NIN-like protein 7 (Liu et al., 2022). The ability to sense soil nitrate allows plants to redirect root growth to sites with higher nitrate concentrations (Krouk et al., 2010; Liu et al., 2020) and also to time seed germination so that the plant emerges in a site with adequate nitrate abundance (Alboresi et al., 2005; Duermeyer et al., 2018). This can be used by seeds of some plants to detect gaps in vegetation where nitrate is not taken up by roots of other plant competitors (Pons, 1989).

However, nitrate does not stimulate the germination of seeds in all plants. While the majority of species studied up to date are stimulated, some are insensitive, and germination of others is even inhibited (Boudell & Stromberg, 2015; Figura et al., 2020; Luna & Moreno, 2009; Ponert et al., 2013). It is not clear what drives these differences. Studies investigating autotrophic plants have concluded that the germination response to nitrate seems to be independent of both phylogenetic relationships and ecological requirements (Boudell & Stromberg, 2015; Luna & Moreno, 2009). Up to date, five eudicot autotrophic species whose germination is inhibited by nitrate have been identified (Boudell & Stromberg, 2015; Luna & Moreno, 2009). However, these results need to be interpreted with caution because (i) nitrate was applied as KNO_3, thus providing also potassium; (ii) the KNO_3 solution treatments were compared with distilled water, which may have adverse effects due to its low osmolarity; and (iii) the nitrate concentrations tested (20–75 mM) were far exceeding those naturally occurring in soil solutions (usually far below 1 mM; see Figura et al., 2020).

Other studies focused on germination of initially mycoheterotrophic orchids (monocots), that is, plants that rely in their initial growth on carbon derived from root-associated mycorrhizal fungi. They tested addition of low naturally occurring nitrate concentrations (~0.01–2 mM) to nutrient solution in vitro, thus eliminating osmotic effect of distilled water (Figura et al., 2020, 2021; Ponert et al., 2013). Orchid germination was found to be generally inhibited by nitrate with clear differences between species—some responding to extremely low concentrations while others being nearly insensitive (Figura et al., 2020). The differences in responses to nitrate observed in vitro were related to species ecological preferences and the soil nitrate content in native sites indicating that the germination response of initially mycoheterotrophic orchids to nitrate may be adaptive (Figura et al., 2020). Mycoheterotrophic orchids generally prefer soils with low available nutrients, and they obtain nitrogen from symbiotic fungi (Fochi et al., 2017; Li et al., 2022), so their negative response to nitrate could help their seeds to detect suitable patches (Figura et al., 2020). However, any other group of mycoheterotrophic plants have not been tested so it is unknown whether such a negative response to nitrate is related to mycoheterotrophy in general or not. Orchid seeds are not only adapted to mycoheterotrophic germination, but they also have very reduced structure including undifferentiated embryo and they lack endosperm (Yeung, 2017). The more negative response of orchids to nitrate in comparison to autotrophic plants might therefore also be a result of a different seed structure.

In this study, we focused on eudicot family Ericaceae which offers unique opportunity to compare responses to nitrate of closely related autotrophic and mycoheterotrophic species. This family consists mainly of autotrophic species with fully developed seeds, but two of its evolutionary lineages have adapted to mycoheterotrophy—the subfamily Pyroloideae comprises initially mycoheterotrophic species (Lallemand et al., 2017; Matsuda et al., 2020) and the subfamily Monotropoideae which comprises fully mycoheterotrophic species (Lallemand et al., 2016; Rose et al., 2018). Species from both of these subfamilies possess reduced dust seeds, similar to orchid seeds but differing in the presence of a minute endosperm (Copeland, 1947; Olson, 1980; Pyykkö, 1968; Takahashi, 1993). We therefore decided to analyse germination responses to nitrate and seed structure of selected species from different lineages of Ericaceae to identify whether the response to nitrate relates to nutritional mode and seed structure. As a control, we also analysed responses to ammonium ion to see whether the observed effects are responses to nitrate itself or to nitrogen in general.

2 | MATERIALS AND METHODS

2.1 | Plant material

We have selected representatives of all four genera of the initially mycoheterotrophic subfamily Pyroloideae (*Chimaphila umbellata*, *Moneses uniflora*, *Orthilia secunda* and *Pyrola minor*), one representative of the mycoheterotrophic subfamily Monotropoideae (*Monotropa uniflora*) and seven species from the autotrophic lineages of Ericaceae to cover the whole phylogenetic range (*Arbutus unedo*, *Enkianthus campanulatus*, *Ledum groenlandicum*, *Kalmia latifolia*, *Oxycoccus macrocarpus*, *Phyllodoce caerulea* and *Vaccinium myrtillus*) (Table S1). As controls, we used (i) the well-known experimental model *Arabidopsis thaliana* (Brassicaceae), which is known to react positively to nitrate (Bouguyon et al., 2015; Zhang et al., 1999), and (ii) orchid *Dactylorhiza majalis*, which is known to react negatively to nitrate (Figura et al., 2020, 2021). The effect of nitrate was also tested on the initially mycoheterotrophic *Pyrola rotundifolia* from subfamily Pyroloideae, but

we were unable to obtain enough seeds to perform all experiments with this species (Table S1). In the species with dry capsules, whole ripe fruits were collected (Table S2), dried at room temperature and manually extracted seeds were stored in the dark and dry conditions at +4°C. In the case of species with berries, the seeds were sown immediately after manual extraction from the fruits (Table S2).

2.2 | Seed disinfection, sowing and cultivation

We used axenic in vitro cultivation system and medium BM-1 (Himedia, Van Waes & Debergh, 1986) supplemented with 0,5 g L^{-1} of activated charcoal, free from inorganic nitrogen which previously turned out to be most suitable for axenic cultivation of pyroloids (Figura et al., 2019) and which we found to be also suitable for autotrophic Ericaceae. Medium pH was adjusted to 5.8 with NaOH, autoclaved at 144 kPa, 121°C (Tuttnauer 2540 EK-N) for 20 min and poured into 5 cm plastic Petri dishes. To test the effect of nitrate and ammonium ions, we added $NaNO_3$ or $(NH_4)_2SO_4$, respectively, before autoclaving so the final concentration was 0, 1, 10 or 100 mg L^{-1} of NO_3^- or NH_4^+, respectively, in the culture medium. These concentrations correspond to naturally occurring nitrate concentrations in soil solutions and have been used in previous studies investigating the effect of nitrate on mycoheterotrophic seed germination (Figura et al., 2019, 2020, 2021; Ponert et al., 2013). The seeds were disinfected in 5 mL syringes and sown as suspension of disinfected seeds in sterile deionised water as described previously (Ponert et al., 2011, 2013). Briefly, seed were pre-incubated in 70% ethanol, washed three times with deionised water (conductivity <0.2 µm cm^{-1}), treated with 2% H_2SO_4 (or not, see Table S1) and subsequently with the solution of $Ca(OCl)_2$ or NaOCl (we had to use NaOCl in *Vaccinium myrtillus* because the disinfection with $Ca(OCl)_2$ solution was not successful and led to high level of contaminations), washed three times with sterile deionised water (time in each disinfectant was optimised for each species, see Table S1) and sown as a suspension of seeds onto culture medium in Petri dishes. $Ca(OCl)_2$ solution was prepared by dissolving 20 g chlorinated lime (Kittfort, Czech Republic) in 100 mL deionised water, filtering through filter paper and adding a drop of Tween 20 following Figura et al. (2019). This solution was used within 30 min after filtering. NaOCl solution was prepared by diluting of Savo Original (commercially available 'household bleach'; Unilever ČR, Czech Republic) to a final NaOCl concentration of 9.4 g L^{-1}. Seven Petri dishes (replicates) sealed with air permeable foil (Parafilm M) were prepared for each experimental variant. The cultures were incubated in the dark at 20°C.

2.3 | Anatomical analysis

Dry seeds of all species were fixed in 4% formaldehyde. Cryosections (20 µm) were prepared on a Shandon cryomicrotome after 24 h incubation of samples in 2% sucrose solution for cryoprotection. Hand sections (app. 100 µm) were prepared on a Leica hand microtome (Soukup & Tylová, 2014). Lipids were detected with Sudan Red 7B (1 h) according to (Brundrett et al., 1991). Proteins were stained with Ponceau 2R in 2% acetic acid (10 min) and Azur II (10 s) according to Gutmann et al. (1996). Starch was detected by staining with Lugol's solution. Sections were mounted in 50% glycerol. An Olympus BX51 microscope (Olympus Corp., Tokyo, Japan) equipped with an Apogee U4000 digital camera (Apogee Imaging Systems, Inc., Roseville, CA, USA) was used for observation. For scanning electron microscopy (SEM), dry seeds were coated with gold (2 nm thin layer) in an ion sputter coater (Bal-Tec SCD 050) and observed with a JEOL JSM-IT 200 microscope.

To measure seed dimensions, longitudinal sections of seeds (in a medial plane) were analysed using NIS Elements AR 3.22.15 (Laboratory Imaging, Prague, Czech Republic). We estimated the total length and width of the seed (including seed coat) and the length and width of the living part of the seed (embryo plus endosperm, if present; excluding seed coat) and used mean values per species ($n = 5$) for further analyses (Table S2).

2.4 | Data analysis

Cultures were observed weekly using a Zeiss Stemi 305 steroeomicroscope (every 3 days in the case of *A. thaliana* which germinates faster). Because different species germinated at different times, the total germination rate was counted at the stage when germination rate did not increase further (see Table S1). To count germination rate, seeds without embryo or with obviously undeveloped embryo were excluded and only well-developed seeds with ruptured testa were considered as germinated seeds following Figura et al. (2019). Pictures of seedlings were taken with a Canon EOS 60D equipped with a Canon EF 100 mm f/2.8 L macro lens.

Statistical analyses were performed in R software (version 3.6.1; R Core Team, 2021, n.d.) and the significance level was $p < 0.05$ in all analyses. The obtained data of germination rates can be analysed in several different ways, so here we decided to use two alternative approaches which were used with the same type of data previously. The first one follows Figura et al. (2021). We treated the four tested nitrogen concentrations (0, 1, 10, 100 mg L^{-1}) as discrete values and tested the significant differences between the four experimental variants as independent groups separately for each species. Normality of data was tested using Shapiro–Wilk test (Shapiro & Wilk, 1965) and homogeneity of variances was tested using Bartlett test (Bartlett, 1937). Differences between the measurements were statistically tested with ANOVA, followed by the Tukey–Kramer test (Kramer, 1956) for homogeneous data with a normal distribution. Non-homogenous data with normal distribution were treated with Welch ANOVA (Welch, 1951) followed by the Games-Howell post hoc test (Toothaker, 1993). Data which did not have normal distribution were analysed using the Kruskal–Wallis test (Kruskal & Wallis, 1952), followed by pairwise comparisons using Wilcoxon's rank-sum test (Table S3).

As the above-mentioned approach treating the tested nitrogen concentrations as discrete values do not allow rigorous comparison

between species, we used another approach treating nitrogen concentration as a continuous variable further. This second approach mostly follows Figura et al. (2020) and allows us to characterise the strength of nitrogen effect on germination rate of respective species (as specified below), to compare the species responses between and to test differences in responses to nitrogen between groups of species. The relationship between the proportion of germinated seeds and the nitrogen content in medium was modelled separately for each species using a generalised linear model (GLM) with quasi-binomial structure of errors and logit link function, as implemented in R software (version 3.6.1; R Core Team, 2021). To compare these modelled species-specific responses to nitrogen between, we used two further approaches to characterise the relationship between the concentration of nitrogen and the germination rate with a single number for each species. (Table S4). First, we used the models to predict germination rates at two nitrogen concentrations: 0 and 50 mg/L, and we used these values to calculate the predicted percentual change in germination rate at nitrogen concentration 50 mg/L compared to germination rate at 0 mg/L (similar as in Figura et al., 2020). The second approach was to directly compare the model parameters characterising the effect of nitrogen concentration (Table S4). Both statistical approaches reached similar conclusions. In both cases, the tested species were categorised using species traits related to seed structure and germination (see Table S4) and the differences in the predicted percentual change in germination rate and the model parameters between the categories of species were tested with the Wilcoxon test (Table S4, category 'autotrophic_dust' contains only two values so it had to be omitted from the statistical comparisons). Classification of seeds as 'dust seeds' was based on their size and followed that of Martin (1946). We included also the category of fruit type because the seeds from berries are likely to be dispersed endozoochorously which might cause a different regulation of their dormancy.

Furthermore, we analysed the relationship between the predicted percentual change in germination rate and seed dimensions. We used only the predicted change in germination rate, in this case because it got very similar results with direct comparison of the model parameters characterising the effect of nitrogen. For seed dimensions, we used mean values per species. We calculated Person's correlation coefficients (r) and coefficients of determination for linear regression (R^2). We analysed not only all species together but we also performed separate analyses for autotrophic and mycoheterotrophic species. However, the number of species was low for correct interpretation of these separate analyses, especially in mycoheterotrophic species (only 5 species tested).

3 | RESULTS

3.1 | Anatomical observations

The seeds of all the initially mycoheterotrophic and mycoheterotrophic species (*Dactylorhiza majalis*, *Monotropa uniflora* and all Pyroloids) contain undifferentiated, globular embryos, while the seeds of all autotrophic species have embryos with well-defined cotyledons (Figure 1; Figures S1 and S2). Endosperm was lacking in *Dactylorhiza majalis*, but visible in all other species. All autotrophic species of Ericaceae (*Enkianthus campanulatus*, *Arbutus unedo*, *Kalmia latifolia*, *Ledum groenlandicum*, *Oxycoccus macrocarpus*, *Phyllodoce caerulea* and *Vaccinium myrtillus*) had a thick multilayered endosperm of variable thickness. Initially mycoheterotrophic (*Chimaphila umbellata*, *Moneses uniflora*, *Orthilia secunda*, *Pyrola minor*) and mycoheterotrophic (*Monotropa uniflora*) representatives of Ericaceae had endosperm consisting of few cells surrounding the globular embryo. The mature seed of *Arabidopsis thaliana* contained a single-cell endosperm layer (Figure 1), as the endosperm is partially absorbed by the growing embryo during the final phase of seed development (see also Verma et al., 2022).

Histochemical detections identified abundant storage lipids and proteins in all analysed seeds (Figures S3 and S4). None of the seeds showed a positive reaction to starch with Lugol's solution (Figures S3 and S4). Seeds of mycoheterotrophic species were generally smaller than those of autotrophic species (Figure S5). The similar but weaker difference was also significant for groups of species with different fruit types (Figure S5). The germination of all autotrophic plants was bipolar, and these plants developed soon after germination two well-developed cotyledons as well as roots (Figure S6). The germination of all initially mycoheterotrophic and mycoheterotrophic species was unipolar, resulting in the development of a protocorm that later formed roots or shoots (Figure S6).

3.2 | Effects of inorganic nitrogen

All species tested germinated successfully, but even the germination rate on medium without inorganic nitrogen varied significantly between species, spanning from less than 0.5% in *Monotropa uniflora* to almost 100% in *Oxycoccus macrocarpus* (Figure 2; Figures S7 and S8). The effects of nitrate and ammonium ions on germination rate varied between species from inhibition to stimulation (Table S4; Figure 2; Figures S7 and S8) and the effects also frequently differed even within the same plant species (Table S4; Figure 2; Figure S8). Both species used as controls reacted to nitrate as expected, that is, germination of *Arabidopsis thaliana* was stimulated (Figure 2; Figure S8) and that of *Dactylorhiza majalis* was inhibited (Figure 2; Figure S8). Within the Ericaceae, germination of all initially mycoheterotrophic and mycoheterotrophic species was inhibited by nitrate, while the response of autotrophic species varied from a weak inhibition to stimulation (Table S4; Figure 2; Figures S7 and S8). Thus, we compared the modelled species responses to nitrate (GLM) between these groups of species and we found this difference being highly significant, regardless of whether the predicted changes in germination rate or the model parameters were used (Figure 3; Figure S9). Interestingly, the difference between species with different fruit types was not significant (Figure 3; Figure S9). We further attempted to separate the autotrophic species into two groups according to seed size (large/dust). However, only two species were available

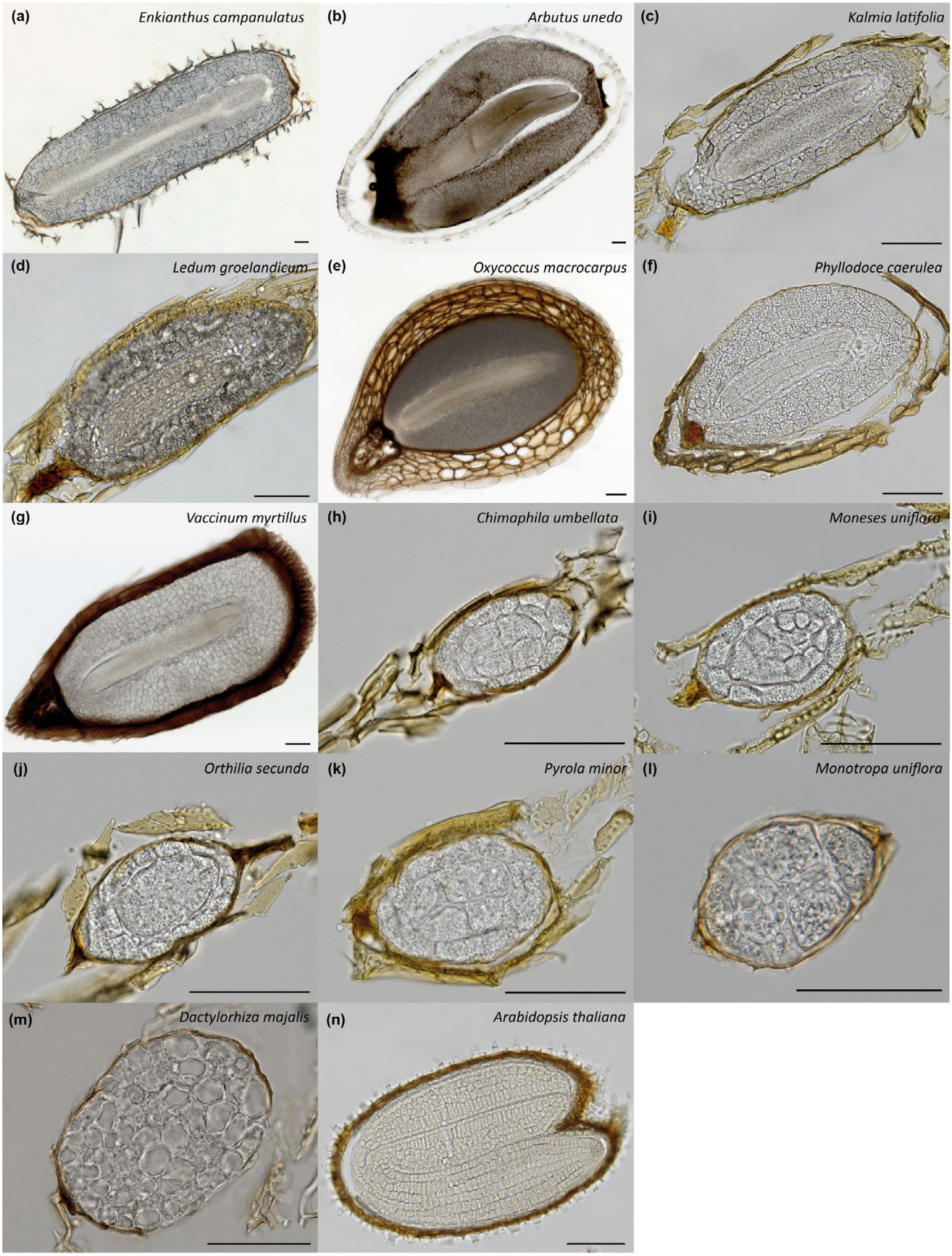

FIGURE 1 Seed anatomy of all studied species. *Enkianthus campanulatus* (a), *Arbutus unedo* (b), *Kalmia latifolia* (c), *Ledum groenlandicum* (d), *Oxycoccus macrocarpus* (e), *Phyllodoce caerulea* (f), *Vaccinium myrtillus* (g), *Chimaphila umbellata* (h), *Moneses uniflora* (i), *Orthilia secunda* (j), *Pyrola minor* (k), *Monotropa uniflora* (l), *Dactylorhiza majalis* (m), *Arabidopsis thaliana* (n). Cryosections (a, c, d, f, h–n), hand sections (b, e, g), bright-field (a–n), scale bars = 100 μm (a–n).

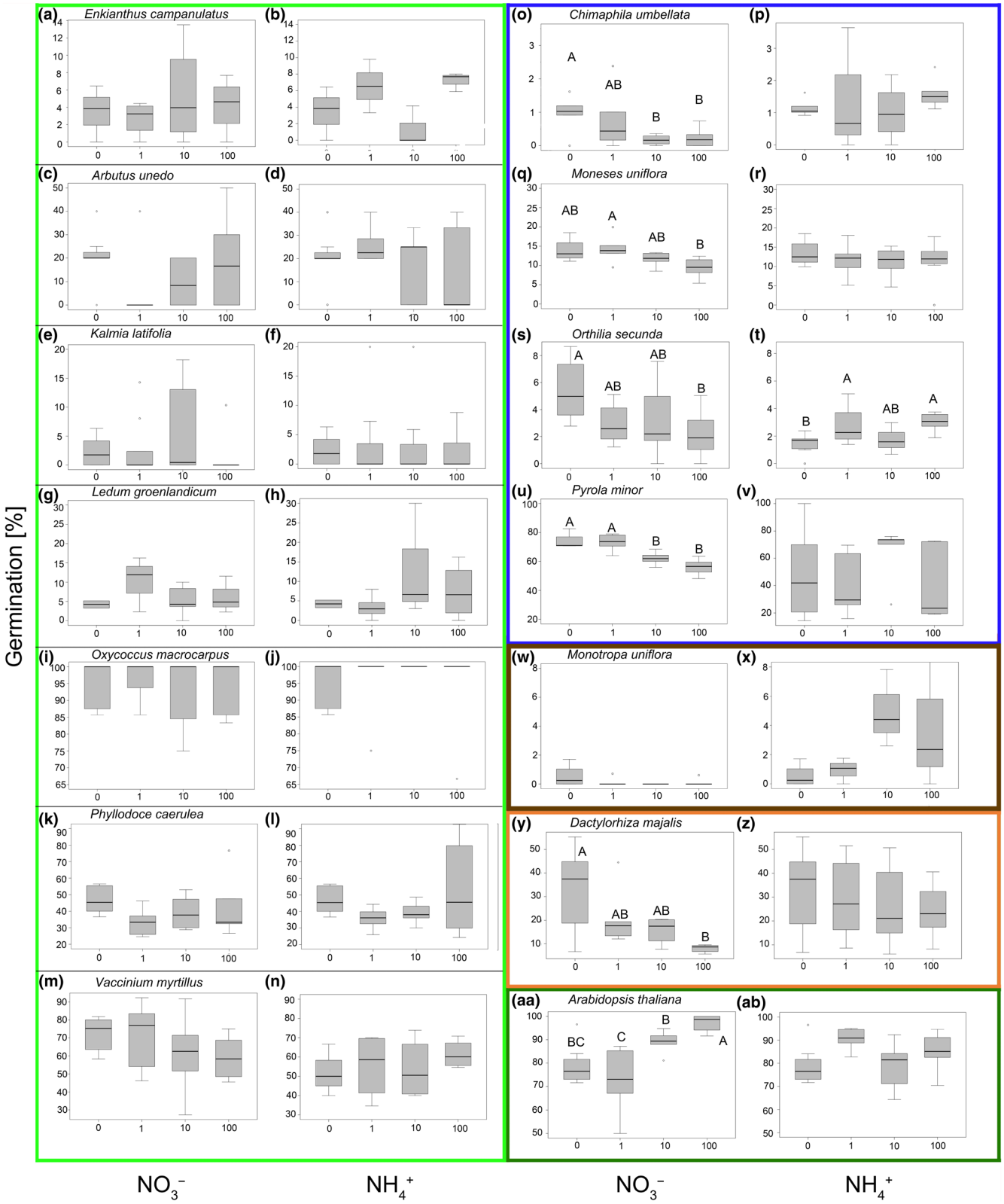

FIGURE 2 Effects of nitrate and ammonium ion on in vitro germination of the studied plants. The four different concentrations of nitrogen are treated here as groups and details of statistical analyses are given in Table S3. Germination rate [%] was counted 3 months after sowing. Concentrations are in mg L^{-1}. Graphs for each species are in pairs: Left graphs represent the effect of nitrate and right that of ammonium ion. *Enkianthus campanulatus* (a, b), *Arbutus unedo* (c, d), *Kalmia latifolia* (e, f), *Ledum groenlandicum* (g, h), *Oxycoccus macrocarpus* (i, j), *Phyllodoce caerulea* (k, l), *Vaccinium myrtillus* (m, n), *Chimaphila umbellata* (o, p), *Moneses uniflora* (q, r), *Orthilia secunda* (s, t), *Pyrola minor* (u, v), *Monotropa uniflora* (w, x), *Dactylorhiza majalis* (y, z) and *Arabidopsis thaliana* (aa, ab). Ericaceaous autotrophic plants are framed pale green (a–n), initially mycoheterotrophic ericaceous plants by blue (o–v), fully mycoheterotrophic ericaceous plant by dark brown (w, x), fully mycoheterotrophic orchid by pale brown (y, z) and non-ericaceous autotrophic plant by dark green (aa–ab).

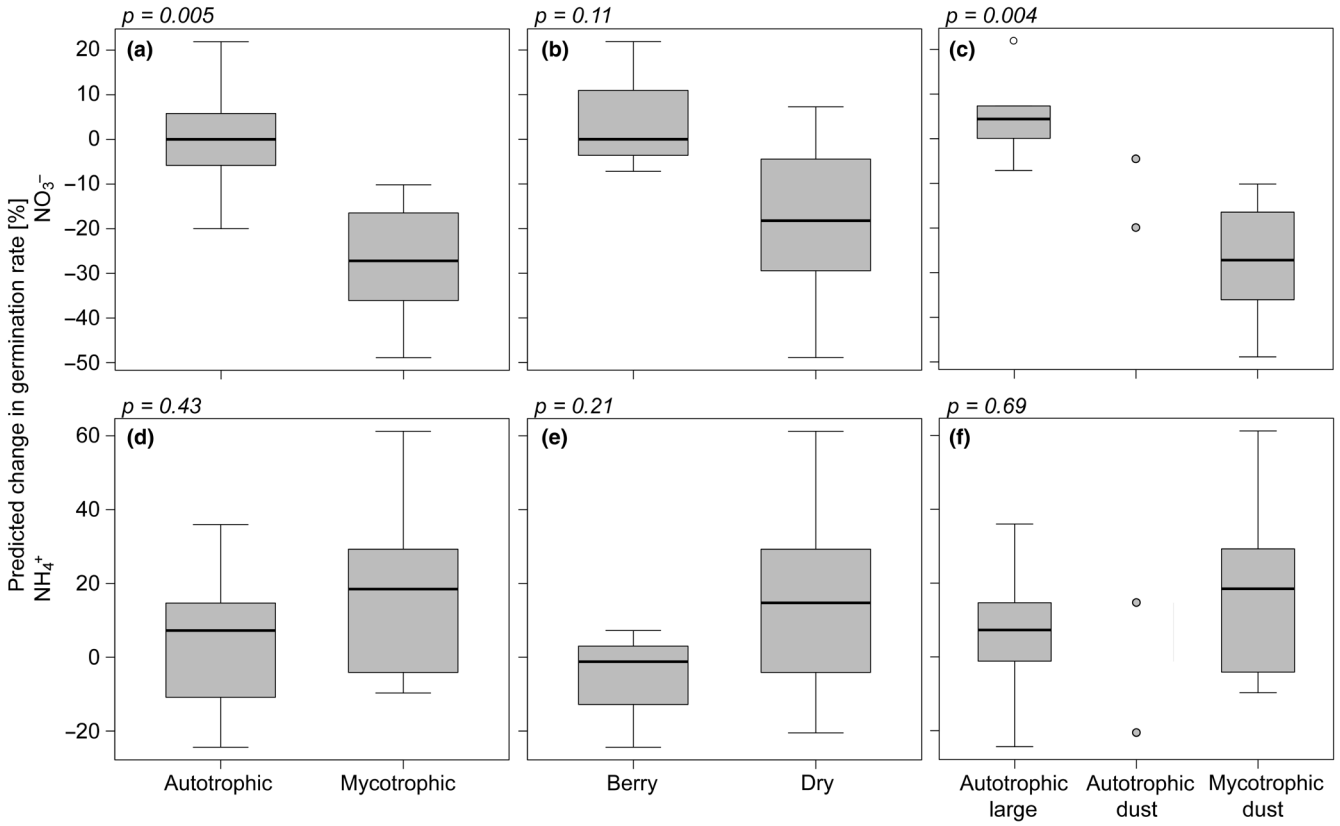

FIGURE 3 Comparison of modelled species responses to inorganic nitrogen between groups of species. Predictions were based on generalised linear models (GLM) with quasi-binomial structure of errors and logit link function (Figure S1). MH—mycoheterotrophic species. The predicted change in germination rate was calculated as a percent change in germination rate at nitrogen concentration 50 mg/L compared to 0 mg/L. Differences between groups we tested with Wilcoxon test. Only two species were available for the group of 'autotrophic dust' in (c) and (f), so this was excluded from statistical comparison. (a, d) Differences between autotrophic and mycoheterotrophic taxa; (b, e) differences between species with dry fruits and with berries; (c, f) the group of autotrophic plant from (a), (d) was further subdivided into species with dust seeds and larger seeds.

from the group of autotrophic species with dust seeds, so this category could not be included in the statistical analyses, but interestingly, the responses of these species were more or less intermediate between the other two categories (Figure 3; Figure S9).

The modelled response of Ericaceae to ammonium ion showed no significant difference between the tested species groups (Figure 3; Figure S9). However, it should be noted that the germination of the fully mycoheterotrophic *Monotropa uniflora* responded slightly positively to ammonium ion, with the highest germination rates ever reported for this species in the presence of high concentrations of ammonium ion (9.2%; Figure 2; Figure S8). The general trend of differences in responses to ammonium ion between the groups of mycoheterotrophic and autotrophic species was opposite to the difference in response to nitrate (Figure 3; Figure S9).

Analyses of the relationships between seed dimensions and the modelled species responses to nitrate showed a strong positive correlation in the case of nitrate, but a relatively weak negative correlation in the case of ammonium ion (Figure 4). When the autotrophic and mycoheterotrophic species were analysed separately, we found a positive correlation for nitrate in the case of autotrophic plants, especially in the dimensions of the living part of the seed (Figure S10). In other cases, correlations were less evident, although it should be noted that the number of species analysed was low (Figure S10).

4 | DISCUSSION

Nitrate is one of the most studied signals breaking seed dormancy. However, its effects are species-specific and it may not only induce germination but also repress it (Boudell & Stromberg, 2015; Figura et al., 2020; Luna & Moreno, 2009; Ponert et al., 2013). Our results obtained in axenic in vitro cultivation system show high variability in the germination responses to nitrate even within members of a single eudicot family, Ericaceae, ranging from stimulation to inhibition. This may not be surprising, as studies investigating eudicots indicated that the germination responses to nitrate are likely to be independent of both phylogenetic relationships and ecological requirements (Boudell & Stromberg, 2015; Luna & Moreno, 2009). However, in orchids (monocots), the sensitivity of seeds to nitrate is likely adaptive, as indicated by its correlation with species pedological requirements (Figura et al., 2020). This could be explained as a difference between eudicots and monocots.

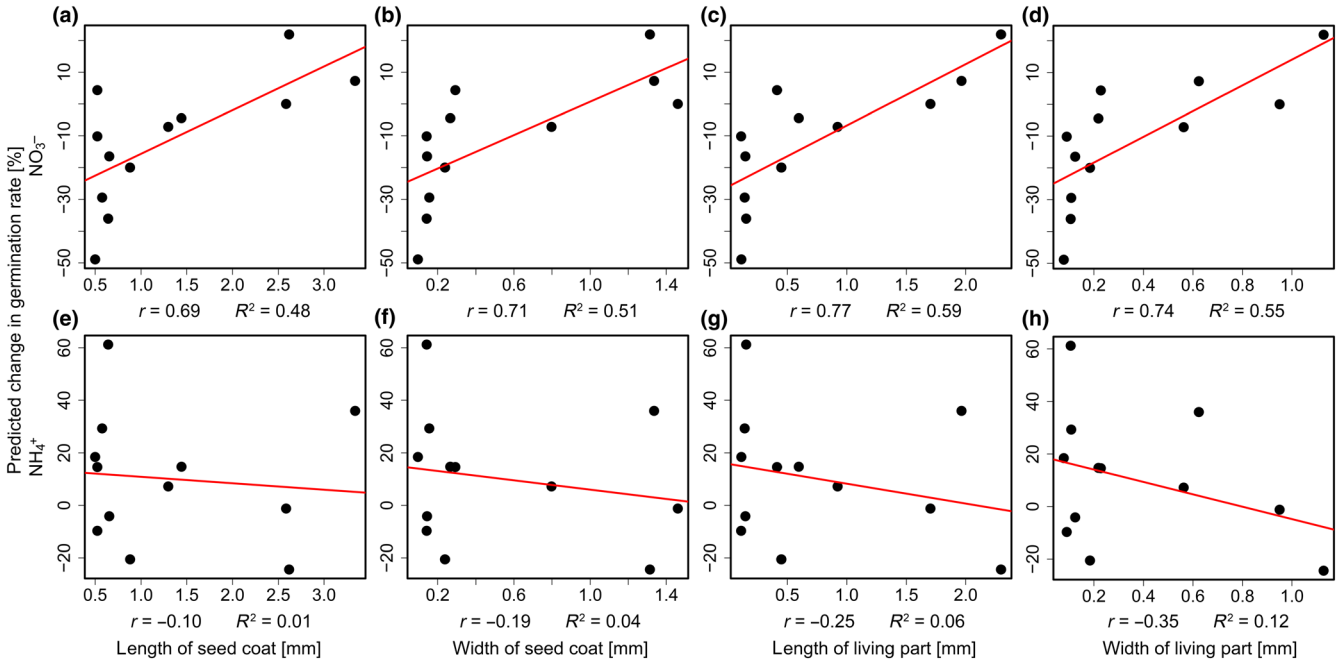

FIGURE 4 Relationships between modelled species responses to inorganic nitrogen and seed dimensions. All studied species are analysed together. The predictions of responses to nitrogen were based on generalised linear models (GLM) with quasi-binomial structure of errors and logit link function (Figure S1). The predicted change in germination rate was calculated as a percent change in germination rate at nitrogen concentration 50 mg/L compared to 0 mg/L.

However, all orchid species studied are initial mycoheterotrophs with extremely reduced seeds lacking endosperm, whereas all eudicot species studied up till now are fully autotrophic plants with normally developed seeds, raising the possibility of another causal factors. It has been proposed that the inhibitory response of orchids to nitrate may be related to their dependence on mycorrhizal symbiosis and the ability to obtain nitrogen from symbiotic fungi (Figura et al., 2020). To address this complex situation, we focused on the family Ericaceae which contains lineages of fully or initially mycoheterotrophic species together with autotrophic ones. We modelled the germination responses to nitrate separately for each species using generalised linear models (GLM), what allows us to compare species responses between groups of species. We found that the germination of fully and initially mycoheterotrophic species of this family is more negatively affected by nitrate than the germination of autotrophic species. Whereas autotrophic species were stimulated by nitrate or insensitive or weakly inhibited, all fully and initially mycoheterotrophic species were inhibited by nitrate and frequently more intensively comparing with autotrophic species. This is similar to the results obtained with initially mycoheterotrophic orchids all of which were at least partially inhibited (Figura et al., 2020). This suggests that the unusually negative germination response to nitrate observed in these two groups may be related to mycoheterotrophy or to some associated factors such as seed structure. Seeds of both above-mentioned mycoheterotrophic groups, orchids and mycoheterotrophic Ericaceae, are reduced to 'dust seeds' with tiny globular embryos. Nevertheless, seeds of Ericaceae have tiny endosperm (e.g. Christoph, 1921; Figura et al., 2019; Fürth, 1920; Olson, 1980; data in this article) while orchid seeds lack it (Arditti & Ghani, 2000). It is therefore likely that the absence of endosperm in orchid seeds is not the cause of their negative reaction to nitrate.

However, it remains unclear whether the more negative response of mycoheterotrophic species is due to their mycoheterotrophic germination or due to their extremely reduced dust seeds. Thus, we compared between not only groups of autotrophic and mycoheterotrophic species but also two other categories related to seed size. Some of the studied species produce berries specialised to endozoochorous dispersal, so we compared them with species-producing dry fruits (Debussche & Isenmann, 1989; Heinken et al., 2002; Jaroszewicz et al., 2023). The difference between these groups was not significant, but all the species strongly inhibited by nitrate were present in category of dry fruits. However, this likely stems from the fact that all mycoheterotrophic species which have been tested have dry fruits (Eriksson & Kainulainen, 2011). The seeds of mycoheterotrophic species are generally much smaller than those of autotrophic ones (Leake, 1994) and we found the same difference within Ericaceae. It could therefore be hypothesised that the size of seeds may predetermine their response to nitrate. To test this hypothesis, we performed two types of analyses. First, some autotrophic Ericaceae (*Kalmia latifolia*, *Ledum groenlandicum*) produce tiny anemochorous seeds, comparable in size to the dust seeds of mycoheterotrophs. Despite the fact that they contain embryos with developed cotyledons, they might also be considered as dust seeds (e.g. Eriksson & Kainulainen, 2011). When we classified

these taxa separately from autotrophic species with large seeds and from mycoheterotrophic species, we found their response to nitrate being intermediate between these groups. Unfortunately, we only studied two species from this group (*Kalmia latifolia* and *Ledum groenlandicum*) which does not allow statistical testing of significances. Thus, we also compared species responses to nitrogen with the measured dimensions of their seeds and we found a strong positive correlation for nitrate while only a weak correlation for ammonium ion. However, because mycoheterotrophic species which are highly sensitive to nitrate produce the smallest seeds, it is possible that this relationship is driven by them. Thus, we further analysed the same relationship separately for autotrophic and mycoheterotrophic species. It should be noted that the number of studied species was relatively low for these separate analyses, especially in mycoheterotrophic species where the differences in seed dimensions are very small, and only the five species tested in this paper are not sufficient in number to correctly identify the correlation. Nevertheless, the available data indicate that the correlation between species responses to nitrate and seed dimensions exists also at least within autotrophic species. Therefore, we cannot exclude the possibility that the seed size itself predetermines seed response to nitrate.

Taken together, two factors may be responsible for the negative response of mycoheterotrophic species of Ericaceae and orchids to nitrate: seed size and mode of carbon acquisition (autotrophic vs. mycoheterotrophic). To disentangle these effects, a significantly larger number of species would need to be studied, including those from other groups of mycoheterotrophic plants. It would be particularly interesting to include some of a few mycoheterotrophic species with relatively large seeds (e.g. Triuridaceae; Suetsugu et al., 2017) or in those with fleshy fruits (e.g. Suetsugu, 2018). Although we are unable to disentangle the effects of these two factors, our results allow interesting conclusions to be drawn. Both groups of mycoheterotrophic plants studied up to date respond more negatively to nitrate than other plants despite their bodies are for yet unknown reasons enriched by nitrogen (Minasiewicz et al., 2023). As nearly all mycoheterotrophic plants have tiny dust seeds (Leake, 1994; Merckx, 2013), it can be expected that germination of nearly all mycoheterotrophic species will be negatively affected by nitrate. Indeed, a recent study by Gomes et al. (2019) showed that tropical mycoheterotrophic plants associated with arbuscular mycorrhizal fungi avoided nitrate-rich patches in the rainforest. It has also been observed that at least some pyroloids prefer sites with low level of available nitrogen at maturity (Jacquemyn et al., 2018; Johansson & Eriksson, 2013). It is, therefore, possible that the inhibitory effect of nitrate on seed germination evolved convergently in dust seeded mycoheterotrophs and could allow these plants to find suitable patches for germination in a similar way to the stimulatory effect of nitrate on eutrophic species. Unfortunately, no representatives of any other group of mycoheterotrophic plants have yet been cultivated in vitro to further test this hypothesis. The unusually negative germination response of mycoheterotrophic plants to nitrate may also suggest that seed germination of mycoheterotrophic plants might be regulated differently from seed germination of autotrophic plants. It has been observed that germination of mycoheterotrophic orchids and Pyroloideae is not stimulated by gibberellins (e.g. Arditti, 1967; Figura et al., 2019; Miura et al., 2024; Rasmussen, 1995). This may suggest that seed germination of mycoheterotrophic plants may have more differences from autotrophic plants. Our results may also have important implications in the context of the global changes of environment. The concentrations of nitrate in soils are unstable in time (Březina et al., 2019) and the changes in agricultural practices combined with increasing atmospheric nitrate deposition are leading towards increasing nitrate levels in many areas of the world (Engardt et al., 2017; Rivett et al., 2008; Singer, 2012). It has been proposed that such an increase in nitrate levels could negatively affect some orchid populations (Figura et al., 2020), and our results indicate that also other mycoheterotrophic plants could be negatively affected. However, it should also be noted that we have not investigated the effects of symbiotic organisms on germination in this article. It has been shown that mycorrhizal fungi can modulate the response of orchids to nitrate (Figura et al., 2021), and this remains to be elucidated in Ericaceae. It would also be interesting to see if nitrate or the reaction to it can affect mycorrhizal symbiosis in mycoheterotrophic Ericaceae. In mycoheterotrophic orchids, seed insensitivity to gibberellins during germination has been shown to be related to inactivation of gibberellins and thus induction of mycorrhizal association (Miura et al., 2024). It cannot be ruled out that a similar mechanism could also operate in the case of nitrate. However, data on its effect on mycorrhiza of mycoheterotrophic Ericaceae are lacking.

AUTHOR CONTRIBUTIONS

Tomáš Figura: Conceptualisation, methodology, formal analysis, investigation, resources, writing—original draft, writing—review & editing, visualisation, funding acquisition. Edita Tylová: Methodology, investigation, writing—original draft, writing—review & editing, visualisation. Y. Matsuda: Resources, writing—review & editing. Martina Janoušková: Writing—review & editing, supervision. Jan Ponert: Conceptualisation, methodology, formal analysis, investigation, resources, writing—original draft, writing—review & editing, visualisation, funding acquisition, supervision.

ACKNOWLEDGEMENTS

We are grateful to the Montreal Botanical Garden for providing seeds of *C. umbellata* and *M. uniflora*, namely Julie Corbeil Walsh and Stephane Marc Bailleul. We would also like to thank Yvetta Šefrnová for help in the laboratory and Iva Jadrná for help with seed collection. The authors acknowledge the Viničná Microscopy Core Facility (VMCF of the Faculty of Science, Charles University) and the MEYS CR (LM2023050 Czech-BioImaging), for their support & assistance in this work. The study was supported by the Czech Science Foundation (project ThiefHunt GN23-05310O) and the Czech Academy of Sciences (long-term research development program RVO 67985939).

CONFLICT OF INTEREST STATEMENT

Tomas Figura reports that financial support was provided by Czech Science Foundation. Other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data deposited in the Dryad Digital Repository: http://doi.org/10.5061/dryad.gtht76hwv (Figura et al., 2024).

ORCID

Tomáš Figura https://orcid.org/0000-0001-5714-7810
Edita Tylová https://orcid.org/0000-0001-7239-8212
Yosuke Matsuda https://orcid.org/0000-0002-7001-3101
Martina Janoušková https://orcid.org/0000-0002-6041-8068
Jan Ponert https://orcid.org/0000-0003-3827-8086

REFERENCES

Alboresi, A., Gestin, C., Leydecker, M., Bedu, M., Meyer, C., & Truong, H. (2005). Nitrate, a signal relieving seed dormancy in Arabidopsis. *Plant, Cell & Environment*, *28*, 500–512. https://doi.org/10.1111/j.1365-3040.2005.01292.x

Arditti, J. (1967). Factors affecting the germination of orchid seeds. *Botanical Review*, *33*, 1–97. https://doi.org/10.1007/BF02858656

Arditti, J., & Ghani, A. (2000). Numerical and physical properties of orchid seeds and their biological implications. *New Phytologist*, *145*, 367–421.

Bartlett, M. (1937). Properties of sufficiency and statistical tests. *Proceedings of the Royal Society of London. Series A: Mathematical and Physical Sciences*, *160*, 268–282.

Boudell, J. A., & Stromberg, J. C. (2015). Impact of nitrate enrichment on wetland and dryland seed germination and early seedling development. *Journal of Vegetation Science*, *26*, 452–463. https://doi.org/10.1111/jvs.12258

Bouguyon, E., Brun, F., Meynard, D., Kubeš, M., Pervent, M., Leran, S., Lacombe, B., Krouk, G., Guiderdoni, E., Zažímalová, E., Hoyerová, K., Nacry, P., & Gojon, A. (2015). Multiple mechanisms of nitrate sensing by Arabidopsis nitrate transceptor NRT1.1. *Nature Plants*, *1*, 15015. https://doi.org/10.1038/nplants.2015.15

Březina, S., Jandová, K., Pecháčková, S., Hadincová, V., Skálová, H., Krahulec, F., & Herben, T. (2019). Nutrient patches are transient and unpredictable in an unproductive mountain grassland. *Plant Ecology*, *220*, 111–123. https://doi.org/10.1007/s11258-019-00906-3

Brundrett, M. C., Kendrick, B., & Peterson, C. A. (1991). Efficient lipid staining in plant material with sudan red 7B or fluoral yellow 088 in polyethylene glycol-glycerol. *Biotechnic & Histochemistry*, *66*, 111–116. https://doi.org/10.3109/10520299109110562

Christoph, H. (1921). Untersuchungen über mykotrophen Verhältnisse der 'Ericales' und die Keimung von Pirolaceen [in German]. *Beihefte Botanisches Centralblatt*, *38*, 115–157.

Copeland, H. F. (1947). Observations on the structure and classification of the Pyroleae. *Madrono*, *9*, 65–102.

Debussche, M., & Isenmann, P. (1989). Fleshy fruit characters and the choices of bird and mammal seed dispersers in a Mediterranean region. *Oikos*, *56*, 327–338.

Duermeyer, L., Khodapanahi, E., Yan, D., Krapp, A., Rothstein, S. J., & Nambara, E. (2018). Regulation of seed dormancy and germination by nitrate. *Seed Science Research*, *28*, 1–8. https://doi.org/10.1017/s096025851800020x

Engardt, M., Simpson, D., Schwikowski, M., & Granat, L. (2017). Deposition of sulphur and nitrogen in Europe 1900–2050. Model calculations and comparison to historical observations. *Tellus B: Chemical and Physical Meteorology*, *69*, 1328945. https://doi.org/10.1080/16000889.2017.1328945

Eriksson, O., & Kainulainen, K. (2011). The evolutionary ecology of dust seeds. *Perspectives in Plant Ecology, Evolution and Systematics*, *13*, 73–87. https://doi.org/10.1016/j.ppees.2011.02.002

Farzadfar, S., Knight, J. D., & Congreves, K. A. (2021). Soil organic nitrogen: An overlooked but potentially significant contribution to crop nutrition. *Plant and Soil*, *462*, 7–23. https://doi.org/10.1007/s11104-021-04860-w

Figura, T., Ponert, J., Tylová, E., Matsuda, Y., & Janoušková, M. (2024). Data from: Nitrate inhibition of germination in Ericaceae relates to seed size and mycoheterotrophy. *Dryad Digital Repository*, https://doi.org/10.5061/dryad.gtht76hwv

Figura, T., Tylová, E., Jersáková, J., Vohník, M., & Ponert, J. (2021). Fungal symbionts may modulate nitrate inhibitory effect on orchid seed germination. *Mycorrhiza*, *31*, 231–241. https://doi.org/10.1007/s00572-021-01021-w

Figura, T., Tylová, E., Šoch, J., Selosse, M.-A., & Ponert, J. (2019). *In vitro* axenic germination and cultivation of mixotrophic Pyroloideae (Ericaceae) and their post-germination ontogenetic development. *Annals of Botany*, *123*, 625–639. https://doi.org/10.1093/aob/mcy195

Figura, T., Weiser, M., & Ponert, J. (2020). Orchid seed sensitivity to nitrate reflects habitat preferences and soil nitrate content. *Plant Biology*, *22*, 21–29. https://doi.org/10.1111/plb.13044

Fochi, V., Chitarra, W., Kohler, A., Voyron, S., Singan, V. R., Lindquist, E. A., Barry, K. W., Girlanda, M., Grigoriev, I. V., Martin, F., Balestrini, R., & Perotto, S. (2017). Fungal and plant gene expression in the *Tulasnella calospora–Serapias vomeracea* symbiosis provides clues about nitrogen pathways in orchid mycorrhizas. *New Phytologist*, *213*, 365–379. https://doi.org/10.1111/nph.14279

Fürth, P. (1920). Zur Biologie und Mikrochemie einiger Pirola. *Sitzungsberichte der Akademie der Wissenschaften Wien, Mathematisch-Naturwissenschaftliche Klasse*, *1*, 559–587.

Glass, A. D. M. (2009). Nitrate uptake by plant roots. *Botany*, *87*(7), 659–667. https://doi.org/10.1139/B09-014

Gomes, S. I. F., van Bodegom, P. M., Merckx, V. S. F. T., & Soudzilovskaia, N. A. (2019). Environmental drivers for cheaters of arbuscular mycorrhizal symbiosis in tropical rainforests. *New Phytologist*, *223*, 1575–1583. https://doi.org/10.1111/nph.15876

Gutmann, M., von Aderkas, P., Label, P., & Lelu, M.-A. (1996). Effects of abscisic acid on somatic embryo maturation of hybrid larch. *Journal of Experimental Botany*, *47*, 1905–1917.

Heinken, T., Hanspach, H., Raudnitschka, D., & Schaumann, F. (2002). Dispersal of vascular plants by four species of wild mammals in a deciduous forest in NE Germany. *Phytocoenologia*, *32*, 627–643. https://doi.org/10.1127/0340-269X/2002/0032-0627

Ho, C. H., Lin, S. H., Hu, H. C., & Tsay, Y. F. (2009). CHL1 functions as a nitrate sensor in plants. *Cell*, *138*, 1184–1194. https://doi.org/10.1016/j.cell.2009.07.004

Jacquemyn, H., Waud, M., & Brys, R. (2018). Mycorrhizal divergence and selection against immigrant seeds in forest and dune populations of the partially mycoheterotrophic *Pyrola rotundifolia*. *Molecular Ecology*, *27*, 5228–5237. https://doi.org/10.1111/mec.14940

Jaroszewicz, B., Coissac, E., Taberlet, P., Czajkowska, M., Świsłocka, M., Kowalczyk, R., & Ratkiewicz, M. (2023). Is endozoochoric seed dispersal by large herbivores an evolutionary adaptation? Revisiting the Janzen's 'foliage is the fruit' hypothesis. *Acta Oecologica*, *118*, 103888. https://doi.org/10.1016/j.actao.2022.103888

Johansson, V. A., & Eriksson, O. (2013). Recruitment limitation, germination of dust seeds, and early development of the mycoheterotrophic *Pyrola chlorantha* (Ericaceae). *Botany*, *91*, 49–54. https://doi.org/10.1139/cjb-2012-0201

Kramer, C. (1956). Extension of multiple range tests to group means with unequal numbers of replications. *Biometrics*, *12*(3), 307–310.

Krouk, G., Lacombe, B., Bielach, A., Perrine-Walker, F., Malinska, K., Mounier, E., Hoyerova, K., Tillard, P., Leon, S., Ljung, K., Zazimalova, E., Benkova, E., Nacry, P., & Gojon, A. (2010). Nitrate-regulated auxin transport by NRT1.1 defines a mechanism for nutrient sensing in plants. *Developmental Cell*, *18*(6), 927–937. https://doi.org/10.1016/j.devcel.2010.05.008

Kruskal, W. H., & Wallis, W. A. (1952). Use of ranks in one-criterion variance analysis. *Journal of the American Statistical Association*, *47*(260), 583–621.

Kuzyakov, Y., & Xu, X. (2013). Competition between roots and microorganisms for nitrogen: Mechanisms and ecological relevance. *New Phytologist*, *198*(3), 656–669. https://doi.org/10.1111/nph.12235

Lallemand, F., Gaudeul, M., Lambourdière, J., Matsuda, Y., Hashimoto, Y., & Selosse, M. A. (2016). The elusive predisposition to mycoheterotrophy in Ericaceae. *New Phytologist*, *212*(2), 314–319. https://doi.org/10.1111/nph.14092

Lallemand, F., Puttsepp, Ü., Lang, M., Luud, A., Courty, P. E., Palancade, C., & Selosse, M. A. (2017). Mixotrophy in Pyroleae (Ericaceae) from Estonian boreal forests does not vary with light or tissue age. *Annals of Botany*, *120*(3), 361–371. https://doi.org/10.1093/aob/mcx054

Leake, J. (1994). The biology of myco-heterotrophic ('saprophytic') plants. *New Phytologist*, *127*(2), 171–216.

Li, M. H., Liu, K. W., Li, Z., Lu, H. C., Ye, Q. L., Zhang, D., Wang, J. Y., Li, Y. F., Zhong, Z. M., Liu, X., Yu, X., Liu, D. K., Tu, X. D., Liu, B., Hao, Y., Liao, X. Y., Jiang, Y. T., Sun, W. H., Chen, J., … Liu, Z. J. (2022). Genomes of leafy and leafless Platanthera orchids illuminate the evolution of mycoheterotrophy. *Nature Plants*, *8*(4), 373–388. https://doi.org/10.1038/s41477-022-01127-9

Liu, B., Wu, J., Yang, S., Schiefelbein, J., & Gan, Y. (2020). Nitrate regulation of lateral root and root hair development in plants. *Journal of Experimental Botany*, *71*(6), 1740–1750. https://doi.org/10.1093/jxb/erz536

Liu, K.-H., Liu, M., Lin, Z., Wang, Z.-F., Chen, B., Liu, C., Guo, A., Konishi, M., Yanagisawa, S., & Wagner, G. (2022). NIN-like protein 7 transcription factor is a plant nitrate sensor. *Science*, *377*(6605), 1419–1425.

Luna, B., & Moreno, J. M. (2009). Light and nitrate effects on seed germination of Mediterranean plant species of several functional groups. *Plant Ecology*, *203*(1), 123–135. https://doi.org/10.1007/s11258-008-9517-8

Maathuis, F. J. (2009). Physiological functions of mineral macronutrients. *Current Opinion in Plant Biology*, *12*(3), 250–258. https://doi.org/10.1016/j.pbi.2009.04.003

Martin, A. C. (1946). The comparative internal morphology of seeds. *American Midland Naturalist*, *36*(2), 513–660. https://doi.org/10.2307/2421457

Matsuda, Y., Yamaguchi, Y., Matsuo, N., Uesugi, T., Ito, J., Yagame, T., Figura, T., Selosse, M.-A., & Hashimoto, Y. (2020). Communities of mycorrhizal fungi in different trophic types of Asiatic *Pyrola japonica* sensu lato (Ericaceae). *Journal of Plant Research*, *133*(6), 841–853. https://doi.org/10.1007/s10265-020-01233-9

Merckx, V. S. F. T. (2013). *Mycoheterotrophy: The biology of plants living on fungi*. Springer. https://doi.org/10.1007/978-1-4614-5209-6

Minasiewicz, J., Zwolicki, A., Figura, T., Novotná, A., Bocayuva, M. F., Jersáková, J., & Selosse, M. A. (2023). Stoichiometry of carbon, nitrogen and phosphorus is closely linked to trophic modes in orchids. *BMC Plant Biology*, *23*, 254. https://doi.org/10.1186/s12870-023-04436-z

Miura, C., Furui, Y., Yamamoto, T., Kanno, Y., Honjo, M., Yamaguchi, K., Suetsugu, K., Yagame, T., Seo, M., Shigenobu, S., Yamato, M., & Kaminaka, H. (2024). Autoactivation of mycorrhizal symbiosis signaling through gibberellin deactivation in orchid seed germination. *Plant Physiology*, *194*(2), 546–563. https://doi.org/10.1093/plphys/kiad517

Olson, A. R. (1980). Seed morphology of *Monotropa uniflora* L. (Ericaceae). *American Journal of Botany*, *67*(6), 968–974.

Ponert, J., Figura, T., Vosolsobě, S., Lipavská, H., & Vohník, M. (2013). Asymbiotic germination of mature seeds and protocorm development of *Pseudorchis albida* (Orchidaceae) are inhibited by nitrates even at extremely low concentrations. *Canadian Journal of Botany*, *91*(9), 662–670. https://doi.org/10.1139/cjb-2013-0082

Ponert, J., Vosolsobě, S., Kmecová, K., & Lipavská, H. (2011). European orchid cultivation—From seed to mature plant. *European Journal of Environmental Sciences*, *1*(2), 95–107. https://doi.org/10.14712/23361964.2015.52

Pons, T. L. (1989). Breaking of seed dormancy by nitrate as a gap detection mechanism. *Annals of Botany*, *63*(2), 139–143. https://doi.org/10.1093/oxfordjournals.aob.a087716

Pyykkö, M. (1968). Embryological and anatomical studies on Finnish species of the Pyrolaceae. *Annales Botanici Fennici*, *5*(2), 153–165.

R Core Team. (2021). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Rasmussen, H. N. (1995). *Terrestrial orchids: From seed to mycotrophic plant*. Cambridge University Press.

Rivett, M. O., Buss, S. R., Morgan, P., Smith, J. W. N., & Bemment, C. D. (2008). Nitrate attenuation in groundwater: A review of biogeochemical controlling processes. *Water Research*, *42*(16), 4215–4232. https://doi.org/10.1016/j.watres.2008.07.020

Rose, J. P., Kleist, T. J., Löfstrand, S. D., Drew, B. T., Schönenberger, J., & Sytsma, K. J. (2018). Phylogeny, historical biogeography, and diversification of angiosperm order Ericales suggest ancient neotropical and east Asian connections. *Molecular Phylogenetics and Evolution*, *122*, 59–79. https://doi.org/10.1016/j.ympev.2018.01.014

Shapiro, S. S., & Wilk, M. B. (1965). An analysis of variance test for normality (complete samples). *Biometrika*, *52*, 591–611.

Singer, S. F. (2012). *The changing global environment*. Springer Netherlands.

Soukup, A., & Tylová, E. (2014). Essential methods of plant sample preparation for light microscopy. In N. J. Clifton (Ed.), *Methods in molecular biology* (pp. 1–23). Humana Press. https://doi.org/10.1007/978-1-62703-643-6_1

Suetsugu, K. (2018). Independent recruitment of a novel seed dispersal system by camel crickets in achlorophyllous plants. *New Phytologist*, *217*, 828–835. https://doi.org/10.1111/nph.14859

Suetsugu, K., Shitara, T., & Yamawo, A. (2017). Seed dispersal by ants in the fully mycoheterotrophic plant *Sciaphila secundiflora* (Triuridaceae). *Journal of Asia-Pacific Entomology*, *20*(3), 914–917. https://doi.org/10.1016/j.aspen.2017.06.011

Takahashi, H. (1993). Seed morphology and its systematic implications in Pyroloideae (Ericaceae). *International Journal of Plant Sciences*, *154*, 175–186. https://doi.org/10.1086/297103

Toothaker, L. E. (1993). *Multiple comparison procedures*. Sage.

Van Waes, J. M., & Debergh, P. C. (1986). *In vitro* germination of some Western European orchids. *Physiologia Plantarum*, *67*(2), 253–261. https://doi.org/10.1111/j.1399-3054.1986.tb02452.x

Verma, S., Attuluri, V. P. S., & Robert, H. S. (2022). Transcriptional control of Arabidopsis seed development. *Planta*, *255*, 90. https://doi.org/10.1007/s00425-022-03870-x

Welch, B. L. (1951). On the comparison of several mean values: An alternative approach. *Biometrika*, *38*, 330–336.

Yeung, E. C. (2017). A perspective on orchid seed and protocorm development. *Botanical Studies*, *58*, 1–14. https://doi.org/10.1186/s40529-017-0188-4

Zhang, H., Jennings, A., Barlow, P. W., & Forde, B. G. (1999). Dual pathways for regulation of root branching by nitrate. *Proceedings of the National Academy of Sciences of the United States of America*, *96*, 6529–6534. https://doi.org/10.1073/pnas.96.11.6529

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1: Seeds of the studied plants.

Figure S2: Details of embryos of studied species.

Figure S3: Histochemical detection of storage compounds in seeds.

Figure S4: Histochemical detection of storage compounds in seeds.

Figure S5: Differences in seed dimensions between groups of studied species.

Figure S6: Seedlings of the studied plants obtained during in vitro experiments.

Figure S7: Effect of nitrate on germination of *Pyrola rotundifolia*.

Figure S8: Effects of nitrate and ammonium ion on germination rate of the studied plants.

Figure S9: Comparison of modelled species responses to inorganic nitrogen between groups of species using the model parameters characterising the effect of nitrogen concentration.

Figure S10: Relationships between modelled species responses to inorganic nitrogen and seed dimensions, analysed separately for autotrophic species and for heterotrophic species.

Table S1: Origin of seeds used in the study, their disinfection and cultivation.

Table S2: Seed dimensions of the studied species.

Table S3: Results of models treating nitrogen concentration as discrete values.

Table S4: Outputs of models treating inorganic nitrogen concentration as a continuous variable and categories of species traits.

How to cite this article: Figura, T., Tylová, E., Matsuda, Y., Janoušková, M., & Ponert, J. (2025). Nitrate inhibition of germination in Ericaceae relates to seed size and mycoheterotrophy. *Functional Ecology*, *39*, 609–620. https://doi.org/10.1111/1365-2435.14710