

The decreasing trend of the competitive advantage of *Epichloë* endophyte-infected *Achnatherum sibiricum* over endophyte-free plants with nitrogen addition was reversed by pathogenic fungi inoculation

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Abstract

Background and aims

According to the nitrogen-disease hypothesis, plant diseases will become more serious with the aggravation of global nitrogen (N) deposition. Numerous studies have indicated that *Epichloë* endophytes can enhance host plant resistance to pathogens. It is unclear how the competitive ability of endophyte-infected (EI) over endophyte-free (EF) plants changes under the interference of N deposition and plant disease.

Methods

In this study, *Achnatherum sibiricum*, native to the Inner Mongolia steppe of China, was used as experimental material. We experimentally manipulated N addition and pathogen inoculation and examined the growth and competition between EI and EF *A. sibiricum*.

Results

The results showed that EI plants had a greater competitive advantage than EF plants under low N conditions, and this advantage decreased with the N supply. When high N and pathogens were both present, pathogen inoculation reversed the adverse effects of high N supply on the competitive advantage of EI *A. sibiricum*. Endophyte infection not only reduced the disease of EI plants but also reduced the disease of neighboring EF plants. Meanwhile, endophytes changed the response of the host disease to nitrogen. The disease index of EF plants increased with increasing leaf N content, while that of EI plants did not change.

Conclusion

This study highlights that under the dual factors of N deposition and pathogen infection, endophytic fungi improve the competitive ability of host plants. Our results show that considering both biotic and abiotic factors is crucial for predicting the dominance of plant-fungal symbionts in the community.

Introduction

Human activities over the last century, such as the excessive use of fertilizers and the combustion of fossil fuels, have released massive amounts of nitrogenous compounds, increasing the level of reactive nitrogen (N) on the Earth's land surface (Galloway et al. 2008; Greaver et al. 2016). On the one hand, increased atmospheric deposition of reactive N can promote the rapid growth of nitrophilous species/varieties in the community and obtain relative advantages in interspecific/intraspecific competition (Bai et al. 2015; Farrer and Suding 2016; Tian et al. 2016). On the other hand, increased leaf

N concentration can increase the resources available to pathogens, resulting in plants that may be more susceptible to disease (Mitchell et al. 2003), thereby enabling disease-resistant species/varieties to have an advantage in competition. In natural ecosystems, the combination of biotic and abiotic factors leads to more complex intra- and interspecific interactions (Borer et al. 2014; Crawford et al. 2021).

Symbiotic microbes also affect the competitive ability of host plants (van der Heijden et al. 1998). There is a symbiotic relationship between cool season grasses (subfamily Pooideae) and *Epichloë* (= *Neotyphodium*) fungal endophytes, and the fitness of these endophytes and their host plants are tightly coupled (Clay and Schardl 2002). Endophyte infection can promote host plant growth (Chen et al. 2020; Guo et al. 2015) and enhance host plant resistance to drought, herbivores, pathogens, etc. (Cibils-Stewart et al. 2022; Decunta et al. 2021; Graff et al. 2020; Rho et al. 2018). Endophyte infection may also influence the intra- and interspecific competitive ability of host plants. Previous studies have demonstrated that endophyte infection can enhance the intraspecific competitive ability of the hosts *Festuca arundinacea* (Hill et al. 1991, 1998; Marks and Cheplick 1991), *Festuca pratensis* (Malinowski et al. 1997), *Bromus benekenii* (Brem and Leuchtmann 2002) and *Leymus chinensis* (Liu et al. 2020). However, a few studies have yielded inconsistent findings. For instance, endophytic fungal infection had no effect on the intraspecific competitive ability of the host *Lolium perenne* (Cheplick et al. 2014) and a negative effect on the intraspecific competitive ability of the host *Brachypodium sylvaticum* (Brem and Leuchtmann 2002).

In studies of agricultural grass species, the beneficial effects of endophytes on the host mostly occur in a high N environment (Ren et al. 2009; Saikkonen et al. 2006). However, some research has demonstrated inconsistent results in natural grasses. For example, some studies indicated that endophyte infection promoted the growth of the hosts *Trisetum spicatum* and *Festuca pratensis* under a relatively high N supply (Buckley et al. 2019; Dirihan et al. 2015), while others indicated that endophyte infection had no effect on the growth of *Achnatherum inebrians* under high N supply but improved its growth under low N conditions (Hou et al. 2021; Wang et al. 2018). To date, no work has reported the effect of N supply on the competitive ability of endophyte-infected plants. In a related study, Sarri and Feath (2012) found that endophyte infection could enhance the intraspecific competitive ability of *Festuca arizonica* under low nutrient conditions, but this competitive advantage disappeared with increasing environmental nutrients.

According to the nitrogen-disease hypothesis, high N supply can directly increase foliar fungal disease severity by altering plant biochemical characteristics and pathogen resources (Mitchell et al. 2003). The nitrogen-disease hypothesis is widely known in agricultural ecosystems (Chen et al. 2007; Guo et al. 2021; Lecompte et al. 2013). In natural ecosystems, Ebeling et al. (2021) investigated plant leaf damage to 153 plant taxa from 27 grassland communities and found that the pathogen damage increased with N content in grasses and legumes. Endophytic fungi are widely distributed in grasses (Song et al. 2016). Many published studies have shown that endophyte infection may enhance the resistance of host grasses to disease (Clarke et al. 2006; Pérez et al. 2020; Zhang et al. 2022). When N addition and pathogens co-occur, how intraspecific competitive ability is affected by endophytes remains unknown.

Understanding the effects of multiple environmental changes on the intraspecific relationships of host plants associated with endophytes is critical to predicting the population dynamics and community diversity of host plants.

Achnatherum sibiricum is normally a subordinate species in grassland and is usually colonized by *Epichloë* endophytes with high infection rates (86%-100%) in natural habitats (Wei et al. 2006). Previous studies have demonstrated that endophytes can promote plant growth (Li et al. 2016; Zhou et al. 2018). To explore the effect of endophytes on the intraspecific competitive ability of host plants in multiple environmental factors, *A. sibiricum* was used as the target plant, and N addition and pathogen inoculation were used as influencing factors. Specifically, we aimed to address the following questions: (1) How do endophytes affect the intraspecific competitive ability of the host *A. sibiricum*? (2) Is the effect of endophytes on the intraspecific competitive ability of the host *A. sibiricum* related to N addition or pathogen infection? (3) When N addition and pathogen infection co-occur, how do endophytes affect the intraspecific competitive ability of *A. sibiricum*?

Materials And Methods

Experimental materials

A. sibiricum is a common perennial grass in the Inner Mongolia grassland of China. It is normally a subordinate species in grasslands but sometimes may become dominant (Ma et al. 1985). The mature seeds of *A. sibiricum* and planting soil used in this study were collected from natural populations in the National Hulunber Grassland Ecosystem Observation and Research Station in northeast China (119.67°E, 49.10°N). At the collection site, 300 *A. sibiricum* plant individuals were selected at random. The harvested seeds were naturally dried and stored at 4°C. One hundred seeds from different plant individuals were randomly selected to detect the endophyte infection frequency of the seeds based on the aniline blue–lactic acid staining method (Latch et al. 1984). All seeds were colonized by endophytes, and the infection rate of seeds was 100%. Half of the collected endophyte-infected (EI) seeds were randomly selected and placed in a convection drying oven for 30 d at 60°C to obtain endophyte-free (EF) seeds. This method has been shown to have no significant effect on the seed germination rate, germination potential and germination index (Li et al. 2012; Ren et al. 2011). A previous investigation by Zhang et al (2009) found that the *A. sibiricum* of different plant individuals in the same geographic population were infected with *Epichloë sibirica* (Es) and *Epichloë gansuensis* (Eg). However, different kinds of endophytic fungi had no significant effect on the growth of *A. sibiricum* and the pathogen (Shi et al. 2020; Zhou et al. 2019). Therefore, this study only considered whether the plants were infected by endophytes and did not distinguish the endophyte species.

Soil from the collection site was sieved (2 mm), homogenized, and then autoclaved twice at 0.11 MPa and 121°C temperature for 30 min, with a 24 h resting period in between. Both EI and EF *A. sibiricum* seeds were surface sterilized with 0.5% NaClO to remove any potential pathogens and cleared with sterile distilled water. The surface sterilized seeds were planted into a plastic pot (20 cm top diameter and 22 cm

deep) that received 2.5 kg of sterilized soil. Two weeks after germination, eight plant individuals were placed in each pot (four EI *A. sibiricum* + four EF *A. sibiricum* individuals in mixtures). Before the experiment, the endophyte infection status of each plant was checked based on the aniline blue–lactic acid staining method to ensure that the plants were infected with endophytes (Latch et al. 1984). The soil-borne fungal pathogen *Rhizoctonia solani* used in this study, a common pathogen, was obtained from the Agricultural Culture Collection of China (ACCC).

The *R. solani* strain was activated on potato dextrose agar (PDA). After three days, mycelium was cut with a sterile 6 mm diameter punch and cultured in liquid medium (PDA medium without agar) with shaking at 120 r/min and 25°C for 3 days. The cultured fungal mass was washed with sterile water three times and homogenized for 30 s using a homogenizer to prepare a suspension. The absorbance of this suspension was measured at a wavelength of 600 nm ($OD_{600} = 2.3$) and stored at 4°C in a refrigerator until use (Zang et al. 2010). Ten weeks after germination, the pathogen suspension was inoculated. The pathogen suspension (5 mL) was injected into the rhizosphere of each plant with a sterile syringe. For the noninoculation pathogen group, 5 mL of sterile water was injected into the rhizosphere of each plant.

Experimental design

A four-factor random block design was adopted. The first factor was N treatment with two levels: low nitrogen (LN) and high nitrogen (HN). The second factor was pathogen inoculation treatment with two levels: pathogen inoculation (P+) and pathogen noninoculation (P-). The third factor was the endophyte infection status of *A. sibiricum* with two levels: endophyte-infected (EI) and endophyte-free (EF). The fourth factor was mixture type: monoculture of EI *A. sibiricum*, monoculture of EF *A. sibiricum*, mixtures of EI *A. sibiricum* and EF *A. sibiricum*. Each combination was replicated six times, resulting in a total of 72 pots. This experiment began on 10 May 2021 and was conducted in the campus experimental field at the College of Life Sciences, Nankai University, Tianjin, China, and lasted for 110 days. During the whole experiment, plants were watered 2–3 times per week to meet the normal growth of plants. The plants were harvested at the end of the experiment and dried for 48 h at 65°C to obtain biomass.

Nitrogen treatment

Different N treatments were controlled by changing the concentration of NH_4NO_3 in the nutrient solution. The nutrient solution included NH_4NO_3 (HN: 10 mM, LN: 2 mM), 5.0 mM $CaCl_2$, 5.0 mM KCl, 2.5 mM $MgSO_4 \cdot 7H_2O$, 2.0 mM KH_2PO_4 , 29 μM Na_2 -EDTA, 20 μM $FeSO_4 \cdot 7H_2O$, 45 μM H_3BO_3 , 6.6 μM $MnSO_4$, 0.8 μM $ZnSO_4 \cdot 7H_2O$, 0.6 μM H_2MoO_4 , and 0.4 μM $CuSO_4 \cdot 5H_2O$, pH 6.0 ± 0.1 . After 40 days of germination, 300 mL of nutrient solution was applied every two weeks, four times in total.

Disease investigation

Three weeks after inoculating *R. solani*, we assessed the disease severity degree at the base of the pseudostem of each plant individual (Institute 1980). Grade 0: no disease spot; Grade 1: the disease spot area is less than 25% of the leaf sheath area. Grade 3: the disease spot area accounts for 25 to 50% of

the leaf sheath area (excluding 50%); Grade 5: the disease spot area accounts for 50 to 75% of the leaf sheath area (excluding 75%); Grade 7: the disease spot is greater than or equal to 75% of the leaf sheath area; Grade 9: the disease spot symptoms penetrate the leaf sheath. The disease index (DI) was calculated as follows:

$$DI = \frac{\sum(x \times t)}{s \times \sum t} \times 100$$

where DI represents the disease index, x represents the degree of disease severity, t represents the total number of leaves at each degree of disease severity, and s represents the highest disease severity observed.

Competitiveness

The aggressivity index (AGR) of EI *A. sibiricum* versus EF *A. sibiricum* was calculated according to McGilchrist and Trenbath (1971):

$$AGR_{mn} = RY_m - RY_n = (DM_{mn} / DM_{mm}) - (DM_{nm} / DM_{nn})$$

where RY is the relative yield of species m or n, using the ratio of the dry weight of a species grown in the mixture (DM_{mn} or DM_{nm}) to the dry weight in the respective monoculture (DM_{mm} or DM_{nn}). $AGR_{mn} = 0$ indicates that the two species have the same competitive ability; $AGR_{mn} > 0$ indicates a higher competitive ability of species m than that of species n; and $AGR_{mn} < 0$ indicates a lower competitive ability of species m than that of species n.

Leaf nitrogen and total phenolic content

The leaf N concentration of *A. sibiricum* was determined using an elemental analyzer (Vario EL/micro cube, Elementar, Hanau, Germany). The total phenolic concentration of *A. sibiricum* was determined by the Folin-Ciocalteu colorimetric method (Chen et al. 2015).

Statistical analyses

All statistical analyses were performed with R version 4.1.1 (R Core Team 2021). To examine the effects of N addition, endophyte infection and plant mixture type on the disease index of *A. sibiricum*, we used three-way analysis of variance (ANOVA). To examine the effects of N addition and pathogen infection on the AGR of *A. sibiricum*, we used a two-way analysis of variance (ANOVA). A three-way analysis of variance (ANOVA) was used to analyze the effects of N addition, endophyte infection and pathogen infection on the leaf N content of plants. A one-sample t test was used to compare the difference between the AGR and zero. Regression models were used to analyze the relationship between the disease index/biomass and leaf N content and the relationship between the disease index and total phenolic content. Shapiro–Wilk and Levene's tests were performed to determine data distribution and homogeneity.

Results

Disease index

Endophyte infection significantly reduced the disease index of the host *A. sibiricum*, while the degree of reduction was related to N supply and plant mixture type. In different N treatments, the disease index of EF *A. sibiricum* increased significantly with the N supply, while the disease index of EI *A. sibiricum* was not significantly affected by nitrogen levels. Therefore, the beneficial effect of endophytes on the disease resistance of host grasses was the most significant under N supply (Fig. 1a). For different plant mixture types, compared with monoculture, the disease index of the EI and EF *A. sibiricum* were significantly decreased in the mixture (Fig. 1b), but the EF *A. sibiricum* showed a greater degree of decline; that is, endophyte infection may not only reduce the disease resistance of host *A. sibiricum* but also reduce the disease resistance of non-host plants with host neighbors.

Figure 1

Competitiveness

One-sample t test results showed that the AGR of *A. sibiricum* was significantly greater than zero under low N uninoculated pathogen and high N inoculated pathogen treatment, that is, the competitive ability of EI *A. sibiricum* was significantly higher than that of EF *A. sibiricum*. The AGR of *A. sibiricum* did not differ from zero under high N uninoculated pathogen and low N inoculated pathogen treatment, that is, EI *A. sibiricum* and EF *A. sibiricum* had the same competitive ability. The competitive advantages of EI *A. sibiricum* compared to EF *A. sibiricum* were affected by the interaction between nitrogen and pathogens (Fig. 2). On the one hand, the effect of N supply on the competitiveness of EI *A. sibiricum* was related to pathogen inoculation. Under the conditions of pathogen noninoculation, high N addition significantly reduced the competitive advantage of EI *A. sibiricum*; under the conditions of pathogen inoculation, high N addition significantly improved the competitive advantage of EI *A. sibiricum*, that is, the negative effect of endophyte-infected *A. sibiricum* over endophyte-free plants on the competitive ability with N addition was reversed by fungal pathogen inoculation. On the other hand, the effect of pathogen inoculation on the competitiveness of EI *A. sibiricum* was related to N supply. Pathogen inoculation reduced the competitive advantage of EI *A. sibiricum* under low N addition; however, pathogen inoculation enhanced the competitive advantage of EI *A. sibiricum* under high N addition (Fig. 2).

Figure 2

Nitrogen content in plants

The leaf N content of *A. sibiricum* was significantly affected by the interaction of N addition, endophyte infection, and pathogen infection. Under the conditions of low N uninoculated pathogen and high N inoculated pathogen, endophyte-infected plants had higher leaf N content than endophyte-free plants (Fig. 3).

Figure 3

The growth and disease index of EI and EF *A. sibiricum* varied with leaf nitrogen content

The biomass of EI *A. sibiricum* increased significantly with increasing leaf N content, while the disease index of EI *A. sibiricum* did not change (Fig. 4a), that is, Endophytic fungi may promote plant growth and have no significant effect on pathogen defense. The biomass and disease index of EF *A. sibiricum* increased significantly with increasing leaf N content, that is, the defenses against pathogens of EF *A. sibiricum* decreased with growth (Fig. 4b).

Figure 4

The relationship between disease index and total phenolic content

Linear regression results showed that whether infected or not, the disease index of *A. sibiricum* decreased significantly with increasing total phenol content (Fig. 5), showing a significant negative correlation.

Figure 5

Discussion

Endophyte effects on host competitive ability depended on nitrogen supply

The relationship of endophyte-grass mutualism was related to nutrient supply, especially N supply (Saikkonen et al. 2006). Endophyte infection is more favorable for the growth of some plants under low N conditions (Hou et al. 2021; Lewis 2004; Ravel et al. 1997; Wang et al. 2018), while it is more favorable for the growth of other plants under high N conditions (Arachevaleta et al. 1989; Ren et al. 2009). To date, no research has discussed the effects of N on the intraspecific competition of endophyte-infected plants. The present study found that the intraspecific competitive ability of EI over EF *A. sibiricum* was related to N supply. Specifically, EI plants had a greater competitive advantage than EF plants under low N conditions, and this advantage decreased with N supply. Sarri and Feath (2012), who controlled the amount of NPK compound fertilizer, found that endophytic fungi could improve the intraspecific competition ability of *F. arizonica* under low nutrient conditions, and this competitive advantage disappeared with the increase in nutrients, which was similar to the results in this study. The reason why EI plants have a greater competitive advantage than EF plants under low N conditions may be that endophytes improve the N absorption of host plants and promote the growth of hosts, while the host plants directly acquire N from the soil in high N environments, reducing the reliance on endophytic fungi and decreasing the competitive advantage of EI plants.

Endophyte infection changed the response of the host disease to nitrogen

Nitrogen addition has been found to aggravate the disease of gramineous plants in both natural and agricultural ecosystems (Guo et al. 2021; Heckman et al. 2016; Lecompte et al. 2013; Mitchell et al. 2003).

In this study, we found that the disease index of EF plants increased with increasing N content, which supported the nitrogen disease hypothesis, but the disease index of EI plants did not change significantly with increasing N content, which did not support the nitrogen disease hypothesis. Our results further found that the total phenolic content of *A. sibiricum* leaves was significantly negatively correlated with the disease index, whether infected or not. Phenolic compounds, an important class of secondary metabolites, play a crucial role in plant disease resistance (Cheynier et al. 2013). The results of this study indicate that endophytes may enhance the disease resistance of host plants by increasing the content of phenolic compounds with increasing soil available N.

Pathogen inoculation effects on the competitiveness of EI relative to EF plants depended on nitrogen

Numerous studies have shown that EI plants have higher disease resistance than EF plants (Iannone et al. 2017; Kou et al. 2021; Wang et al. 2015). In a previous study on *A. sibiricum* by Shi et al (2020) reported that endophytes may improve the resistance of the host to both necrotrophic and biotrophic pathogens. However, no studies have reported whether endophytes can affect the disease resistance of neighboring non-host plants and thus further affect the competition between host and non-host plants. This study found that endophyte infection may not only reduce the disease of EI plants but also reduce the disease of neighboring EF plants, only the beneficial effect on EI plants was higher. Moreover, the effects of pathogen inoculation on the competition between EI and EF plants were related to N supply. Although endophytes significantly reduced the disease index of host plants, they had no significant effect on the competitive ability of host plants under low N supply. However, endophytes not only significantly reduced the disease index of host plants but also enhanced the competitive ability of host plants under high N supply.

Epichloë endophytes change the growth-defense trade-off of the host grass

According to a trade-off between growth and defense theory, plants tend to divert more resources to defense at the expense of normal growth when encountering biotic stresses such as pathogens (Lind et al. 2013). Although the trade-off between growth and defense seems to be ubiquitous in plants (Kneitel et al., 2004), the uncoupling of growth and defense functions has also been reported. For instance, Cao et al. (1998) found that overexpression of NPR1 (nonexpressor of pathogenesis-related gene 1) enhanced the resistance of *Arabidopsis thaliana* to pathogens with no obvious detrimental effect on the plants. In grass-*Epichloë* symbiosis, Bastías et al. (2021) found that when plant leaves were damaged by insect herbivores, endophytes promoted the growth of host plants and improved host plant resistance to herbivores by producing alkaloids. In the present study, *Epichloë* endophytes enhanced host plant resistance to pathogens and promoted the growth of host plants under high N conditions. The promotion of endophytes on the growth of *A. sibiricum* may be related to the upregulated expression of the auxin signaling pathway and photosynthesis-related genes (Shi et al. 2022), and the improvement of endophytes on the resistance of the host *A. sibiricum* may be related to the increase in phenolic compounds.

Conclusion

In conclusion, the impact of endophytes on the intraspecific competitive ability of host plants was affected not only by N supply but also by pathogens. The competitive advantage of *A. sibiricum* relative to EF *A. sibiricum* decreased with N supply, while pathogen inoculation reversed the adverse effects of high N supply. In grassland ecosystems, on the one hand, N supply will increase with global N deposition (Crawford et al. 2021). On the other hand, plant diseases have always been one of the important reasons for the degradation of grasslands (Cappelli et al. 2020; Mitchell et al. 2003). The results of this study suggest that the advantage of EI over EF grasses would increase in the future.

Declarations

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Author contributions Junzhen Zhang and Anzhi Ren conceived the ideas and designed methodology; Junzhen Zhang, Xinhe Yu, Yaobing Qu, Xinjian Shi and Luoyang He collected the data; Junzhen Zhang, Xianqin Wei and Nianxi Zhao analyzed the data; Junzhen Zhang, Lei Chen and Anzhi Ren led the writing of the manuscript. All authors read and approved the final manuscript.

Data Availability Data supporting the findings of this study are available from the corresponding author on the reasonable request.

Conflict of interest The authors declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

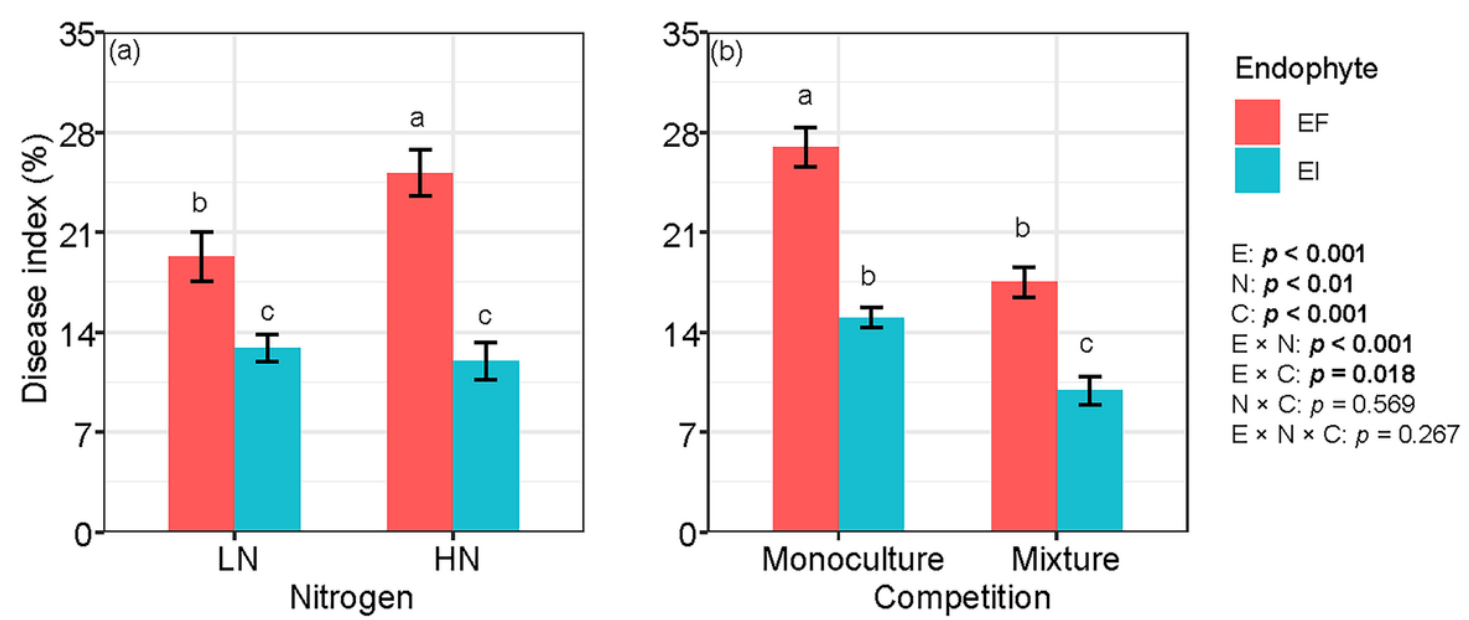


Figure 1

Effects of nitrogen addition (N), endophyte infection (E) and plant mixture type (C) on the disease index for *Achnatherum sibiricum*. Different letters indicate significant differences ($p < 0.05$). EI: endophyte infection; EF: endophyte free; LN: low nitrogen treatment; HN: high nitrogen treatment. Monoculture, EI and EF *A. sibiricum* grow separately in pots; mixture, EI and EF *A. sibiricum* grow together in pots

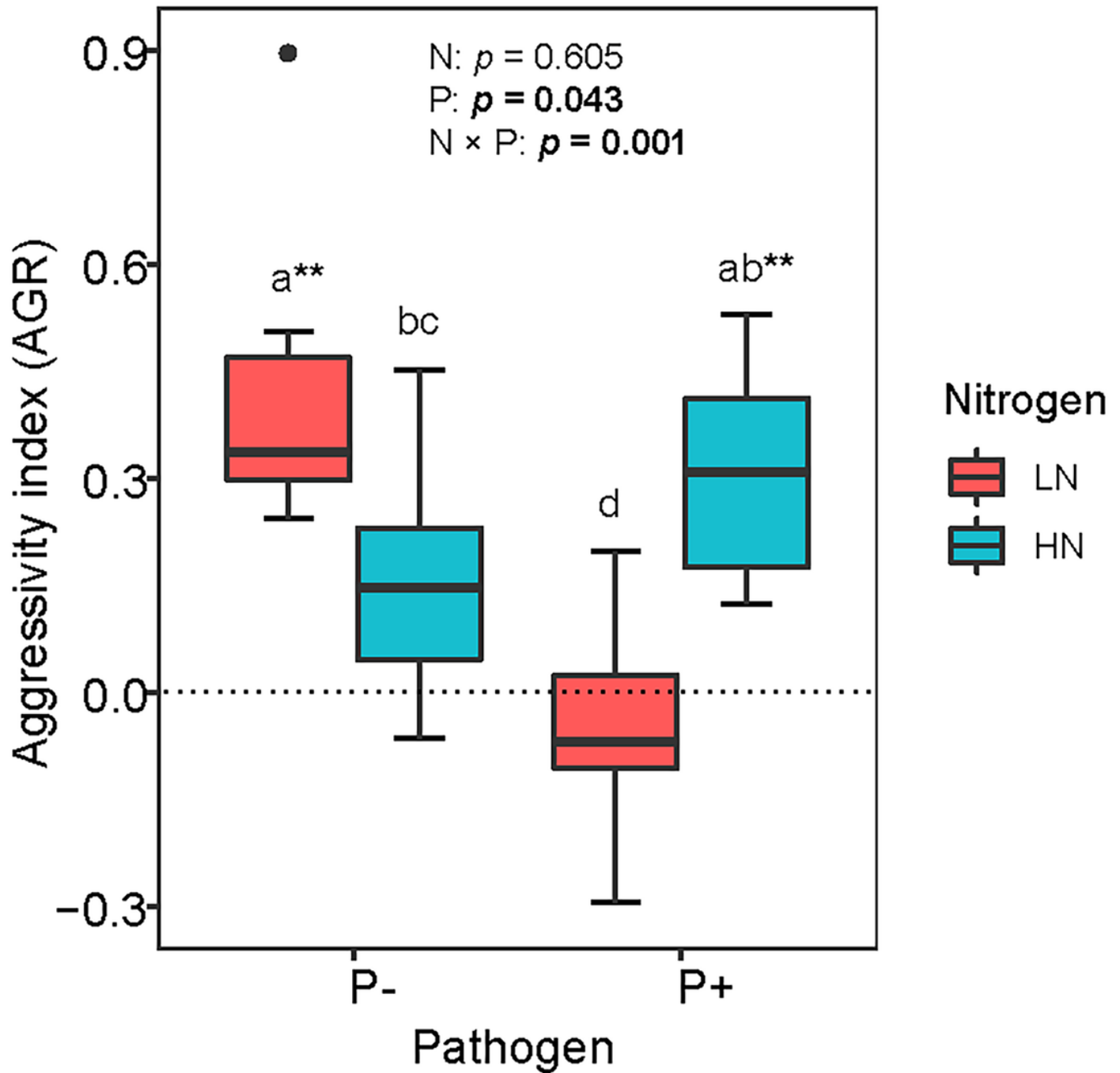


Figure 2

Effects of nitrogen addition and pathogen infection (P) on the aggressivity index (AGR) for *Achnatherum sibiricum*. * represents a significant difference between the AGR and 0 (* $p < 0.05$, ** $p < 0.01$). Different letters indicate significant differences ($p < 0.05$). P-: pathogen noninoculation; P+: pathogen inoculation; LN: low nitrogen treatment; HN: high nitrogen treatment

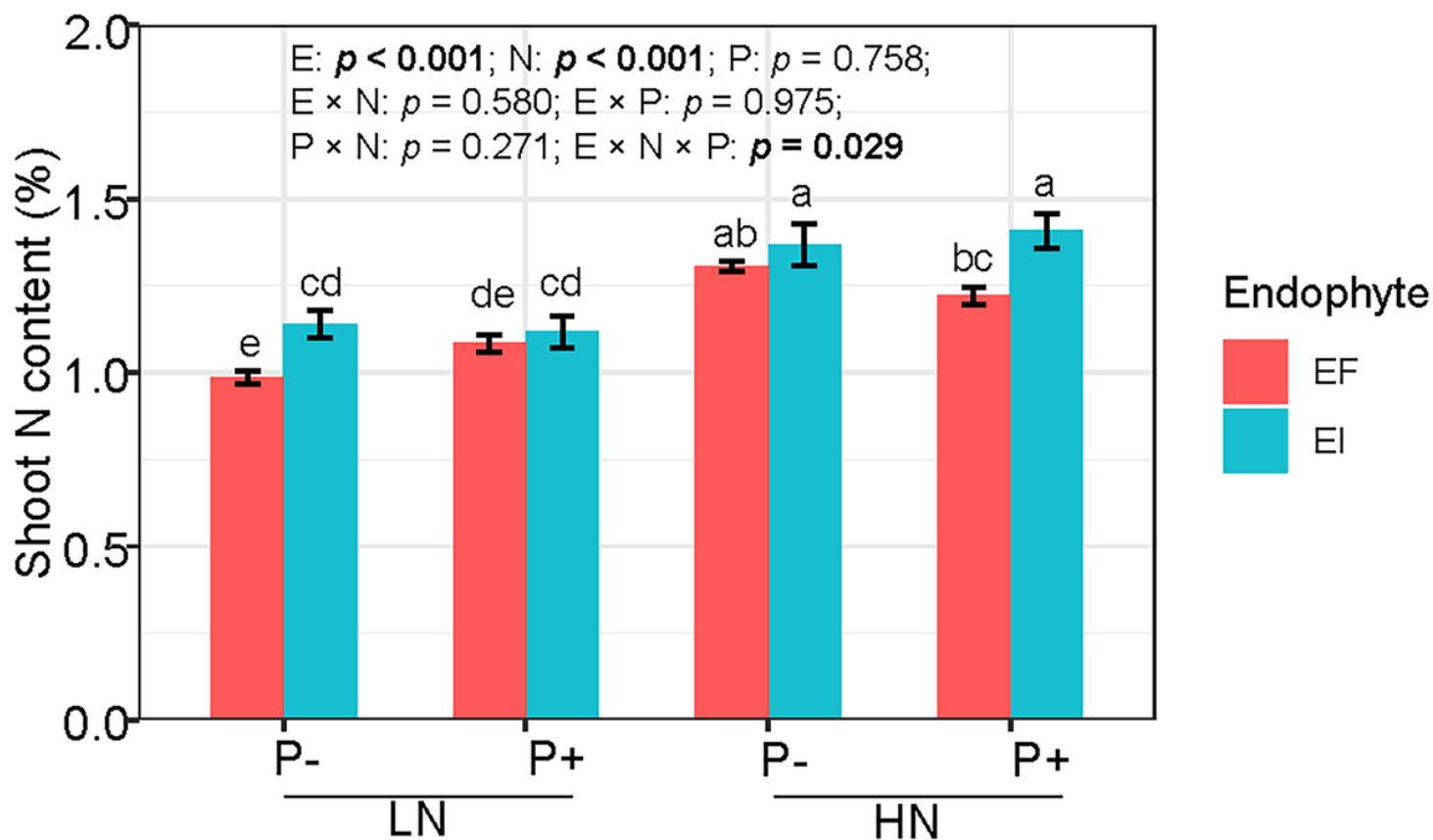


Figure 3

Effects of nitrogen addition and pathogen infection on the leaf nitrogen content for endophyte-infected and endophyte-free *Achnatherum sibiricum*. Different letters indicate significant differences ($p < 0.05$). EI: endophyte infection; EF: endophyte free; P-: pathogen noninoculation; P+: pathogen inoculation; LN: low nitrogen treatment; HN: high nitrogen treatment

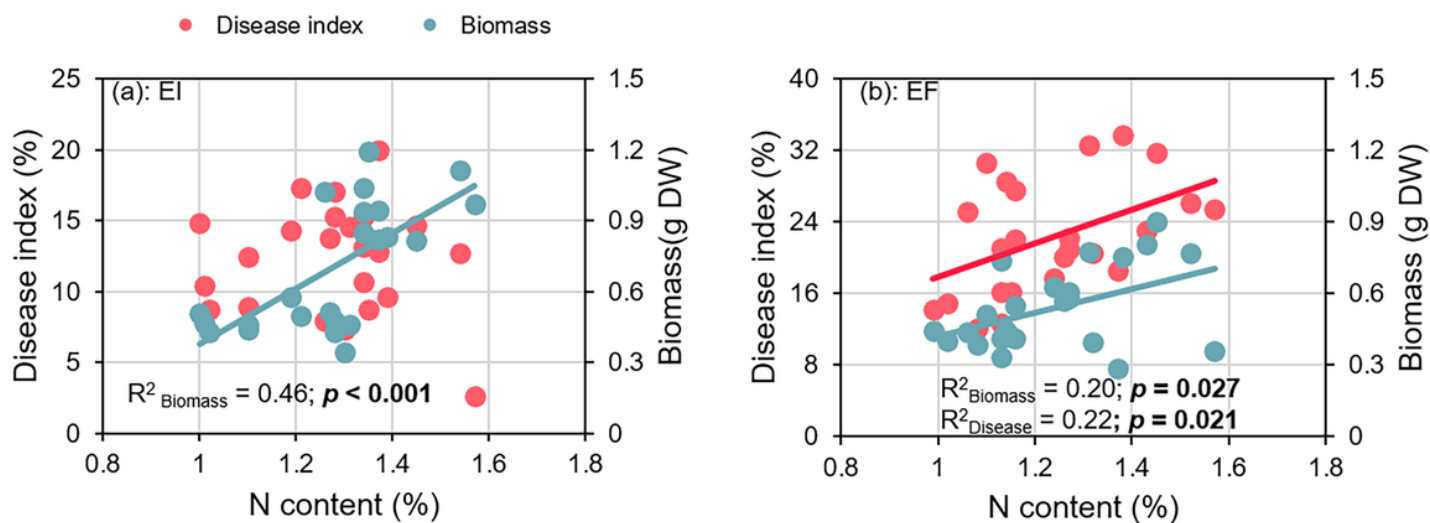


Figure 4

The relationship between disease index/biomass of endophyte-infected (a) and endophyte-free (b) *Achnatherum sibiricum* and leaf nitrogen content

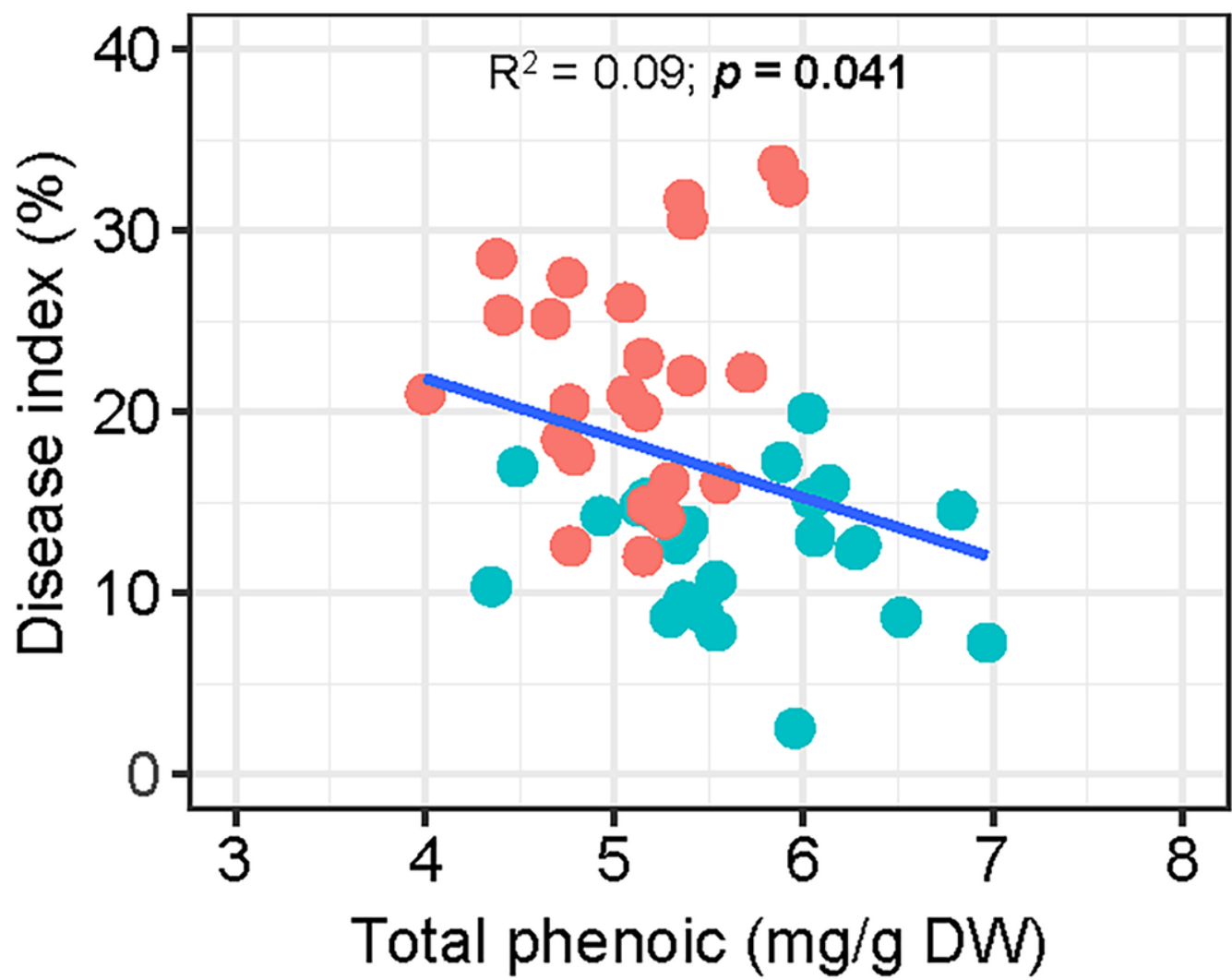


Figure 5

The relationship between the disease index of endophyte-infected and endophyte-free *Achnatherum sibiricum* and total phenolic content