Endophyte-Mediated Effects on the Growth and Physiology of *Achnatherum sibiricum* Are Conditional on Both N and P Availability

Xia Li, Anzhi Ren*, Rong Han, Lijia Yin, Maoying Wei, Yubao Gao

College of Life Sciences, Nankai University, Tianjin, P. R. China

Introduction

Many grasses are infected by clavicipitaceous fungal endophytes that occur in aboveground plant tissues. Asexual endophytes live asymptomatically within the host tissues, receiving protection and nutrients, and are vertically transmitted to the next plant generation via host seeds. Based on numerous studies using tall fescue and perennial ryegrass in agronomic, fertilized soils, this symbiosis has been considered strongly mutualistic—mainly because endophyte infection may improve herbivore resistance of the host grasses due to production of alkaloids [1], and increase plant vigor and tolerance to a wide range of abiotic environmental conditions [e.g. drought] [2–3]. There is increasing evidence that the benefits from endophyte infection depend largely on the availability of other resources, in particular nutrients [4]. Resource limitation can increase the cost of supporting some endophytes [5–6], potentially changing the interaction from mutualism to parasitism or commensalism [7]. In fact, many of the studies that have found improved growth in endophyte-infected (EI) grasses were done under benign conditions of moderate to high soil nutrient availability [8–11].

Studies on endophyte-related responses of grasses to nutrient acquisition have focused on the influence of nitrogen (N), since this element is not only a constituent of alkaloids in infected plants but also one of the most important limiting resources for plant growth in nature. In the plant the photosynthetic apparatus is the largest sink of N [12]. Photosynthetic capacity and photosynthetic N use efficiency (PNUE) correlates strongly with N allocation to the photosynthetic machinery [13]. Small changes in N allocation can greatly influence light-saturated photosynthetic rate (P\(_{\text{max}}\)) and PNUE, and therefore plant performance [14–16]. Consequently, leaf N allocation to photosynthesis is an important factor explaining differences in P\(_{\text{max}}\) and PNUE [14]. Published reports of the effects of endophyte infection on N use efficiency of grass-endophyte associations are inconsistent. Arachavaleta et al. [17] found beneficial effects of endophyte infection in tall fescue only at high N concentrations, and this result was further supported by our previous study in perennial ryegrass [11]. In contrast, Ravel et al. [18] found an advantage of EL plants over EF (endophyte-free) plants was greater at low N levels. It has been documented that increased N availability may also change the relative availability of other nutrients such as phosphorus (P) [19–20]. Therefore, we asked whether the inconsistent results are caused, in part, by other nutrients such as P.

Similar to N nutrition, P availability also influences ergot alkaloid production in EI grasses [21]. However, published reports...
of the effects of endophyte infection on P use efficiency of grass-endophyte associations are limited [22]. Malinowski et al. [23] found that EI tall fescue expressed an increased root absorption area through reduced root diameter and increased root hair length compared with the EF counterpart. The Fe^{3+} reducing activity on the root surface and total phenolic concentration in roots also increased dramatically in response to endophyte infection [24]. N addition may change the relative availability of P. On one hand, N addition could stimulate phosphatase activity of the root [25], which could potentially promote P uptake from bound-P. In fact, the production and excretion of acid phosphatase is considered to be one component of a plant phosphate-starvation rescue system [26]. On the other hand, a high N:P supply ratio could result in P starvation in the plant [27]. Populations previously limited by N can switch to limitation by P after receiving high N [19–20]. N:P stoichiometry in plant tissues, especially leaves, is related to growth strategy and can be an indicator of vegetation composition, functioning and nutrient limitation at the community level [20]. Until now, however, the effect of both N and P availability on grass-endophyte associations has received little attention.

Endophytic fungi not only occur in agronomic grasses but also in almost all habitats where grasses are common [29]. In our previous survey in the permanent grasslands of northern China, 25 of 41 species of grasses surveyed (61%) were infected by Neotyphodium endophytes [30]. However, most of the work for endophyte-plant interactions has been based upon endophyte-plant studies of two, economically important, artificially selected and non-native grass species [31–32]. Few studies exist to predict the significant effect on germination rate, germination potential and potential effects of the heat treatment on seed germination and seedling growth. After a 30-d heat treatment, none of the seedlings were infected. Moreover, high temperature treatment had no significant effect on germination rate, germination potential and germination index [41].

Materials and Methods

Ethics statement
No specific permissions were required since in this study we only collected a limited amount of seeds from a native grassland, and this grassland is not privately-owned or protected in any way. Our field study did not involve any endangered or protected species.

Study System

Achnatherum sibiricum is a perennial, sparse bunch grass that is native to the Inner Mongolia Steppe of China. It is usually a companion species in the grassland and can sometimes become a dominant species. High incidences of Neotyphodium endophyte infection (86–100%) in A. sibiricum were recorded in seven native populations in our previous study [30]. In the present study, seeds of A. sibiricum were collected from natural population in Hailar in Northeast China (119.67° E, 49.10° N), where the annual mean temperature is around –2°C and annual precipitation about 367 mm. This meadow steppe belongs to a transitional type of habitat between forest and steppe. Achnatherum sibiricum within this area is less preferred by mammalian herbivores compared to other dominant species in the community [38]. In the sampled area the dominant species included Stipa bescuculiflora Roshev. and Leymus chinensis L., with A. sibiricum and Koeleria cristata (Linn.) Pers. as common species. Within this population, we collected seeds in August 2008 and stored them at 4°C.

Detection of endophytes using the aniline blue staining method [39] showed that endophyte infection frequency of the Hailar population was 100%. To eliminate the endophyte, we heat treated a subset of randomly chosen seeds in a convection drying oven according to Kamadon and Rudgers [40]. Because disinfection procedures have not been established for this species, we initially treated seeds for 0, 5, 10, 15, 20, 25 or 30 d at 60°C to determine the optimal treatment time. Then all treated seeds were planted in plastic pots filled with vermiculite in November 2008. To assess treatment effectiveness, we examined three leaf peels from each plant under a microscope [39]. In addition, we assessed potential effects of the heat treatment on seed germination and seedling growth. After a 30-d heat treatment, none of the seedlings were infected. Moreover, high temperature treatment had no significant effect on germination rate, germination potential and germination index [41].

Experimental Design

The plants used in this experiment were cloned from 100 plants grown from seeds that were not heat treated (endophyte-infected, EI) and 100 plants from seeds that were heat treated for 30 d (endophyte-free, EF), multiplied and selected for uniformity in spring of 2009 and 2010. During this period, the plants were clipped repeatedly and kept in vegetative growth. This procedure allowed the subsequent assessment of plant performance to be separated from the initial heat treatment by a round of vegetative reproduction, and is commonly used in endophyte studies [40,42]. On June 2010, we randomly chose 100 EI and 100 EF tillers (one tiller from each plant), of approximately equal size, and transplanted them evenly into 40 white plastic pots (20 EI and 20 EF pots, five tillers per pot). One pot was 23 cm in diameter and 25 cm in depth and filled with 5 kg of sand. The design of the experiment was completely randomized and a 2 x 2 factorial, with infection status (EI vs. EF), N availability (N+ vs. N−, i.e. supply vs. deficiency), and P availability (P+ vs. P−, i.e. supply vs. deficiency) as the variables. There were five replicates per treatment group. The experiment lasted 49 d, from 5 August to 23 September 2010, and was carried out at the campus.
Growth and Biomass Measurements of tiller number, leaf number and shoot height of the plants were recorded at the beginning and end of the experiment. The positions of the pots were randomly rotated each week to minimize location effects.

Nutrient Treatment
We established four treatments in which nutrient availability was varied, i.e. N\textsuperscript{+}P\textsuperscript{+}, N\textsuperscript{+}P\textsuperscript{2}, N\textsuperscript{2}P\textsuperscript{+} and N\textsuperscript{2}P\textsuperscript{2}. Ramets from each EI and EF group were grown under all combinations of nutrient availability. The nutrients were supplied by the addition of complete Hoagland nutrient solution. The composition of the nutrient solution was 5.0 mM Ca(NO\textsubscript{3})\textsubscript{2}, 5.0 mM KNO\textsubscript{3}, 2.5 mM MgSO\textsubscript{4} \(\cdot\) 7H\textsubscript{2}O, 2.0 mM KH\textsubscript{2}PO\textsubscript{4}, 2 \(\cdot\) 9 Mn\textsubscript{2-}EDTA, 20 \(\mu\)M FeSO\textsubscript{4} \(\cdot\) 7H\textsubscript{2}O, 45 \(\mu\)M H\textsubscript{3}BO\textsubscript{3}, 6.6 \(\mu\)M MnSO\textsubscript{4}, 0.8 \(\mu\)M ZnSO\textsubscript{4} \(\cdot\) 7H\textsubscript{2}O, 0.6 \(\mu\)M H\textsubscript{2}MoO\textsubscript{4}, 0.4 \(\mu\)M CuSO\textsubscript{4} \(\cdot\) 5H\textsubscript{2}O and pH 6.0 \(\pm\) 0.1. For N\textsuperscript{2} treatment, 5.0 mM CaCl\textsubscript{2} and 5.0 mM KCl were added instead of Ca(NO\textsubscript{3})\textsubscript{2} and KNO\textsubscript{3}. For P\textsuperscript{2} treatment, 2.0 mM KCl was added instead of KH\textsubscript{2}PO\textsubscript{4}. The pH was adjusted to 6.0 \(\pm\) 0.1.

During the experiment, 0.8 L of nutrient solution was added twice a week per pot, and 15 times in total. Plants were subjected to ambient light and temperature regimes. The positions of the pots were randomly rotated each week to minimize location effects.

Growth and Biomass Measurements of tiller number, leaf number and shoot height of the longest tiller were made on all ramets at the beginning and end of the experiment. At the end of the experiment, leaves, sheaths and culms were harvested from each tiller, dried and weighed. The positions of the pots were randomly rotated each week.

Table 1. Three-way ANOVA for vegetative growth of endophyte-infected (EI) or endophyte-free (EF) *Achnatherum sibiricum*.

| Tiller No. | Leaf No. | SLA | Shoot biomass | Root biomass | Total biomass |
|-----------|----------|-----|---------------|-------------|--------------|
| df        | MS       | F   | P             | MS          | F   | P   | MS     | F   | P    | MS     | F   | P    |
| Endophyte (E) | 1 1334 | 13.39 | <0.01 | 11730 | 10.99 | <0.01 | 53.94 | 5.426 | 0.026 | 28.80 | 26.58 | <0.01 |
| Nitrogen (N) | 1 8208 | 82.41 | <0.01 | 61701 | 57.83 | <0.01 | 88.66 | 8.917 | <0.01 | 383.2 | 353.6 | <0.01 |
| Phosphorus (P) | 1 5700 | 5.723 | 0.023 | 1918 | 1.798 | 0.189 | 13.17 | 1.324 | 0.258 | 87.38 | 80.65 | <0.01 |
| E x N | 1 7.225 | 0.073 | 0.789 | 24.03 | 0.023 | 0.882 | 0.390 | 0.039 | 0.844 | 0.445 | 0.411 | 0.526 |
| E x P | 1 420.3 | 422.0 | 0.521 | 1113 | 1.043 | 0.315 | 10.44 | 1.050 | 0.313 | 0.702 | 0.648 | 0.427 |
| N x P | 1 2070 | 2079 | 0.159 | 2352 | 0.220 | 0.642 | 4.323 | 0.435 | 0.514 | 3.091 | 2.833 | 0.101 |
| E x N x P | 1 2862 | 2874 | 0.100 | 60.03 | 0.056 | 0.814 | 0.206 | 0.021 | 0.886 | 11.13 | 10.27 | <0.01 |
| Residual | 32 | 99.60 | 1067 | 9.942 | 1.083 | 0.430 | 2.266 |

doi:10.1371/journal.pone.0048010.t001
and roots were harvested separately. Ten fully expanded leaves growing on vegetative tillers per pot were chosen to measure the area and were weighed separately for determination of specific leaf area (SLA). Roots were washed free of soil. Then all plant parts, including leaf blades, sheaths, roots and senescent leaves were separately oven-dried at 60°C.

**Gas Exchange**

At the end of the treatments, gas exchange measurements (see below) were made on the youngest fully expanded attached leaf in a pot with a LI-COR 6400 infrared gas analyzer (LI-Cor, Lincoln, NE, USA). The same leaf was also used for measurements of SLA and N content. In this way, differences among leaves of the same plant could be avoided when the relationships among the variables were analyzed.

Photosynthesis-light responses of plants were assessed under 400 μmol mol⁻¹ CO₂. Net photosynthetic rate (Pn) was measured at 1500, 1200, 1000, 800, 600, 400, 300, 200, 150, 100 and 50 μmol m⁻² s⁻¹ PPFD (photosynthetic photon flux density). From the Pn–PPFD curve, Pmax and saturation PPFD were determined.

Photosynthesis-CO₂ responses of plants were assessed under saturation PPFD, 1200 μmol m⁻² s⁻¹. Pn was measured at 1500, 1200, 1000, 800, 600, 400, 300, 200, 150, 100 and 50 μmol mol⁻¹ CO₂ in the reference chamber. The leaf temperature was held constant at 25°C by the equipment. From the Pn–Ci (internal CO₂ concentration) curve, the parameters needed to calculate the fraction of leaf N allocated to the photosynthetic machinery were determined. The calculation details are as follows.

The Pn–Ci curve was fitted with a linear equation (Pn = kCi + i) within 50–200 μmol mol⁻¹ Ci [43]. Maximum carboxylation rate (Vc max) and dark respiration rate (Rd) were calculated according to Farquhar and Sharkey [44] as follows:

\[
V_{c\ max} = k \left( C_{i} + K_{c} \left(1 + O_{i}/K_{o}\right)\right)^{2}/\left(\Gamma + K_{c} \left(1 + O_{i}/K_{o}\right)\right)
\]

\[
R_{d} = V_{c\ max} (C_{i} - \Gamma')/\left[C_{i} + K_{c} \left(1 + O_{i}/K_{o}\right)\right]^{-(i+C_{i}+i)}
\]

where Kc and Kω are the Michaelis–Menten constants of Rubisco for carboxylation and oxidation, respectively, and calculated according to Niinemets and Tenhunen [45]. \(\Gamma\) is the CO₂

**Table 2.** Biomass allocation of endophyte-infected (EI) or endophyte-free (EF) *Achnatherum sibiricum* under various conditions of N and P availability.

| Treatment | Shoot biomass (g) | Root biomass(g) | Total biomass(g) | Root: Shoot |
|-----------|-------------------|-----------------|------------------|-------------|
| P+ N+     | 14.15 ± 1.299a    | 5.14 ± 0.846a   | 19.30 ± 1.999a   | 0.36 ± 0.042c |
|           | 13.56 ± 1.501a    | 4.70 ± 0.691a   | 18.26 ± 1.929a   | 0.35 ± 0.049b |
| P− N−     | 9.36 ± 0.775b     | 4.95 ± 0.428a   | 14.32 ± 1.088b   | 0.53 ± 0.042 b|
|           | 7.09 ± 0.579c     | 3.76 ± 0.437b   | 10.85 ± 0.790c   | 0.53 ± 0.069 b|
| P+ N+     | 13.07 ± 1.271a    | 5.24 ± 0.547a   | 18.31 ± 1.748a   | 0.40 ± 0.024c |
|           | 9.84 ± 1.247b     | 3.74 ± 0.915b   | 13.58 ± 1.897b   | 0.38 ± 0.078c |
| P− N−     | 5.06 ± 0.424d     | 3.46 ± 0.494bc  | 8.52 ± 0.446d    | 0.69 ± 0.142a |
|           | 4.37 ± 0.672d     | 2.78 ± 0.701c   | 7.15 ± 1.332d    | 0.63 ± 0.088a |

Note. Values are means ± SE. Significant differences (*P* < 0.05) for each variable are indicated by lowercase letters for variables where N, P availability and endophyte infection were analyzed together.

doi:10.1371/journal.pone.0048010.t002

**Table 3.** Three-way ANOVA for photosynthetic parameters, N allocation and acid phosphatase activity of endophyte-infected (EI) or uninfected (EF) ramets of *Achnatherum sibiricum*.

|                  | Nₐ   | Pmax | PNUE | Pᵢ | Acid phosphatase activity |
|------------------|------|------|------|----|--------------------------|
|                  | df   | MS   | F    | P  | df   | MS   | F    | P  | df   | MS   | F    | P  | df   | MS   | F    | P  |
| Endophyte (E)    | 1    | 0.567| 90.06| <0.01|31.91|17.67|<0.01|895.2|83.08|<0.01|0.003|18.35|<0.01|
| Nitrogen (N)     | 1    | 4.332|687.6 |<0.01|215.1|119.1|<0.01|771.8|104.7|<0.01|0.029|185.1|<0.01|
| Phosphorus(P)    | 1    | 0.050|7.945 |<0.01|3.869|2.142|<0.01|18.99|6.803|<0.01|0.035|2.628|0.125|
| E* N*            | 1    | 0.001|0.080 |0.780|5.946|3.292|0.082|209.3|74.97|<0.01|0.434|32.56|<0.01|
| E* P             | 1    | 0.045|7.112 |0.012|4.095|2.267|0.145|19.26|6.901|0.015|0.000|0.015|0.905|
| N* P             | 1    | 0.017|2.646 |0.114|0.581|0.322|0.576|21.24|7.609|0.011|0.118|8.816|<0.01|
| E*N* P           | 1    | 0.030|4.688 |0.038|5.115|2.832|0.105|0.647|0.232|0.635|0.027|1.996|0.177|
| Residual         | 32   | 0.006|1.806 |2.791|0.013|0.000|          |        |        |        |        |        |        |

Note. Nₐ, total leaf nitrogen content; Pmax, maximum net photosynthetic rate; PNUE, photosynthetic nitrogen use efficiency; Pᵢ, the fraction of leaf nitrogen allocated to all components of the photosynthetic machinery.

doi:10.1371/journal.pone.0048010.t003
Table 4. N allocation and maximum photosynthetic rate of endophyte-infected (EI) or endophyte-free (EF) *Achnatherum sibiricum* under various conditions of N and P availability.

| Treatment | \(N_A\) | \(P_T\) | \(P_{\text{max}}\) |
|-----------|---------|--------|-----------------|
| \(P^+\)   | \(N^+\) | 0.92±0.084c | 0.719±0.059c | 15.58±1.323a |
|           | \(N^+\) | 1.28±0.100a | 0.474±0.034d | 12.80±1.068b |
| \(N^-\)   | \(N^+\) | 0.35±0.036f | 1.240±0.227b | 10.60±0.708c |
|           | \(N^+\) | 0.61±0.081d | 0.614±0.124cd | 7.95±0.844d |
| \(P^-\)   | \(N^+\) | 1.02±0.114c | 0.571±0.037cd | 15.23±1.223a |
|           | \(N^-\) | 1.13±0.080b | 0.471±0.051d | 12.29±2.773bc |
| \(N^-\)   | \(N^-\) | 0.25±0.075f | 1.529±0.116a | 8.12±0.463d |
|           | \(N^-\) | 0.49±0.023e | 0.758±0.139c | 8.50±0.972d |

Note. \(N_A\), total leaf nitrogen content in g m\(^{-2}\); \(P_T\), the fraction of leaf nitrogen allocated to all components of the photosynthetic machinery in g g\(^{-1}\); \(P_{\text{max}}\), maximum net photosynthetic rate in \(\mu\text{mol m}^{-2} \text{s}^{-1}\). Values are means ± SE. Significant differences (\(P<0.05\)) for each variable are indicated by lowercase letters for variables N, P availability and endophyte infection were analyzed together.

doi:10.1371/journal.pone.0048010.t004

The acid phosphatase secreted by the roots was measured according to the method of McLachlan [49]. At harvest, the sand was washed from the roots, water was removed with tissue paper, and 2.0 g of fresh roots (representative sub-sample) were added to sodium acetate-acetic acid buffer with para-nitrophenyl phosphate (PNPP). The concentration of para-nitrophenol in the solution was determined in a spectrophotometer by measuring the absorbance at 405 nm. Phosphatase activity was calculated as the amount of para-nitrophenol produced per g fresh root mass and per hour.

Statistical Analyses

All statistical analyses were performed with SPSS 10.0 (SPSS, Chicago). For some variables (tiller number, leaf number and biomass allocation), natural log transformation was used to homogenize variance and to obtain a normal distribution of residuals. Effects of N availability, P availability and endophyte infection were analyzed using a three-way analysis of variance (ANOVA). Differences between the means of different treatments and endophyte infection were compared using Duncan’s multiple-range tests at \(P<0.05\).

Results

Shoot Growth and Biomass Allocation

At the beginning of the experiment, there were no significant differences between the EI and EF plants in tiller number (\(F=0.073, P=0.999\)), leaf number (\(F=0.279, P=0.958\)) and

Figure 2. Photosynthetic nitrogen use efficiency (PNUE) of endophyte-infected (EI) or endophyte-free (EF) *Achnatherum sibiricum* under various conditions of N and P availability. Bars are means±1 SE. An asterisk denotes significance at \(P<0.05\). doi:10.1371/journal.pone.0048010.g002

Endophytic Benefits and Nutrients Availability

Other Response Variables

The youngest fully expanded leaves were collected for measuring photosynthetic pigment content [47]. N and P concentrations. Dried roots were sampled for measuring N and P concentrations. N concentrations of the plant were analyzed using the Kjeldahl method, and P concentrations were measured by molybdenum–antimony colorimetric method [48].

Figure 3. Acid phosphatase activity of endophyte-infected (EI) or endophyte-free (EF) *Achnatherum sibiricum* under various conditions of N and P availability. Bars are means±1 SE. An asterisk denotes significance at \(P<0.05\). doi:10.1371/journal.pone.0048010.g003
shoot height of the longest tiller ($F=0.266, P=0.963$). Endophyte presence significantly increased tiller number, leaf number and SLA of A. sibiricum irrespective of N or P availability (Table 1, Fig. 1). Total biomass was significantly affected by main effects of endophyte status, N and P availability, and the interaction of endophyte ×N×P (Tables 1 and 2). Under N+P− and N−P+ conditions, EI plants had significantly higher shoot, root and total biomass than EF plants. Under N+P+ and N−P− conditions, however, there were no significant differences in biomass between EI and EF plants. Under P+ condition, the biomass of both EI and EF plants decreased with N deficiency; however, the degree of decrease was lower for EI than EF plants. At the same time, both EI and EF plants allocated more resources to roots and thus the root:shoot ratio increased with N deficiency. Under N+ condition, when compared with P supply, the total biomass of EI plants was maintained with P deficiency; for EF populations, however, the biomass decreased significantly with P deficiency (Table 2).

### N Allocation and Photosynthesis

Area-based leaf N content ($N_{LA}$) was significantly affected by endophyte infection, N and P availability as well as their interaction (Table 3). In all treatments, $N_{LA}$ of EI was lower than that of EF plants (Table 4). $N_{LA}$ of EI was significantly affected by N supply but not by P supply. For $N_{LA}$ of EF, however, it was significantly affected by both N and P supply. When N allocation was considered, there were differences between EI and EF plants and/or among different treatments. With N supply, EI plants had similar or slightly higher N fractions allocated to the photosynthetic machinery ($P_{1}$) when compared with their EF counterparts. With N deficiency, however, the above N fraction in EI plants was significantly higher compared to their EF counterparts.

The maximum net photosynthetic rate for EI tended to be higher than that of EF, but there was a significant difference only in N+P− and N−P+ treatments (Table 4). When PNUE was considered, it was significantly higher for EI compared to EF plants in all treatments (Fig. 2).

### Acid Phosphatase Activity

Acid phosphatase activity was significantly affected by main effects of endophyte status, N and P availability and the interaction N×P (Table 3). In all treatments, the acid phosphatase activity of EI plants tended to be higher than that of EF plants, but the difference was significant only in N+P− treatment (Fig. 3).

### Plant N and P Concentrations

Both leaf and root N and P concentrations of A. sibiricum were significantly affected by N and P availability as well as endophyte infection (Table 5). Leaf N concentration was significantly lower for EI compared to EF plants. Under N+ conditions, EI leaf N concentration was not affected by P deficiency, while EF leaf N decreased significantly with P deficiency. Under P+ conditions, both EI and EF leaf P concentrations decreased significantly with N deficiency. Endophyte infection had no effect on leaf P concentration but significantly increased root P concentration. Total N concentration (N concentration of the whole plant) was significantly decreased, while total P concentration (P concentration of the whole plant) was significantly increased by endophyte infection, and thus N:P ratio was significantly lower for EI compared to EF plants (Table 6).

### Discussion

The present study demonstrated that a beneficial interaction between the native grass A. sibiricum, and its associated fungal endophyte (Nostocphillum sp.) depended on both N and P availability. When only N or P was limited, EI plants accumulated significantly more aboveground biomass and total biomass than EF plants. When both N and P were limited, however, the benefits of endophyte infection declined. These findings are in agreement with reports on the response of perennial ryegrass to N deficiency [18] and tall fescue to P deficiency [24], in which only one element (N or P) was deficient; and also in agreement with previous research that EI plants have no advantage over EF plants at low nutrient availabilities [3,50]. We did not find a significant advantage of EI plants over their EF counterparts when N and P were supplied – this has been reported previously in tall fescue [3]. A possible explanation for this difference is that the nutrients in the medium where A. sibiricum grew were not sufficiently high. McCormick et al. [4] also found EI Danthonia spicata did not have a performance advantage relative to EF plants under fertilized conditions, in which the medium where D. spicata grew was extremely nutrient poor even in the fertilized treatment. Overall, our results suggested that the benefits from endophyte infection depended largely on the supply of N and/or P. When both N and P were limited simultaneously, the benefits from endophyte infection disappeared.
When N was supplied, EI plants had similar N concentration and total biomass regardless of P status, while EF plants had significantly lower N and total biomass in P deficiency compared to P supply. There were no significant differences in P concentration between shoots of EI and EF plants in all treatments. This suggests that the beneficial effect of endophyte infection on biomass production of the host plants was more strongly regulated by the availability of N rather than P [51]. With N supply, endophyte infection may help the host grass in maintaining biomass regardless of P status. With N deficiency, even with P supply, the biomass of both EI and EF plants decreased; however, EI biomass decreased slowly.

Current knowledge suggests that leaf N content is correlated with photosynthetic capacity [52]. In the present experiment, N concentration was lower for EI compared to EF plants; however, EI plants allocated significantly higher fractions of N to photosynthetic machinery with N deficiency. EI plants had significantly lower leaf N concentration but significantly higher maximum photosynthetic rate, PNUE, and total biomass than did EF plants in the N−P+ treatment. It has been reported that organisms with a greater growth advantage in nutrient-poor environments are those able to modify their body nutrient content and increase efficiency of nutrient use without major decreases in their growth rates [53–54]. Under N−P+ conditions, EI plants grew better than EF plants by lowering their N concentration while increasing their N allocation to photosynthetic machinery. Therefore, it is N allocation to photosynthetic machinery instead of leaf N concentration itself that was more highly correlated with plant growth [55–56].

In N+P− treatment, P concentration in the shoot of EI and EF plants was similar but EI roots had significantly higher P concentration than EF roots, and similar results were reported by Zabalgogeazcoa et al. [57] on the response of *Festuca rubra* grown in low nutrient soil. Higher root P concentration here was attributed to higher acid phosphatase activity of EI roots. Phosphatase is an enzyme excreted by plant roots, fungi and bacteria and may contribute to as much as 65% of the annual P uptake of grasses [58]. A series of studies have shown that phosphatase activity was increased by AM fungal colonization [59]. Thus, high acid phosphatase activity of EI roots related to AM colonization? Endophytes in grasses have been reported to reduce mycorrhizal colonization of host roots as well as spore densities in the soil [60–61]. In our sampled area in the Inner Mongolia Steppe, Bao [62] found that AM infected over 80% of Gramineae; however, the average infection rate was relatively low (i.e. about 28%). AM infection was not found in the *Achnatherum* genus. In the present study, although we did not measure AM colonization of the roots, the plants were grown from seeds collected in the natural grassland where AM colonization was not found in the *Achnatherum* genus, so it is reasonable to assume they were not colonized by mycorrhizae. Therefore, in the N+P− treatment in the present study, it is endophyte infection that significantly improved acid phosphatase activity of the host grass, which led to higher root P concentration and further higher total biomass in EI compared to EF plants.

The results presented here agreed with the initial prediction that beneficial interaction between the native grass *A. sibiricum* and its associated fungal endophyte depended on both N and P availability. The results further suggested that the beneficial effect of endophyte infection was more conditional on N than P. Under N+P− conditions, endophyte infection significantly improved acid phosphatase activity of EI plants, and so biomass of EI plants was not affected by P deficiency, and resulted in a greater P concentration and more biomass in EI than EF plants. Under

### Table 6. N and P concentration of endophyte-infected (EI) or endophyte-free (EF) *Achnatherum sibiricum* under various conditions of N and P availability.

| Treatment | N concentration (g/kg) | P concentration (g/kg) | N:P ratio |
|-----------|------------------------|------------------------|-----------|
|           | Leaf | Root | Total | Leaf | Root | Total | Leaf | Root | Total |
| N−P−      |       |       |       |       |       |       |       |       |       |
| N−EI      | 22.61±0.132c | 6.01±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c | 6.02±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c |
| P−EI      | 22.61±0.132c | 6.01±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c | 6.02±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c |
| N+P−      |       |       |       |       |       |       |       |       |       |
| N−EI      | 22.61±0.132c | 6.01±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c | 6.02±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c |
| P−EI      | 22.61±0.132c | 6.01±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c | 6.02±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c |
| N+P+      |       |       |       |       |       |       |       |       |       |
| N−EI      | 22.61±0.132c | 6.01±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c | 6.02±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c |
| P−EI      | 22.61±0.132c | 6.01±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c | 6.02±0.145d | 28.62±0.132c | 6.20±0.145d | 34.82±0.325c |

Note: Values are means ± SE. Significant differences (P<0.05) for each variable are indicated by lowercase letters for variables N, P availability and endophyte infection were analyzed together.
N−P+ conditions, both EI and EF biomass decreased compared with N+P+ conditions. EI plants had decreased leaf N concentration but allocated higher fractions of N to photosynthetic machinery compared to EF plants, which resulted in a slow decrease of EI growth—thus EI plants had significantly more biomass than EF plants. Under N−P− conditions, both EI and EF plants allocated higher fractions of N to photosynthesis and had a greater P concentration in roots, but there was no significant difference in biomass between EI and EF plants. Additionally, we did not find a clear cost of endophyte infection even in the N−P− treatment. Admittedly, the duration of the field pot experiment was short in comparison with the natural life span of the grass host and our results should be interpreted with caution. We propose that future studies should examine a wider range of native grass-endophyte systems in long-term field studies to better understand the general role of defensive mutualism in endophyte-plant interactions.

**Author Contributions**

Conceived and designed the experiments: AR. Performed the experiments: XI, RH LYMW. Analyzed the data: XI, AR. Contributed reagents/materials/analysis tools: YG. Wrote the paper: AR.

**References**

1. Cheplick GP, Clay K (1988) Acquired chemical defences in grasses: the role of fungal endophytes. Oikos 52:389–388.
2. Elmi AA, West CP (1995) Endophyte infection effects on stomatal conductance, osmotic adjustment and drought recovery of tall fescue. New Phytol 131: 61–67.
3. Hesse U, Schoberlein W, Wittenmayer L, Forster K, Warmtloff E, et al. (2003) Effects of *Nostopodium* endophytes on growth, reproduction and drought-stress tolerance of three *Lolium* L. genotypes. Grass Forage Sci 58: 407–415.
4. McCormick MK, Gross KL, Smith RA (2001) *Dunaliella spicata* (*Prasinaceae*) and *Aphanocapsa baikalensis* (*Balaniasis*): environmental dependence of a symbiosis. *Am J Bot* 88: 907–909.
5. Cheplick GP (2007) Costs of fungal endophyte infection in *Lolium perenne* genotypes from Eurasia and North Africa under extreme resource limitation. *Environ Exp Bot* 60:202–210.
6. Muller CB, Krauss J (2005) Symbiosis between grasses and arbuscular fungal endophytes. *Curr Opin Plant Biol* 8:430–436.
7. Marks S, Clay K (2007) Low resource availability differentially affects the growth of host grasses infected by fungal endophytes. *Int J Plant Sci* 168:1269–1277.
8. Marks S, Clay K, Marks S (1989) Interactions between infection by endophyte fungi and nutrient limitation in the grasses *Lolium perenne* and *Festuca arundinacea*. *New Phytol* 111: 89–97.
9. Ravil C, Balfourier F, Guillaumier JJ (1999) Enhancement of yield and persistence of perennial ryegrass inoculated with one endophyte isolate in France. *Agronomie* 19:635–644.
10. Lewis GC (2004): Effects of biotic and abiotic stress on the growth of three genotypes of *Lolium perenne* with and without infection by the fungal endophyte *Nostopodium loli.* Ann Appl Biol 144: 53–63.
11. Ren AZ, Gao YB, Wang W, Wang JL, Zhao NX (2009) Influence of nitrogen content of perennial ryegrass. *J Integr Plant Biol* 51: 75–83.
12. Poorter H, Evans JR (1998) Photosynthetic nitrogen-use efficiency of species that differ inherently in specific leaf area. *Oecologia* 116: 26–37.
13. Niinemets U, Vallades F, Csaulemann R (2003) Leaf-level photosynthetic activity and plasticity of invasive *Rhododendron ponticum* and non-invasive *Elyx arbutifolius* co-occurring at two contrasting European sites. *Plant Cell Environ* 26:941–956.
14. OuYang T, Hikosaka K, Hase T (2004) Allocation of nitrogen to cell wall decreases photosynthetic nitrogen-use efficiency. *Funct Ecol* 18:419–425.
15. Feng YL, Fu GL, Zheng YL (2008) Specific leaf area relates to the differences in leaf cell construction cost, photosynthesis, nitrogen allocation and use efficiencies between invasive and noninvasive alien congens. *Planta* 229:383–390.
16. FengYL, Lei YB, Wang W, Raaway RM, Valiente-Banuet A, et al. (2009) Evolutionary tradeoffs for nitrogen allocation to photosynthesis versus cell walls in an invasive plant. *PNAS* 106:853–1856.
17. Arachevala M, Bacon CW, Howland CS, Radcliffe E (1989) Effect of the tall fescue endophyte on plant response to environmental stress. *Agron J* 81: 83–90.
18. Ravel C, Courcy C, Goutret A, Pachart G (1997). Beneficial effects of *Nostopodium loli* on the growth and the water status in perennial ryegrass cultivated under nitrogen deficiency or drought stress. *Agronomie* 17: 173–181.
19. Mohren GMJ, Van Den Burg J, Burger FW (1986) Phosphorus deficiency induced by nitrogen input in Douglas fir in the Netherlands. *Plant Soil* 95:191–200.
20. Van Der Woude BJ, Pegtel DM, Bakker JP (1994) Nutrient limitation after long-term nitrogen-fertilizer application in cut grasslands. *J Appl Ecol* 31: 405–412.
21. Malinowski DP, Belesky DP, Baligar VC, Felders JMJ (1998b) Influence of phosphorus on the growth and ergot alkaloid content of *Nostopodium endophytes infected tall fescue (Festuca arundinacea).* *Plant Soil* 190: 53–61.
22. Ren AZ, Gao YB, Zhou F (2007) Response of *Neotyphodium lolii* to phosphorus deficiency. *Plant Soil* 205:1–12.
23. Malinowski DP, Allouche GA, Belesky DP (1998) Evidence for chemical changes on the root surface of tall fescue in response to infection with the fungal endophyte *Neotyphodium cyanosema*. *Plant Soil* 205:1–12.
24. Fujita Y, Robberecht BJM, de Ruiter PC, Heil GW, Wassen MJ (2010) Increased N affects P uptake of eight grassland species: the role of root surface phosphatase activity. *Oikos* 119:1665–1673.
52. Hikosaka K (2004) Interspecific difference in the photosynthesis-nitrogen relationship: patterns, physiological causes, and ecological importance. J Plant Res 117: 481–494.
53. Elser JJ, Acharya K, Kyle M, Comer J, Makino W, et al. (2003) Growth rate-stoichiometry couplings in diverse biota. Ecol Lett 6: 936–943.
54. Mulder K, Bowden WB (2007) Organismal stoichiometry and the adaptive advantage of variable nutrient use and production efficiency in Daphnia. Ecol Modell 202: 427–440.
55. González AL, Kononoski JS, Danger M, Ishida S, Isai N, et al. (2010) Can ecological stoichiometry help explain patterns of biological invasions? Oikos 119: 779–790.
56. Jeyasingh PD, Weider LJ, Sterner RW (2009) Genetically-based tradeoffs in response to stoichiometric food quality influence competition in a keystone aquatic herbivore. Ecol Lett 12:1229–1237.
57. Zabalgogeazcoa I, Ciudad AG, Vázquez de Aldana BR, Criado BG (2006) Effects of the infection of the fungal endophyte Epichloe festucae on the growth and nutrient content of Festuca rubra. Eur J Agron 24: 374–384.
58. Kroehler CJ, Linkins AE (1988) The root surface phosphatases of Eriophorum vaginatum: effects of temperature, pH, substrate concentration and inorganic phosphorus. Plant Soil 103: 3–10.
59. Allen EB, Allen MF, Helm DJ, Trappe JM, Moliva R, et al. (1995) Patterens and regulation of mycorrhizal and fungal diversity. Plant Soil 170: 47–62.
60. Chu-chou M, Guo B, An ZQ, Hendrix JW, Ferriss RS, et al. (1992) Suppression of mycorrhizal fungi in fescue by the Acremonium coenophialum endophyte. Soil Biol Biochem. 24: 633_637.
61. Mack KML, Rudgers JA (2008) Balancing multiple mutualists: asymmetric interactions among plants, arbuscular mycorrhizal fungi, and fungal endophytes. Oikos 117: 310–320.
62. Bao YY (2004) Diversity and ecological distribution of arbuscular mycorrhizal association in the grassland and desert of Inner Mongolia. Ph.D thesis, Inner Mongolia Agricultural University, China.