Endophytic fungi facilitate the coexistence of host grasses and neighboring legumes by changing rhizobial abundance

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Research Article

Keywords: *Achnatherum sibiricum*, endophyte, rhizobia species, plant interaction, *Medicago ruthenica*, plant productivity

Posted Date: November 29th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1038076/v1>

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Abstract

Purpose: Grass-endophyte and legume-rhizobium symbionts coexist in grasslands. However, the effects of endophyte infection on legume-rhizobium symbionts remain poorly understood, especially in natural grasslands.

Methods: In this study, *Achnatherum sibiricum*-*Epichloë* endophytes and *Medicago ruthenica*-rhizobia were selected as materials to investigate whether and how endophyte infection affected the growth of legume-rhizobia symbionts. It was hypothesized that endophytes can facilitate the coexistence of grass-legume systems.

Results: The results demonstrated that endophyte infection affected the growth of both rhizobia and *M. ruthenica*-rhizobia symbionts, and the results depended on rhizobial identity. Endophyte infection inhibited the growth of *Mesorhizobium ciceri*, which significantly promoted the growth of *M. ruthenica*, and promoted *Sinorhizobium meliloti*, which had no significant effect on the growth of *M. ruthenica*. Endophyte infection also changed the interaction between *A. sibiricum* and *M. ruthenica*. When inoculated with *M. ciceri*, endophyte infection weakened the promoting effect of *A. sibiricum* on *M. ruthenica*, while when inoculated with *S. meliloti*, endophyte infection enhanced the promoting effect. Endophyte infection affected the growth of *M. ruthenica*-rhizobia symbionts by affecting rhizobia abundance in roots and nitrogen content in plant leaves.

Conclusion: In conclusion, endophyte infection was beneficial to biomass accumulation and species coexistence in grass-legume mixed planting systems. In this study, it was proposed that endophyte

23 infection may change the growth of legume-rhizobia symbionts by affecting the growth and nitrogen

24 fixation of rhizobia.

25 **Abbreviations**

26 Arbuscular mycorrhizal fungi (AMF); *Lotus tenuis* (*L. tenuis*); *Achnatherum sibiricum* (*A. sibiricum*);

27 *Medicago ruthenica* (*M. ruthenica*); *Mesorhizobium cicero* (*M. ruthenica*); *Sinorhizobium meliloti* (*S.*

28 *meliloti*); A real-time quantitative PCR (qPCR); Relative interaction intensity index (RII)

Introduction

Plant microbiota refers to a large and diverse collection of microorganisms on the surface of plants (including aboveground and underground parts) and in plants in nature (Müller et al. 2016). These interactions play a crucial role in plant growth and development, plant community structure and ecosystem function (Gong and Xin 2020; van der Putten et al. 2009; Wagg et al. 2011). At present, great progress has been made in the study of the interactions between plants and underground microbial rhizobia and arbuscular mycorrhizal fungi (AMF) (Oldroyd et al. 2013; Philippot et al. 2013), but the interactions between aboveground and underground microorganisms and their effects on plant growth are relatively poorly understood.

Endophytic fungi are ubiquitous fungi that inhabit the aboveground tissues of healthy plants without causing obvious disease (Arnold et al. 2000). Endophytic fungi and grasses form a mutualistic symbiosis (Clay and Schardl 2002). In this symbiosis, the host grass provides shelter and photosynthates to the endophytes (Schardl et al. 2004). However, endophytes can promote the growth and development of host grasses (Clay 1990; Saikkonen et al. 2004) and improve the ability of host grasses to resist biotic stresses such as herbivory (Burns and Fisher 2006; Clay et al. 2005), pathogen infection (Rúa et al. 2013; Wang et al. 2021), and abiotic stresses such as drought (Hesse et al. 2003; Worchel et al. 2013) and nutrient deficiency (Malinowski and Belesky 2000; Saari and Faeth 2012).

An increasing number of studies have shown that endophyte infection can affect not only the growth and resistance of host plants but also the soil microbial community in host habitats such as AMF (Iqbal et al. 2012; Slaughter et al. 2019). Focusing on economically important cultivated grasses such as *Lolium arundinaceum* Darbyshire ex. Schreb. (Tall fescue) and *Lolium perenne* L. (Perennial ryegrass), these studies observed that the presence of endophytes generally reduced mycorrhizal colonization (Chu-Chou et al. 1992; Müller 2003; Omacini et al. 2006). However, for native grasses,

the effects of endophyte infection on AMF have been reported to range from negative (Zhou et al. 2018) to neutral (Zhou et al. 2016) to positive (Novas et al. 2005). Additionally, the effects of endophyte infection on AMF also depend on AMF identity (Larimer et al. 2012; Liu et al. 2011).

Rhizobia are widespread in grassland ecosystems (Charpentier and Oldroyd 2010; Hirsch et al. 2001). Rhizobia plays an important role in improving soil fertility and grassland productivity (Chen et al. 2004; Coêlho et al. 2019; Coskun et al. 2017). Does endophyte infection affect rhizobia and legume-rhizobia symbionts in host habitats? At present, limited reports have focused on the effect of endophyte-cultivated grasses such as tall fescue and perennial ryegrass. The negative effects of endophytes on nodule number have been reported in *Medicago sativa* L. (Alfalfa), *Trifolium pratense* L. (Red clover) and *Trifolium repens* L. (White clover) when grown with endophyte-infected (E+) tall fescue (Liu et al. 2021; Staley and Belesky 2004) and in white clover when grown with E+ perennial ryegrass (García Parisi et al. 2015). Regarding the effects of endophyte infection on the growth of legume plants, negative effects have been reported in alfalfa, red clover, *Lotus tenuis* (*L. tenuis*) and white clover when grown with E+ tall fescue (Hoveland et al. 1999; Liu et al. 2021; Malinowski et al. 1999; Petigrosso et al. 2020), white clover when grown with E+ perennial ryegrass (Sutherland and Hogland 1989), and red clover, *Lotus corniculatus* L. (Bird's foot trefoil), *Trifolium subterraneum* L. (Subterranean clover), *Plantago lanceolata* L. (Ribwort plantain) when grown with E+ *Festuca rubra* L. (Red fescue) (Vázquez-de-Aldana et al. 2013). In addition, the neutral (Malinowski et al. 2011; Prestidge et al. 1992) or positive effects of E+ grasses on legume growth (Eerens et al. 1998) have been documented. In natural grasslands, the diversity of legumes and rhizobia is higher than that in artificial grasslands. Does endophyte infection in native grasses affect the growth of legume-rhizobia symbionts? No studies have been reported thus far.

In this study, endophyte-infected and endophyte-free *Achnatherum sibiricum* (L.) Keng (*A. sibiricum*) and *Medicago ruthenica* L. (*M. ruthenica*), a common legume in its natural habitat, were used as materials to explore the following scientific questions: (1) Does endophyte infection affect the rhizobial growth of *M. ruthenica* in a mixture with *A. sibiricum*? (2) Does endophyte infection change the growth of legume-rhizobia symbionts by affecting the growth of rhizobia? (3) Do these effects of endophyte infection depend on rhizobial species identity?

Materials and Methods

EXPERIMENTAL MATERIALS

Achnatherum sibiricum (L.) Keng is a caespitose perennial grass. It is distributed in northeastern, northern and northwestern China and Siberia of Russia, and it is normally a subordinate species in grasslands (Ma et al. 1985). The results of field investigations showed that the infection rate of endophytes was close to 100% (Wei et al. 2006). *A. sibiricum* can be infected by either *Epichloë gansuensis* (Li et al. 2004) or *Epichloë sibirica* (Zhang et al. 2009) in different plants. According to previous studies, there was no significant difference between the effects of the two endophyte species on pathogenic fungi (relevant data have not been published), AMF (Wu et al. 2018) or rhizobial growth (Appendix). Therefore, only the influence of endophyte infection on the *Medicago ruthenica*-rhizobia symbiont was discussed.

Endophyte-infected (E+) seeds of *A. sibiricum*, seeds of *M. ruthenica* and cultivated soil were collected from natural grassland in the National Hulunber Grassland Ecosystem Observation and Research Station (119.67°E, 49.10°N) in northeast China, where the annual mean temperature is roughly -2 °C and the mean annual precipitation is approximately 367 mm. Endophyte-free (E-) seeds

were obtained from E+ seeds treated at 60 °C for 30 days, and this disinfection method is an ideal method for constructing the E- population of *A. sibiricum* (Li et al. 2010). The collected soil was homogenized and sieved (passed through a 2 mm mesh) and autoclaved at 1 atm pressure and 121 °C for 20 min three times with 24 h intervals before use.

After surface sterilization with 0.5% sodium hypochlorite solution for 10 min and washing with sterilized water, seeds were planted in plastic pots (220 mm in diameter, 170 mm in depth) filled with 2.5 kg of the soil. Each pot has 10 plant individuals. At the beginning of the experiment, the aniline blue staining method was used to analyze the endophyte infection frequency of the seeds (Latch et al. 1984). The results showed that the infection rates of E+ and E- plants were 100% and 0%, respectively.

Two rhizobial species were chosen, *Mesorhizobium cicero* (*M. cicero*) and *Sinorhizobium meliloti* (*S. meliloti*), purchased from Beijing Beina Chuanglian Biotechnology Company, which are common species in Inner Mongolia steppe (Bi et al. 2009). Before use, the tested strains were first activated on YMA plates and cultured at 28 °C for 3 days. Then, they were transferred to liquid medium and cultured on a shaking bed (150 r·min⁻¹, 28 °C, 30 h). In the first three days, each seedling of *M. ruthenica* was inoculated with a 100 µL suspension of *M. cicero* or *S. meliloti* (bacterial count >10⁹ cuf·mL⁻¹) and simultaneously treated with 100 µL sterile water (the control)

EXPERIMENTAL DESIGN

A randomized block design with three factors was used. The first factor was the endophyte infection status of *A. sibiricum*, which was divided into two levels: E+ and E-. The second factor was rhizobial inoculation treatment, including *M. cicero* (Mc), *S. meliloti* (Sm) and a nonrhizobial (NR) control. The third factor was plant mixture type, including monoculture and mixture (10 plant

individuals per plant species for monocultures and eight *A. sibiricum* + two *M. ruthenica* individuals in mixtures). Note that *A. sibiricum* was not colonized by rhizobia, and there were no rhizobial inoculation treatments for *A. sibiricum* monoculture. Thus, there were 11 combinations. Each combination was replicated five times, yielding a total of 55 pots. The experiment began in August 2020 and was conducted in the campus experimental field at Nankai University, Tianjin, China, lasting 4 months. During the experiment, each pot was watered as needed. The position of the pots was randomly changed every week to eliminate the position effect.

At the end of the experiment, the roots of *M. ruthenica* were rinsed clearly, and a certain amount was taken to assess the root rhizobia abundance. The remaining aboveground and underground plants were dried at 80 °C for 24 h and weighed to obtain the biomass. The biomass per plant was calculated according to the number of plants per pot. The contents of C and N in aboveground subsamples of *M. ruthenica* after drying were determined by elemental analysis (Vario EL/Micro Cube, Elementar, Hanau, Germany), and the flavonoid content was determined by UV spectrophotometry (Bo et al. 2008).

RATERHIZOBIA ABUNDANCE

A real-time quantitative PCR (qPCR) system was established for quantification of rhizobia in the plant tissue of *M. ruthenica*. Genomic DNA (gDNA) was extracted from approximately 100 mg of fresh plant root tissue and 5 ml of rhizobial suspension. DNA extraction was performed with the NuClean Plant Genomic DNA Kit and TIANamp Bacteria DNA Kit. The concentration and purity of extracted gDNA were estimated by using a NanoDrop2000. Quantitative PCR was performed with an UltraSYBR Mixture kit using a Mastercycler EP Realplex 96-well reaction plate (Eppendorf, Hambury,

Germany). *NodC* (forward: 5'-ATGCTCAAGGCGGGTTTC-3'; reverse: 5'-TCGCTATTGCTCAATGTACAAAG-3') of *M. cicero* and *mlcC6601* (forward: 5'-CTCGCGTCGGACTATGCA-3'; reverse: 5'-CCACTTCTCGGTCAGCTCTT-3') of *S. meliloti* were selected for quantitative analysis (Han et al. 2020). The qPCR protocol started at preincubation with one cycle of 95 °C for 10 min, followed by amplification with 40 cycles of 95 °C for 15 s, 64 °C for 30 s, and 72 °C for 32 s. The standard curve was constructed by gradient dilution of rhizobial gDNA suspension.

THE RELATIVE INTERACTION BETWEEN *A. SIBIRICUM* AND *M. RUTHENICA*

The relative interaction intensity index (RII) is commonly used to reflect the interaction strength between plant species (Armas et al. 2004) and is expressed as

$$RII = (DM_{ij} - DM_{ii}) / (DM_{ij} + DM_{ii})$$

DM_{ij} is the dry weight of species *i* under mixed planting, and DM_{ii} is the dry weight of species *i* under monoculture. When the value is 0 to +1, it indicates a positive interaction (facilitation) between plants. Values of -1 to 0 indicate negative interactions (competition) between plants.

STATISTICAL ANALYSES

All statistical analyses were performed with SPSS software (Version 20.0, SPSS, Chicago, USA). A two-way analysis of variance (ANOVA) was used to analyze the effects of endophyte infection (E) and rhizobia (R) on the root rhizobia abundance, RII, flavonoid content of *M. ruthenica* and pot biomass, while the biomass of *M. ruthenica* was analyzed by means of a two-way ANOVA considering the Factors R and plant mixture type (C). Linear regression was used to test the relationship between

root rhizobia abundance and biomass, between root rhizobia abundance and nitrogen concentration, and between nitrogen concentration and biomass of *M. ruthenica*. A single sample T test was used to compare the significance of the difference between the *M. ruthenica* RII and 0. The differences between the means among different factors were compared using Duncan's multiple-range tests at $P < 0.05$.

Results

PLANT EFFECTS OF ENDOPHYTE INFECTION ON ROOT RHIZOBIA ABUNDANCE OF *MEDICAGO RUTHENICA*

The effect of endophyte infection on the rhizobial abundance of *M. ruthenica* depended on rhizobial species identity. When inoculated with Mc, the rhizobial abundance of *M. ruthenica* mixed with E+ *A. sibiricum* was significantly lower than that mixed with E- *A. sibiricum*. Mc is inhibited by endophyte infection. When inoculated with Sm, the rhizobial abundance of *M. ruthenica* mixed with E+ *A. sibiricum* was significantly higher than that of E- *A. sibiricum*, indicating that Sm is promoted by endophyte infection (Table 1, Fig. 1).

(Fig. 1)

EFFECTS OF ENDOPHYTE INFECTION AND RHIZOBIA INOCULATION ON THE BIOMASS OF *M. RUTHENICA*

In the mixture with *A. sibiricum*, Mc inoculation significantly promoted the growth of *M. ruthenica*, while Sm inoculation did not affect the growth of *M. ruthenica* when compared with NM

treatment. That is, Mc was beneficial, while Sm was neutral to the growth of *M. ruthenica* in the mixture. The effect of endophyte infection on the growth of *M. ruthenica* was related to rhizobial status. In the NM treatment, endophyte infection had no significant effect on the growth of *M. ruthenica*. When inoculated with beneficial Mc, endophyte infection inhibited the growth of *M. ruthenica*. When inoculated with neutral Sm, endophyte infection improved the growth of *M. ruthenica* (Table 1, Fig. 2).

(Fig. 2)

RELATIONSHIP AMONG THE BIOMASS, LEAF NITROGEN CONCENTRATION AND ROOT RHIZOBIA ABUNDANCE OF *M. RUTHENICA*

There was a significant positive correlation between the biomass and rhizobial abundance of *M. ruthenica* regardless of rhizobial species (Fig. 3a). There was a significant positive correlation between leaf nitrogen concentration and root rhizobia abundance of *M. ruthenica* (Fig. 3b). There was also a significant positive correlation between the biomass of *M. ruthenica* and leaf nitrogen concentration (Fig. 3c); that is, rhizobial abundance had an impact on biomass by affecting leaf nitrogen concentration.

(Fig. 3a; Fig. 3b; Fig. 3c)

EFFECTS OF ENDOPHYTE INFECTION AND RHIZOBIA INOCULATION ON RELATIVE INTERACTION INTENSITY(RII) BETWEEN *A. SIBIRICUM* AND *M. RUTHENICA*

When *M. ruthenica* was mixed with *A. sibiricum*, the RIIs were all greater than 0, which indicated

that *A. sibiricum* could promote the growth of *M. ruthenica*. When rhizobia were not inoculated, endophyte infection did not change the promoting effect of *A. sibiricum*. When inoculated with rhizobia, the effect of endophyte infection on promoting the effect of *A. sibiricum* was dependent on rhizobial species identity. Inoculation with beneficial Mc, endophyte infection reduced the promoting effect of *A. sibiricum* on *M. ruthenica*. Inoculation with neutral Sm, endophyte infection enhanced the promoting effect of *A. sibiricum* on *M. ruthenica* (Table 1, Fig. 4a).

(Fig. 4a)

For the total biomass of the mixed planting system, the biomass of E+ *A. sibiricum*-*M. ruthenica* mixed planting system was significantly higher than that of E- *A. sibiricum*-*M. ruthenica* mixed planting system regardless of rhizobial status (Table 1, Fig. 4b), i.e., endophyte infection promoted biomass accumulation in the grass-legume mixed planting system, and only the degree of promotion was related to rhizobial status.

(Fig. 4b)

EFFECTS OF ENDOPHYTE INFECTION AND RHIZOBIA INOCULATION ON FLAVONOID CONTENT OF *M. RUTHENICA*

The effect of endophyte infection on the flavonoid content of *M. ruthenica* depended on rhizobial species identity. Under Mc inoculation, the accumulation of flavonoids was reduced by endophyte infection. Under Sm inoculation, the accumulation of flavonoids was promoted by endophyte infection

(Table 1, Fig. 5).

(Fig. 5)

Discussion

Endophytes exist in the aboveground part of host plants. At present, the effect of endophytes on underground AMF has been widely studied in cultivated grasses as well as native grasses (Arrieta et al. 2015; Buyer et al. 2011; Liu et al. 2020; Mack and Rudgers 2008; Vignale et al. 2016;). Similar to AMF, rhizobia are also an underground beneficial microorganism in grasslands. Regarding the effect of endophyte infection on rhizobia in the host habitat, only the effect of cultivated grass, such as tall fescue and perennial ryegrass, on nodule number has been reported. In a mixed system of tall fescue with white clover and perennial ryegrass with white clover, endophyte infection significantly reduced the nodulation number of legume plants (García Parisi et al. 2015; Liu et al. 2021). In native grasslands, the diversity of both endophytes and rhizobia is higher than that of cultivated grassland. Therefore, is the influence of endophyte infection on rhizobia dependent on the species of rhizobia? Thus far, no study has investigated this relationship. This study showed that the effect of endophyte infection on rhizobia depended on rhizobial species identity. Endophyte infection had an inhibitory effect on Mc, which promoted the growth of *M. ruthenica*, but had a promotive effect on Sm, which had no significant effect on the growth of *M. ruthenica*.

Currently, the mechanism of the effect of endophytes on rhizobia has not been identified. One possible reason is that endophyte infection can change rhizobial infection of legumes by affecting the flavonoid content in neighboring legumes. Flavonoids are important chemical signals in the

establishment of legume-rhizobium symbionts and plant-AMF symbionts (Abdel-Lateif et al. 2012; Hassan and Mathesius 2012). In the study of AMF, it was found that the higher the concentration of flavonoids in root exudates of *Triadica sebifera* L. (Tallow tree), the higher the AMF colonization rate (Tian et al. 2020). In this study, when Mc was inoculated, endophyte infection reduced both the flavonoid content and rhizobial abundance of neighboring legumes. When Sm was inoculated, endophyte infection increased the content of both flavonoids and rhizobia. Another possible reason is that endophyte infection changes the community of soil microorganisms associated with plant roots (Bell-Dereske et al. 2017; Ju et al. 2020; Rojas et al. 2016), thus affecting the interaction between rhizobia and other soil microorganisms. Han et al. (2020) found that *Sphingomonas* was beneficial to the growth of *Mesorhizobium* but harmful to the growth of *Sinorhizobium* in the rhizosphere soil of *Glycine max* cv. Williams (Soybean). Using high-throughput sequencing technology, the V5-V7 region of 16S rRNA of the bacteria in the rhizosphere soil of E+ and E- *A. sibiricum* was sequenced in our laboratory, and it was found that endophyte infection significantly reduced the content of *Sphingomonas* in the rhizosphere soil of *A. sibiricum* (relevant data have not been published). In this study, endophyte infection may decrease the content of Mc and increase the content of Sm in the roots of neighboring legumes by decreasing the content of *Sphingomonas* in the rhizosphere soil.

Studies on cultivated grass such as tall fescue and perennial ryegrass showed that endophyte infection tended to inhibit nodule formation in neighboring legumes. Can endophyte infection further affect the growth of legumes by affecting nodule formation? Studies using E+ tall fescue in mixture with alfalfa, red clover (Staley and Belesky 2004) and white clover (Liu et al. 2021) have found that endophyte infection reduces the number of legume root nodules as well as the growth of legume plants. However, García Parisi et al. (2015) found that although E+ perennial ryegrass reduced the nodule

number of white clovers, it had no significant impact on the biomass of white clovers. In the present study, the rhizobial abundance of *M. ruthenica* roots was quantitatively analyzed by qPCR, and we found that there were significantly positive correlations between biomass and leaf nitrogen concentration and between leaf nitrogen concentration and rhizobial abundance of *M. ruthenica* regardless of rhizobial status. Therefore, it was speculated that endophyte infection changed the biomass of *M. ruthenica* by affecting rhizobial abundance and leaf nitrogen concentration. García Parisi et al. (2015) found that the endophyte in perennial ryegrass reduced the nodule number of white clovers but had no significant impact on the nitrogen concentration and biomass of white clovers. The effects of endophyte infection on leaf nitrogen concentration and biomass were similar, which was consistent with the results. However, there was no relationship between the effects of endophyte infection on root nodule number and leaf nitrogen concentration, which was inconsistent with the results, indicating that nodule number was not an ideal indicator to reflect the nitrogen fixation ability of legumes (Mothapo et al. 2013).

Studies on cultivated grass showed that endophyte infection affected host grass-legume interactions. In the mixed system of red fescue with red clover, endophyte infection intensified the inhibitory effect of red fescue on red clover (Vázquez-de-Aldana et al. 2013). In the mixed system of tall fescue and red clover, endophyte infection weakened the promoting effect of tall fescue on red clover (Malinowski et al. 1999). In this study, the growth of *M. ruthenica* was generally promoted by *A. sibiricum*, and endophyte infection could affect the interaction between host *A. sibiricum* and *M. ruthenica*. This result was dependent on rhizobial species identity. When beneficial Mc was inoculated, endophyte infection weakened the promoting effect of *A. sibiricum*. When inoculated with neutral Sm,

endophyte infection enhanced the promotion effect of *A. sibiricum*. Simultaneous infections with endophytes and rhizobia are beneficial to the coexistence of host grasses and legumes.

Endophytes can affect host grass-legume interactions, so what is the effect of endophyte infection on the productivity of the host grass-legume interaction system? García Parisi et al. (2015) found that in the mixed system of E+ perennial ryegrass and white clover, although endophyte infection reduced the nodule number of white clovers, it had no effect on the growth of white clovers, and the total biomass of the system increased. Petigrosso et al. (2020) found that in a mixed system of E+ tall fescue and *L. tenuis*, the increase in tall fescue yield compensated for the decrease in *L. tenuis* yield, and the total biomass of the system increased. In this study, the results showed that regardless of whether the endophytes inhibited or promoted the growth of *M. ruthenica*, in terms of mixture system biomass, endophyte infection significantly increased the biomass of the system regardless of rhizobial status, which may have resulted from the effective differentiation of these two symbionts in resource utilization and space use (Nyfeler et al. 2009).

In conclusion, using the native grasses *A. sibiricum* and *M. ruthenica* as materials, the study demonstrated that the effect of endophyte infection on the rhizobia of neighboring legumes was dependent on rhizobial species identity. The beneficial Mc was inhibited by endophyte infection, while neutral Sm was promoted by endophyte infection. Endophyte infection changed the growth of legumes by affecting rhizobia in roots and nitrogen content in leaves. Endophyte infection was not only beneficial to biomass accumulation in grass-legume systems but also facilitated the coexistence of grass and legumes in the system. Therefore, E+ grasses are expected to be an important resource of grass species for grassland improvement.

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517 **Table 1.** Analyses of variance (ANOVA) for root rhizobia abundance, RII, flavonoid content of *Medicago ruthenica* and pot biomass

518

	<i>dMathef</i>	Root rhizobia abundance		RII		Pot biomass		Flavonoid content	
		<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Endophyte(E)	1	5.850	.024	5.298	.030	45.202	<.001	4.294	.049
Rhizobia (R)	2	134.394	<.001	12.717	<.001	6.207	.007	11.697	<.001
E × R	2	120.229	<.001	11.583	.001	3.687	.040	10.551	.001

519 Significant *P* -values (*P* < 0.05) are in bold.

Fig. 1 Effect of the interaction between endophyte infection and rhizobia on the root rhizobia abundance of *Medicago ruthenica*. Bars represent $M \pm SE$ ($n = 5$). The different letters above bars denote means that are significantly different ($P < 0.05$). E⁻, endophyte-free *Achnatherum sibiricum*; E⁺, endophyte-infected *Achnatherum sibiricum*; NM, nonrhizobial treatment; Mc, inoculated *Mesorhizobium cicero*; Sm, inoculated *Sinorhizobium meliloti*

Fig. 2 Effect of the interaction between endophyte infection and rhizobia on the biomass of *Medicago ruthenica*. Bars represent $M \pm SE$ ($n = 5$). The different letters above bars denote means that are significantly different ($P < 0.05$). E⁻, endophyte-free *Achnatherum sibiricum*; E⁺, endophyte-infected *Achnatherum sibiricum*; NM, nonrhizobial treatment; Mc, inoculated *Mesorhizobium cicero*; Sm, inoculated *Sinorhizobium meliloti*

Fig. 3 The relationship between biomass, leaf nitrogen concentration and rhizobial abundance of *Medicago ruthenica* ($n=20$)

Fig. 4 Effects of the interaction between endophyte infection and rhizobia on the relative interaction intensity (RII) for *Medicago ruthenica* (a) and pot biomass (b). Bars represent $M \pm SE$ ($n = 5$). The different letters above bars denote means that are significantly different ($P < 0.05$). E⁻, endophyte-free *Achnatherum sibiricum*; E⁺, endophyte-infected *Achnatherum sibiricum*; NM, nonrhizobial treatment; Mc, inoculated *Mesorhizobium cicero*; Sm, inoculated *Sinorhizobium meliloti*

Fig. 5 Effect of the interaction between endophyte infection and rhizobia on the flavonoid content of

542 *Medicago ruthenica*. Bars represent $M \pm SE$ ($n = 5$). The different letters above bars denote means that
 543 are significantly different ($P < 0.05$). E⁻, endophyte-free *Achnatherum sibiricum*; E⁺,
 544 endophyte-infected *Achnatherum sibiricum*; NM, nonrhizobial treatment; Mc, inoculated
 545 *Mesorhizobium cicero*; Sm, inoculated *Sinorhizobium meliloti*

546

547 **Appendix**

548 **Fig. S1** Effect of *Epichloë* endophyte culture filtrate on the growth of *Mesorhizobium cicero* (a) and
 549 *Sinorhizobium meliloti* (b). Bars represent $M \pm SE$ ($n = 5$). The different letters above bars denote
 550 means that are significantly different ($P < 0.05$). CK, PDA liquid broth; Eg, *E. gansuensis*; Es, *E.*
 551 *sibiricum*

Fig. 1

554 **Fig. 2**

555

556 Fig. 3

560 Fig. 4

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566

567