

Effect of *Epichloë gansuensis* endophyte on rhizosphere bacterial communities and nutrient concentrations and ratios in the perennial grass species *Achnatherum inebrians* during three growth seasons

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Abstract. *Achnatherum inebrians* is an invasive perennial grass widespread in natural grasslands of north-west China and plays an important role in grassland ecological restoration. The presence of the seed-borne endophytic fungus *Epichloë gansuensis* in *A. inebrians* promotes grass growth, increases resistance to abiotic stress, and affects the rhizosphere microbial community of host plants. However, the relationships among *E. gansuensis*, rhizosphere bacteria and plant contents of carbon (C), nitrogen (N), phosphorus (P) and potassium (K) during different growing seasons are not clear. We examined changes in the rhizosphere bacterial community and in nutrient contents and ratios in *A. inebrians* with (E+) and without (E−) *E. gansuensis* in May, August and December. The Shannon diversity index was higher for rhizosphere bacteria of E+ than E− plants in the three different seasons. Leaf C, N and P contents and root P and K contents were higher in E+ than E− plants in May, and leaf K and root C were higher in E+ than E− plants in August. Leaf C : N ratios were lower in E+ than E− plants in December, and leaf C : K ratios were lower in E+ than E− plants in August and December. In addition, our results indicate significant interactions among rhizosphere bacteria, C, N, P and K contents, and endophyte treatment in three different seasons. In conclusion, *E. gansuensis* enhanced the C, N, P and K contents of host plants, and affected nutrient ratios of *A. inebrians* probably by increasing rhizosphere bacterial diversity and altering rhizosphere bacterial community structure. This study provides new findings on the ecological function of the endophyte *E. gansuensis*, including its potential role in enhancing soil fertility. The improvements in soil fertility were utilised in extrapolating to forage grass–endophyte associations.

Keywords: different growth phase, endophyte, rhizosphere bacterial diversity, stoichiometry.

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Introduction

Maintaining high agricultural productivity and forage yield in the face of increased global food demand and natural resource consumption is a principal problem (Cassman 1999; Tilman *et al.* 2002). Over the last few decades, crop yields have not increased in proportion with increasing fertiliser inputs, resulting in increasing environmental risks and inefficient use of nutrients (Zhang *et al.* 2010a). The need to develop strategies to overcome this challenge has drawn attention to the importance of harnessing the biological potential inherent in

rhizosphere processes for improving the use efficiency of crop nutrients. In the rhizosphere zone, important interactions occur among the plant, soil and microorganisms, and rhizosphere microbes can be regarded as an important factor for plant health. Rhizosphere microbial communities could govern plant nutrient chemistry and regulate plant growth and root health (Marschner *et al.* 2004; Dotaniya and Meena 2015). In addition, rhizosphere microorganisms can also impact nutrient uptake and plant growth by releasing growth-inhibiting or growth-stimulating substances that influence

root structure and root physiology (Calvo *et al.* 2017), and stimulate hormonal regulation (Nihorimbere *et al.* 2011). Marschner *et al.* (2004) used PCR-DGGE (denaturing gradient gel electrophoresis) technology to analyse specific microbial groups in the microbial community, and found that many different factors contribute to shaping species composition and numerical predominance of specific bacteria; these factors include pH, soil type, soil fertility, plant nutrition status, plant species and plant age. Therefore, plant microbial ecologists are interested in the extent to which these communities are associated with changes in various environmental factors (Marschner *et al.* 2004; Ryu *et al.* 2005; Govindasamy *et al.* 2009). The biological activity of both the microorganisms and the root structure could play a central role in nutrient availability (Marschner 2012).

The rhizosphere microbial communities of different plants in the same soil are distinct (Ibekwe and Kennedy 1998; Schlatter *et al.* 2019). Much evidence has shown that the rhizosphere bacterial community will change during the growth and development of the plant (Houlden *et al.* 2008; Ziegler *et al.* 2013), which indicates that the plant growth phase has a key function in regulating the structure of the rhizosphere soil bacterial community (Kourtev *et al.* 2002; Fierer *et al.* 2013). Studies also showed that rhizosphere microbial structure and function are influenced by discrepancies of rhizodeposition and root exudation in the different zones in root, related to soil types, cultural practices, crop rotation and tillage (Miller *et al.* 1989; Westover *et al.* 1997; Grayston *et al.* 1998; Horwath *et al.* 1998; Lupwayi *et al.* 1998; Latour *et al.* 1999; Maarastawi *et al.* 2018; Man *et al.* 2020). Interactions between rhizosphere bacteria and plants also have an important effect on the mineral content of plants (Pii *et al.* 2015), and nutrient deficiencies might be overcome by rhizosphere bacteria (Zhang *et al.* 2010a). Microorganisms such as plant-growth-promoting rhizobacteria and mycorrhizal fungi decompose organic matter by releasing hydrolytic enzymes, thereby increasing soil nutrient availability (Miransari 2011; Ollivier *et al.* 2011; Meena *et al.* 2017). If the benefits of rhizosphere communities to plants are to be positively manipulated, a better understanding of the importance of plant factors and soil for the rhizosphere microbiome is required.

Plant nutrients play an important function in stoichiometry, ecosystem nutrient cycling and their relationship (Yang and Luo 2011; Reed *et al.* 2012). Carbon (C) accounts for 99% of the living biomass of plants. Nitrogen (N), potassium (K) and phosphorus (P) are indispensable macronutrients in the process of plant growth; however, in ecosystems they are usually a major factor limiting plant growth and development (Reef *et al.* 2010). Nitrogen, P, and K govern both larger scale biogeochemical patterns and physiological constraints of organisms (Sternner and Elser 2002). The demand for these elements is not fully understood in different plant growth stages and plant organs (Ågren 2008; Yang and Luo 2011). Some studies demonstrated that plant age and tissue are key factors regulating stoichiometry and nutrient levels (Niklas and Cobb 2006; Yang and Luo 2011). In addition, increasing plant age alters the distribution pattern of metabolites, which

leads to changes in nutrient ratios and levels (Ågren 2008; Niklas and Cobb 2006; Yang and Luo 2011). The P : C, K : C and N : C ratios of leaves all decrease over a growth season (Ågren 2008).

Achnatherum inebrians (Hance) Keng (*Stipa inebrians* Hance) is a perennial grass species, and is widespread in natural grasslands of north-west China, including the subalpine and alpine grasslands of Gansu, Inner Mongolia, Xinjiang, Tibet and Qinghai (Li *et al.* 2004b). In western China, *A. inebrians* has become a dominant invasive species and plays an important role in grassland ecological restoration. A major characteristic of *A. inebrians* is that almost all plants are hosts of asexual endophytic fungi transmitted by seeds. This finding led to ongoing research into this grass–endophyte association, and included the initial assigning of the name *Neotyphodium gansuense* to the endophyte (Li *et al.* 2004b; Nan and Li 2004). In 2015, a re-examination of the endophytes in *A. inebrians* found that there were in fact two species, and these were assigned the names *Epichloë gansuensis* and *E. inebrians* (Chen *et al.* 2015). Li *et al.* (2004a) reported the characteristics of the *Epichloë gansuensis* endophyte: conidia solitariae, subglobosae, ellipsoidea, semiglobularea, reniformia, hyalina, saepe transversaliter affixa, hyalinae, laterales vel intercalares.

Extensive research has examined the consequences of the presence of the endophyte on *A. inebrians* and the ecosystem. The root system of *A. inebrians* is very developed, including the length of primary root and the numbers of lateral and fibrous roots. Xia (2018) showed that the root surface area of *A. inebrians* with endophyte (E+) was significantly greater than of *A. inebrians* without endophyte (E−). The root surface area of *A. inebrians*, both E+ and E−, increased first and then decreased with soil depth, and the maximum superficial area of root was at 10–30 cm soil depth under drought stress. The endophyte *E. gansuensis* dramatically promoted growth over the total root length in *A. inebrians* plants, with the greatest root length of both E+ and E− plants at 10–30 cm soil depth (Xia 2018). *Epichloë gansuensis* improved *A. inebrians* growth under low-N stress by modulating stoichiometry and increasing N-utilisation efficiency of the plant (Wang *et al.* 2018b). *Epichloë gansuensis* significantly affects bacterial diversity in *A. inebrians* rhizosphere soil; Li (2017) found the dominant bacteria of *A. inebrians*–E+ rhizosphere soil to be *Bacillus*, *Hydrogenophaga* and *Pseudomonas* spp. Several studies have demonstrated that the presence of *E. gansuensis* increases the tolerance of *A. inebrians* to biotic and abiotic stresses, including resistance to herbivores, diseases and pests (Li *et al.* 2007; Zhang *et al.* 2012), cadmium stress (Zhang *et al.* 2010b), NaCl stress (Wang *et al.* 2019, 2020a, 2020c), cold stress (Chen *et al.* 2016), drought stress (Xia *et al.* 2018), and low N stress (Wang *et al.* 2018a, 2018b). In addition, *Epichloë* symbionts in other grasses increase tolerance to abiotic stresses, including salt tolerance in tall fescue (*Festuca arundinacea*) (Reza and Mirlohi 2010) and *Hordeum brevisubulatum* (Song *et al.* 2015a), drought tolerance in tall fescue (White *et al.* 1992), and waterlogging tolerance in *H. brevisubulatum* (Song *et al.* 2015b). One study demonstrated that the interaction of grazing and *Epichloë* endophytes affected the contents of P,

silicon and phenolic compounds in meadow fescue (*Schedonorus pratensis*), with silicon content ~16% higher in E+ than E- plants (Huitu *et al.* 2014).

Epichloë endophytes have powerful biological functions that affect the economic and ecological value of artificial and natural grasslands; therefore, further study to explore possible mechanisms and applications is warranted. Recent studies demonstrated that *E. gansuensis* influences rhizosphere bacterial community structure and root-associated fungal community structure, and clarified the effect of this endophyte on the relationship between soil nutrients and rhizosphere soil (Zhong *et al.* 2018; Ju *et al.* 2020). However, whether the presence of *E. gansuensis* can affect both the rhizosphere bacterial community structure and the leaf and root nutrient contents (C, N, P and K) in *A. inebrians* is poorly understood. The above information indicates that the rhizosphere microbial community plays an important role in nutrient absorption by plants. How *E. gansuensis* influences rhizosphere bacteria and regulates nutrient accumulation by the host grass is little understood. The effect of *E. gansuensis* on the rhizosphere bacterial community in host *Achnatherum inebrians* during different seasons of the yearly growing period remains unclear. Therefore, to study the effect of *E. gansuensis* on rhizosphere bacteria and nutrient accumulation by host grasses during different growth seasons, it is useful to gain understanding of the symbiotic relationship between *A. inebrians* and *E. gansuensis*.

The aims of this study are to explore: (i) the influence of *E. gansuensis* on rhizosphere bacterial community structure and diversity under *A. inebrians* in spring (May), autumn (August) and early winter (December); (ii) the influence of *E. gansuensis* on C, N, P and K contents in *A. inebrians* leaves and roots in May, August and December; and (iii) the influence of *E. gansuensis* on the relationship between rhizosphere bacteria and plant nutrients during the three growth seasons.

Materials and methods

Experimental site and rhizosphere sample collection

The experimental site was established at Yuzhong campus of Lanzhou University in 2011. Two regions of a site were established: one *A. inebrians* with *E. gansuensis* (E+), the other *A. inebrians* without *E. gansuensis* (E-). The seeds of E+ and E- *A. inebrians* plants derive from a single E+ plant. Half of the seeds were maintained as E+ for use in the study. The other half were treated with a 100-fold dilution of the fungicide thiophanate-methyl (active ingredient 70% methyltobizine; Jiangsu Longdeng Chemical Co., Ltd) for 2 h, and then the seeds were washed with sterile water. This is an effective method to kill the seedborne fungal endophyte of *A. inebrians* (Li *et al.* 2016; Hou *et al.* 2020). In order to ensure that the field plants in the E+ and E- plots were as required, aniline blue staining was used to check the infection of seedlings from the fungicide-treated and untreated seeds by microscopic examination of leaf-sheath pieces. The existence of endophyte on plants in the experimental areas was also evaluated by aniline blue staining to observe the presence of characteristic hyphae of *E. gansuensis* in leaf sheaths.

The study had three independent replicates. Each replicate comprised two blocks (one E+ and one E-) of dimensions 1.05 m by 2.45 m. Each block had 12 plants arranged in six rows \times two lines, all 35 cm apart. Each plant represented one plot. The distance between E+ and E- blocks in a replicate was 2.5 m. There are 36 plants of each of E+ and E- per replicate, respectively. In the 6 years after establishment, the experimental area was affected by only natural environmental conditions, with no impact of livestock or wildlife. Three rhizosphere soil samples were collected from plants in E+ and E- plots in 2016; all samples from E+ plots showed E+ plants, and all samples from E- plots showed E- plants. The mean annual temperature was 11.6°C, and the annual rainfall was 400 mm year⁻¹ in 2016.

Rhizosphere soil, leaves and roots were collected on 15 May, 15 August and 15 December 2016. The leaves and roots were dried at 80°C to constant weight, and were used to measure contents of C, N, P and K. Samples were transferred to sterile and dry polyethylene bags with a sterilised spoon. Five individual E+ or E- *A. inebrians* plants were sampled for the rhizosphere soil (0–20 cm soil depth) and root samples in five random plots, and these five individual samples were blended into a composite E+ or E- sample. The leaves from the same five individual E+ or E- *A. inebrians* plants were blended into a composite E+ or E- leaf sample. Sampling was performed with three independent repetitions. All samples of rhizosphere soil were sieved through a 2-mm sieve, and stored 4°C for subsequent isolation of culturable rhizosphere soil bacteria.

Field soil nutrient compositions during the two respective growth seasons of May and August were found in our recently published paper (Hou *et al.* 2020), and the field soil nutrient compositions in December were as follows (mean \pm s.e.): organic C 0.45 \pm 0.05% in E+ soil, and 0.48 \pm 0.03% in E- soil; total N (mg g⁻¹) 0.35 \pm 0.01 in E+ soil, and 0.34 \pm 0.01 in E- soil; total P (mg g⁻¹) 0.63 \pm 0.08 in E+ soil, and 0.59 \pm 0.02 in E- soil; nitrate-N (mg kg⁻¹) 1.57 \pm 0.01 in E+ soil, and 1.48 \pm 0.12 in E- soil; ammonium-N (mg kg⁻¹) 29.30 \pm 1.87 in E+ soil, 23.34 \pm 1.47 in E- soil.

Isolation and identification of rhizosphere bacteria

The culture media used in the study are shown in Table 1. Each rhizosphere soil sample (1 g) was added to 100 mL of the different sterilised liquid media, and cultured with constant shaking (150 rpm) at 28°C. After 12 h, the soil suspension was plated on the corresponding agar culture media using the dilution plate method, and incubated for another 24 h at 28°C until colonies appeared. Each colony was transferred to fresh liquid medium of the same type to establish a pure culture. After 12 h of constant shaking (150 rpm) at 28°C, bacterial suspensions were plated onto corresponding agar media, and then incubated for another 24 h at 28°C until colonies appeared. For preservation of bacteria, each colony from the different agar media was transferred to 20 mL KB liquid medium, which was subsequently cultured with constant shaking (150 rpm) at 28°C. After 24 h, 700 μ L each bacterial suspension and 700 μ L 50% glycerol were mixed for storage at -80°C. Identification of rhizosphere soil bacteria was performed by PCR with 16S primers 27F (5'-

Table 1. Composition of the culture media

Culture medium	Composition of the medium (per L)	References
KB medium	Glycerol (20.0 mL), MgSO ₄ ·7H ₂ O (1.5 g), K ₂ HPO ₄ (1.5 g), peptone (20.0 g), agar (15.0 g)	Wang <i>et al.</i> (2015)
NA medium	Beef extract (3.0 g, NaCl (5.0 g), tryptone (10.0 g), pH 7.4, agar (15.0 g)	Yang (2012)
Modified Gao medium NO.1	Starch soluble (20.0 g), K ₂ HPO ₄ (0.5 g), KNO ₃ (1.0 g), NaCl (5.0 g), FeSO ₄ (0.01 g), MgSO ₄ ·7H ₂ O (0.5 g), pH 7.6, agar (15.0 g)	Yang (2012)
Mongina organic culture medium	(NH ₄) ₂ SO ₄ (0.5 g), glucose (10.0 g), MgSO ₄ ·7H ₂ O (0.3 g), NaCl (0.3 g), FeSO ₄ ·7H ₂ O (0.03 g), KCl (0.3 g), CaCO ₃ (1.0 g), MnSO ₄ ·4H ₂ O (0.03 g), yeast extract (0.5 g), lecithin (0.2 g), pH 7.0, agar (15.0 g)	Zhang (2008)
PKO medium	Glucose (10.0 g), NaCl (0.3 g), (NH ₄) ₂ SO ₄ (0.5 g), KCl (0.3 g), FeSO ₄ ·7H ₂ O (0.036 g), MgSO ₄ ·7H ₂ O (0.3 g), Ca ₃ (PO ₄) ₂ (2.0 g), MnSO ₄ ·4H ₂ O (0.03 g), pH 7.0, agar (15.0 g)	Zhang (2008)
Silicate bacteria medium	Sucrose (0.5 g), CaCO ₃ (0.1 g), Na ₂ HPO ₄ (0.5 g), FeCl ₃ (0.05 g), MgSO ₄ ·7H ₂ O (0.5 g), glass powder (0.1 g), pH 7.0, agar (15.0 g)	Zhang (2008)
NFM medium	Malic acid (5.0 g), MgSO ₄ ·7H ₂ O (0.2 g), NaCl (0.1 g), K ₂ HPO ₄ (0.5 g), CaCl ₂ ·2H ₂ O (0.02 g), NaMoO ₄ ·2H ₂ O (0.002 g), bromothymol blue (5 mL 0.5%), KOH (4.5 g), biotin (10.0 g), pH 7.0, agar (15.0 g)	Zhang (2008)
Peptone medium	Peptone (5.0 g), MgSO ₄ ·7H ₂ O (0.5 g), K ₂ HPO ₄ (0.5 g), agar (15.0 g)	Yang (2012)
Stephenson medium	(NH ₄) ₂ SO ₄ (2.0 g), MnSO ₄ ·4H ₂ O (0.01 g), NaH ₂ PO ₄ (0.25 g), MgSO ₄ ·7H ₂ O (0.03 g), K ₂ HPO ₄ (0.75 g), CaCO ₃ (5.0 g), agar (15.0 g)	Yang (2012)
Defined medium	Glucose (10.0 g), CaCl ₂ ·6H ₂ O (0.5 g), K ₂ HPO ₄ (0.5 g), KNO ₃ (1.0 g), agar (15.0 g)	Yang (2012)

AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTCCTTGTTACGACTT-3') (Wang *et al.* 2015). The PCR products were sequenced, and the 16S rDNA sequences were compared with the GenBank databases by using BLAST.

Assay of plant nutrient contents

Total organic C in leaves and roots was measured according to the method of Sparks and Bartels (1996). Total P and N contents in the leaf and root were measured according to Song *et al.* (2015a), and K content in leaves and roots was measured using the method of Bao *et al.* (2016). Data on the contents of C, N, P and K from the leaves and roots were used to calculating C:N, C:P, N:P, N:K and P:K ratios of the leaves and roots.

Calculations and statistical analyses

Calculation of relative frequency of a taxon was by the formula of the number of colonies in a particular taxon divided by the total number of colonies in all taxa (Su *et al.* 2010). The diversity was assessed with the Shannon index (H'):

$$H' = - \sum_{i=1}^k P_i \times \ln P_i$$

where k is the total number of each replicate in the genus level, and P_i is the relative frequency of each replicate in the genus level (Su *et al.* 2010).

SPSS 17.0 software (SPSS, Chicago, IL, USA) was used to analyse data. The significance difference between E+ and E- plant rhizosphere soils for all of the parameters was determined by independent t -tests. Two-way analysis of variance (ANOVA) was used to analyse the effects of endophyte and growth seasons on the Shannon index. Three-way ANOVA was used to analyse the effects of endophytic fungi, growth seasons and plant tissue on the contents of plant C, N), P and K. Three-way ANOVA tested the effects of endophyte, growth season and tissue on

plant C : N, C : P, C : K, N : P, N : K and P : K ratio. Statistical significance was defined at the $P = 0.05$ confidence level, and means are evaluated with standard errors. RDA was performed using Canoco 4.5 software and PCA was performed using Statistic 6.0.

Results

Composition of rhizosphere bacterial community of E+ and E- plants during different growth seasons

The composition of the culturable rhizosphere bacterial communities for the different growth seasons of E+ and E- plants is shown in Supplementary Material, Table S1 (available at the journal's website). In total, 201 rhizosphere bacterial colonies were obtained from E+ and E- *A. inebrians* plant rhizosphere soil sampling in May, of which 116 were from E+ samples, and 85 from E- samples. In August, 275 rhizosphere bacterial colonies were obtained, of which 170 were from E+ samples and 105 from E- samples. In December, 225 rhizosphere bacterial colonies were obtained, of which 127 were from E+ samples, and 98 from E- samples.

The isolates of rhizosphere bacteria from E+ plants in May were classified into eight orders and 11 genera. The orders (with genera in parentheses) were Actinomycetales (*Paenarthrobacter*, *Microbacterium*), Streptomycetales (*Streptomyces*), Rhizobiales (*Rhizobium*), Enterobacterales (*Enterobacter*), Pseudomonadales (*Acinetobacter*, *Pseudomonas*), Bacillales (*Bacillus*), Sphingobacteriales (*Sphingobacterium*) and Flavobacteriales (*Chryseobacterium*, *Flavobacterium*) (Supplementary Table S1). Isolation of rhizosphere bacteria from E- plants in May found five orders and nine genera: Actinomycetales (*Pseudarthrobacter*, *Microbacterium*), Rhizobiales (*Rhizobium*, *Ochrobactrum*), Xanthomonadales (*Stenotrophomonas*), Pseudomonadales (*Pseudomonas*) and Bacillales (*Bacillus*, *Brevibacillus*, *Lysinibacillus*) (Supplementary Table S1). *Bacillus*, *Rhizobium* and *Microbacterium* were the common genera that

occurred in rhizosphere soil of E+ and E- plants at the May sampling (Supplementary Table S1).

Rhizosphere bacteria from E+ plants in August belonged to nine orders and were classified into 19 genera (Supplementary Table S1). The orders and genera were Actinomycetales (*Glutamicibacter*, *Arthrobacter*, *Pseudarthrobacter*, *Microbacterium*), Corynebacteriales (*Rhodococcus*), Rhizobiales (*Rhizobium*), Burkholderiales (*Variovorax*), Enterobacterales (*Enterobacter*, *Erwinia*, *Pantoea*, *Serratia*), Pseudomonadales (*Acinetobacter*, *Pseudomonas*), Xanthomonadales (*Stenotrophomonas*), Bacillales (*Bacillus*, *Brevibacillus*, *Lysinibacillus*, *Paenibacillus*) and Sphingobacteriales (*Olivibacter*). By contrast, the rhizosphere bacteria from E- plants in August belonged to seven orders and were classified into 11 taxa: Actinomycetales (*Arthrobacter*, *Microbacterium*), Corynebacteriales (*Rhodococcus*), Rhizobiales (*Ensifer*), Enterobacterales (*Enterobacter*, *Kluyvera*, *Pantoea*), Pseudomonadales (*Acinetobacter*, *Pseudomonas*) and Xanthomonadales (*Stenotrophomonas*) (Supplementary Table S1). *Kluyvera* and *Ensifer* were unique to the E-plant rhizosphere soil in August. *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Enterobacter*, *Microbacterium*, *Pantoea*, *Pseudomonas* and *Stenotrophomonas* were the common genera that occurred in rhizosphere soil of E+ and E- plants in the August sampling (Supplementary Table S1).

At the December sampling, bacteria in the rhizosphere soil of E+ plants belonged to four orders and 13 genera. These were: Actinomycetales (*Arthrobacter*, *Microbacterium*, *Paenarthrobacter*), Burkholderiales (*Massilia*, *Limnobacter*), Enterobacterales (*Enterobacter*, *Leclercia*, *Erwinia*, *Pantoea*), Pseudomonadales (*Pseudomonas*, *Acinetobacter*) and Bacillales (*Bacillus* and *Lysinibacillus*). The rhizosphere bacteria from E- plants in December belonged to five orders and 10 genera: Actinomycetales (*Paenarthrobacter*, *Glutamicibacter*), Burkholderiales (*Massilia*), Enterobacterales (*Enterobacter*, *Leclercia*, *Citrobacter*, *Kosakonia*), Pseudomonadales (*Acinetobacter*, *Pseudomonas*) and Bacillales (*Bacillus*). *Acinetobacter*, *Bacillus*, *Enterobacter*, *Leclercia*, *Massilia*, *Paenarthrobacter* and *Pseudomonas* were the genera that were present in rhizosphere soil of both E+ and E- plants at the December sampling (Supplementary Table S1). Isolates of *Arthrobacter*, *Erwinia*, *Pantoea*, *Microbacterium*, *Limnobacter* and *Lysinibacillus* were confined to rhizosphere soil of E+ plants in December. Isolates of *Glutamicibacter*, *Kosakonia* and *Citrobacter* were isolated only in rhizosphere soil of E- plants during this sampling season.

In summary, the presence of *E. gansuensis* markedly influenced the *A. inebrians* rhizosphere soil bacterial community, and *Bacillus* was dominant genus in rhizosphere soil of E+ plants and E- plants during the three different growth seasons.

Shannon index of rhizosphere bacteria of E+ and E- plants during different growth seasons

The Shannon index is used to reflect the alpha diversity and can be used to estimate the microbial diversity in the sample.

The larger the Shannon index, the higher diversity of the community. Here, use of the Shannon index can reflect the richness and uniformity of each species in the community. Our data showed that two genera, *Pseudomonas* and *Bacillus*, both had high relative frequency in rhizosphere soil of E+ and E- plants in May (Fig. 1a), and that the relative frequency of *Pseudomonas* was highest (27.51%) and *Acinetobacter* lowest (2.59%) in rhizosphere soil of E+ plants in May (Fig. 1a). The relative frequency of *Bacillus* was highest (49.38%) and *Pseudarthrobacter* lowest (3.53%) in rhizosphere soil of E- plants in May (Fig. 1a). Two genera, *Acinetobacter* and *Bacillus*, both had high relative frequency in rhizosphere soil of E+ and E- plants in August (Fig. 1b), and the relative frequency of *Bacillus* was highest (14.71%) and *Olivibacter* lowest (1.82%) in rhizosphere soil of E+ plants in August (Fig. 1b). The relative frequency of *Arthrobacter* was highest (18.00%) and *Ensifer* lowest (2.82%) in rhizosphere soil of E- plants in August (Fig. 1b). Three genera, *Pseudomonas*, *Bacillus* and *Enterobacter*, had high relative frequency in rhizosphere soil of E+ and E- plants in December (Fig. 1c), and the relative frequency of *Bacillus* was highest (19.60%) and *Massilia* lowest (2.36%) in rhizosphere soil of E+ plants in December (Fig. 1c). The relative frequency of *Pseudomonas* was highest (28.74%) and *Citrobacter* lowest (3.30%) in rhizosphere soil of E- plants in December (Fig. 1c).

We also found that the Shannon index was different between the rhizosphere bacteria communities of E+ and E- *A. inebrians* in May, August and December. The Shannon index of the rhizosphere bacteria of E+ plants was higher than that of E- plants in all seasons, and was enhanced by 28.5% in May, 21.4% in August and 11.4% in December compared with E- plants (Fig. 1d). ANOVA showed that endophyte treatment had a significant ($P < 0.001$) impact on the Shannon index, as did season (Table 2). There was also a significant ($P = 0.007$) endophyte \times season interaction effect on the Shannon index (Table 2). In summary, *E. gansuensis* significantly increased the Shannon index of the community of rhizosphere soil bacteria in *A. inebrians* during the three different growth seasons.

Contents of C, N, P and K in leaves and roots of E+ and E- plants during the different growth seasons

The presence of *E. gansuensis* had significant effects on the C, N, P and K contents of leaves and C, P and K contents of roots during one or more of the growth seasons (Fig. 2). Leaf C content was significantly different between E+ and E- plants in May, being 18.9% higher in E+ plants (Fig. 2a). Similarly, E+ leaves had a higher content of N than E- leaves in May and December, with N content enhanced by 11.9% in May and 41.0% in December (Fig. 2b). Leaf P content was significantly different between E+ and E- plants in May and December, being higher in E+ than E- plants, with a relative increase of 20.8% in May (Fig. 2c). Leaf K content was significantly different between E+ and E- plants in August, being 12.5% higher in E+ plants (Fig. 2d). Root C content of E+ plants was higher than of E- plants in only August, with a relative

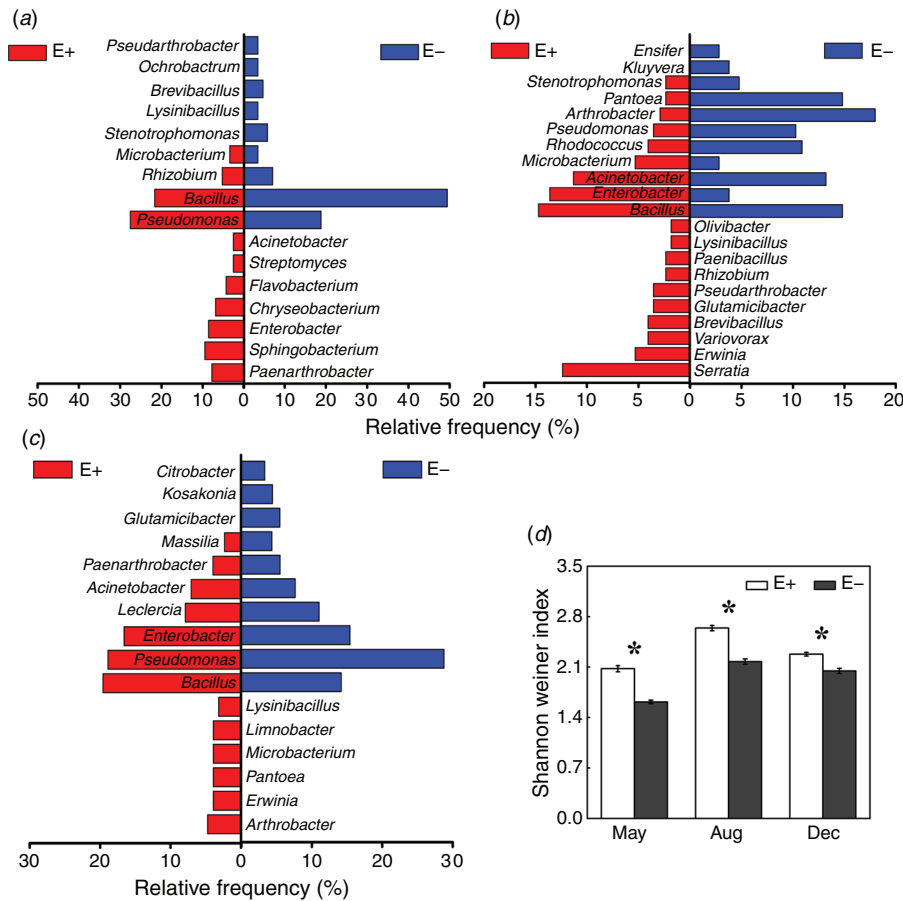


Fig. 1. Relative frequencies of different bacterial community structures at the genus level in rhizosphere soil of *A. inebrians* with (E+) and without (E-) *E. gansuensis* endophyte in (a) May, (b) August and (c) December. (d) Shannon index of the rhizosphere bacteria at the different growth periods of E+ and E- *A. inebrians*. Data are the mean of three replicates, capped lines are standard errors. * $P < 0.05$: significant difference between E- and E+ by independent *t*-tests.

Table 2. Two-way ANOVA for the effects of endophyte presence (with, without) and growth season (May, August, December) on Shannon index of rhizosphere bacterial community

Treatment	d.f.	F-value	P-value
Endophyte (E)	1	193.561	<0.001
Season (S)	2	136.899	<0.001
E × S	2	7.664	0.007

enhancement of 9.8% (Fig. 2e). Root P and K contents of E+ plants were increased by 35.0% and 22.6%, respectively, compared with E- plants in May (Fig. 2g, h). However, there was no clear difference in root N content between E+ and E- plants in any season (Fig. 2f). Three-way ANOVA showed a significant ($P = 0.002$) main effect of endophyte treatment on content of N (but not C, P or K), whereas season and tissue effects were significant ($P < 0.001$) on contents of all four elements (Table 3). Significant two-way interaction effects included endophyte × season on N content ($P = 0.001$); endophyte × tissue on N ($P < 0.001$) and K ($P = 0.030$) contents; and season × tissue on N ($P < 0.001$), P ($P < 0.001$)

and K ($P < 0.001$) contents (Table 3). The three-way interaction effect of endophyte × season × tissue was significant on K content ($P < 0.001$, Table 3). In summary, *E. gansuensis* had significantly different effects on the contents of C, N, P and K in *A. inebrians* leaves and roots during the three growth seasons.

Ratios among C, N, P, and K contents leaves and roots of E+ and E- A. inebrians during the different growth seasons

The ratios C:N, C:P and C:K increased over the growing season. The C:K ratio of E+ leaves was significantly ($P < 0.05$) higher than of E- leaves in May, but lower in August and December. The C:P ratios of E+ and E- leaves were not significantly different at any sampling. In December, the C:N ratio of E+ leaves was remarkably ($P < 0.05$) lower than of E- leaves (Fig. 3a-c). The N:K ratio of E+ leaves was higher than of E- leaves in May and December, and the P:K ratio of E+ leaves was higher than of E- plants in May (Fig. 3e, f). The C:N and C:K ratios of E+ roots were significantly higher than of E- roots in August (Fig. 3g, i). Three-way ANOVA showed no significant main effect of endophyte treatment on the ratios

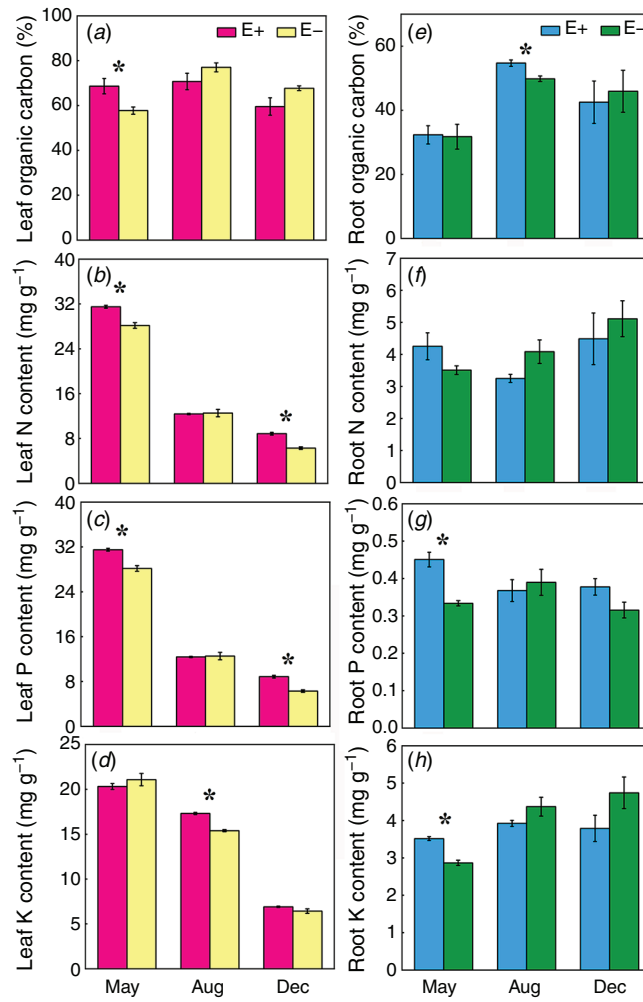


Fig. 2. Influence of *E. gansuensis* endophyte (E+, E-) on *A. inebrians* nutrient contents in the different growth seasons: (a) leaf organic C, (b) leaf N, (c) leaf P, (d) leaf K, (e) root organic C, (f) root N, (g) root P, (h) root K. Data are means \pm standard error. * $P < 0.05$: significant difference between E- and E+ by independent *t*-tests in the same growth season.

Table 3. Three-way ANOVA for the effects of endophyte presence (with, without), growth season (May, August, December) and plant tissue (root or leaf) on plant carbon, nitrogen, phosphorus and potassium contents

P-values in bold are significant

Treatment	d.f.	Carbon		Nitrogen		Phosphorus		Potassium	
		<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value
Endophyte (E)	1	0.015	0.905	11.670	0.002	1.006	0.326	0.716	0.406
Season (S)	2	18.202	<0.001	711.679	<0.001	44.227	<0.001	509.533	<0.001
Tissue (T)	1	130.709	<0.001	2571.522	<0.001	498.375	<0.001	3879.380	<0.001
E \times S	2	2.514	0.102	8.901	0.001	3.040	0.067	3.039	0.067
E \times T	1	0.197	0.662	19.158	<0.001	0.108	0.745	5.298	0.030
S \times T	2	2.9327	0.073	792.937	<0.001	40.075	<0.001	662.650	<0.001
E \times S \times T	2	2.29	0.123	2.382	0.114	1.731	0.199	10.948	<0.001

C : N, C : P, C : K, N : P, N : K and P : K; however, season and tissue effects were significant on C : N, C : P, C : K, N : P and P : K (Table 4). Significant two-way interaction effects included endophyte \times tissue on N : P ratio ($P = 0.039$), and

season \times tissue on C : N, C : K, N : P, N : K and P : K ratios (Table 4). The three-way interaction effect of endophyte \times season \times tissue was significant on C : K ratio ($P = 0.043$, Table 4).

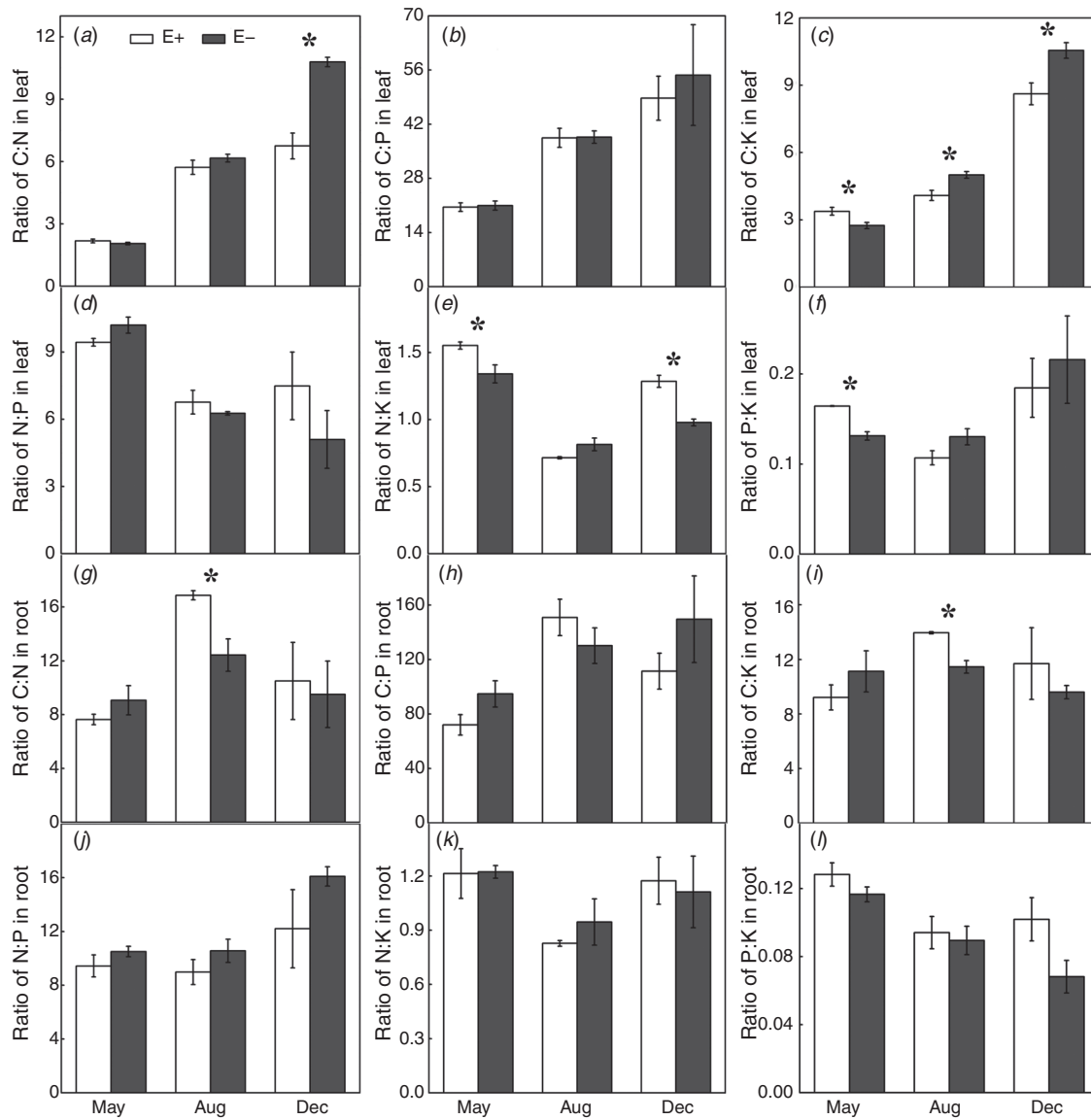


Fig. 3. Effect of *E. gansuensis* endophyte (E+, E–) on *A. inebrians* nutrient ratios in the different growth seasons: (a) leaf C : N, (b) leaf C : P, (c) leaf C : K, (d) leaf N : P, (e) leaf N : K, (f) leaf P : K, (g) root C : N, (h) root C : P, (i) root C : K, (j) root N : P, (k) root N : K, and (l) root P : K. Data are means ± standard error. **P* < 0.05: significant difference between E– and E+ by independent *t*-tests in the same growth season.

Table 4. Three-way ANOVA for the effects of endophyte presence (with, without), growth season and plant tissue (root, leaf) on plant C : N, C : P, C : K, N : P, N : K and P : K ratios
P-values in bold are significant

Treatment	d.f.	C : N		C : P		C : K		N : P		N : K		P : K	
		<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value	<i>F</i> -value	<i>P</i> -value
Endophyte (E)	1	0.007	0.934	1.142	0.296	0.019	0.891	1.238	0.277	1.206	0.283	0.197	0.661
Season (S)	2	19.587	<0.001	12.354	<0.001	13.481	<0.001	3.814	0.036	30.321	<0.001	4.636	0.020
Tissue (T)	1	58.691	<0.001	124.991	<0.001	97.236	<0.001	32.121	<0.001	0.347	0.561	27.540	<0.001
E × S	2	2.265	0.126	1.719	0.201	0.557	0.580	0.028	0.972	2.657	0.091	0.773	0.473
E × T	1	3.917	0.0596	0.590	0.450	2.191	0.152	4.774	0.039	2.270	0.145	1.267	0.271
S × T	2	9.751	<0.001	2.475	0.105	16.046	<0.001	11.443	0.000	3.720	0.039	7.801	0.002
E × S × T	2	2.387	0.113	1.261	0.301	3.603	0.043	1.798	0.187	0.448	0.644	1.391	0.268

Redundancy analysis of E. gansuensis, the different growth seasons, and C, N, P and K contents of leaves and roots

The first axis of the redundancy analysis explained 28.6% of the variance, and the second axis explained 27.4% of the variance (Fig. 4). According to the analysis, E+ was an important factor affecting the N, P and K contents of leaves and the P content of roots, whereas E- was a key factor affecting the C, N and K contents of roots and the C content of leaves (Fig. 4). Further, our results showed that May was positively correlated with the N, P and K contents of leaves and with P and N contents of roots; however, August was positively correlated with the C and K contents of leaves and with C and K contents of roots. December had a positive correlation with the C, N and K contents of roots (Fig. 4). The presence of *E. gansuensis* clearly affected the different nutrients of leaves and roots, and the growth season affected the different nutrients of leaves and roots in *A. inebrians*.

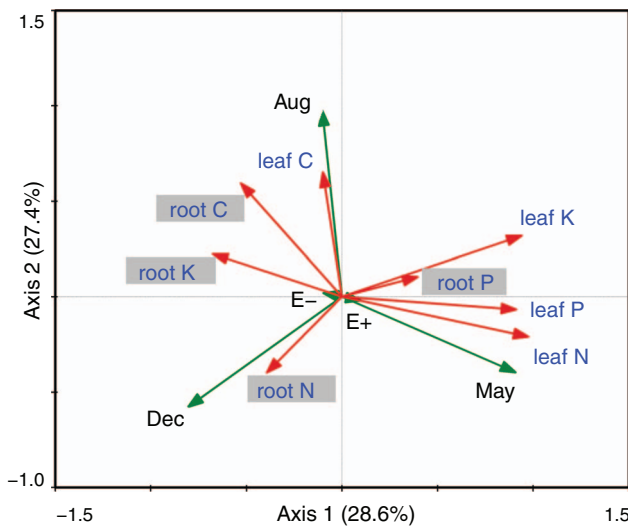


Fig. 4. Redundancy analysis of *E. gansuensis* endophyte (E+, E-), growth season, and leaf and root nutrient contents (C, N, P and K).

Correlations of the composition of the rhizosphere bacterial community with C, N, P and K contents of E+ and E- A. inebrians during different growth seasons

Effects of all of the genera of rhizosphere bacteria of E+ and E- *A. inebrians* on the plant contents of C, N, P and K during the different growth seasons were estimated.

We found significant effects of rhizosphere bacteria on the C, N, P and K contents of leaves and roots between E+ and E- *A. inebrians* plants in May (Fig. 5). For example, the C, N, P and K contents of leaves of E+ plants were positively correlated with *Bacillus*, *Acinetobacter*, *Paenarthrobacter* and *Streptomyces*; and the N and P contents of leaves of E- plants were positively correlated with *Bacillus* and *Stenotrophomonas* (Fig. 5). The C and N contents of roots of E+ plants were positively correlated with *Microbacterium* and *Flavobacterium*; and the N and K contents of roots of E- plants were positively correlated with *Bacillus* and *Stenotrophomonas* (Fig. 5). The P content of roots of E+ plants was positively correlated with *Pseudomonas*, *Enterobacter*, *Sphingobacterium*, *Chryseobacterium* and *Microbacterium*; and the P content of roots of E- plants was positively correlated with *Pseudomonas* (Fig. 5).

Similarly, our results also demonstrated significant effects of rhizosphere bacteria on the contents of C, N, P and K in leaves and roots between E+ and E- *A. inebrians* plants in August (Fig. 6). For example, the C and P contents of E+ leaves were positively correlated with *Arthrobacter*, *Pantoea*, *Stenotrophomonas*, *Rhizobium*, *Paenibacillus* and *Bacillus*; and the C, N and P contents of E- leaves were positively correlated with *Microbacterium*, *Arthrobacter*, *Acinetobacter* and *Ensifer* (Fig. 6). The N content of E+ leaves was positively correlated with *Olivibacter* and *Lysinibacillus*. The K content of E- leaves was positively correlated with *Pseudomonas*, *Bacillus*, *Pantoea* and *Rhodococcus* (Fig. 6). The C, N and K contents of E+ roots were positively correlated with *Variovorax*, *Brevibacillus* and *Rhodococcus*. The N and P contents of E- roots were positively correlated with *Microbacterium*, *Arthrobacter*, *Acinetobacter* and *Ensifer* (Fig. 6). The P content of E+ roots was positively

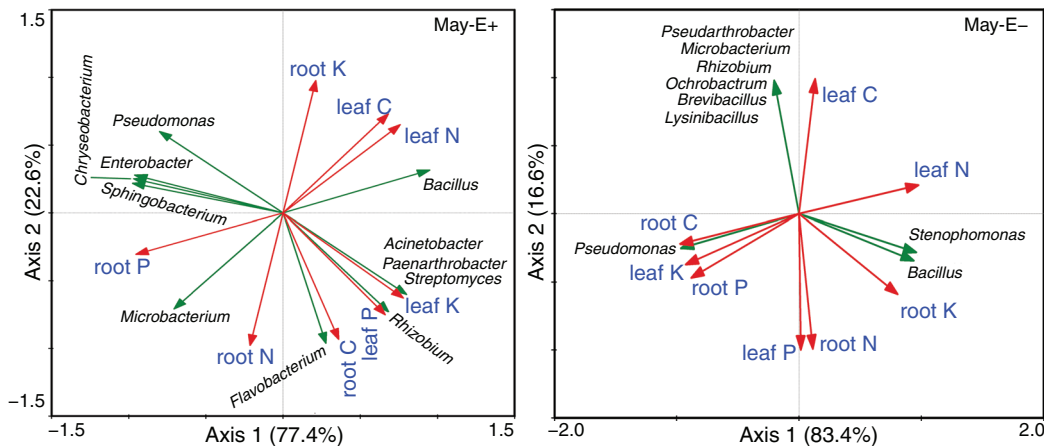


Fig. 5. Redundancy analysis of rhizosphere bacteria and the leaf and root nutrient contents (C, N, P and K) of *A. inebrians* with (E+) and without (E-) *E. gansuensis* endophyte in May.

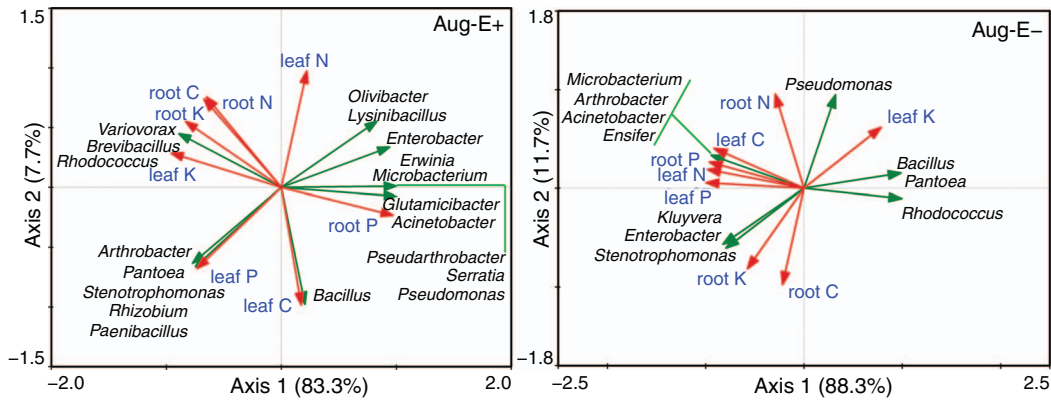


Fig. 6. Redundancy analysis of rhizosphere bacteria and the leaf and root nutrient contents (C, N, P and K) of *A. inebrians* with (E+) and without (E-) *E. gansuensis* endophyte in August.

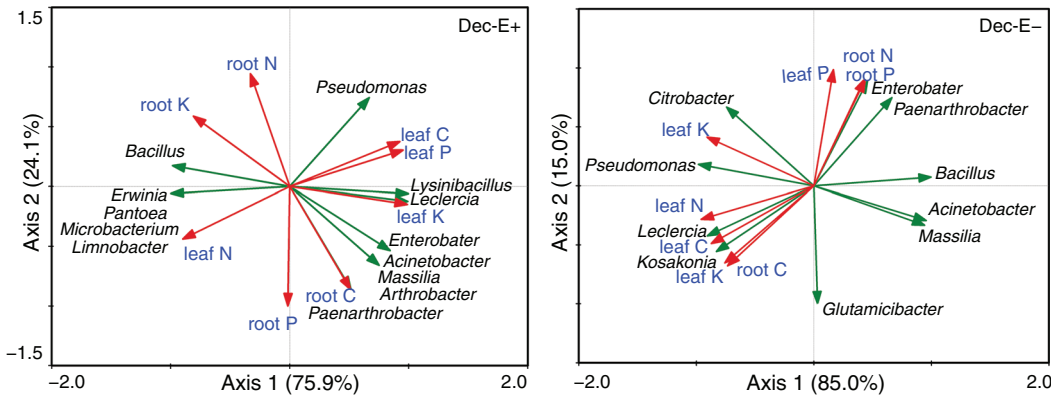


Fig. 7. Redundancy analysis of rhizosphere bacteria and the leaf and root nutrient contents (C, N, P and K) of *A. inebrians* with (E+) and without (E-) *E. gansuensis* endophyte in December.

correlated with *Glutamicibacter* and *Acinetobacter*. The C and K contents of E- roots were positively correlated with *Kluyvera*, *Enterobacter* and *Stenotrophomonas* (Fig. 6).

Significant effects of rhizosphere bacteria on the C, N, P and K contents of leaves and roots between E+ and E- *A. inebrians* plants were also evident in December (Fig. 7). For instance, the C, P and K contents of E+ leaves were positively correlated with *Pseudomonas*, *Lysinibacillus* and *Leclercia* in December; and the C, N and K contents of E- leaves were positively correlated with *Citrobacter*, *Pseudomonas* and *Leclercia* (Fig. 7). The N content of E+ leaves was positively correlated with *Bacillus*, *Erwinia*, *Pantoea*, *Microbacterium*, *Limnobacter* and *Leclercia*. The P content of E- leaves was positively correlated with *Enterobacter* and *Paenarthrobacter* (Fig. 7). Further, the C and P contents of E+ roots were positively correlated with *Enterobacter*, *Acinetobacter*, *Massilia*, *Arthrobacter* and *Paenarthrobacter*. The N and P contents of E- roots were positively correlated with *Enterobacter* and *Paenarthrobacter*. The N and K contents of E+ roots were positively correlated with *Bacillus* and *Pseudomonas*. The C and K contents of E- roots were positively correlated with *Leclercia* and *Kosakonia* (Fig. 7).

Figure 8 summarises results of principal component analysis for endophyte treatment in May, August and

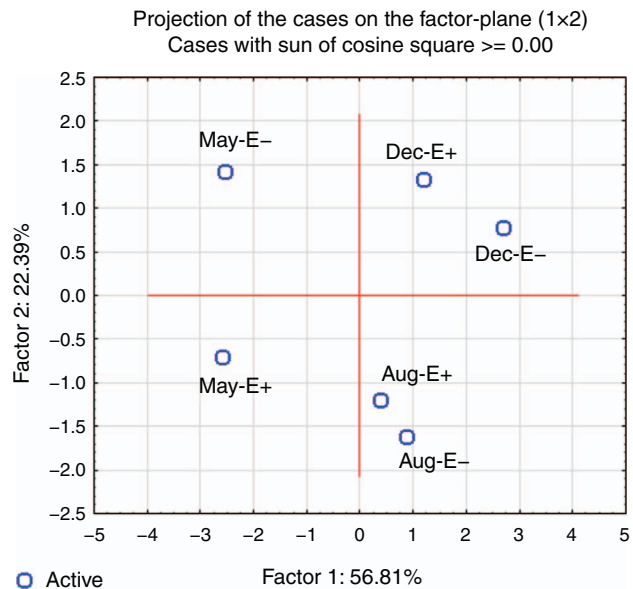


Fig. 8. Principal component analysis of each site (*A. inebrians* with (E+) and without (E-) *E. gansuensis* endophyte) and season on the factor plane.

December; it shows that factors 1 and 2 explained >78% of total variance.

Discussion

Endophytes of the genus *Epichloë* have been found in many cool-season grasses including *Achnatherum inebrians*, *Elymus dahuricus*, *Festuca sinensis*, *F. elata*, *Hordeum brevisubulatum* and *Lolium perenne* (Scharndl *et al.* 2004; Song *et al.* 2015a; Wang *et al.* 2020a). A previous study showed that the relationships between *Epichloë* endophytes and grasses are considered mutually beneficial (Müller and Krauss 2005). Studies have reported that the relative abundances of the fungal component were dramatically distinct in different endophyte-treated bulk soils or rhizosphere soils, and that the presence of endophyte influences the structure of soil fungal community, but not the fungal total biomass in fescue soils (Rojas *et al.* 2016). Similarly, Zhong *et al.* (2018) found that *A. inebrians* infected with wild *E. gansuensis* endophyte had higher root-associated fungal diversity than *A. inebrians* infected with cultivated *E. gansuensis*; however, the presence of *E. gansuensis* obviously decreased root-associated fungal diversity compared with E– *A. inebrians*. Guo (2014) reported that, compared with other endophytes, *Epichloë coenophiala* AR584E+ and *E. coenophiala* CTE+ increased soil organic C content in rhizosphere soil. Franzluebbers *et al.* (1999) also showed that *Neotyphodium coenophialum* (*Epichloë coenophiala*) endophyte leads to small differences in soil microbial community structure between tall fescue plants with 0% and 100% endophyte infection.

In our study, communities of rhizosphere bacteria, and *A. inebrians* nutrients, were mediated by *E. gansuensis* endophyte infection and these are critical for ecosystem function and productivity. This research provides insights into how the *A. inebrians*–*E. gansuensis* symbiosis influences the rhizosphere microbial community, the C, N, P and K contents of the host grass, and the relationship between rhizosphere bacterial community and nutrient content leaves and roots of *A. inebrians* during three seasons of the growth period. *Epichloë gansuensis* increased the Shannon index of the rhizosphere bacterial community and changed the rhizosphere bacterial community (Fig. 1, Table 1). Zhong *et al.* (2018) reported that the interaction between soil disturbance and *E. gansuensis* leads to changes in fungi communities in the root association. Ju *et al.* (2020) demonstrated by using Illumina pyrosequencing that *E. gansuensis* increased the Shannon index of the *A. inebrians* rhizosphere soil bacterial community, which was consistent with our results. Further, the study of Hou *et al.* (2020) showed that *E. gansuensis* increased the activities of soil invertase, alkaline phosphatase and urease, and the contents of soil organic C, total N, available N and available P. Therefore, *E. gansuensis* perhaps regulates soil enzyme activity through reprogramming soil microbial structure. Meanwhile soil enzymes participate in the soil nutrient cycle, which probably increases nutrient accumulation of host grasses to improve plant growth. Bansal *et al.* (2014) showed that with the increase in microbial diversity and

richness, the activity of soil urease was also enhanced, and this increased N cycling, which is beneficial to the accumulation of host grass nutrients and plant growth. Similarly, our results indicated that *E. gansuensis* increased the uptake of nutrients (N, P and K) during the different growth seasons (Fig. 2). The study of Wang *et al.* (2020b) also indicated that beneficial bacteria (plant-growth promoting rhizobacteria, PGPR) activate nutrients (N, P and K) and promote wheat growth under reduced fertiliser application conditions. These PGPR function as biofertilisers; and likewise, the presence of *E. gansuensis* has a role as a biofertiliser because it enhances the presence of bacterial genera regarded as PGPR. *Pseudomonas* and *Bacillus* are dominant genera in May, *Acinetobacter* and *Bacillus* in August, and *Pseudomonas*, *Bacillus* and *Enterobacter* in December (Fig. 1).

The effect of an *Epichloë* endophyte on root-associated and rhizosphere microorganisms differs in different grass–*Epichloë* associations. The presence of an *Epichloë* endophyte in perennial ryegrass (*Lolium perenne*) and *A. sibiricum* suppressed arbuscular mycorrhizal fungi (AMF) colonisation, although this effect depended on endophyte strain, ryegrass cultivar and P supply (Liu *et al.* 2011; Zhou *et al.* 2016). A previous study demonstrated that *Acremonium coenophialum* (*E. coenophiala*) endophyte modulated N metabolism in tall fescue (Lyons *et al.* 1990). It is also reported that under salt stress and soil-water deficit conditions, the presence of an *Epichloë* endophyte affected the N and P contents in shoot and root of *H. brevisubulatum* and *A. inebrians*, and altered the stoichiometry of host grasses (Song *et al.* 2015a; Wang *et al.* 2019; Xia *et al.* 2018). Similarly, we found that *E. gansuensis* infection influenced C, N, P and K contents in leaves and roots of *A. inebrians* in May, August and December (Fig. 2), and changed the stoichiometry (elemental ratios) of host grasses (Fig. 3). Therefore, *E. gansuensis* could promote growth of host *A. inebrians* probably by regulating soil microbial community structure, soil enzyme activity and nutrient content, to increase nutrient accumulation. We found that *E. gansuensis* increased the N content of leaves compared with E– leaves in December (Fig. 2). However, in a previous study, endophyte infection had no clear influence on N-use efficiency of chewings fescue (*Festuca rubra*) (Richardson *et al.* 1999). Li *et al.* (2012) found that the benefits to *A. sibiricum* from endophyte infection largely depended on the supply of N and/or P, and in the case of P supply and N deficiency, there was no dramatic difference of N content in E+ and E– roots. According to our results, there were different *E. gansuensis* treatment effects on the contents of C, N, P and K in leaves and roots during the three different growth seasons (Fig. 2). Ju *et al.* (2020) reported that soil moisture had a clear influence on soil nutrients, especially nitrate-N, available N and P and total N; and *E. gansuensis* had obvious impacts on soil nutrients, especially available P and nitrate-N. Differences in soil moisture and temperature in the different growth seasons would likely have regulated soil nutrient content, and may be why *E. gansuensis* has seasonal effects on the nutrient content of the host grass.

Plants take up CO₂, soil available N, available K, available P and other nutrient elements, and synthesise metabolites

required for plant growth. The C, N, P and K contents of leaves and roots and their respective ratios changed with the plant growth season (Fig. 3). Owing to changes in C, N, P and K contents in different growth seasons and tissues, the C : N, C : P and C : K ratios in the leaves increased with growth stage. It is possible that the reduction in leaf C : N ratio of E+ plants was due to increased synthesis of N content compared with E- plants in December. Many researchers have proposed the growth-rate hypothesis, and this is one of the important paradigms for ecological stoichiometry (Elser et al. 1996, 2010). Low ratios of C : P and N : P exist in high-growth-rate species (Main et al. 1997). Our results show that the C : P ratios of leaves and roots decreased in May, supporting the growth-rate hypothesis. The N : P ratio of leaves is a key indicator to define the nutrient element limitations (Koerselman and Meuleman 1996). Our results reflected that the stoichiometry of E+ and E- plants should consider other elements such as K, and its elemental ratios N : K, C : K and K : P, which may respond to changing environment (Rivas-Ubach et al. 2012). Calvo et al. (2017) demonstrated that a mixture of four strains of plant-growth-promoting *Bacillus* spp. could enhance nutrient contents (N, P and K) by influencing root morphology at the early stages of maize (*Zea mays*) growth. A previous study by Xia (2018) indicated that *E. gansuensis* can regulate the root-system structure of *A. inebrians*. Therefore, our research implies that *E. gansuensis* has the role of promoting plant growth and stimulating the absorption of nutrients for the host grass, probably in part by regulating the root morphology of *A. inebrians*.

Epichloë endophytes have important biological and ecological function (Franzuebbers et al. 1999; Reza and Mirlohi 2010; Zhang et al. 2010b, 2012; Song et al. 2015a, 2015b; Rojas et al. 2016; Tian et al. 2017; Xia et al. 2018; Wang et al. 2020a). It was reported that root-associated fungi of uncultivated, wild, endophyte-infected *A. inebrians* show positive correlation with soil properties, but root-associated fungi of cultivated *A. inebrians* show negative correlation with soil properties (Zhong et al. 2018). However, there was no previous study into the relationship between rhizosphere bacteria and plant nutrients under *E. gansuensis* endophyte infection during the different growth seasons, and this led to the present study. Our results showed that E+ was positively correlated with the N, P and K contents of leaves and the P content of roots; however, E- was positively correlated with the C, N and K contents of roots and the C content of leaves (Fig. 4). Interestingly, Ju et al. (2020) reported that Proteobacteria was positively related with pH, available N and K, and nitrate-N in the rhizosphere, and negatively related with soil organic C and total N in the rhizosphere. Therefore, *E. gansuensis* probably influenced the rhizosphere soil bacterial community, and the microbial community is highly correlated with soil nutrients, and this would influence the absorption of nutrients for plants. A critical characteristic of the association between grass and *Epichloë* is that the endophyte is systemic within host grasses, but importantly, hyphae of these endophytic fungi are not present in roots (Christensen et al. 2008). The evidence from this present study is that *E. gansuensis* is an important

factor positively affecting the nutrient content of leaves, with the exception of C.

There was a significant difference of relationship between rhizosphere bacteria and nutrient content of E+ and E- plants in the three different growth seasons. The grass *A. inebrians* grows in poor soil environments and E+ plants are becoming increasingly abundant on the barren grassland in north-west China, with nearly all *A. inebrians* plants being endophyte-infected. This suggests that the endophyte is beneficial, or at least not disadvantageous, to the host grass. Our previous study also showed that *E. gansuensis* improved tolerance to low N by regulating enzyme activity related to N metabolism and glucose-6-phosphate dehydrogenase activity (Wang et al. 2018a; Wang et al. 2018b). The present results confirm that *E. gansuensis* also provides an advantage by enhancing rhizosphere bacterial diversity, which probably further influenced enzyme activity and nutrient contents of soils, and positively affects absorption of nutrients by plants in natural conditions. As noted earlier, *E. gansuensis* enhances the activity of soil enzymes and contents of available N and P (Hou et al. 2020), and influences the diversity of the community of rhizosphere soil bacteria (Ju et al. 2020). Importantly, a study investigating response of rhizosphere soil properties, enzyme activities and microbial diversity to intercropping types on the Loess Plateau of China found that activities of soil invertase, soil phosphatase and soil urease had a positive correlation with the diversity of rhizosphere bacteria (Gong et al. 2019). The growing conditions of *A. inebrians* are often harsh with low nutrient availability. The present research shows that *E. gansuensis* increased rhizosphere bacterial diversity and enabled the *A. inebrians* host grass to have better growth status by accumulating greater quantities of nutrient elements when exposed to natural conditions. A study involving tall fescue indicated that *Epichloë* endophyte could increase adaptability of the grass to a P-deficient environment by regulating nutrient absorption (Malinowski et al. 1999, 2000). Feedback in the plant-soil system shows that plants affect characteristics of the rhizosphere, and then the rhizosphere affects the growth of the same or other plants (Ehrendfeld et al. 2005). Further, positive plant-soil feedback could come from increased availability of nutrients (Wardle et al. 1999; Chapman et al. 2006), or the accumulation of a mutualistic symbiont in the rhizosphere (Klironomos 2002). Until now, the key focus of studies into plant-soil feedback has been the effects of different plant species on soil properties (Burke et al. 1998), or on soil community composition (Vandenkoornhuysen et al. 2003; De Deyn et al. 2004; Innes et al. 2004; Kennedy et al. 2005). Therefore, *E. gansuensis* probably plays a significant role in positive plant-soil feedback by changing rhizosphere bacterial communities, which in turn enhances the nutritional status of *A. inebrians*.

Rhizosphere bacterial composition in *A. inebrians* was significantly related to the nutrient content of leaves and root (Figs 5 7). Many studies have confirmed the effect of rhizosphere microorganisms on plant nutrients; for example, Höflich et al. (1995) isolated two rhizosphere bacteria (*Pseudomonas* and *Rhizobium*) from soil that stimulated root length and branching, and promoted shoot and root dry

weight of pea (*Pisum sativum*). Inoculation of PGPR enhanced wheat height and biomass, and enhanced the content of N, P and K in wheat shoots; further, after combined application of N fertiliser and PGPR, the speed of increase in nutrient accumulation and wheat yield was even higher (Abbasi *et al.* 2011). This was consistent with our results. Inoculations of *Bacillus cereus*, *Brevibacillus reuszeri* and *Rhizobium rubi* with manure enhanced plant weight, chlorophyll content, and N, K, and P content of broccoli compared with control (Yildirim *et al.* 2011). The present research also indicated that *Bacillus* had a positive correlation with N content of leaves of E+ and E- plants at May (Fig. 5). *Bacillus* promotes the recovery and absorption of K in Sudan grass (*Sorghum vulgare* Pers. var. *sudanense*) (Basak and Biswas 2009). The mechanism by which rhizosphere bacteria promote nutrient absorption may be related to their function in nutrient availability, because some rhizosphere bacteria have special biological functions. For example, *Brevibacillus reuszeri* and *Ochrobactrum cytisi* have P-solubilising function; *Bacillus cereus*, *Bacillus*, *Pseudomonas* and *Rhizobium* have N₂-fixing function; and *Rhizobium rubi* has the of P-solubilising and N₂-fixing functions (Glick 2012; Yildirim *et al.* 2011; Meena *et al.* 2017). All of these functions can be beneficial for promoting nutrient absorption. In addition, some rhizosphere bacteria can produce indole-3-acetic acid (IAA), for example, strains WPR-32, WPR-42 and WPR-51 from wheat rhizosphere (Abbasi *et al.* 2011), and IAA can contribute to the growth of plants. Our results also showed that *Pseudomonas* had a positive correlation with P content of roots of E+ plants in August (Fig. 6). Strains of *Pseudomonas* and *Bacillus* significantly increased the content of N, P and K in the roots and leaves of cotton (*Gossypium* sp.) and peas, and both can produce IAA (Egamberdiyeva and Höflich 2004). However, one study proved that some rhizosphere microorganisms are beneficial to plant yield and growth but they do not increase plant nutrient absorption; for example, some of the P-solubilising rhizobacteria isolated from field-grown crops can increase plant height and seed yield of canola but cannot promote P absorption of plants (De Freitas *et al.* 1997). *Pseudomonas alcaligenes* PsA15, *Mycobacterium phlei* MbP18 and *Bacillus polymyxa* BcP26 have a much better stimulatory role on N, P and K absorption of maize in nutrient-deficient soil than fertile soil (Egamberdiyeva 2007). Betaproteobacteria are very important in the process of plant nutrient cycling and growth; different growth periods strongly affect communities of Betaproteobacteria in potato rhizospheres, and different potato cultivars have differing rhizosphere effects on their communities (Inceoglu *et al.* 2010). In our study, *E. gansuensis* increased P content of leaves and roots in May, but had no effects on P content of leaves and roots in August and December, possibly because *E. gansuensis* activity and quantity were greater in May and the host *A. inebrians* plants were experiencing rapid growth. The development and structure of the rhizosphere bacterial community are affected by different factors such as plant species, plant age and soil type (Canbolat *et al.* 2006; Roesti *et al.* 2006; Qiao *et al.* 2017). This is consistent with our results, with the diversity and community structure of rhizosphere soil being different at

the three sampling dates. The N level of plant tissue and soil N availability were significantly related to the change of rhizosphere bacterial community structure. One study found that at the peak of plant growth, the absorption rate of N by plants was inversely proportional to the level of microbial biomass N, which caused a change of bacterial community structure in the rhizosphere (Bell *et al.* 2015). This may explain the differences observed in our study of the rhizosphere bacterial community between E+ and E- plants in different growth seasons, which led to changes of plant nutrients.

Important findings of this study are that *E. gansuensis* enhanced *A. inebrians* rhizosphere bacterial community diversity at three different growth phases, with the Shannon index highest in August. Contents of C, N, P and K in E+ leaves and of C, P and K in E+ roots were also greater than of E- plants during one or more of the growth seasons. Moreover, the N, P and K contents of leaves and the P content of roots were positively correlated with *E. gansuensis* endophyte infection, whereas the C, N and K contents of roots and the C content of leaves were positively correlated with E-. There were significant impacts of rhizosphere bacteria on the C, N, P and K contents of E+ and E- *A. inebrians* leaves and roots in May, August and December. *Bacillus* had positive correlation with N content of leaves of E+ and E- host grasses in May; *Pseudomonas* had positive correlation with P content of roots of E+ plants in August; *Pseudomonas* had positive correlation with the C content of leaves and roots in E+ and E- plants in December. Overall, our results indicate that the mutualistic grass-*Epichloë* symbiosis has potential to affect the community structure of rhizosphere bacteria and promote host-grass nutrient uptake, improving the yield and quality of grass. The results suggest that host grasses-*Epichloë* endophyte symbiosis could enhance the competitive ability of host growth and the economic value of grasses in natural rangelands.

Author contributions

Jianfeng Wang and Zhibiao Nan designed the research. Jianfeng Wang, Wenpeng Hou and Zhibiao Nan performed the research. Jianfeng Wang, Zhibiao Nan, Chao Xia, Tao Chen, Xiuzhang Li and Wenpeng Hou analysed the data. Jianfeng Wang, Wenpeng Hou and Michael J. Christensen wrote the paper.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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