The Zoige pioneer plant *Leymus secalinus* has different endophytic bacterial community structures to adapt to environmental conditions

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Background. *Leymus secalinus* is a pioneer plant grown in the Zoige desertified alpine grassland, and it is also one of the dominant plant species in environmental remediation. *L. secalinus* plays a large role in vegetation reconstruction in sandy land, but the abundance and diversity of its endophytes have not been investigated until now.

Objectives. The study was performed to investigate the changes in endophytic bacterial community structure in *L. secalinus* in different ecological environments and to analyze the effects of environmental changes and different plant tissues on the *L. secalinus* endophytes.

Methods. Leaf, stem, and root tissue samples of *L. secalinus* were collected from Zoige and the laboratory. DNA was extracted, and the 16S ribosomal DNA was amplified. The sequence library was sequenced on an Illumina MiSeq platform and clustered by operational taxonomic units (OTUs). α-diversity and β-diversity analyses, species diversity analyses, functional prediction, and redundancy (RDA) analyses for the soil physicochemical properties were conducted. Results. α-Diversity and β-diversity analyses showed that the endophytic bacteria in *L. secalinus* varied in different areas and tissues. The abundances of *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, which are related to nitrogen fixation, in *L. secalinus* grown in Zoige increased significantly.

Moreover, the abundance of nutrition metabolism and anti-stress abilities increased in functional prediction in the desert samples. The soil physicochemical properties had an insignificant influence on bacterial diversity.

Conclusion. The changes in endophytic bacterial community structure in *L. secalinus* were significant. They were caused by the choice of environment and plant. The endophytic bacteria in *L. secalinus* grown in alpine sandy land may have anti-stress properties and the ability to fix nitrogen, which has potential value in environmental remediation and agricultural production.

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Abstract

Background. *Leymus secalinus* is a pioneer plant grown in the Zoige desertified alpine grassland and it is also one of the dominant plant species used for environmental remediation. *L. secalinus* plays a large role in the reconstruction of vegetation in sandy soils, but the abundance and diversity of its endophytic bacteria have not yet been investigated.

Objectives. This study investigated the changes in the endophytic bacterial community structure of *L. secalinus* under different ecological environments. The effects of environmental changes and different plant tissues on *L. secalinus* endophytic bacteria were also studied.

Methods. Leaf, stem, and root tissues were sampled from *L. secalinus* growing in the Zoige Grassland (Alpine sandy land) and an open field nursery (Control). DNA was extracted and the 16S ribosomal DNA was amplified. The sequence library was analyzed using an Illumina MiSeq and clustered by operational taxonomic units (OTUs). α-diversity and β-diversity analyses, species diversity analyses, functional prediction, and redundancy (RDA) analyses for the soil physicochemical properties were conducted.

Results. α-diversity and β-diversity analyses showed that the endophytic bacteria in *L. secalinus* varied in different areas and tissues. The abundance of *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, which is related to nitrogen fixation, increased significantly in the *L. secalinus* found in the Zoige Grassland. Functional prediction showed that the nutrition metabolism and anti-stress abilities increased in the desert samples. The soil physicochemical properties had an insignificant influence on bacterial diversity.

Conclusion. The changes in the endophytic bacterial community structure in *L. secalinus* were significant and were caused by environmental alterations and plant choice. The endophytic bacteria in *L. secalinus* grown in alpine sandy land may have greater anti-stress properties and the ability to fix nitrogen, which has potential value in environmental remediation and agricultural production.

Introduction

The Zoige alpine grassland, located on the eastern edge of the Qinghai Tibet Plateau (QTP), is an important part of the Jeurre Prairie in China. Its altitude is approximately 3,500 meters, and its monthly average temperature is -9.6 ℃ to 11.2 ℃. It is a water conservation area of the Yellow River and the Yangtze River, and is important in maintaining the area's ecological balance and animal husbandry. Owing to climate changes (e.g., increasing temperature and decreasing precipitation) (Hu et al., 2015; Niu et al., 2008) and anthropogenic factors (e.g., over-grazing, drainage of water systems) (Dong et al., 2009), degradation of these grasslands began in the 1970s and peaked in 2000 (Hu et al., 2018). The degradation of the grasslands was controlled by the establishment of biological and sand barriers, as well as a prohibition on cattle grazing (Liu et al.,

2020; Luo et al., 2019; Yan & Lu, 2015). The Zoige Grassland has been increasingly used as an experimental site for the establishment of multiple ecosystem governance (Jian et al., 2022; Liu et al., 2020; Luo et al., 2019).

The Zoige Grassland is characterized by high-altitude, cold temperatures, and barren soil, all of which present problems for the reconstruction of vegetation. *Leymus secalinus* is a pioneer plant grown in the Zoige's sandy soil due to its powerful vitality. Moreover, *L. secalinus* has the ability to meet the nutritional requirements of livestock. *L. secalinus* is a perennial herb belonging to the family Poaceae, the dominant species that grows in active dunes (Kang et al., 2017). *L. secalinus* reproduce both sexually through seed and clonally via the vegetative growth of rhizomes that can develop into a new individual in a short time. These characteristics make it beneficial for the rapid remediation of sandy lands. The proportion of spreading ramets in *L. secalinus* is increased in low-nutrient soils (Ye et al., 2006), and the rhizome biomass, length, diameter, and adventitious roots at the rhizome nodes are enhanced under drought conditions (Zheng et al., 2021). Furthermore, all of the ramets are changed as described above owing to clonal integration (Sui et al., 2011), which helps them to manage stressful conditions. These qualities make *L. secalinus* an ideal plant for phytoremediation in alpine sandy land.

The endophytic bacteria found in plants may play an important role in stress resistance, during periods of salt stress (Khan et al., 2020), low temperature (Subramanian et al., 2015), and drought (Ullah et al., 2019). Moreover, many studies have shown that endophytic bacteria can promote plant growth (Adhikari & Pandey, 2020) and increase the absorption of mineral elements (Afzal et al., 2019) by the host to enhance its viability. Plants may develop unique endophytic communities in environmental extremes (Przemieniecki et al., 2019; Yang et al., 2020), which are highly valuable for research and application.

Many studies have shown the symbiotic relationship between bacteria and plant tissues. Our understanding of endophytic bacteria has improved due to high-throughput technology. With it we have also discovered a two-way relationship between endophytic bacteria and plants. For example, the plant roots secrete compounds that attract or repel bacteria, and the bacteria secrete pectinases or cellulases to colonize the roots and even move to the stem (Santi et al., 2013). The diversity of plant endophytic bacteria is related to the plant species (Jian et al., 2022), soil type (Ling et al., 2020), growth stage (Mohr et al., 2021), and climatic conditions (Zhang et al. 2018). The understanding of endophytic bacteria is advancing rapidly with the development of high-throughput sequencing. Research on the endophytic bacteria of plants on the QTP's unique environments has received great attention. Previous studies have focused on the unique and dominant plant species of an area (Liang et al., 2021; Wei et al., 2022; Yang et al., 2020). However, the plants used in phytoremediation in the QTP have yet to be adequately studied.

Endophyte diversity and biological functions are often distinctive under high-altitude, cold, and barren conditions. For example, *Burkholderia* is highly abundant in the arctic pioneer plant mountain sorrel (*Oxyria digyna*). Many studies have proven that *Burkholderia* has a variety of growth-promoting functions, such as nitrogen fixation (Bhattacharjee et al., 2008) and phosphate solubilization (Kour et al., 2021). In another study, the seed bacterial community of eight native alpine grassland plants growing in the QTP was dominated by γ-Proteobacteria, which can comprise up to 99% of the endophytic community (Liang et al., 2021). Based on these characteristics, we explored the endophytic bacterial community structure of *L. secalinus* in different ecological environments. The effects of environmental changes and various plant tissues

93 on the endophytic bacteria of *L. secalinus* was also studied. These variations may explain the
94 vitality of *L. secalinus* grown in the Zoige alpine sandy soil and provide a resource for
95 environmental remediation and agricultural production.

96

Materials & Methods

97

Collection and preparation of sample materials

98

99 The *L. secalinus* (Georgi) Tzvelev collected from the Zoige alpine sandy soil was designated
100 DL, and the samples collected in the open field nursery in the laboratory were designated L. The
101 sampling site in the Zoige sandy soil (Fig. 1) is located in the Zoige alpine grassland
102 (33º41′0″N,102º56′7″E; altitude: 3,500 m), which has a mean annual precipitation > 600 mm. The
103 average monthly temperature during the winter ranges from -9.6 to -3.9 °C. The open field nursery
104 is located at Sichuan University (Chengdu, China) (30º38′2″N,104º4′35″E; altitude: 500 m) with
105 the mean annual precipitation > 800 mm; the average monthly temperature during the winter
106 ranges from 5.6 to 7.9 °C. The entire plant was dug up at each location, and the rhizospheric soils
107 were removed. Five plants were randomly selected and considered to be one sample. Three parallel
108 samples were collected from the roots (LR and DLR, without rhizomes), stalk (LS and DLS), and
109 leaves (LL and DLL) and then stored in liquid nitrogen.

110 The plant tissues were surface sterilized in a laminar flow hood. The plant tissues were
111 sterilized for 30 s in 75% ethanol, washed with sterile water once, and then soaked in 0.1%
112 mercuric chloride ($HgCl_2$) for 5 min. After that, the plant tissues were washed through surface
113 sterilization three times with sterile water. Water from the final wash was plated on NB agar plates
114 to ensure that the surface was sterilized.

DNA extraction

115

116 All the DNA of samples was extracted using a PowerSoil DNA Isolation Kit (MoBio
117 Laboratories, Carlsbad, CA, USA) following the manufacturer's instructions. Then, the above
118 genomic DNA was developed on 1% agarose gels using a NanoDrop spectrophotometer (Thermo
119 Fisher Scientific, Waltham, MA, USA) to ensure a high purity and quality.

PCR amplification

120

121 All of the primers used in this study are shown in Table 1. The 5' end of the forward and
122 reverse primers had an 8-digit barcode sequence added to differentiate the different samples. The
123 PCR was performed as previously described using 25 μl reaction volumes on a Mastercycler
124 Gradient (Eppendorf, Hamburg, Germany). The reaction volumes followed those used by Lu et al.
125 (2022). The cycling parameters were modified slightly from Chen et al. (2022), which included 95
126 °C for 5 min to lead to the DNA melting, followed by 30 cycles of 95 °C for 20 s, 55 °C for 30 s,
127 and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

High-throughput sequencing and data analyses

128

129 Deep sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA,
130 USA) at the Allwegene Company (Beijing, China). The above amplicons were pooled to an

131 equimolar ratio. According to standard protocols, paired-end sequencing was performed on the
132 Illumina MiSeq (2×300). Raw paired-end reads were joined to generate clean merged reads after
133 filtering adaptors, low-quality reads, host sequences, and barcodes. All of the analysis are based
134 on clean merged reads. Clean merged reads were clustered into OTUs using the UPARSE
135 algorithm of Vsearch (v2.7.1) with a 97% similarity (Edgar, 2013). All of the OTUs were sorted
136 into different taxonomic groups using the Ribosomal Database Project (RDP) classifier tool
137 against the SILVA128 database (Cole et al., 2009).

138 The species abundance bar plot diagram analysis was performed using R (v3.6.0) and based
139 on taxonomic annotation and relative abundance results. The principal coordinates analysis
140 (PCoA) and principal coordinate analysis (PCA) were also analyzed using R (v3.6.0) and were
141 based on the OTUs collected from each sample to examine the similarity between different samples
142 (Wang et al., 2012). The unweighted pair group method with arithmetic mean (UPGMA)
143 clustering tree described the dissimilarity (1 - similarity) between multiple samples. These values,
144 based on the evolutionary distances between the microbial communities from each sample, were
145 calculated using Bray-Curtis algorithms (Jiang et al., 2013).

146 *LEfSe analysis*

147 Linear discriminant analysis (LDA) effect size (LEfSe) (Segata et al., 2011) (LDA score >4)
148 was performed with the LEfSe tool to identify species that have significantly different abundances
149 between groups.

150 *Function prediction*

151 16S rRNA sequences from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database
152 was used for functional prediction in PICRUSt2. Using the information in the KEGG database, the
153 abundance of each functional category for all samples was illustrated as heatmaps (Hidalgo et al.,
154 2021).

155 *Soil physicochemical properties*

156 The physicochemical properties of the non-rhizosphere soil from the Zoige Grassland and
157 control samples from the laboratory were documented to evaluate the influence of soil nutrient
158 conditions on the endophytic bacteria. The soil pH was determined with a ratio of 1:5 soil:
159 ultrapure water (v/v) using a PHS-3S pH-meter (INESA & Scientific Instrument, Shanghai, China)
160 (Jian et al., 2022). The soil organic carbon (SOC), total nitrogen (TN), total phosphorus (TP), total
161 potassium (TK), available nitrogen (N), available phosphorus (AP), and available potassium (AK)
162 were determined following Zhang et al. (2015). Indophenol blue colorimetry was used to
163 determine the ammonium nitrogen (NH_4-N) content, and dual-wavelength ultraviolet
164 spectrophotometry was used to determine nitrate nitrogen (NO_3-N). The relationship between the
165 endophytic bacterial community abundances and the soil physicochemical properties was
166 measured using redundancy analysis (RDA).

167

168 **Results**

169 *Analysis of the sequencing data*

170   A total of 2,219,372 raw reads were obtained from 18 samples of *L. secalinus* (roots, stems,
171   and leaves). After adjusting for quality control filtration and removing chimera, non-specific
172   amplicons, and host genes, a total of 1,852,014 high-quality sequences were produced. Each
173   sample had an average of 102,889 reads. A total of 11,736 OTUs were obtained from high-quality
174   sequences with a 97% similarity, and the number of OTUs of each sample ranged from 140 to
175   1,591 (Table 2).

176   *α-Diversity analysis*

177   The α-diversity of all the samples is shown in Table 2. All of the samples of plant tissue had
178   a mean coverage index above 85%, which suggests that the samples were sequenced to an adequate
179   depth. The Chao1, Shannon, and Simpson indices showed significant differences in *L. secalinus*
180   endophytic bacteria in different ecological environments. The α-diversity of the L group was
181   higher compared with that of the DL group in the roots, stems, and leaves; this may be due to a
182   higher level of nutrients available in L group soil (Shi et al., 2014). An increase in α-diversity
183   indicates high stability, which is more favorable for plant growth. In different tissues, the Chao1
184   and Shannon indices of the root samples (LR and DLR) were significantly higher than those of the
185   stems (LS and DLS) and leaves (LL and DLL). This suggests that the endophytic bacteria in the
186   roots had a greater diversity and mean abundance compared with those in the stems and leaves.
187   Most endophytic bacteria can enter plants through the roots and then move toward the stems and
188   leaves (Santi et al., 2013).

189   *β-diversity analysis*

190   The PCoA analysis is shown in Fig. 2A. These results indicate that the distance between the
191   roots and stems and leaves was much longer than the distance between the stems and leaves. We
192   hypothesized that most of the endophytic bacteria of the stems and leaves spread from the roots.
193   There is an enormous difference between the roots and stems, and leaves, as evidenced by the
194   endophytic bacteria that moved and may be screened by *L. secalinus*. The hierarchical clustering
195   results (Fig. 2C) agreed with those of the PCoA, which showed DLR and LR in the same branch
196   while the others were in another branch.

197   Figure 2B describes the PCA analysis based on the abundance of OTUs, which noted that the
198   L and DL groups could be differentiated from the coordinate axes. All of the DL groups were on
199   the positive PC2 axis, and the L groups were on the negative PC2 axis. Although the PCA analysis
200   is based on Euclidean distances, it is consistent with the results of PCoA, which also suggested
201   that the endophytic bacteria in the roots differed substantially compared with those from the stems
202   and leaves. This significant difference could have resulted from the different primers used and the
203   selectivity of *L. secalinus*.

204   ***Species diversity***

205   *Bacterial community structure and diversity*

206   Based on the OTU data, species abundance bar graphs (Fig. 3) were plotted using the R
207   language tool. The endophytic bacteria for the DL group were classified into 35 phyla, 81 classes,
208   189 orders, 264 families, and 465 genera. The L group was classified into 35 phyla, 82 classes,
209   192 orders, 270 families, and 500 genera.

At the phylum level (Fig. 3A, Table S1), the endophytic bacteria were primarily Proteobacteria. The proportions of Proteobacteria in the DLR, DLS, and DLL samples were 44.0%, 46.3%, and 39.6%, respectively. The proportions in LR, LS, and LL samples were 48.3%, 84.3%, and 58.2%, respectively, which were similar to those of rice (*Oryza sativa*) as shown by Kumar et al. (2021). The other major phyla of all the samples included Actinobacteriota, Bacteroidetes, Firmicutes, Patescibacteria, Myxococcota, and Gemmatimonadota. The abundance of Actinobacteriota in the DLR and DLS increased significantly compared with those of the LR and LS.

At the genus level (Fig. 3B, Table S2), *Pseudomonas, Stenotrophomonas, Methylibium, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Sphingomonas, Brevundimonas, Acidibacter, Serratia,* and *Sphingobium* had relative abundances of more than 1.5% in the L group. Moreover, *Chryseobacterium, Pseudomonas, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Clavibacter, Brevundimonas, Bacillus, Devosia, Agreia, Variovorax, Rhodococcus, Lechevalieria,* and *Sphingomonas* had relative abundances of more than 1.5% in the DL group.

Changes in the bacterial community structure

The amounts of Proteobacteria and Actinobacteriota were significantly different between the DL and L groups (Fig. 3C). We found that the relative abundance of Proteobacteria was reduced in all samples of the DL group compared with the L group (Fig. 3A, Table S1), and its abundance was significant in the stems (P=0.036). The relative abundance of Actinobacteriota increased in all samples of the DL group compared with the L group (Fig. 3A, C, and Table S1), and it was significant in the roots (P=0.006) and stems (P=0.047). For the other phyla, Firmicutes significantly increased (P=0.024), and Acidobacteriota significantly decreased (P=0.039) in the DLR compared with the LR (Fig. 3A, Table S1).

A series of changes at the phylum level could be related to the nitrogen balance. A study by Fonseca et al. (2018) showed that *Eucalyptus*, which was reported to have a negative N balance, had more than 50% Actinobacteriota in its rhizosphere microbiome. They also found that Firmicutes decreased and Proteobacteria increased as the soil's N content increased through the application of nitrogen or culture with legumes. The relationship between the N content (Table 3) and bacterial abundance in this study agreed with their results. Acidobacteria was reported to have a poor ability to fix nitrogen or solubilize phosphate, which may be due to alterations in the ecological environment.

At the genus level, the abundances of *Clavibacter* and *Brevundimonas* increased in the DLL compared with the LL, while the abundances of *Agreia, Chryseobacterium, Variovorax,* and *Rhodococcus* increased in the DLS compared with the LS (Fig. 3B, Fig. S1, and Table S2). The abundances of *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium, Polaromonas, Nocardioides, Microbacterium,* and *Paenibacillus* increased in the DLR compared with the LR (Fig. 3B, Fig. S1, and Table S2). All the genera described above were common among endophytic bacteria, and most of them contained species that could fix nitrogen or solubilize phosphate.

Function prediction

The functions were predicted based on the KEGG database that utilized the 16S rRNA

251 sequencing data (Fig. 4). We found that the DL group had a higher abundance of starch and sucrose
252 metabolism than the L group, which was due to its low content of organic carbon (Table 3). In
253 addition, the abundance of peroxisome and nucleotide excision repair in the DL group was greater
254 than that in the L group, which was related to the ecological environment. The DL group was
255 collected from the Zoige alpine sandy soil, which was exposed to strong UV rays and low
256 temperatures. The endophytic bacteria in that environment had to respond to cell damage caused
257 by stress. Regarding cell movement, the bacteria in the DL group moved substantially less than
258 those in the L group, as reflected by its flagellar assembly and bacterial chemotaxis. In previous
259 studies, bacteria have had increased locomotor activity under low nutrient conditions in order to
260 obtain more nutrients (Amsler et al., 1993). However, we found that the functional abundance cell
261 movement was reduced under low nutrient conditions (DL group) (Fig. 4 and Fig. S2), which may
262 be due to insufficient nutrition to fuel movement in those bacteria.

263 ***RDA analysis of soil physicochemical properties***

264 The relationship between soil physicochemical properties and species abundance was
265 analyzed using an RDA analysis. The soil physicochemical properties whose p-values were less
266 than 0.01 were filtered out, as shown in Fig. 5. At the phylum level (Fig. 5A), RDA1 and RDA2
267 only explained 5.86% of the variation in the endophytic bacterial community structure, and RDA1
268 and RDA2 explained 8.92% of the variation in the endophytic bacterial community structure at
269 the genus level. We considered that the soil physicochemical properties did not adequately
270 interpret the changes in the bacterial community. In our previous study, the soil physicochemical
271 factors explained 93.5% (phylum level) and 93.47% (genus level) of the variation in the
272 rhizosphere bacterial community structure (Jian et al., 2022). The RDA results may indirectly
273 prove that the effects of the selectivity of plants were more potent than those of the soil
274 physicochemical factors on the bacterial community in *L. secalinus*.

275 At the phylum level (Fig. 5A), the contribution of pH, TP, AP, NO_3^-, and TN were 31.2%,
276 26.7%, 26.0%, 14.0%, and 2.0%, respectively. The proteobacteria were the most affected by AP.
277 Actinobacteriota and Firmicutes were positively correlated with pH and negatively correlated with
278 N, and P. There was little impact on Bacteroidetes with pH, TP, AP, NO_3^-, and TN. At the genus
279 level (Fig. 5B), the pH had insignificant effects on the endophytic bacteria, whereas TP, TK, TN,
280 N, and SOC showed significant effects. TK, TP, N, SOC, and TN contributed 61.9%, 16.6%,
281 10.8%, 7.7%, and 3.1%, respectively. The genera with ascending abundance in the L group
282 (compared with the DL group) were positively correlated with TP, TK, TN, N, and SOC and
283 included *Stenotrophomonas* and *Sphingomonas*. *Chryseobacterium*,
284 *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium* and others, which were relatively
285 higher in abundance in the DL group than the L group, were negatively correlated with TP, TK,
286 TN, N, and SOC.

287

288 **Discussion**

289 ***Endophytic nitrogen-fixing bacteria increased in barren environments***

290 Nitrogen is an essential nutrient for plant growth and development and is the main component
291 of many plant components, including chlorophyll, amino acid, and ATP (Banik et al., 2019). There

292 were significant differences in soil nutrient composition between the Zoige sandy soil and the open
293 field stock nursery in the laboratory, as shown in Table 3. The total nitrogen (16-fold difference)
294 and available nitrogen (22-fold difference) showed significant differences between these soil
295 types. We hypothesized that these gaps led to increased nitrogen-fixing bacteria in the DL group.
296 *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium*, which are in the Rhizobiaceae, are
297 among the most important nitrogen-fixing bacteria (You et al., 2021). A previous study showed
298 that *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium* promoted the growth of the
299 aboveground parts of the plant (Pang et al., 2021). However, they are not a well-studied endophytic
300 bacteria. The abundance of *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium* increased
301 in barren environments, which accounted for 6.99% in the DLS and only 1.46% in the LS (Table
302 S2). In addition, an association has been shown between Actinobacteriota and nitrogen-fixing
303 bacteria. Many endophytic nitrogen-fixing bacteria were isolated in previous studies. In the
304 phylum Actinobacteria, *Microbacterium*, *Rhodococcus*, *Nocardioides*, and *Clavibacter* increased
305 in the DL group. *Rhodococcus* and *Nocardioides* were reported to fix nitrogen (Kukla et al., 2014;
306 Lim et al., 2014). *Microbacterium* and *Clavibacter* did not show the ability to fix nitrogen in
307 previous studies, but have other growth-promoting abilities, such as the ability to dissolve
308 phosphorus (Borah et al., 2021; Kumar et al., 2016). Of the genera that increased in the DL group,
309 *Brevundimonas*, *Chryseobacterium*, and *Paenibacillus* have also been reported to fix nitrogen (do
310 Carmo Dias et al., 2021; Herrera-Quiterio et al., 2020; Naqqash et al., 2020).

311 *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* was found in all the samples we
312 collected but was not found in rhizosphere soil (Jian et al., 2022). These bacteria may form
313 symbiotic associations with *L. secalinus*, which help *L. secalinus* to adapt to low-N conditions.
314 Other studies have not shown symbiotic associations between *Allorhizobium-Neorhizobium-*
315 *Pararhizobium-Rhizobium* and *L. secalinus*, so this hypothesis remains to be directly proven. *L.*
316 *secalinus* will recruit the N-fixing community as endophytic bacteria in a low N environment. The
317 changes in endophytic nitrogen-fixing bacteria are concentrated in the roots and stems. In this
318 study, we hypothesize that the roots and stems of *L. secalinus* are a major site of nitrogen fixation,
319 similar to what occurs in rice (Ito et al., 1980).

320 ***Endophytic bacteria have multiple pro-growth functions***

321 Phosphorus is the second most significant limiting element of plant growth, which may
322 become the first limiting element under specific ecological contexts (Hinsinger, 2001). Bacteria
323 may release the precipitated P from the soil through natural solubilization (Bhattacharyya & Jha,
324 2012) and endophytic fungi can increase the host response to phosphate starvation to protect the
325 host in the low P environment (Hacquard et al., 2016). TP and AP were lower in the Zoige alpine
326 sandy soil compared to the open field stock nursery (Table 3), hindering the reconstruction of
327 vegetation. In addition to *Microbacterium* and *Clavibacter*, *Agreia* (Sheng et al., 2011),
328 *Chryseobacterium* (Kaur et al., 2022), and *Brevundimonas* (Jiang et al., 2022), whose abundance
329 increased in the DL group, may also be able to solubilize phosphate. These genera negatively
330 correlate with TP in RDA analysis. These phosphate-solubilizing endophytic bacteria can help *L.*
331 *secalinus* to survive in the low P environment.

332 The other pro-growth functions also are reported in the species whose abundance increased
333 in the DL group. *Clavibacter* (Kumar et al., 2016), *Rhodococcus* (Kukla et al., 2014),
334 *Brevundimonas* (Jiang et al., 2022), and *Chryseobacterium* (Herrera-Quiterio et al., 2020) have
335 IAA producing ability. IAA enhances plant growth and can increase the rate of nitrogen fixation

336 of endophytic bacteria (Defez et al., 2017). ACC deaminase (ACCD) positive bacterial strains are
337 present in *Rhodococcus* (Kukla et al., 2014) and *Polaromonas* (Jasim et al., 2015), which can
338 significantly reduce reactive oxygen species (ROS) and lipid peroxidation levels (Srinivasan et al.,
339 2021). In addition, ACCD can substantially increase the host's biomass, chlorophyll content, and
340 photosynthetic activity in low-N conditions (Srinivasan et al., 2021).

341 ***Changes in the endophytic bacteria are related to the environment***

342 There were significant differences between the endophytic bacterial community structure of
343 *L. secalinus* under different ecological environments. Different tissues of plants in the same
344 ecological environment also indicated some differences. An analysis of the α-diversity showed
345 that even the diversity of endophytic bacteria decreased in cold, barren environments. β-diversity
346 analysis showed that the endophytic bacteria in the roots were more enriched and diverse than
347 those in the stems and leaves, suggesting that there is a closer phylogenetic proximity of stems to
348 leaves. The soil physicochemical properties only explained <10% of the variation in the
349 endophytic bacterial community structure, which is indirect proof of the selectivity of plants.
350 Climate may be the primary reason for the change in endophytic bacterial communities, rather than
351 the soil physicochemical properties.

352 We did not collect data on the UV index and temperature, but they are important factors
353 affecting the endophytic bacteria. The species in which abundance increased in the DL group are
354 tolerant of cold. For example, *Agreia* and *Polaromonas* are common genera in cold surroundings
355 (Franzetti et al., 2013; Sheng et al., 2011), and *Polaromonas* has been shown to have UV resistance
356 (Ciok et al., 2018). Some species of *Clavibacter* can significantly attenuate chilling-induced
357 electrolyte leakage, lipid peroxidation, and the accumulation of ROS as endophytic bacteria (Ding
358 et al., 2011) to improve the ability of plants to resist cold. An increased abundance of these species
359 is strongly associated with local climatic conditions.

360 Functional prediction showed that the abundance of nutrient metabolism related to stress
361 resistance increased in the DL group. This evidence illustrated that the species whose abundance
362 increased in the DL group have a stronger ability to adapt to the environment or to promote plant
363 growth. These species had the ability to improve the stress tolerance of *L. secalinus* to cold, strong
364 UV, and barren soil to some extent. They have substantial potential in environmental remediation
365 and agricultural production. However, the endophytic bacteria in *L. secalinus* in the Zoige alpine
366 sandy soil still need to be isolated, identified, and investigated.

367

Conclusions

369 Our study explored the changes to the endophytic bacterial community structure in *L.*
370 *secalinus* under different ecological environments using 16s rRNA high-throughput sequencing.
371 The results showed that endophytic bacteria affect different tissues of *L. secalinus* in different
372 ecological environments. The relative abundance of the endophytic bacteria such as
373 *Allorhizobium−Neorhizobium−Pararhizobium−Rhizobium* and their predicted functions
374 increased in *L. secalinus* in the Zoige alpine sandy soil, indicating that they are designed to
375 accommodate the high-altitude, cold, strong UV, and barren environment. The physicochemical
376 properties of the soil did not explain the changes in endophytic bacteria. However, other factors,

377 such as temperature, UV light intensity, and the selectivity of *L. secalinus*, may have a stronger
378 influence on the endophytic bacteria. Our study demonstrated that *L. secalinus* is a passive and
379 active part of a differentiated endophytic bacterial community. *L. secalinus* has a limited capacity
380 to adapt to its environment. Endophytic bacteria of *L. secalinus* in the Zoige alpine sandy soil may
381 promote plant growth, which helps *L. secalinus* survive under harsh environments. The endophytic
382 bacteria in *L. secalinus* in the Zoige alpine sandy soil have the potential to improve environmental
383 remediation and agricultural production.

384

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577

Figure 1

Sampling location and organism

Figure 2

β-diversity data.

(A) Scatter plot of PCoA based on UniFrac and Bray-Curtis. (B) Scatter plot of the PCA score based on the Euclidean distance. (C) Hierarchical clustering. PCA, principal component analysis; PCoA, principal coordinates analysis.

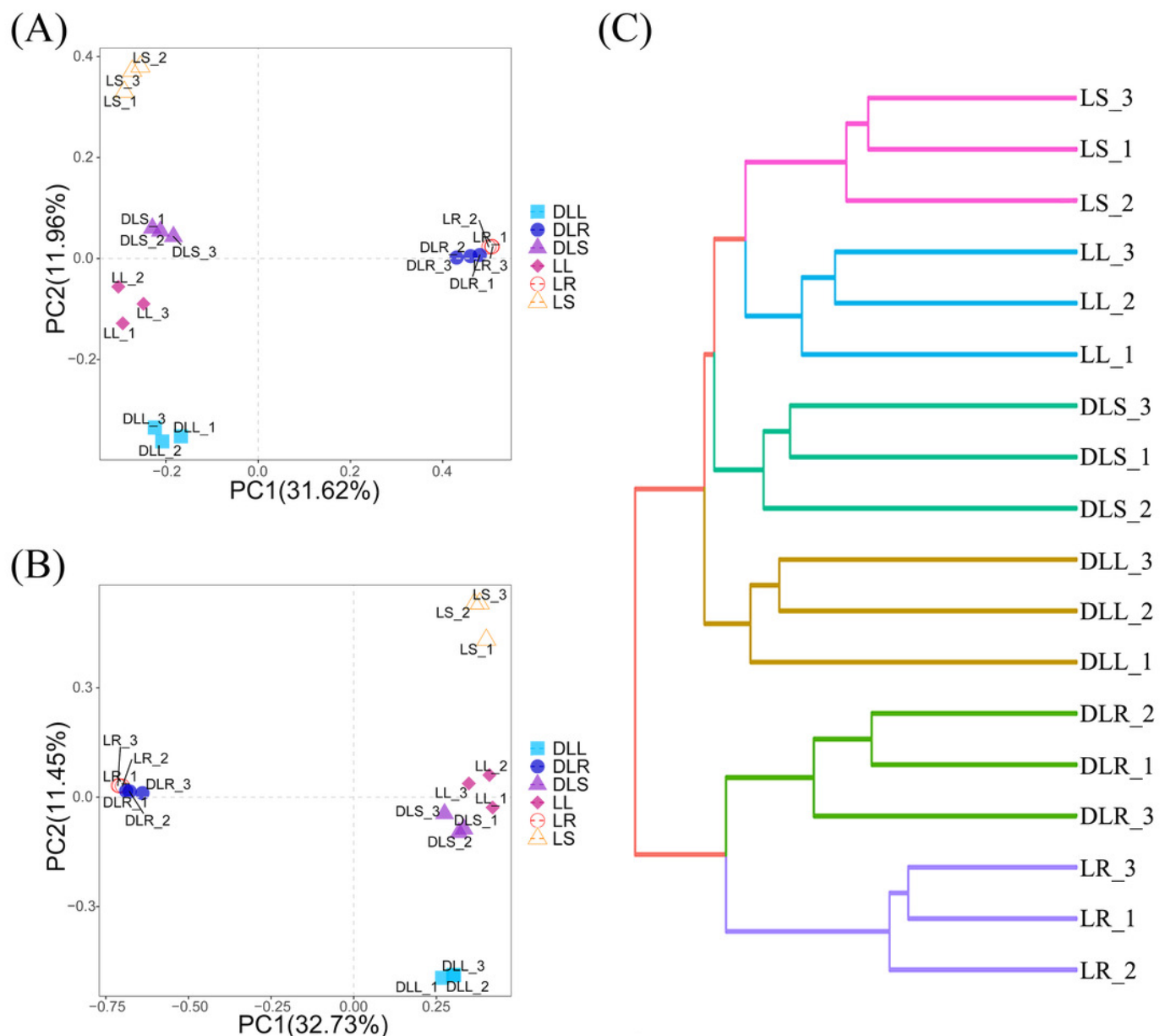



Figure 3

Relative abundance of endophytic bacteria.

Endophytic bacteria communities associated with *Leymus secalinus* at the phylum level (A) and genus level (B) and LEfSe analysis between DL and L groups (C).LEfSe, linear discriminant analysis (LDA) effect size.

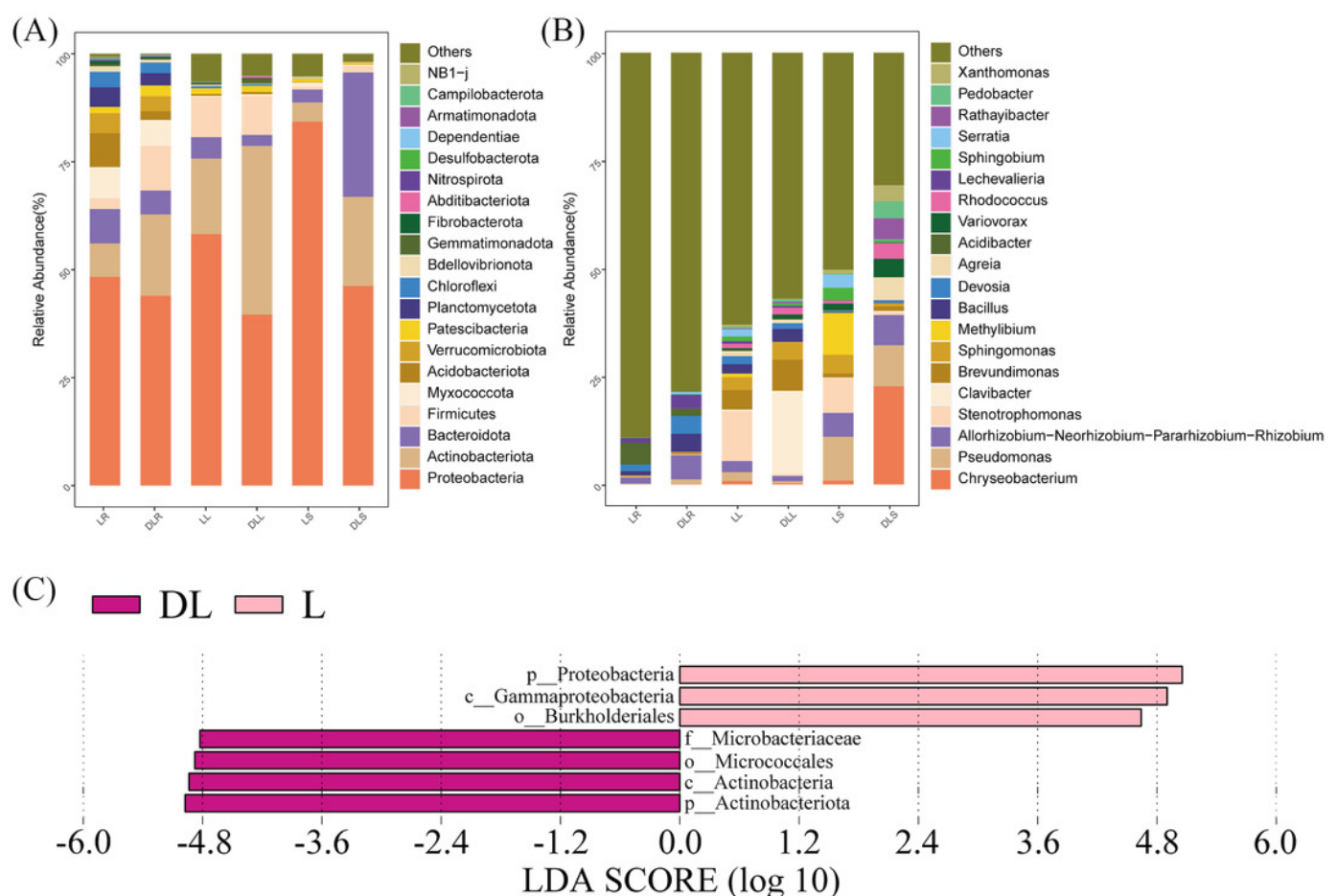



Figure 4

Abundance heatmap of the KEGG function of endophytic communities.

KEGG, Kyoto Encyclopedia of Genes and Genomes.

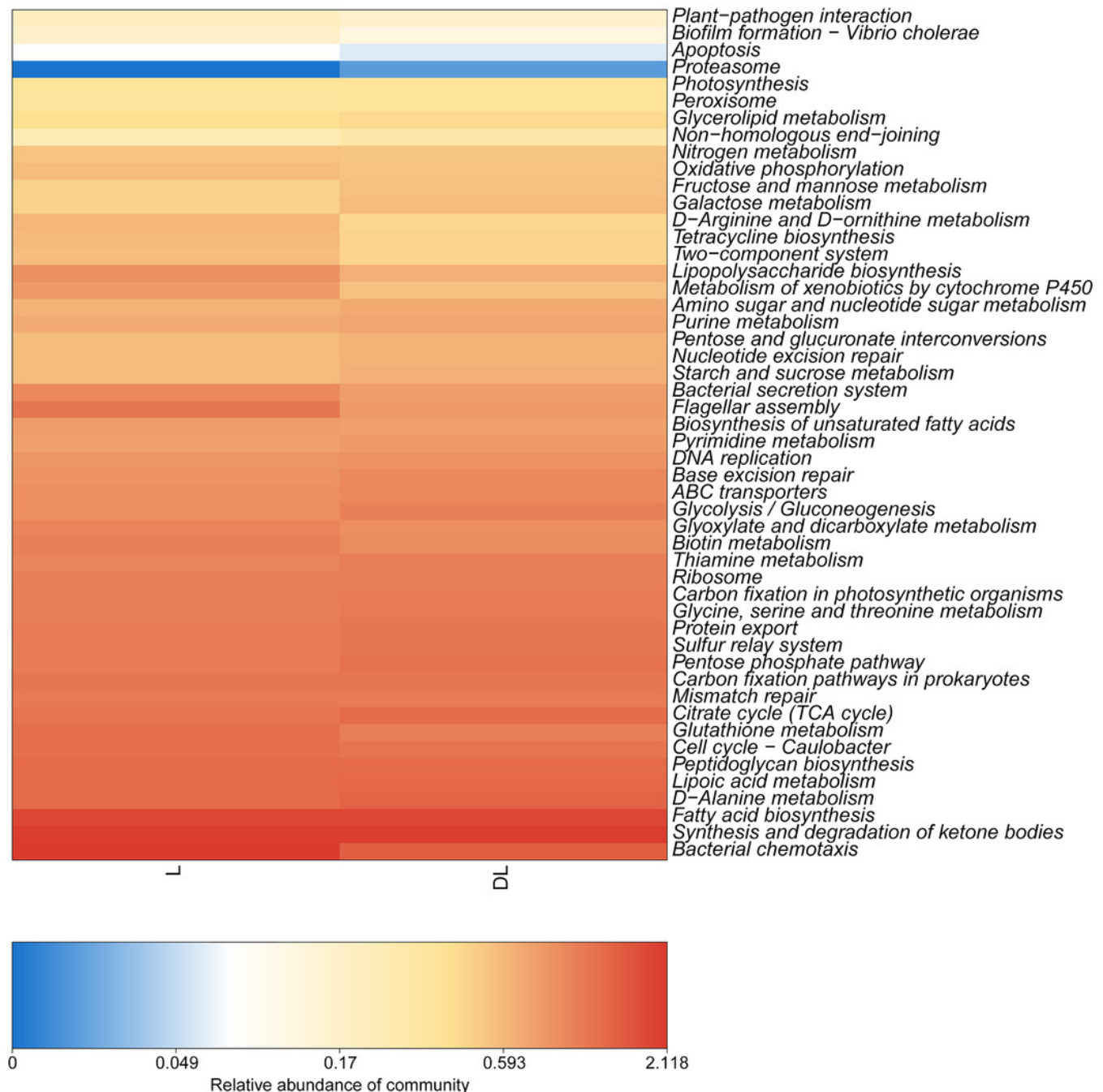
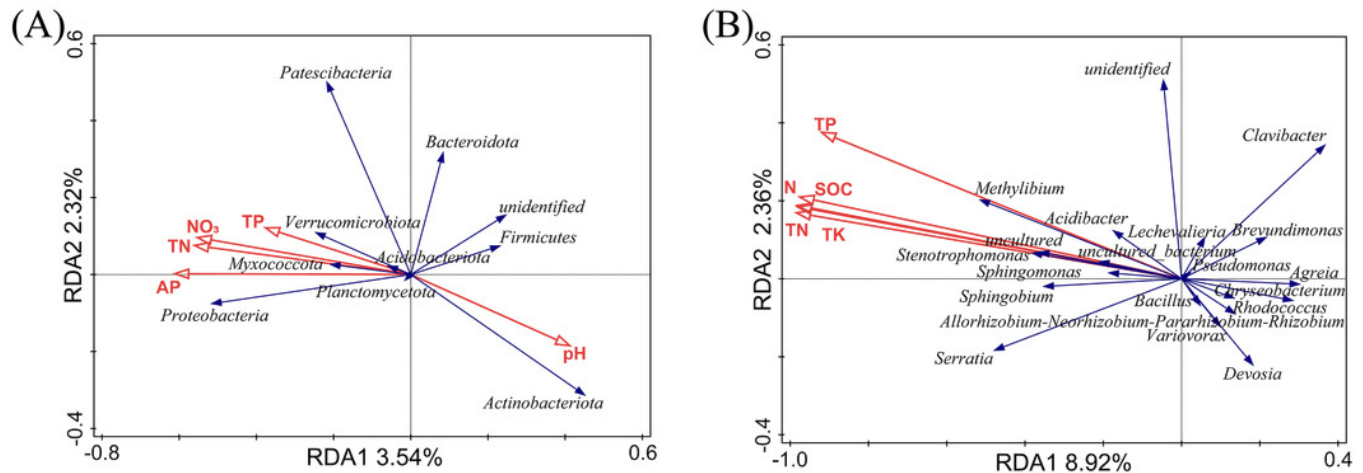



Figure 5

RDA analysis at phylum level (A) and genus level (B).

RDA, redundancy analysis.

All the primers used in this study.

Organ	Position	Direction	Primer name	Sequence 5′–3′	Reference
Root	16S V5-V7	forward	779F	AACMGGATTAGAT ACCCKG	Chelius and Triplett, 2001 (Chelius & Triplett 2001)
		reverse	1193R	ACGTCATCCCCACC TTCC	Bodenhausen et al., 2012 (Bodenhausen et al. 2013)
Stem and leaf	16S V3-V4	forward	332F	ACGGHCCARACTC CTACGGHA	Chen et al., 2022 (Chen et al. 2022)
		reverse	796R	CTACCMGGGTATC TAATCCKG	

2 Bodenhausen N, Horton MW, and Bergelson J. 2013. Bacterial communities associated with the leaves
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α-Diversity of endophytic bacteria in DL and L.

Group name	High-quality sequence	OTUs	Chao1 index	Coverage	Shannon index	Simpson index
LR	162795±100359b	1500±52.4d	2740±204.3 d	0.89±0.0100 a	9.00±0.084 d	0.99±0.0000 a
DLR	259121±67815c	1209±143.5 c	2208±234.9 c	0.91±0.0115 b	8.29±0.450 cd	0.99±0.0058 a
LL	41912±4054a	369±23.3b	476±18.8b	0.99±0.0000 c	6.89±0.121 bc	0.97±0.0058 a
DLL	38072±12171a	349±42.5b	448±54.3ab	0.99±0.0058 c	6.11±1.755 ab	0.89±0.1704 a
LS	43034±13666a	286±23.1b	347±25.8ab	0.99±0.0000 c	5.63±0.596 ab	0.95±0.0200 a
DLS	72405±22092ab	149±11.5a	218±34.3a	0.99±0.0058 c	4.55±1.006 a	0.86±0.1617 a

1 Note: Data are presented as mean±SE, (n=3). Small letters reflect significant differences between different

2 plant tissue samples (p < 0.05). OTUs, operational taxonomic units.

Physical and chemical properties of the soil.

	pH	SOC (g/kg)	TN (g/kg)	TP (g/kg)	TK (g/kg)	N (mg/kg)	AP (mg/kg)	AK (mg/kg)	NH$_4^+$ (mg/g)	NO$_3^-$ (mg/kg)
L	8.1	52.3	4.15	1.13	3.62	174.9	63.10	117.3	20.3	27.06
DL	8.5	2.61	0.25	0.80	2.14	7.67	20.82	39.14	0.69	7.71

1 Note: AK, available potassium; AP, available phosphorus; N, available nitrogen; NH$_4^+$, ammonium; NO$_3^-$,

2 nitrate; SOC, soil organic carbon; TK, total potassium; TN, total nitrogen; TP, total phosphorus.