Nickel mine soil is a potential source for soybean plant growth promoting and heavy metal tolerant rhizobia

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Abstract

Mine soil is not only barren but also contaminated by some heavy metals. It is unclear whether some rhizobia survived under extreme conditions in the nickel mine soil. Therefore, this study tries to isolate some effective soybean plant growth promoting and heavy metal resistant rhizobia from nickel mine soil, and to analyze their diversity. Soybean plants were used to trap rhizobia from the nickel mine soil. A total of 21 isolates were preliminarily identified as rhizobia, which were clustered into eight groups at 87% similarity level using BOXA1R-PCR fingerprinting technique. Four out of the eight representative isolates formed nodules on soybean roots with effectively symbiotic nitrogen-fixing and plant growth promoting abilities in the soybean pot experiment. Phylogenetic analysis of 16S rRNA, four housekeeping genes (*atpD-recA-glnII-rpoB*) and *nifH* genes assigned the symbiotic isolates YN5, YN8 and YN10 into *Ensifer xinjiangense* and YN11 into *Rhizobium radiobacter*, respectively. They also showed different tolerance levels to the heavy metals including cadmium, chromium, copper, nickel, and zinc. It was concluded that there were some plant growth promoting and heavy metal resistant rhizobia with the potential to facilitate phytoremediation and alleviate the effects of heavy metals on soybean cultivation in nickel mine soil, indicating a novel evidence for further exploring more functional microbes from the nickel mine soil.

Keywords: Rhizobia, Nickel mine soil, Soybean, Plant growth promoting, Diversity, Heavy metal tolerance

Introduction

Heavy metal contamination in mining related soils affects both the mining site and the surrounding environment. Heavy metals in soil can originate from natural minerals, yet anthropogenic activities are the main source (Lebrazi & Fikri-Benbrahim 2018). Heavy metal contamination is a risk to food security, ecological environment, and even to human health through bioaccumulation in the food chain (Zhang et al. 2012; Zhou et al. 2013; Long et al. 2021; Qin et al. 2021; Tauqeer et al. 2022). Therefore, areas with severe heavy metal-contaminations need to be remediated before being used for the cultivation of crops, and the selected crops should not accumulate contaminants when growing on the slightly-contaminated areas. Remediating the contaminated soils requires efficient and economical methods such as bioremediation, which is considered eco-friendly, secondary contamination-free, and suitable for non-point source contamination (Shao et al. 2020; Zhang et al. 2020; Yu et al. 2021).

Phytoremediation, especially *in-situ* enhanced phytoremediation, is widely used for the bioremediation of heavy metal-contaminated soil (Thakare et al. 2021). The growth of plants in contaminated soil can be facilitated by utilizing the biological nitrogen fixation (BNF) ability of legume-rhizobia symbionts (Hao et al. 2014; Yu et al. 2017; Wang et al. 2019; Salmi & Boulila 2021). For example, soybean (*Glycine max* L. Merrill) is applicable in remediating heavy metal-contaminated sites (Li et al. 2019). In the symbiosis, rhizobia induce the formation of nodules on the roots of the host plant. Inside the nodules, rhizobia fix atmospheric nitrogen into ammonia which serves as a N source for the legume (Lindstrom & Mousavi 2020; Wang et al. 2020). Inoculation of effective N fixing rhizobial strains leads to the growth promotion of legumes (Catroux et al. 2001). It has been proposed that strains suitable for legume-rhizobia phytoremediation can be isolated from the contaminated sites (Limcharoensuk et al. 2015; Fan

et al. 2016; Balakrishnan et al. 2017; Dhuldhaj & Pandya 2020). Rhizobia include strains with heavy metal resistance and are capable to promote plant growth under heavy metal stress (Grandlic et al. 2009; Fan et al. 2018b). It showed that a copper-resistant *S. meliloti* strain promoted the growth of alfalfa under copper stress (Duan et al. 2019). Rhizobial strains resistant against several heavy metals have the potential to be applied in the *in-situ* bioremediation of soils contaminated with multiple heavy metals (Grandlic et al. 2009; Abd-Alla et al. 2012; Yu et al. 2014; Hao et al. 2015; Yu et al. 2017; Ke et al. 2021). However, indigenous rhizobia resources that could be applied in *in situ* phytoremediation are still scarce in Southwest China.

We hypothesized that heavy metal-contaminated soil could be a putative source for such strains. Thus, soybean plants were used to trap rhizobia from nickel mine soil in Xichang, Sichuan Province, China. The isolates were identified using molecular methods, and soybean growth-promoting abilities and heavy metal resistance of these strains were tested. The results provide better understanding of the potential of using indigenous microbial resources for the alleviation of heavy metal contamination.

Materials and Methods

Sampling and soil analysis

Soil samples were collected from a nickel mine (26° 46′ 54.2″ N, 102° 06′ 53.2″ E) in Xichang, Sichuan Province, China. Three sampling sites spaced 50 to 100 m apart were randomly selected within the area. From the sampling points, five topsoil (0-20 cm) subsamples spaced at least 5 m apart were collected and mixed to make one composite sample per site. The composite samples were quartered and stored on ice before being taken to the laboratory. The soil samples were ground, passed through a 2 mm nylon sieve, and air-dried. Soil water content

in the fresh soil was determined by measuring the weight difference after soil samples had been dried at 105 °C for eight hours. Soil pH was measured using a PHS-3C pH meter (Shanghai Yoke, China) in a 2.5:1 water-soil slurry which had been left to settle overnight. Soil organic carbon content was determined using the $K_2Cr_2O_7$-H_2SO_4 method (Schumacher, 2002). Total nitrogen, phosphorus and potassium contents in the soil samples were determined using Kjeldahl method (Kjeltec 8400, FOSS, Sweden), Mo-Sb colorimetric method (WFJ2100, UNICO, China) and flame spectrophotometry (FP6410, Shanghai Precision & Scientific, China), respectively (Murphy & Riley 1962; Page et al. 1982; Yu et al. 2021). Available nitrogen content was determined using the alkali N-proliferation method; soil available phosphorus and potassium were extracted with sodium bicarbonate solution and NH_4AC solution, respectively, and measured using the previously described method (Wu et al. 2021). The contents of heavy metals were determined after digestion using mixed acid (HNO_3: $HCLO_4$= 3: 1) using inductively coupled plasma optical emission spectrometry (ICP-OES; IRIS Intrepid II, Thermo Electron Corporation, USA).

Trapping and isolation of rhizobia

The indigenous rhizobial strains in the nickel mine soil were trapped using soybean cultivar Nandou No.12 bred by Nanchong Institution of Agricultural Sciences, Sichuan, China. The soybean seeds were sterilized by dipping into 95% alcohol for 3 min and 1% $HgCl_2$ for 5 min, followed by rinsing with sterile water (Yu et al. 2017). The soybean seeds were germinated in a pot filled with sterilized moist vermiculite in the dark for 24 h, and transplanted into pots filled with soil collected from the nickel mine site (S_N). The soybean plants were harvested after 90 days. Three root nodules per soybean plant were selected and sterilized using the above-mentioned methods. The nodules were incubated on beef extract peptone agar at 28 °C, and the

surface sterilization was considered successful when no colonies appeared in 24 h. After that, nodules were crushed in plastic tubes and inoculated on yeast-extract mannitol agar (yeast extract 1.5 g L^{-1}, mannitol 1.0 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4$ $7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, Congo red 0.04 g L^{-1}, agar 20 g L^{-1}). The plates were maintained at 28 °C for 7 to 10 days. During the incubation period, single colonies were selected based on colony morphology and purified by repeated streaking (Yu et al. 2017). Purified isolates with round, plump, milky white, mucilaginous and smooth-edged colonies were examined using light microscopy after Gram staining. Gram-negative isolates with rod-shaped cells were preserved in 20% (*w/v*) glycerol at -80 °C.

Phylogenetic diversity analysis

The isolates were grown in YM liquid medium (yeast extract 1.5 g L^{-1}, mannitol 1.0 g L^{-1}, K_2HPO_4 0.5 g L^{-1}, $MgSO_4$ $7H_2O$ 0.2 g L^{-1}, NaCl 0.1 g L^{-1}, Congo red 0.04 g L^{-1}) for 24 h at 28 °C in a shaking incubator, and DNA was extracted using TIANamp Bacteria DNA Kit (TIANGEN, China). The genetic diversity of the isolates was assessed using BOX-A1R PCR fingerprinting with the primer 5'-CCTCGGCAAGGACGCTGACG-3' (Chen et al. 2014). Amplification was done in a 10 μL volume system containing 5 μL of 2 × PCR mix, 0.2 μL of the primer (10 μmol L^{-1}), 1 μL of template DNA (50 ng mL^{-1}), and 3.8 μL double distilled water (ddH_2O). The BOX-A1R PCR thermal profile included initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 65 °C for 8 min, and a final extension at 65 °C for 16 min. The amplified fragments (8 μL) from the amplification mixture and the 200 bp DNA ladder were separated in 2 % (w/v) agarose gel with ethidium bromide at 80 V for 2.5 h, and were visualized under UV light with the patterns recorded. Based on the patterns, a BOX-A1R PCR cluster tree diagram was created using the

unweighted pair group method with arithmetic averages (UPGMA) in NTSYSpc 2.1 (Yu et al. 2014).

Plant growth promotion ability test

Based on the BOX-A1R PCR fingerprints, eight representative rhizobial isolates were selected for the test of plant growth promotion ability using Leonard jars. Leonard jar and nitrogen-free nutrient solution were prepared as previously described (Kang et 2018; Yu et al. 2017). Soybean seeds were surface-sterilized and germinated as described above. Three sterilized seeds were transferred into the topsoil of a Leonard jar, which was then covered with a layer of approximately 3 cm moist vermiculite. When the soybean seedlings were 2-3 cm tall, the rhizobia culture in exponential phase (OD_{600nm} 0.6~0.8) was inoculated around the roots, and then the topsoil was covered with a layer of 1 cm autoclaved quartz sand. The non-inoculated soybean supplemented with nitrogen-free nutrient solution was set as the negative control, while the N^+ treatment with 1g L^{-1} KNO_3 as the nitrogen source in the nutrient solution was the positive control. Each treatment and control included three replicates. The soybean plants were kept in a greenhouse with artificial light-dark cycles: 17 h light, temperature 25 °C and humidity 80% (to simulate daytime); 7 h dark, temperature 17 °C and humidity 85% (to simulate nighttime). The jars were replenished with nutrient solution when needed. The soybean plants were harvested after 55 days. The number of nodules, root length, and plant height and weight were measured. The plant samples were dried at 105 °C for 30 min to deactivate enzymes, and then at 55 °C until constant weight. The nitrogen, phosphorus, and potassium contents of the dried roots and shoots were measured.

Sequence analysis

147 The isolates that nodulated soybean plants were further characterized using sequence
148 analyses. The almost full length 16S rRNA gene was amplified using the primer pair 27F/1492R
149 (Yu et al. 2017), the housekeeping genes *atpD*, *recA*, *glnII,* and *rpoB* using primer pairs
150 *atpD*255F/ *atpD*782R, *recA*63F/ *recA*555R, *glnII*12F/ *glnII*tsR, and *rpoB*454F/ *rpoB*1364R,
151 respectively (Tang et al. 2012; Zhao et al. 2014), and the nitrogen fixation gene *nifH* using the
152 primer pair *nifH*F/ *nifH*I (Laguerre et al. 2001). All of the primers we used in this study were
153 listed in Table S2. Amplification was done in a 30 μL volume system containing 15 μL of 2 ×
154 PCR mix, 0.15 μL of each primer, one μL of template DNA (50 ng mL^{-1}), and 13.7 μL ddH_2O.
155 For the amplification of 16S rRNA, *atpD*, *recA*, *glnII* and *rpoB* genes, the thermal cycling
156 conditions included an initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C
157 for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72
158 °C for 10 min. For the amplification of *nifH*, the thermal cycling conditions included an initial
159 denaturation at 95 °C for 3 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C
160 for 1 min, extension at 72 °C for 5 min, and a final extension at 72 °C for 6 min. The amplified
161 products were sequenced by Sangon Biotech (Shanghai, China). The fragments of *atpD* (274 bp),
162 *recA* (245 bp), *glnII* (413bp) and *rpoB* (534 bp) were concatenated for multilocus sequence
163 analysis (MLSA). The sequences were matched against reference sequences of rhizobia in the
164 GenBank of NCBI database using BLAST. The reference sequences of different rhizobia species
165 we used for the phylogenetic analysis of the three genes (16SrRNA, housekeeping genes, and
166 *nifH*) were separately listed in Table S3, Table S4, and Table S5. The sequences of the isolates
167 and the reference sequences were subjected to phylogenetic analysis using neighbor joining
168 method in MEGA7.0. The phylogenetic trees were bootstrapped with 1,000 replications for each
169 sequence to evaluate the reliability of the tree topologies (Saitou & Nei 1987).

Heavy metal resistance test

For assessing the heavy metal-resistance ability of the isolates, 10 g L^{-1} stock solutions of cadmium ($CdCl_2$ $2.5H_2O$), chromium ($K_2Cr_2O_7$), copper ($CuCl_2$ $2H_2O$), nickel ($NiSO_4$), and zinc ($ZnSO_4$ $7H_2O$) were prepared by dissolving the salts in ultrapure water. The isolates were grown in 5 mL YM liquid medium with 0, 4, 8, 12, 16, 20, 40, 60, 80, 100 mg L^{-1} of each metal in an orbital shaker at 28 °C for 72 h, after which the optical density ($OD_{600\ nm}$) of the cultures were measured using a spectrophotometer (UV-3300, Shanghai MAPADA, China) (Abd-Alla et al. 2012).

The minimum inhibitory concentrations (MIC) were determined by comparing the OD_{600nm} value of the cultures spiked with metals to those without metals. MIC was defined as the lowest metal concentration resulting in a visually observable decrease in the growth curve of the isolates. Minimum lethal concentration (MLC) was defined as the lowest metal concentration resulting in an OD_{600nm} value lower than 0.1.

Statistical analysis

Statistical differences in soil properties and soybean growth parameters were tested using one-way ANOVA and Fisher's protected LSD (least significant difference) test at $P \leq 0.05$ in IBM SPSS statistics 22.0. The differences in MIC and MLC values were not tested due to their zero variance.

Results

Isolation of rhizobia from nickel mine soil

The soil in the nickel mining area was slightly acidic (pH 6.35), and the organic matter content was low (1.07 %). The contents of total N, P and K were 261.35, 479.85, and 5869.73 mg kg^{-1}, respectively, and 15.08 %, 2.93 %, and 0.38 % of them were available fragments (Table 1). Both nickel and lead contents were approximately 20 mg kg^{-1}, and cadmium, chromium, copper, iron, manganese and zinc were also detected (Table 1).

As a result of trapping rhizobial strains from the nickel mine soil using soybean as the host plant, 21 isolates were preliminarily identified as rhizobia based on colony morphology, microscopic examination of cell shape, and Gram staining. The BOXA1R-PCR fingerprinting (Fig. 1) of the isolates showed that sixteen distinct fingerprint patterns were found, indicating that these isolates from nickel mine soil were genetically diverse. The isolates were clustered into 8 groups at 87% similarity level.

Plant growth promotion ability of selected isolates

Based on the fingerprinting, we selected 8 isolates (YN1, YN5, YN8, YN10, YN11, YN13, YN27, YN30) for the plant growth promotion ability test. Only four isolates (YN5, YN8, YN10, YN11) nodulated soybean plants, with nodule numbers ranging from 50 to 54 per plant (mean value of six plants). The biomass of soybeans inoculated with the four symbiotic isolates (YN5, YN8, YN10, YN11) and isolate YN13 was significantly higher than that in the N^- treatment (P = 0.00) (Fig. 2a). The biomass of soybeans inoculated with isolate YN8 was on the same level as in the N^+ treatment. The shoots of soybeans inoculated with the 4 symbiotic isolates (YN5, YN8, YN10, YN11) and isolate YN1 were significantly longer than those in the N^- treatment (P=0.00, 0.01, 0.00, 0.00, 0.00). The roots of soybeans inoculated with the symbiotic isolates YN8, YN10, and YN11 were significantly longer than those in the N^- treatment (P=0.00) (Fig. 2b). The shoot

N content of soybean plants inoculated with the symbiotic strains was at the same level as in the N^+ treatment, and the shoot N content of soybeans inoculated with the symbiotic strains of YN5, YN8, YN10, and YN11 as well as the isolate YN1 was significantly higher than that in the N^- treatment (P=0.00) (Fig. 3a). The root N content of inoculated soybean plants was higher than that in the N^- treatment (Fig. 3a). The root P content of soybeans inoculated with the symbiotic isolates and isolate YN30 was lower than that in the N^- treatment (Fig. 3b). The shoot K content of soybeans inoculated with the isolate YN10 was higher than that in the N^- treatment (Fig. 3c). The root K content of soybeans inoculated with the isolates YN5, YN8, YN10, YN13, YN27 and YN30 was higher than that in the N^- treatment (Fig. 3c).

Sequence analyses of symbiotic isolates

As a result of the sequence analysis of the almost full length 16S rRNA gene, the symbiotic isolates were assigned to the genera *Ensifer* (formerly designated as *Sinorhizobium*) and *Rhizobium* (Fig. 4). The isolates YN5, YN8, and YN10 were respectively grouped together with the type strains *E. fredii* USDA205, *E. americanum* CFNEI156 and *E. xinjiangense* CCBAU110, and isolate YN11 with *R. radiobacter* ICMP 5856 (formerly *Agrobacterium tumefaciens* ICMP 5856). Based on the multilocus sequence analysis (MLSA) of the concatenated fragments of *atpD* (274 bp), *recA* (245 bp), *glnII* (413bp) and *rpoB* (534 bp), the isolates YN5, YN8, and YN10 were identified as *E. xinjiangense* strains, and YN11 as *Rhizobium radiobacter* strain (Fig. 5). The *nifH* sequences from YN5, YN8, and YN10 were 100% similar with those from *E. fredii* CCBAU23314 and *E. xinjiangense* CCBAU110, and the *nifH* sequence from YN11 with that from *R. radiobacter* (Fig. 6).

Heavy metal resistance ability of symbiotic isolates

In general, *E. xinjiangense* YN5 and *R. radiobacter* YN11 tolerated higher levels of heavy metals compared to *E. xinjiangense* YN8 and YN10 (Fig. 7, Supplementary Table S1). For Cd^{2+}, the MIC and MLC values of *E. xinjiangense* YN5 and *R. radiobacter* YN11 were the highest. For Cr^{6+}, the MLC of all the strains was 16 mg L^{-1}. For Cu^{2+}, the MIC and MLC values of *E. xinjiangense* YN8 were the lowest. For Ni^{2+}, the MLC values of YN5 and YN11 were higher than those of YN8 and YN10. For Zn^{2+}, the MLC value of *E. xinjiangense* YN5 was 300 mg L^{-1}, i.e., over three times higher than that of *R. radiobacter* YN11 and over 18 to 75 times higher than those of YN11 and YN8, respectively.

Discussion

We trapped rhizobia from nickel mine soil using soybean plants in Xichang, Sichuan Province, China, to find effectively plant growth promoting and heavy metal resistant strains for the enhancement of phytoremediation of heavy metal contaminated soil and for the promotion of soybean growth on slightly contaminated farmland. The low organic matter, N, P and K implied that the soil was barren (Zhang et al. 2012; Wu et al. 2021), yet the rhizobia-trapping plants were nodulated, and the isolates from nodules were diverse based on the BOXA1R-PCR fingerprints. However, when inoculated on soybeans, only four out of the eight representative isolates formed nodules on the roots. Similar to rhizobia isolated from *Glycyrrhiza* spp. (Li et al. 2012), the four non-nodulating isolates may have been sporadic symbionts or other endophytes that had entered the nodules along with a genuine symbiont. Similar to the model inoculant of soybean, *Bradyrhizobium diazoefficiens* USDA110 (Sibponkrung et al. 2020), inoculation with the symbiotic isolates resulted in over two times higher biomass than in the non-inoculated control; the higher biomass was accompanied with higher shoot nitrogen content. In addition, even the non-nodulating isolates showed some plant growth promoting abilities. Especially,

257 inoculation with all the representative isolates resulted in higher root N content than in the
258 nitrogen-free control. In most of the inoculated plants, the increase in root N content was
259 accompanied with lower P content.

260 As a host plant, soybean is promiscuous and may be nodulated with both fast- and slow-
261 growing rhizobia (Chen et al. 2021). Based on the 16S rRNA gene analysis, three of our isolates
262 were identified as *Ensifer americanum, E. fredii or E. xinjiangense*, i.e., species with closely
263 related 16S rRNA genes (Peng et al. 2002; Wang et al. 2013). Further analysis using MLSA of
264 the four housekeeping genes assigned the symbiotic isolates into the fast-growing rhizobial
265 species *Ensifer xinjiangense* and *Rhizobium radiobacter*, strains of which have been identified as
266 plant growth promoting symbionts of soybean plants (Peng et al. 2002; Iturralde et al. 2019). To
267 our knowledge, neither *E. xinjiangense* (formerly *Sinorhizobium xinjiangense*) nor *R.*
268 *radiobacter* (formerly *Agrobacterium tumefaciens*) strains have been applied as a single-
269 inoculant plant growth promoter in bioremediation, yet co-inoculation of an *A. tumefaciens* strain
270 with *S. meliloti* promoted the growth of *Medicago lupulina* under Cu and Zn stresses (Jian et al.
271 2019). *R. radiobacter* is traditionally considered as a plant pathogen and is a free-living nitrogen
272 fixer (Kanvinde et al. 1990). In our study, the amplification and sequencing of the *nifH* gene,
273 which encodes nitrogenase iron protein, showed that both the *Ensifer* strains and *R. radiobacter*
274 YN11 had the genetic potential for nitrogen fixation. The nodulation and plant growth promotion
275 capacity of *R. radiobacter* YN11 added to the growing body of evidence that when carrying the
276 nodulation genes, *Rhizobium* (*Agrobacterium*) clade strains can be legume-nodulating symbionts
277 (Cummings et al. 2009).

278 In the soil from mining areas, the concentrations of heavy metals vary considerably from
279 below the background values to hundreds of times higher than the average values for all soils (Li

et al. 2014). Bacteria inhabiting the heavy metal-contaminated sites include legume nodulating strains with high tolerance against the metals (Mohamad et al. 2017). In our study, the symbiotic strains showed varied heavy metal resistance; *E. xinjiangense* YN5 outperformed the other *E. xinjiangense* isolates and the resistance levels of *R. radiobacter* YN11 fell in-between. Compared to the rhizobia isolated directly from V-Ti magnetite mine tailing soil and those from the nodules *Robinia pseudoacacia* in a Pb-Zn mining area (Yu et al. 2014; Fan et al. 2018), our isolates tolerated lower levels of Cd^{2+} and Cu^{2+}. One possible explanation is the level of contamination on the sites where the strains were isolated; the V-Ti magnetite and Pb-Zn mining areas were seriously contaminated (Yu et al. 2014; Fan et al. 2018a), but only Zn content in the nickel mine soil was higher than in the background value for soils in China (Li et al. 2014). The levels of heavy metals tolerated are approximately 10 to 100 times lower in liquid medium than on solid medium (Hassen et al. 1998). It is also important to take into account the different testing methods for the Zn tolerance of *E. xinjiangense* YN5. The V-Ti magnetite and Pb-Zn mining area isolates were tested on solid media (Yu et al. 2014; Fan et al. 2018a) but our isolates in liquid medium, yet *E. xinjiangense* YN5 tolerated a higher level of Zn^{2+}.

Conclusions

Our study used soybean pot experiment to trap 21 rhizobia strains from nickel mine soil. As a result, we selected three *Ensifer xinjiangense* strains (YN5, YN8, and YN10) and one *Rhizobium radiobacter* (YN11) with good nitrogen fixing ability, which can significantly improve the soybean plant height, root length, and biomass yield. Moreover, these 4 strains carried the symbiotic gene *nifH* that can encode dinitrogenase reductase enzyme, which further confirmed their abilities to form root nodules and fix nitrogen. *E. xinjiangense* YN5 and *R. radiobacter* YN11 tolerated higher levels of heavy metals than *E. xinjiangense* YN8 and YN10.

Taken together, the results showed that the nickel mine soil is a potential source for plant growth promoting rhizobia strains, which could be applied as indigenous inoculants in the phytoremediation of slightly contaminated farmland and in the alleviation of the adverse effects of heavy metals on soybean cultivation.

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CAPTION OF TABLES AND FIGURES

Table 1 The physicochemical properties and heavy metal contents of the nickel mine soil collected in Xichang, Sichuan Province, China. Note: WC, water content; OM, soil organic matter content; TN, soil total nitrogen content; AN, soil available nitrogen content; TP, soil total phosphorus content; AP, soil available phosphorus content; TK, soil total potassium content; AK, soil available potassium content. Ni, nickel; Cu, copper; Cr, chromium; Pb, lead; Mn, manganese; Fe, iron; Zn, zinc; Cd, cadmium. The values are expressed as mean ± standard deviation (n=3).

Figure 1 Dendrogram of the BOX-A1R PCR fingerprints of 21 isolates trapped by soybean from the nickel mine soil collected in Xichang, Sichuan Province, China.

Figure 2 Biomass (a) and shoot and root lengths (b) of soybean plants inoculated with isolates trapped from the nickel mine soil collected in Xichang, Sichuan Province, China. Different letters above the bars indicate significant statistical difference at $p<0.05$ (n=3). Comparison between shoot lengths is in normal font and comparison between root lengths is in bold. Tissues: (▨) shoot, (■) root.

Figure 3 Nitrogen (a), phosphorus (b) and potassium (c) contents in the shoots and roots of soybean plants inoculated with the isolates trapped from the nickel mine soil collected in Xichang, Sichuan Province, China. Different letters above bars indicate significant statistical

498 difference at $p<0.05$ (n=3). Comparison between shoot lengths is in normal font and comparison
499 between root lengths is in bold. Tissues: (▨) shoot, (■) root.

500 **Figure** 4 16S rRNA gene phylogeny of the soybean symbiotic isolates from the nickel mine soil
501 collected in Xichang, Sichuan Province, China (in bold), and reference strains. GenBank
502 accession numbers are in parentheses. Bootstrap values >50% are shown at the branch points.
503 The scale bar denotes 5% substitutions per site.

504 **Figure** 5 Multilocus sequence analysis (MLSA) of the soybean symbiotic isolates from the
505 nickel mine soil collected in Xichang, Sichuan Province, China (in bold), and reference strains.
506 MLSA was done using the concatenated fragments of *atpD* (274 bp), *recA* (245 bp), *glnII* (413bp)
507 and *rpoB* (534 bp). GenBank accession numbers are in parentheses. Bootstrap values > 50% are
508 shown at the branch points. The scale bar denotes 5% substitutions per site.

509 **Figure** 6 *nifH* phylogeny of the soybean symbiotic isolates from the nickel mine soil collected at
510 Xichang, Sichuan Province, China (in bold), and reference strains. GenBank accession numbers
511 are in parentheses. Bootstrap values >50% are shown at the branch points. The scale bar denotes
512 5% substitutions per site.

513 **Figure** 7 The growth of the soybean symbiotic isolates from the nickel mine soil collected in
514 Xichang, Sichuan Province, China, at increasing concentrations of Cd^{2+} (a), Cr^{6+} (b), Cu^{2+} (c),
515 Ni^{2+} (d), and (e) Zn^{2+}. Minimum inhibitory concentrations (MIC) are indicated with green and
516 minimum lethal concentrations (MLC) with red. Isolates: (—●—) YN5, (···□···) YN8, (—×—)
517 YN10, and (—▲—) YN11.
518

Table 1(on next page)

The physicochemical properties and heavy metal contents of the nickel mine soil collected in Xichang, Sichuan Province, China.

Note: WC, water content; OM, soil organic matter content; TN, soil total nitrogen content; AN, soil available nitrogen content; TP, soil total phosphorus content; AP, soil available phosphorus content; TK, soil total potassium content; AK, soil available potassium content. Ni, nickel; Cu, copper; Cr, chromium; Pb, lead; Mn, manganese; Fe, iron; Zn, zinc; Cd, cadmium. The values are expressed as mean ± standard deviation (n=3).

Properties	pH	WC (%)	OM (%)	TN (mg kg^{-1})	AN (mg kg^{-1})	TP (mg kg^{-1})	AP (mg kg^{-1})	TK (mg kg^{-1})	AK (mg kg^{-1})
Average value	6.35± 0.01	7.71± 0.00	1.07± 0.06	261.35± 22.74	39.21± 3.04	479.85± 0.00	140.69± 1.44	5869.73± 0.05	22.68±2.91
Properties	**Ni (mg kg^{-1})**	**Cu (mg kg^{-1})**	**Cr (mg kg^{-1})**	**Pb (mg kg^{-1})**	**Mn (mg kg^{-1})**	**Fe (mg kg^{-1})**	**Zn (mg kg^{-1})**	**Cd (mg kg^{-1})**	
Average value	19.69± 0.44	9.79± 0.10	12.78± 0.08	20.13± 0.03	8.57± 0.04	744.20± 4.10	80.07± 1.04	0.46± 0.01	

1

2

Figure 1

Dendrogram of the BOX-A1R PCR fingerprints of 21 isolates trapped by soybean from the nickel mine soil collected in Xichang, Sichuan Province, China.

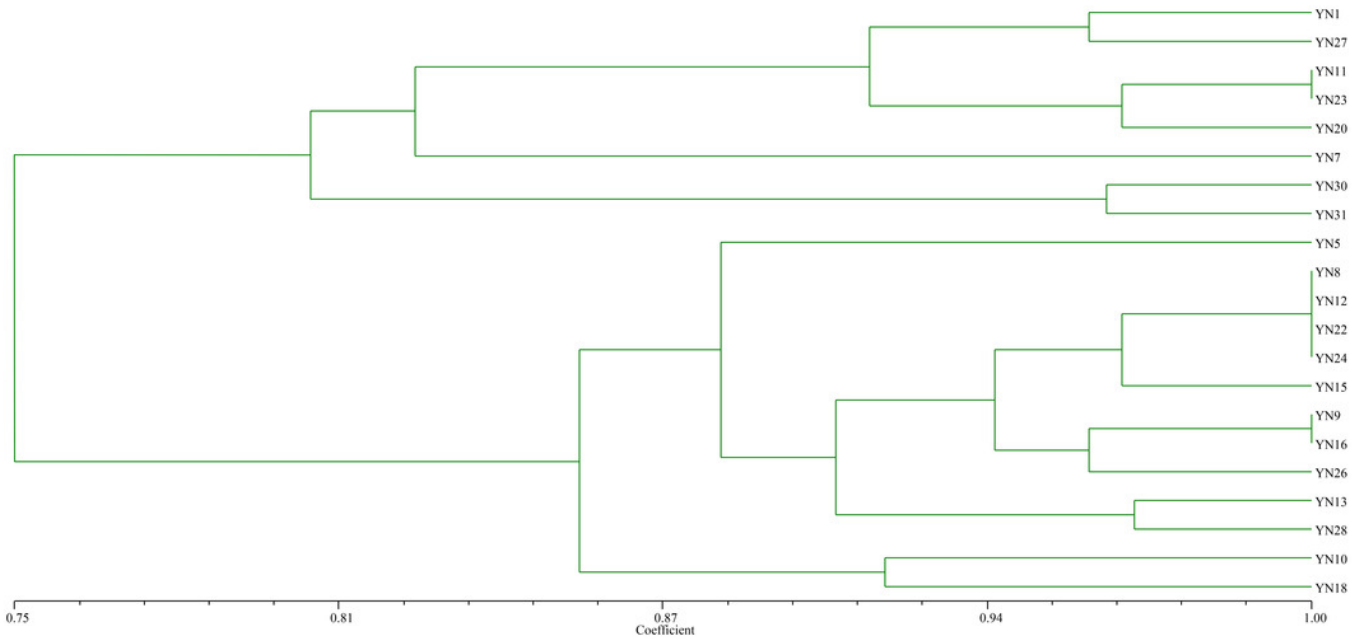


Figure 2

Biomass (a) and shoot and root lengths (b) of soybean plants inoculated with isolates trapped from the nickel mine soil collected in Xichang, Sichuan Province, China.

Different letters above the bars indicate significant statistical difference at $p<0.05$ ($n=3$). Comparison between shoot lengths is in normal font and comparison between root lengths is in bold.

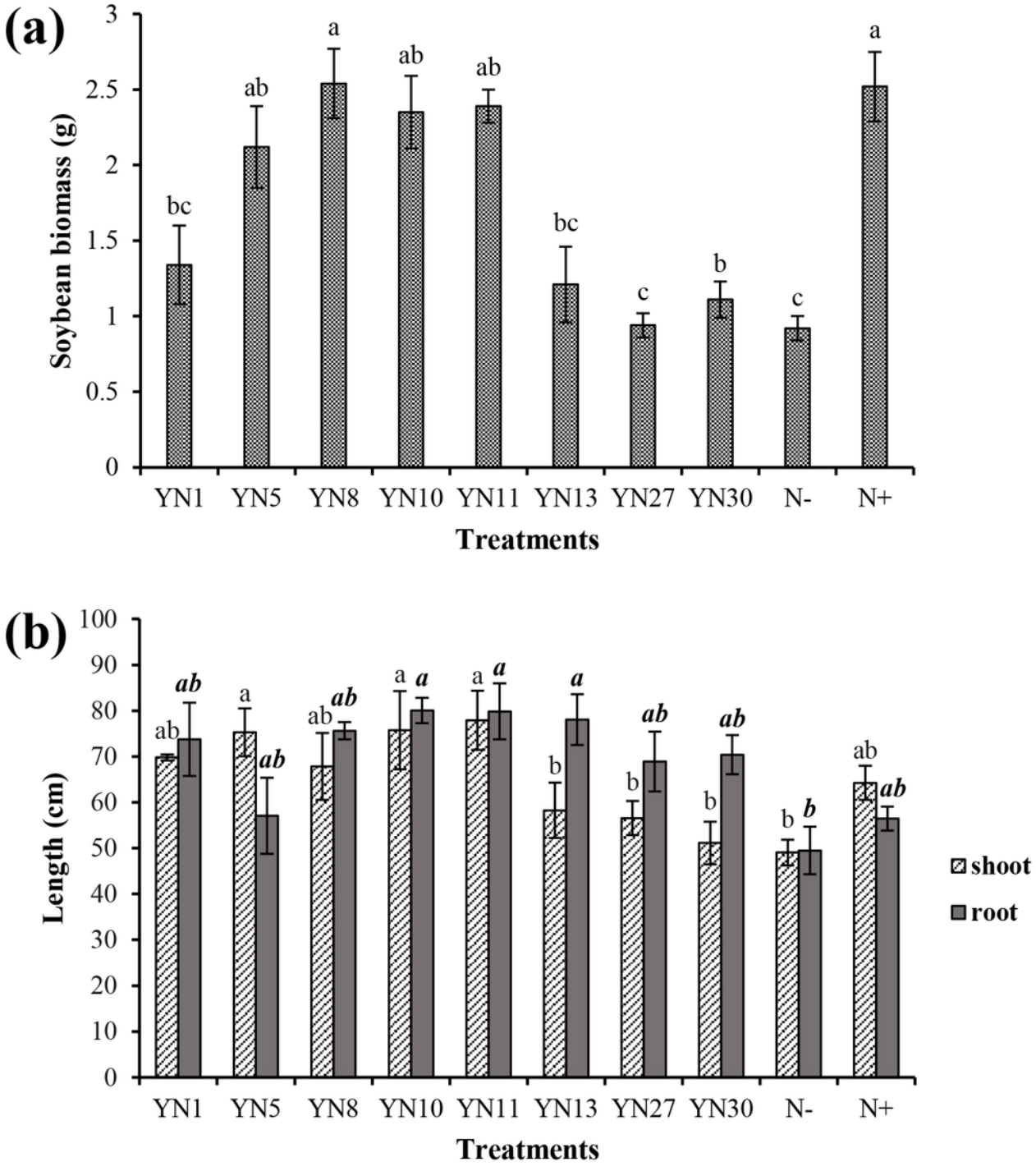

(a)
Soybean biomass (g)
3
2.5
2
1.5
1
0.5
0
bc
ab
a
ab
ab
bc
c
b
c
a
YN1
YN5
YN8
YN10
YN11
YN13
YN27
YN30
N-
N+
Treatments
(b)
Length (cm)
100
90
80
70
60
50
40
30
20
10
0
ab
ab
a
ab
ab
ab
a
a
a
a
b
a
b
ab
b
ab
b
b
ab
ab
YN1
YN5
YN8
YN10
YN11
YN13
YN27
YN30
N-
N+
Treatments
shoot
root

Figure 3

Nitrogen (a), phosphorus (b) and potassium (c) contents in the shoots and roots of soybean plants inoculated with the isolates trapped from the nickel mine soil collected in Xichang, Sichuan Province, China.

Different letters above bars indicate significant statistical difference at $p<0.05$ (n=3).

Comparison between shoot lengths is in normal font and comparison between root lengths is in bold.

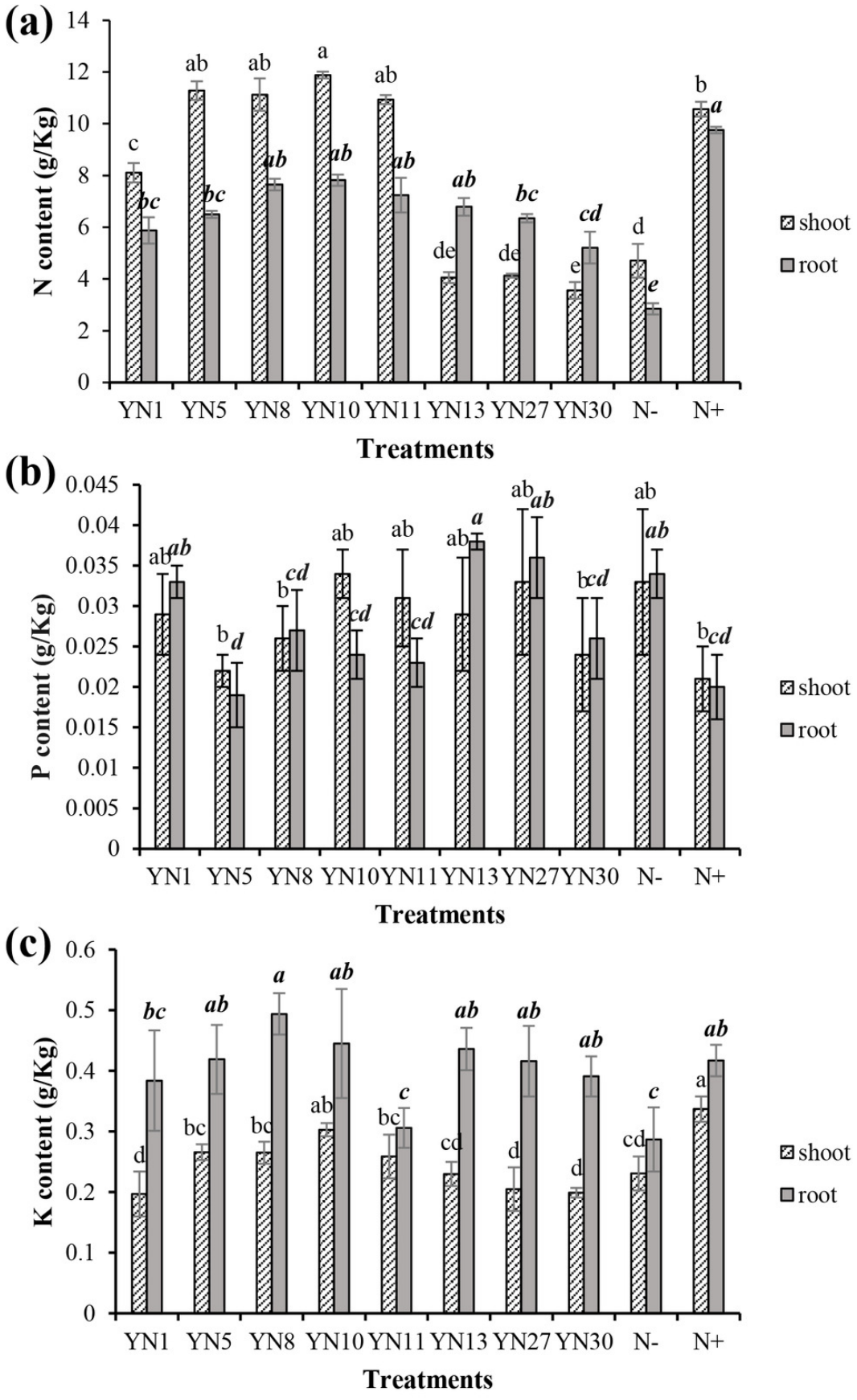

(a)
N content (g/Kg)
14
12
10
8
6
4
2
0
YN1 YN5 YN8 YN10 YN11 YN13 YN27 YN30 N- N+
Treatments
shoot
root
(b)
P content (g/Kg)
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
YN1 YN5 YN8 YN10 YN11 YN13 YN27 YN30 N- N+
Treatments
shoot
root
(c)
K content (g/Kg)
0.6
0.5
0.4
0.3
0.2
0.1
0
YN1 YN5 YN8 YN10 YN11 YN13 YN27 YN30 N- N+
Treatments
shoot
root

Figure 4

16S rRNA gene phylogeny of the soybean symbiotic isolates from the nickel mine soil collected in Xichang, Sichuan Province, China (in bold), and reference strains.

GenBank accession numbers are in parentheses. Bootstrap values >50% are shown at the branch points. The scale bar denotes 5% substitutions per site.

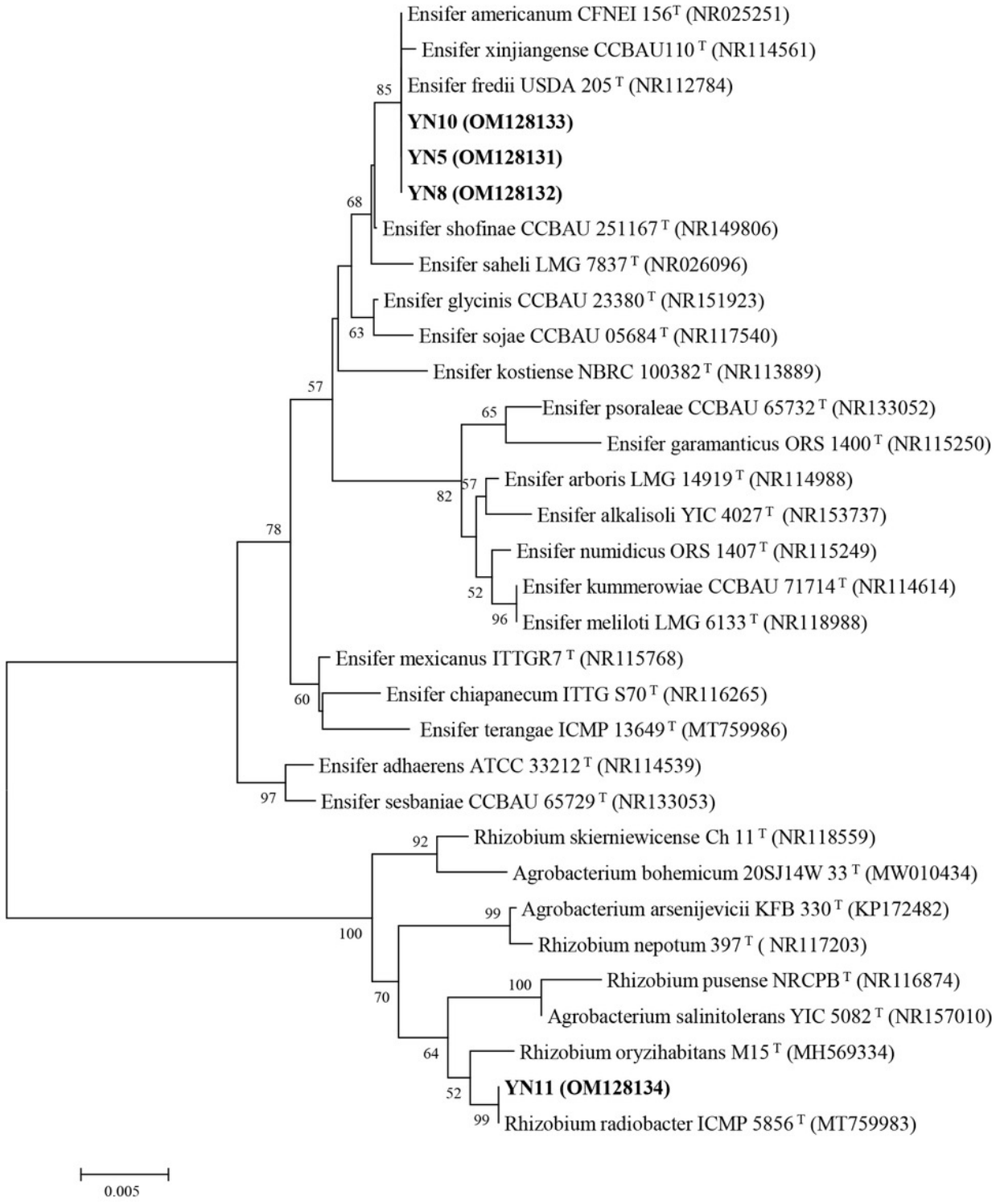

Ensifer americanum CFNEI 156T (NR025251)
Ensifer xinjiangense CCBAU110T (NR114561)
Ensifer fredii USDA 205T (NR112784)
YN10 (OM128133)
YN5 (OM128131)
YN8 (OM128132)
Ensifer shofinae CCBAU 251167T (NR149806)
Ensifer saheli LMG 7837T (NR026096)
Ensifer glycinis CCBAU 23380T (NR151923)
Ensifer sojae CCBAU 05684T (NR117540)
Ensifer kostiense NBRC 100382T (NR113889)
Ensifer psoraleae CCBAU 65732T (NR133052)
Ensifer garamanticus ORS 1400T (NR115250)
Ensifer arboris LMG 14919T (NR114988)
Ensifer alkalisoli YIC 4027T (NR153737)
Ensifer numidicus ORS 1407T (NR115249)
Ensifer kummerowiae CCBAU 71714T (NR114614)
Ensifer meliloti LMG 6133T (NR118988)
Ensifer mexicanus ITTGR7T (NR115768)
Ensifer chiapanecum ITTG S70T (NR116265)
Ensifer terangae ICMP 13649T (MT759986)
Ensifer adhaerens ATCC 33212T (NR114539)
Ensifer sesbaniae CCBAU 65729T (NR133053)
Rhizobium skierniewicense Ch 11T (NR118559)
Agrobacterium bohemicum 20SJ14W 33T (MW010434)
Agrobacterium arsenijevicii KFB 330T (KP172482)
Rhizobium nepotum 397T (NR117203)
Rhizobium pusense NRCPBT (NR116874)
Agrobacterium salinitolerans YIC 5082T (NR157010)
Rhizobium oryzihabitans M15T (MH569334)
YN11 (OM128134)
Rhizobium radiobacter ICMP 5856T (MT759983)
85
68
63
57
65
57
82
78
52
96
60
97
92
99
100
70
100
64
52
99
0.005

Figure 5

Multilocus sequence analysis (MLSA) of the soybean symbiotic isolates from the nickel mine soil collected in Xichang, Sichuan Province, China (in bold), and reference strains.

MLSA was done using the concatenated fragments of *atpD* (274 bp), *recA* (245 bp), *glnII* (413bp) and *rpoB* (534 bp). GenBank accession numbers are in parentheses. Bootstrap values > 50% are shown at the branch points. The scale bar denotes 5% substitutions per site.

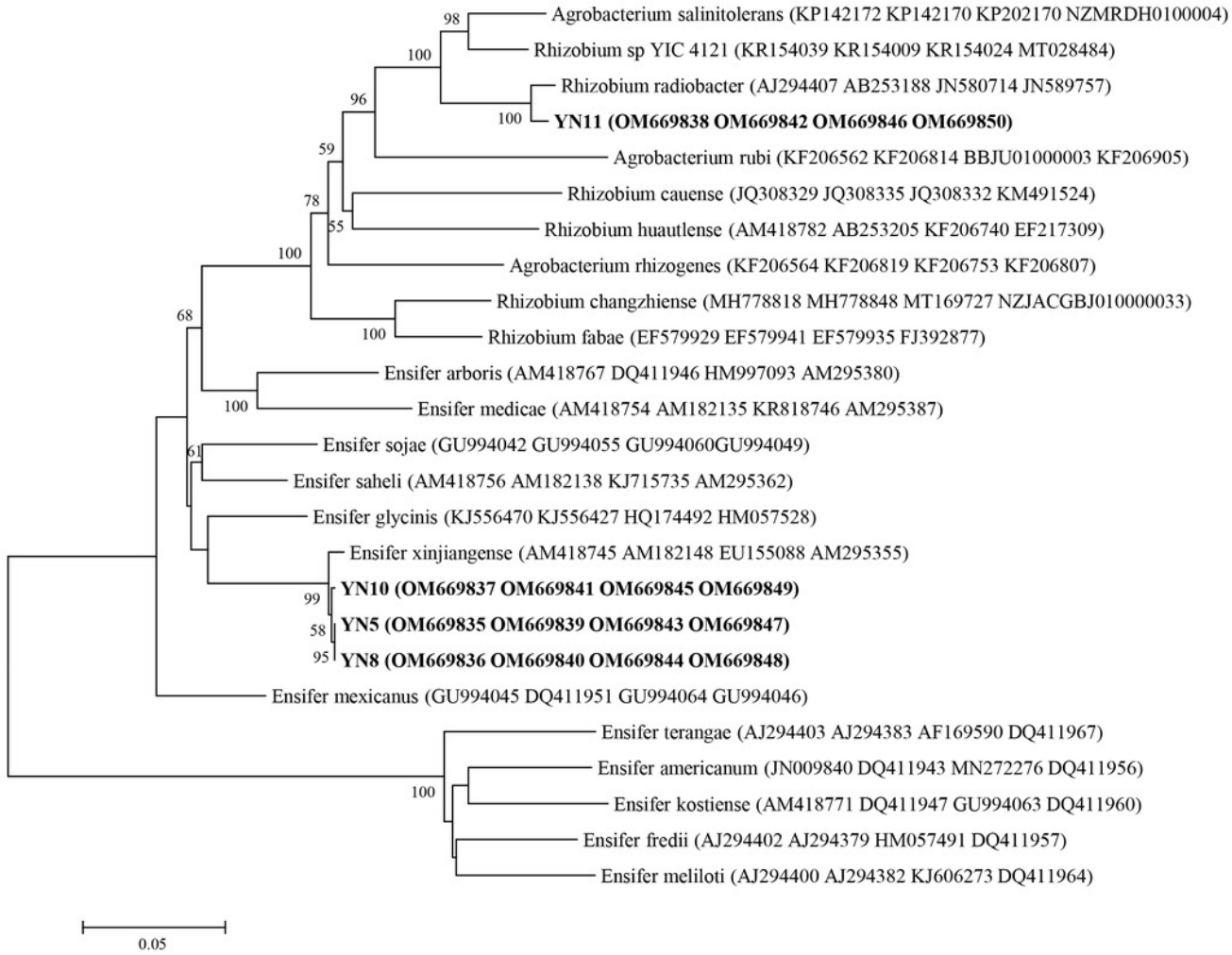


Figure 6

nifH phylogeny of the soybean symbiotic isolates from the nickel mine soil collected at Xichang, Sichuan Province, China (in bold), and reference strains.

GenBank accession numbers are in parentheses. Bootstrap values >50% are shown at the branch points. The scale bar denotes 5% substitutions per site.

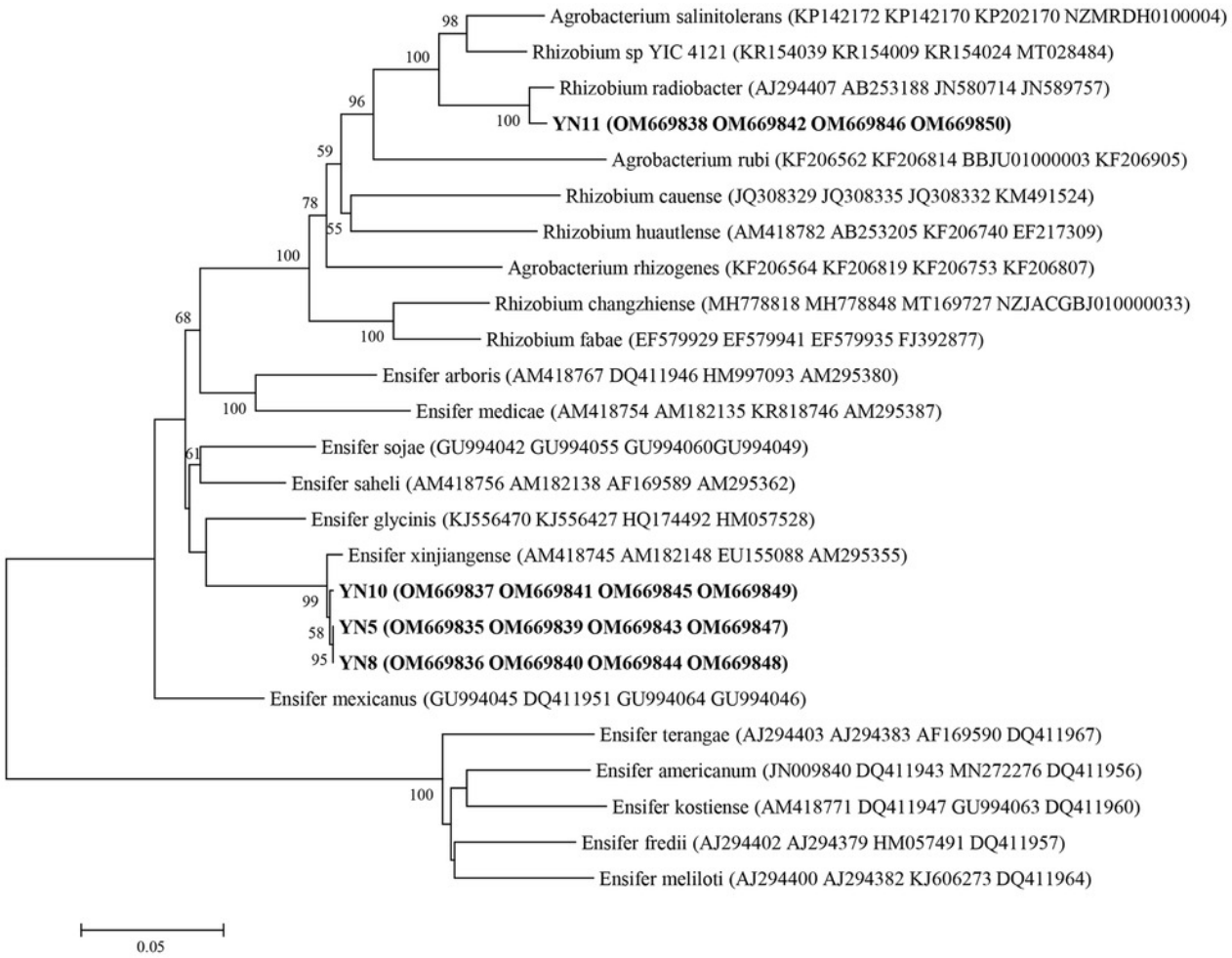
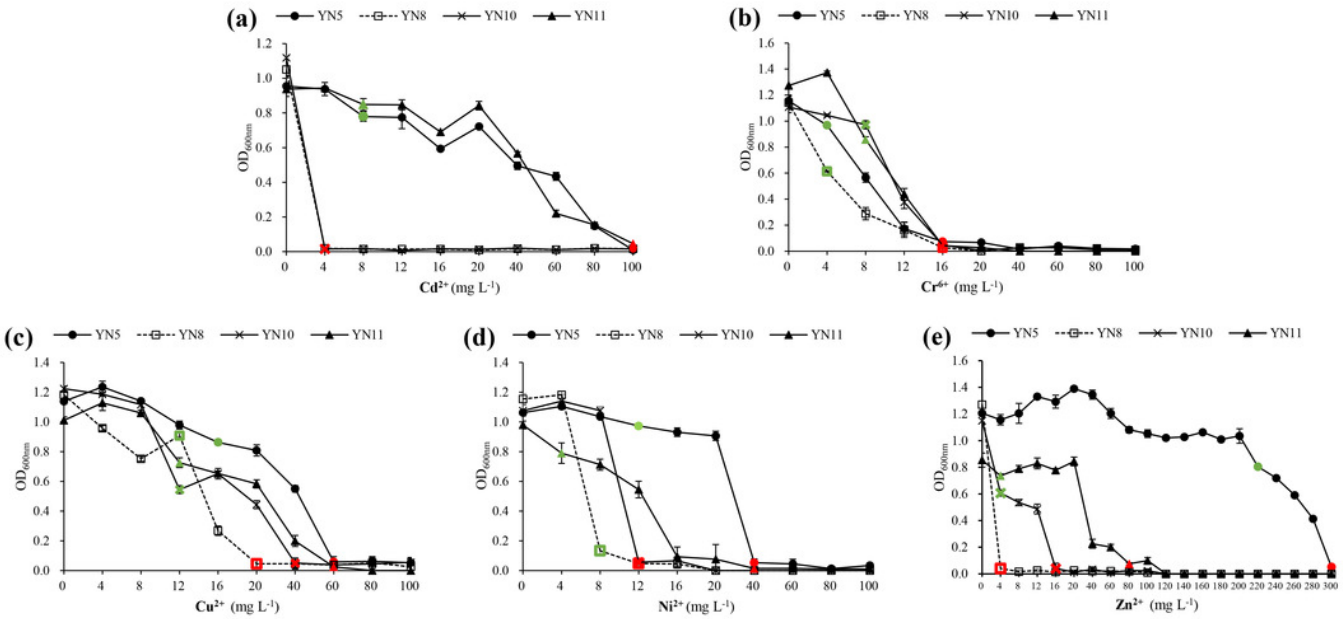


Figure 7

The growth of the soybean symbiotic isolates from the nickel mine soil collected in Xichang, Sichuan Province, China, at increasing concentrations of Cd^{2+} (a), Cr^{6+} (b), Cu^{2+} (c), Ni^{2+} (d), and (e) Zn^{2+}.

Minimum inhibitory concentrations (MIC) are indicated with green and minimum lethal concentrations (MLC) with red.