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Original Article

Bacillus and microalgae biofertilizers improved quality and biomass of *Salvia miltiorrhiza* by altering microbial communities

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ABSTRACT

Objective: Biofertilizers are reliable alternatives to chemical fertilizers due to various advantages. However, the effect of biofertilizers on *Salvia miltiorrhiza* yield and quality and the possible mechanisms remain little known. Here, an experiment was conducted in *S. miltiorrhiza* field treated with two kinds of biofertilizers including *Bacillus* and microalgae.

Methods: A field experiment was conducted on *S. miltiorrhiza* of one year old. The biofertilizers were applied at six treatments: (i) control check, CK; (ii) microalgae, VZ; (iii) *Bacillus*, TTB; (iv) microalgae + *Bacillus* (1:1), VTA; (v) microalgae + *Bacillus* (0.5:1), VTB; (vi) microalgae + *Bacillus* (1:0.5), VTC. Here, high-throughput sequencing, ICP-MS and UPLC were employed to systematically characterize changes of microbial diversity and structure composition, heavy metals content and bioactive compounds, respectively.

Results: Compared to CK, root biomass increased by 29.31%–60.39% ($P < 0.001$). Meanwhile, bioactive compounds were higher than CK after the application of the biofertilizers, peculiarly in TTB and VTB. However, the content of Pb contents in roots significantly reduced by 46.03% and 37.58% respectively in VTC and TTB ($P < 0.05$). VTA application notably increased the available nitrogen content by 53.03% ($P < 0.05$), indicating the improvement of soil fertility. Significantly, bacterial and fungal Chao I diversity indices showed an increasing trend with biofertilizer application ($P < 0.05$), and biofertilizer amendment enriched the rhizosphere soil with beneficial microorganisms that have abilities on promoting plant growth (*Achromobacter* and *Penicillium*), adsorbing heavy metal (*Achromobacter* and *Beauveria*), controlling plant pathogen (*Plectosphaerella, Lechevalieria*, *Sorangium, Phlebiopsis* and *Beauveria*) and promoting the accumulation of metabolites (*Beauveria* and *Phoma*).

Conclusion: Bacillus and microalgae biofertilizers improved the quality and biomass of *S. miltiorrhiza* by altering microbial communities in soil.

1. Introduction

Currently, agricultural plantations of various crops and medicinal plants depend heavily on chemical fertilizers. However, excessive and indiscriminate application of chemicals has significantly intensified the accumulation of toxic compounds in soils, the absorption of chemical compounds in plants and the increase in soil acidity (Alori & Babalola, 2018). Thus, chemical mismanagement adversely affects soil quality and plant health and further threatens human health. The application of biofertilizers has recently attracted widespread attention because it would substantially reduce the use of chemical fertilizers.

Biofertilizers, usually consisting of various beneficial bacteria, fungi and algae such as *Rhizobium*, nitrogen-fixing bacteria, *Bacillus*, photosynthetic bacteria, cellulolytic bacteria, *Lactobacillus*, yeasts, actinomycetes, and microalgae (Alori & Babalola, 2018; Shang et al., 2017; Zhu, Li, Shen, & Jiang, 2005), are reliable alternatives to chemical fertilizers. The beneficial microbial agents can affect host biomass and quality by improving soil fertility and regulating rhizospheric microorganism. The microalgae biofertilizers (VZ) used in this study mainly contain *Anabaena*, *Tolypothrix tenuis* and *Chlorella pyrenoidosa*. *Anabaena* is a cyanobacteria with strong nitrogen-fixing ability that can improve soil fertility and promote crop growth by converting atmospheric nitrogen into soil available forms. The nitrogen content in the soil significantly increased by up to 416%, and the yield of wheat and tomatoes notably improved

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within 90 days following treatment with *Anabaena azotica* 119 (Kong et al., 2016). The use of biofertilizers with *Anabaena, Trichoderma* or *Azotobacter* sp. resulted in a two–three fold increase in photosynthetic pigments and a 50%−90% increase in soil available N (Simranjit et al., 2019). *Anabaena* also has the ability to adsorb heavy metals and is used to reduce heavy metal accumulation in plants and to remedy soil. *Anabaena variabilis* showed 71.4% Pb removal from aqueous solutions at a concentration of 15 mg/L after 16 d of incubation (Abd El-Hameed, Abuarab, Abdel Mottaleb, El-Bahbohy, & Bakeer, 2018). *Anabaena* sp. reduced the accumulation of 60 μmol/L As(III) and As(V) in rice shoots by 49% and 23%, respectively, when cocultured with rice seedlings (Ranjan et al., 2018). *Chlorella pyrenoidosa*, as another important key member in microalgae fertilizer, showed great advantages in degrading organic pollutants (Shi et al., 2017). *Bacillus* biofertilizer (TTB) is enriched in *Bacillus amyloliquefaciens, B. licheniformis* and *B. subtilis*. So far, *Bacillus* sp. has been widely reported to be an important plant growth promoting microorganism (PGPM) and is used in a number of practical applications. *Bacillus megaterium* H3 improved quality and decreased Cd and Pb uptake in the edible tissues of two vegetables (*Brassica campestris* L.var. *aijiaohuang* and *Brassica rapa* L. var. *shanghaiqing*) along with changing soil environment (Wang, Zhang, He, & Sheng, 2018). The application of microbial agents (which mainly contains *Bacillus amyloliquefaciens* FZB42) significantly increased the total saponin content in *Panax notoginseng* roots by 51.49% and promoted the accumulation of biomass both aboveground and underground (Zuo et al., 2015). *P. ginseng* yield was enhanced by 17.0%–19.1%, and ginsenoside (Rg_1 and Rb_1) contents were improved after biofertilizer application (with *Actinomyces*, *Bacillus* and *Aspergillus*) (Dong et al., 2019).

Salviae Miltiorrhizae Radix et *Rhizoma* (SMRR, Danshen in China), the dried root and rhizome of *Salvia miltiorrhiza* (Bge.), is one of the most commonly used traditional herbal medicines and famous for its pharmacological activities on significant promotion of blood circulation and amelioration of blood stasis. Tanshinones (such as cryptotanshinone, tanshinone I and tanshinone IIA) and salvianolic acid (such as salvianolic acid B, salvianolic acid A and salvianolic acid C) are the most important active compounds in SMRR. Previous studies have indicated that *S. miltiorrhiza* is an appropriate model medicinal plant for herbal medicine research (Song et al., 2013; Wang et al., 2009). Previous studies have certified that microbial inoculants had significant effect on improving *S. miltiorrhiza* biomass and quality under abiotic stress. When *S. miltiorrhiza* was under Cd stress, relative to the control, the utilization of microbial inoculant (which mainly contained *Bacillus*) increased the accumulation of total tanshinones by 40.45%, and the treatment combined with garbage enzyme reduced the Cd uptake efficiently by 37.90% (Wei et al., 2020). However, the possible mechanism how the microbial inoculants improved *S. miltiorrhiza* quality and quantity remains little known. Besides, continuous cropping obstacles have resulted in severe economic losses and hinder the sustainable development of *S. miltiorrhiza* industry. Pot experiments have proven that 1-, 2-, 3-, and 4-year continuous cropping soil not only reduced the quality and yield of *S. miltiorrhiza* but also affected its active ingredient content obviously (Liu et al., 2020). A study showed the richness and diversity of bacterial community in the rhizosphere soil of *S. miltiorrhiza* were decreased as the continuous cropping years increased (Zhou et al., 2019). Rhizosphere soil microorganisms play a key role in agricultural systems and are directly involved in regulating the ability of plants to acquire nutrients and the nutrient cycles occurring in soils (Igiehon & Babalola, 2018; Shen & Zhao, 2015). Thus, it is of great importance to reveal the potential changes of soil microbial community to explore the principle of interactions. Therefore, *S. miltiorrhiza* was selected as the experimental object in this study. Bioactive compound contents were chosen as the index to evaluate the effect of biofertilizers on the quality. Soil physicochemical properties and rhizosphere microbial communities were chosen as the index to evaluate the effect of biofertilizers on the soil improvement.

In this study, we investigated the impacts of specific biofertilizers (*Bacillus* and microalgae) on the biomass accumulation and quality improvement of *S. miltiorrhiza*, and assessed the effects of biofertilizer amendment on the soil physicochemical properties and rhizosphere microbial communities. The findings in this research will provide vital information for guiding biofertilizer application in the cultivation of *S. miltiorrhiza* and contribute to the sustainable production of medicinal plants.

2. Materials and methods

2.1. Field description and experimental design

Two types of biofertilizers including VZ biofertilizer (Active microalgae nutrient solution soil remediation solution, Neimenggu Alger Life Science Co., ltd., Ulanqab, China) and TTB biofertilizer (Titubang microbial agent, Beijing Bailing Biotechnology Co., ltd., Beijing, China), were used in the present study. The manufacturer states that the VZ biofertilizer contains an algae single cell content exceeding 4.0×10^6 propagules/mL. The TTB biofertilizer is enriched in *Bacillus amyloliquefaciens, B. licheniformis* and *B. subtilis* and contains polyglutamic acid at concentrations exceeding 4200 mg/L according to the manufacturer.

A field experiment was initiated in April 2019 at the *S. miltiorrhiza* plantation in Weifang City, Shandong Province (N 36°31′58″, E 118°59′45″), China. The experimental site has a temperate monsoon climate with hot summers and fairly cool winters and a mean temperature of 12.3 °C. The field area is a traditional farmland that was previously cultivated with *S. miltiorrhiza*. Prior to planting, soil samples were taken from the 0 to 20 cm depth and analyzed for selected physical and chemical properties, including organic matter (OM, 13.70 g/kg), total N (TN, 0.92 g/kg), available N (AN, 115.60 mg/kg), available P (AP, 29.90 mg/kg), available K (AK, 84.07 mg/kg), Pb (23.03 mg/kg), Cd (0.07 mg/kg), and Hg (0.04 mg/kg). This field area used a ridged cultivation pattern of 40 cm and was subdivided into 18 plots. Each plot was 2 m wide and 10 m long, and plots were 3 m apart from each other. The experiment was conducted in a split plot pattern according to a completely randomized block design with three replications for six treatments, including treatment with VZ biofertilizer (VZ), treatment with TTB biofertilizer (TTB), combined treatment with VZ and TTB biofertilizer at a ratio of 1: 1 (VTA), combined treatment with VZ and TTB biofertilizer at a ratio of 0.5: 1 (VTB), combined treatment with VZ and TTB biofertilizer at a ratio of 1: 0.5 (VTC), and no biofertilizer treatment (CK). Combination treatments with different ratios of VZ and TTB were uniformly mixed prior to use. *S. miltiorrhiza* were obtained by root breeding. The breeding roots were planted in autumn in 2018, then the seedlings showed uniform growth were transplanted to field on April 10, 2019. Plants first inoculated with different biofertilizers on April 28, 2019, and the amounts of biofertilizer applications were shown in Table 1. Then irrigation was performed once a month for two months during the entire cultivation period of seven months.

2.2. Harvesting, plant biomass and soil sampling

Experimental plants were harvested after seven months (Fig. 1) and then separated into aboveground (stems, leaves) and underground (roots) parts to determine the above- and underground biomasses. In addition, underground parts were dried in an oven at 45 °C and analyzed using HPLC. Four *S. miltiorrhiza* seedlings

Table 1
Treatments and the amount of biofertilizer applications.

Groups	Treatments	Amount of application (mL/m^2)
VZ	Microalgae	1.5
TTB	*Bacillus*	10
VTA	Microalgae + *Bacillus* (1:1)	1.5 + 10
VTB	Microalgae + *Bacillus* (0.5:1)	0.75 + 10
VTC	Microalgae + *Bacillus* (1:0.5)	1.5 + 5
CK	Water	–

were randomly selected in each plot to determine plant biomasses and for soil sampling. Plant seedlings were carefully removed from the field, and the roots were gently shaken for collection of the loosely adhering soil. This loosely adhering soil was sampled for analysis of the soil physicochemical properties. The soil remaining attached to the plant roots (<1 mm in thickness from the surface of the root system) was considered to be rhizosphere soil. Rhizosphere soils were brushed and pooled into sterile plastic bags and were transported from the experimental site to the laboratory using a constant temperature box containing dry ice (CO_2). Rhizosphere soils were sifted through a 50 mesh sieve and stored at −80 °C before molecular analysis.

2.3. Bioactive compounds in S. miltiorrhiza roots

The contents of four major bioactive compounds (salvianolic acid B, tanshinone I, tanshinone II_A, and cryptotanshinone) in roots were determined using HPLC (Flexar, PerkinElmer, MA, USA). The dried root samples were ground to fine powders (50 mesh) and stored at room temperature prior to subsequent analysis. The standards for salvianolic acid B, tanshinone I, tanshinone II_A, and cryptotanshinone were purchased from the National Institutes for Food and Drug Control (Beijing, China). The purity of each compound was over 98%, as determined by HPLC analysis. HPLC-grade acetonitrile, aqueous phosphoric acid (Fisher, Emerson, IA, USA) and Wahaha purified water were used for HPLC analysis. Other chemicals and solvents were of analytical grade and obtained from Siaopharm Chemical Reagent (Beijing, China). Then, the samples and standards were prepared in accordance with guidelines set out by the Chinese Pharmacopoeia (Pharmacopoeia Committee of P. R. China, 2015). HPLC analysis was performed on a PerkinElmer HPLC-Class system (Flexar, PerkinElmer, MA, USA) consisting of a binary solvent delivery pump, an auto sampler, and photodiode array detector. An Agilent ZORBAX Extend-C_{18} column (4.6 mm × 250 mm, 5 μm) was applied for all analyses, and the column temperature was maintained at 26 °C. For tanshinones, the mobile phase consisted of acetonitrile (solvent A) and water (solvent B) at the following gradient program: 61%–73% A (0–8 min), 73% A (9–11 min), 73%–74% A (12 min), 74%–90% A (13–18 min), 90%–61% A (19–24 min). The flow rate was 1.2 mL/min, and the PDA detection wavelength was set at 270 nm. Moreover, samples were injected with 10 μL of solvent. For alvianolic acid B, the mobile phase consisted of acetonitrile (solvent A) and 0.1% aqueous phosphoric acid (solvent B) at the gradient program: 0–16 min, 22% A. The flow rate was 1.2 mL/min, and the PDA detection wavelength was set at 286 nm. Moreover, samples were injected with 20 μL of solvent.

2.4. Heavy metal contents in roots

The contents of Pb, Cd and Hg in roots were determined by inductively coupled plasma mass spectrometry (ICP-MS, Thermo, iCAPQ, MA, USA). Sample preparation and standard solutions were performed following the Chinese Pharmacopoeia (Pharmacopoeia Committee of P. R. China, 2015). In brief, the oven-dried roots were ground to fine powders by an agate mortar and pestle and passed through a 1 mm sieve. Then, root powder was put into a Teflon digestion tank and digested with plasma pure HNO_3 using a microwave-accelerated reaction system (ETHOS 1, MILESTONE, M, Italy). The residual solutions were then diluted with deionized water prior to analysis.

2.5. Extraction of rhizosphere soil DNA and Illumina HiSeq sequencing

Community DNA from soil samples was extracted from 0.25 g of each rhizosphere soil sample in accordance with the manufacturer's instructions using a MoBio Powersoil DNA Kit (MoBio Laboratories, Carlsbad, CA, USA). The V3 – V4 region of the bacterial 16S rRNA genes was amplified using the bacterial primers 341F (5′- CCTAYGGGRBGCASCAG)/806R (5′- GGACTACNNGGGTATCTAAT) (Møller and Søborg et al., 2013), and the ITS region of the fungal rRNA gene was amplified using the fungal-specific primer pair ITS1F (5′- CTTGGTCATTTAGAGGAAGTAA)/ITS2R (5′- GCTGCGTTCTTCATCGATGC) (Mueller and Paula et al., 2014). A previously described protocol for polymerase chain reaction (PCR) amplification and purification was followed (Rodrigues and Pellizari et al., 2013). The purified PCR products were quantified using

Fig. 1. Experimental filed at Weifang City, Shandong Province, China.

a QuantiFluor™-ST system (Promega, WI, USA), and the amplicons were pooled in equimolar ratios for sequencing. Pooled DNA products were used to construct an Illumina paired-end library and subsequently paired-end sequenced (2 × 250) on an Illumina HiSeq 2500 platform (Shanghai Biozeron Co., Ltd., Shanghai, China) following standard protocols. The analysis of metagenomics data was performed following the principle described in our previous article (Wei et al., 2020; Wei et al., 2020).

2.6. Soil physicochemical properties

Loosely adhering soil was air-dried at room temperature and then homogenized by being passed through a 50 mesh sieve for analysis of the soil physicochemical properties, including soil organic matter (OM), total nitrogen (TN), available nitrogen (AN), available phosphorus (AP), and available potassium (AK). The determination of soil OM, TN, AN, AP and AK contents was accordant with guidelines set out by the People's Republic of China Forestry Industry Standards, LY/T 1237–1999, LY/T 1228–2015, LY/T 1228–2015, LY/T 1234–2015 and LY/T 1232–2015, respectively.

2.7. Data analysis and statistics

The data across treatment groups were analyzed using one-way analysis of variance (ANOVA) and *t*-tests, which were performed using the statistical package SPSS Version 21.0. The values are given as the mean ± standard error (SE) of three replicates (n = 3 or 12). Significant differences versus the control were marked with $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$, respectively.

3. Results

3.1. Biofertilizer application promoted plant biomass accumulation

Above and underground biomass accumulation of *S. miltiorrhiza* responded significantly to the different biofertilizer treatments (Fig. 2). All five biofertilizer treatments resulted in a significant increase in underground biomass accumulation. Approximately 29.31%, 32.58%, 60.39%, 37.26% and 54.18% increases were observed in the VZ, TTB, VTA, VTB and VTC treatments, respectively, in comparison to the CK ($P < 0.001$). The VZ and VTA treatments significantly increased the aboveground biomass by 16.27% and 17.66% ($P < 0.05$), respectively, compared to the CK. However, no significant difference in aboveground biomass was found between the TTB, VTB, and VTC treatments in comparison to the CK. Thus, both the highest relative increases in above- and underground biomass occurred with VTA. Biofertilizer amendment had a positive impact on the plant biomass accumulation of *S. miltiorrhiza*.

3.2. Impacts of different biofertilizers on the concentrations of bioactive compounds in roots

The impacts of different biofertilizer applications on bioactive compound accumulation in *S. miltiorrhiza* roots were evaluated in terms of the contents of total tanshinones (TTs, including cryptotanshinone, tanshinone I and tanshinone IIA,) and salvianolic acid B (SA) which were analyzed by HPLC. The calibration equations, correlation coefficients, and linear ranges for all the standard solutions, tanshinones and salvianolic acid B were shown in Table 2. All of the marker substances showed good linearity over the investigated concentration ranges (determination coefficients, R^2, 0.9996–0.9998). The quantitative results of SA and tanshinones in the experimental *S. miltiorrhiza* samples were shown in Fig. 3. The highest contents of cryptotanshinone were detected in the TTB treatment, followed by the VTB treatment and were 3.32 and 2.37 times higher than that of CK ($P < 0.01$), respectively. However, VZ, VTA and VTC had no significant impact on the cryptotanshinone concentrations in roots (Fig. 3A). The highest concentration of tanshinone II_A was also observed in the TTB treatment, followed by the VTB treatment, which significantly increased by 71.24% ($P < 0.01$) and 33.43% ($P < 0.05$), respectively, in comparison to the CK (Fig. 3C). However, all five biofertilizer treatments caused no significant difference in tanshinone I contents (Fig. 3B). Like the cryptotanshinone and tanshinone II_A contents, roots of *S. miltiorrhiza* treated with TTB possessed the highest contents of tanshinones, followed by those treated with VTB, with 89.63% ($P < 0.01$) and 40.97% ($P < 0.05$) increases observed, respectively (Fig. 3D). Except TTB, which SA concentration had a 1.92% increase relative to the CK ($P < 0.05$), other four treatments did not show significant impact on SA contents (Fig. 3E). It was clear that amendment with TTB and VTB biofertilizers significantly promoted the accumulation of bioactive compounds, improving the quality of the SMRR.

3.3. Biofertilizer application resulted in a reduction in heavy metal contents in SMRR

The contents of Pb, Cd and Hg were determined in roots by the ICP-MS method. The results indicated that both the Cd and Hg concentrations in all tested root samples were below the detection limit. Importantly, it was clear from Fig. 4 that the Pb concentration in roots treated with all five biofertilizers was lower than that in CK roots. The lowest Pb content was observed in the VTC treatment, followed by the TTB treatment. The application of VTC and TTB resulted in 46.03% and 37.58% reductions in Pb contents, respectively ($P < 0.05$). Other treatments caused no significant impact on Pb contents.

3.4. Effect of biofertilizers on soil physicochemical properties

The application of all five biofertilizers had no significant impact on soil OM, TN, AN, AP and AK, with the exception of the VTA treatment, which significantly increased the AN content by 53.03% ($P < 0.05$) (Fig. 5). The OM, TN, AN, AP (expect for VZ) and AK (expect for VTC) contents were higher in all the biofertilizer treatments (VZ, TTB, VTA, VTB and VTC) than in CK ($P > 0.05$). Surprisingly, all the highest soil OM, TN, AN and AK contents were determined in soil treated with VTA.

3.5. Biofertilizers altered rhizosphere soil microbial community of S. Miltiorrhiza

3.5.1. Sequencing data

After the removal of low-quality reads, a total of 1,047,489 classifiable bacterial 16S rRNA genes and 1,073,723 ITS sequences were acquired from 18 rhizosphere soil samples for further testing. The number of high-quality sequence reads per sample ranged from 46,259 to 67,519 (mean ± SD, 58194 ± 7608) for the bacterial community (dominant length: 401–450) and from 50,047 to 73,161 (mean ± SD, 59651 ± 7531) for the fungal community (dominant length: 201–250). All high-quality sequences were classified taxonomically (phylum to genus) at a 97% sequence similarity cut off using the default settings of QIIME. OTU composition and structure differed obviously among soils treated with different biofertilizers, as indicated by the Venn diagram (Fig. 6). A total of 88,708 bacterial and 9843 fungal OTUs were detected from 18 rhizosphere soil samples, and the number of bacterial and fungal OTUs per sample varied from 4423 to 5258 and from 396 to 703, with an average number of 4928 and 547, respectively. It is clear from the Venn diagram that the number of bacteria shared by CK, VZ, TTB, VTA, VTB and VTC was 4157 and that the numbers of OTUs

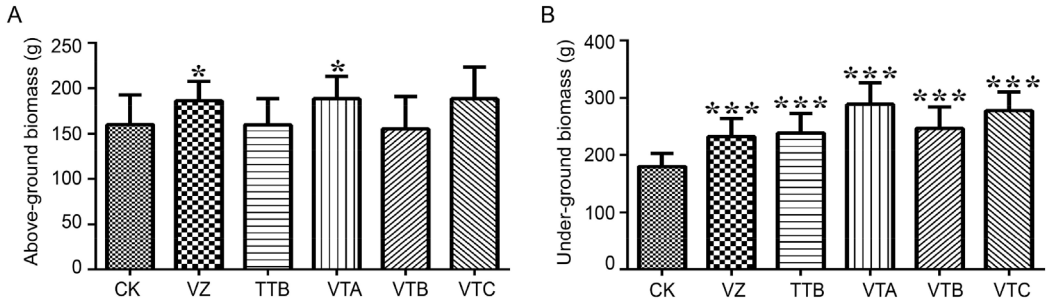

Fig. 2. Effect of biofertilizers on aboveground biomass (A) and underground biomass (B). All values are presented as the means ± SE (n = 3). *, $P \leq 0.05$, **, $P \leq 0.01$ and ***, $P \leq 0.001$ *vs* CK group, respectively.

Table 2
Calibration equations, correlation coefficient (R^2) and linear range of four compounds.

Compounds	Calibration equations	R^2	Test range (μg)
Cryptotanshinone	y = 38966655x − 6214	0.9996	0.02–1.00
Tanshinone I	y = 12427835x + 7442	0.9996	0.02–2.00
Tanshinone IIA	y = 30757185x + 31659	0.9996	0.02–2.00
Salvianolic acid B	y = 8709014x − 187639	0.9998	0.5–10.00

found exclusively in CK, VZ, TTB, VTA, VTB and VTC were 86, 84, 103, 101, 118 and 166, respectively (Fig. 6A). In contrast, the Venn diagram showed that the CK, VZ, TTB, VTA, VTB and VTC treatments shared 345 fungal OTUs, whereas 76, 70, 84, 84, 79 and 62 OTUs were featured exclusively in the CK, VZ, TTB, VTA, VTB and VTC treatments, respectively (Fig. 6C). Rarefaction curves of bacterial (Fig. 6B) and fungal (Fig. 6D) communities, which were used to evaluate OTU saturation, demonstrated that the sequencing efforts were sufficient for this study as the number of OTUs was close to saturation.

3.5.2. Diversity analysis

Given the sensitivity of alpha diversity to biofertilizer application, the impacts of biofertilizer on bacterial and fungal diversity indexes were calculated, and the Chao1 and Shannon (H') indexes were determined with a 97% similarity level. Bacterial and fungal diversities showed an increasing trend after biofertilizer amendment in comparison to the CK (Fig. 7). As shown in Fig. 7A, the amendment of all biofertilizers significantly altered the bacterial Chao1 index, with the exception of VTA treatment. The application of the VZ, TTB, VTB and VTC biofertilizer treatments significantly increased the bacterial Chao1 values by 10.26%, 5.97%, 5.69% and 12.82%, respectively, relative to the CK (P < 0.05). Approximately 29.29% and 37.12% increases in the fungal Chao1 index were observed in the VTB and VTC treatments (P < 0.05), respectively. However, there were no significant changes in fungal Chao I values between the VZ, TTB, VTA treatments and the CK (Fig. 7B). Moreover, none of the five treatments affected the bacterial and fungal H' indexes (Fig. 7C and D).

3.5.3. Microbial community structure

All bacterial 16S rRNA gene sequences were classified into 39 phyla, accounting for 93.21% of the total bacterial sequences. The bacterial profiles were presented in Fig. 8A. Dominant phyla across all treatment soils were Actinobacteria (25.93%–31.37%), Proteobacteria (22.24%–27.94%), Acidobacteria (15.79%–22.48%), Chloroflexi (8.71%–12.97%), Gemmatimonadetes (3.50%–4.74%), Bacteroidetes (2.16%–3.04%), Verrucomicrobia (1.30%–3.11%) and Rokubacteria (0.90%–1.38%), with average relative abundances (RAs) of 28.84%, 24.93%, 19.08%, 10.53%, 3.82%, 2.63%, 2.08% and 1.28%, respectively. Furthermore, significant changes in RAs of dominant phyla were observed in the VTA, TTB and VTC treatments. The application of TTB resulted in a 21.09% reduction in the RA of Gemmatimonadetes (P < 0.05) compared to the CK. VTA treatment significantly increased the RA of Chloroflexi by

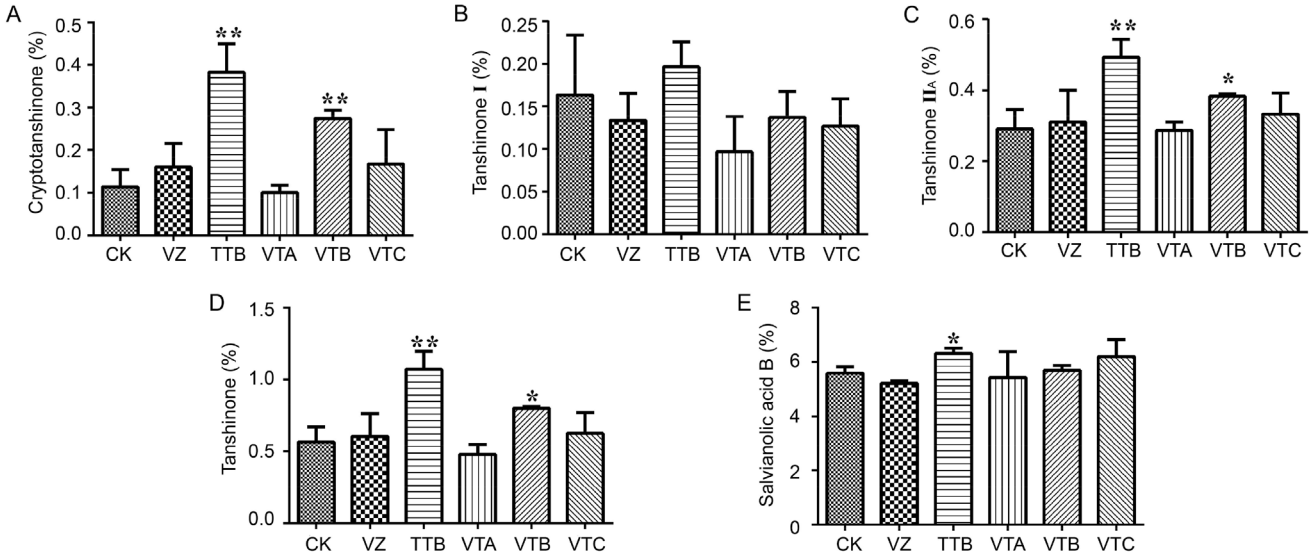

Fig. 3. Effect of biofertilizers on contents of cryptotanshinone (A), tanshinone I (B), tanshinone II_A (C), total tanshinone (D) and salvianolic acid B (E) in roots of *S. miltiorrhiza*.

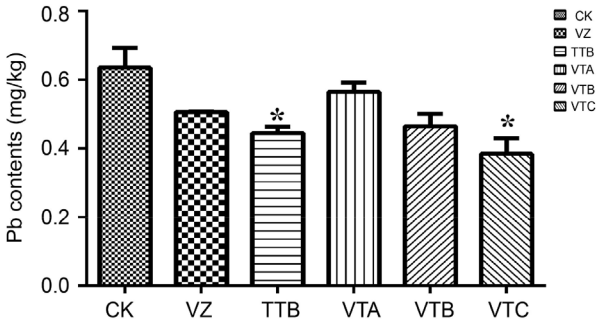

Fig. 4. Pb contents in *S. miltiorrhiza* roots treated with bioferetilizers.

29.46% but notably decreased the RA of Gemmatimonadetes by 26.18% ($P < 0.05$). The amendment of VTC resulted in a 25.54% reduction in the RA of Gemmatimonadetes but an approximately 45.85% increase in the RA of Rokubacteria ($P < 0.05$) compared to CK. It can be concluded that TTB, VTA and VTC notably reduced the RA of Gemmatimonadetes by>20%.

Taxonomic information of fungi at the class level was exhibited in Fig. 8B. The fungal community among all treatments was dominated by 31 classes that contained 96.43% of the total fungal sequences, including Sordariomycetes (47.51%–63.68%), Mortierellomycetes (11.03%–12.81%), Dothideomycetes (7.84%–15.37%), Pezizomycetes (1.13%–21.22%), Eurotiomycetes (1.44%–7.55%), Ascomycota_norank (1.39%–3.51%), Tremellomycetes (1.86%–2.89%) and Agaricomycetes (0.69%–3.47%), with average RAs of 54.30%, 12.21%, 10.63%, 9.78%, 2.66%, 2.64%, 2.44% and 1.77%, respectively. Similar to the results of bacteria, the RAs of certain fungal taxa notably changed with the TTB, VTA and VTC biofertilizer treatments. The application of TTB and VTA biofertilizers caused approximately 38.70% and 19.43% reductions in the RAs of Dothideomycetes and Sordariomycetes ($P < 0.05$), respectively, compared to CK. In addition, VTC amendment significantly reduced the RA of Sordariomycetes by 19.77%, but the RA of Pezizomycetes was 15.60 times higher than that of CK ($P < 0.05$). Clearly, both the VTA and VTC treatments had a significant impact and resulted in a decreasing trend in the RA of Sordariomycetes.

3.5.4. LEfSe analysis

The LEfSe algorithm identified specific bacterial genera from all OTUs whose RAs varied notably among the treatments (Fig. 9). *Lechevalieria*, *Opitutus* and *Achromobacter* were significantly enriched in the TTB, VTA and VTB treatments compared to the other treatments ($P < 0.05$, LDA > 2.0), and the RAs were 2.61, 3.26 and 3.14 times higher than those of the CK, respectively. *Vulgatibacter* and *Sorangium* were notably enriched in soils treated with VTC biofertilizers relative to other treatments ($P < 0.05$, LDA > 2.0), with relative rates of increases in abundance of 2.42 and 3.36 times. Furthermore, *Ohtaekwangia* was found to be more abundant in the CK than in the other treatments ($P < 0.05$, LDA > 2.0). Additionally, a 32.07% increase in the RA of the genus *Bacillus* was observed in the VTB treatment ($P < 0.05$).

Similarly, LEfSe analysis was also employed to detect fungal genera that showed significant differences in RAs between biofertilizer amendment treatments and the CK (Fig. 10). *Phlebiopsis* and *Hyphodermella* were significantly enriched in the VZ treatment relative to the other treatments. In addition, *Dichotomopilus, Beauveria, Setophaeosphaeria, Aaosphaeria* and *Monodictys* were notably enriched in the VZ treatment compared with the other treatments ($P < 0.05$, LDA > 3.0), and the RAs were 4.40, 28.07, 3.27, 1.80 and 6.24 times greater under VZ than under the CK. The application of the TTB biofertilizer caused a significant enrichment in the RAs of *Phoma*, *Melanconiella* and *Paraphoma* ($P < 0.05$, LDA > 3.0), with RAs that were 1.25, 1.84 and 5.74 times higher than that of the CK. With the VTA biofertilizer treatment, *Penicillium* and *Poaceascoma* were found to be enriched in comparison to other treatments ($P < 0.05$, LDA > 3.0). VTB biofertilizer resulted in 0.49%, 73.53% and 19.13% increases in the RAs of *Plectosphaerella, Arxiella* and *Fusariella*, which were significantly enriched in VTB in comparison to other treatments ($P < 0.05$, LDA > 3.0). LEfSe analysis indicated that *Pseudaleuria, Botryotrichum, Cephaliophora, Collariella, Acaulium, Testudomyces, Corynascella* and *Ascodesmis* were significantly enriched in VTC compared to other treatments ($P < 0.05$, LDA > 3.0).

4. Discussion

4.1. Biofertilizers exhibited a good ability to improve S. miltiorrhiza biomass

With the increasing global population and demand for crops, chemical fertilizers gradually play a increasingly important role in maintaining crop productivity, and herbicides and pesticides are of great significance for maintaining crop health. Currently, environmental pollution and food security issues caused by the

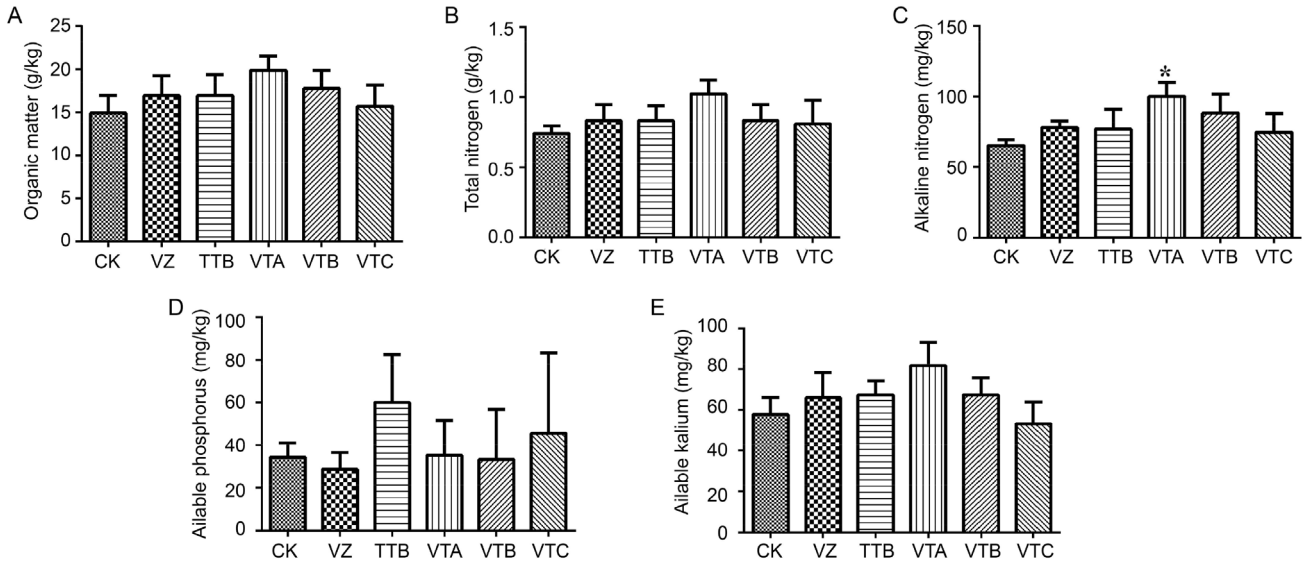

Fig. 5. Effects of biofertilizer application on soil OM (A), TN (B), AN (C), AP (D) and AK (E).

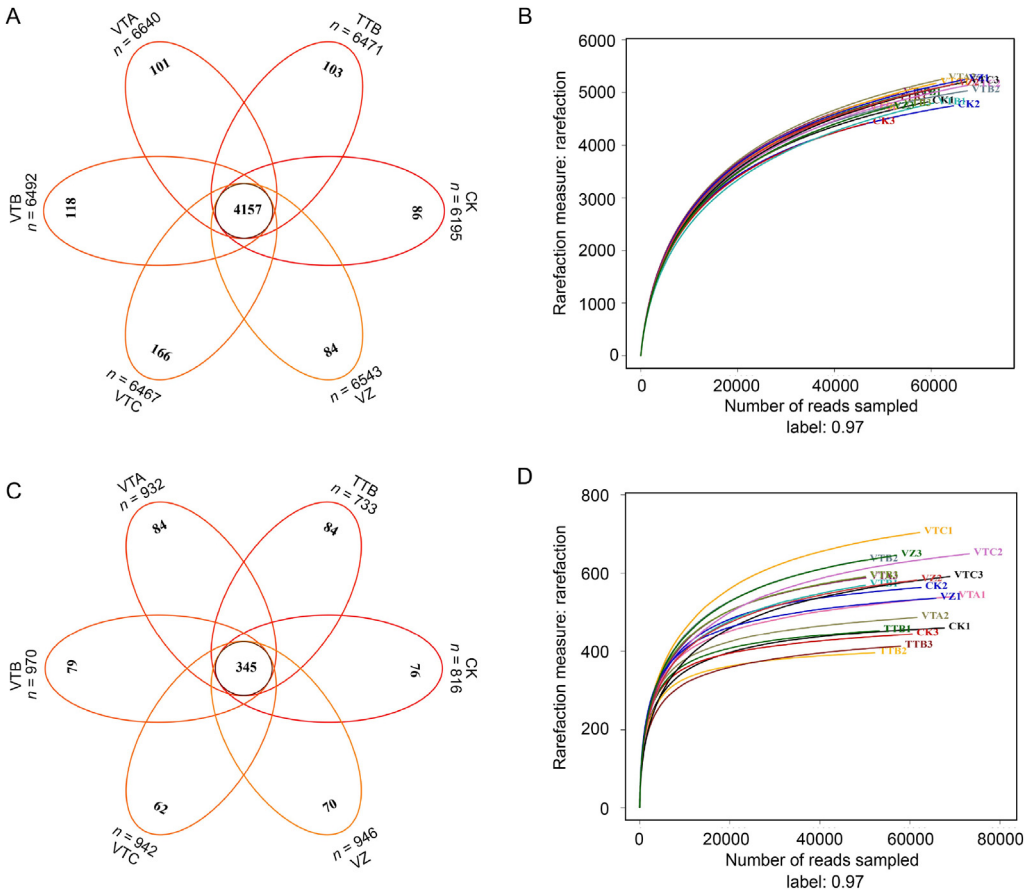

Fig. 6. Veen diagram for bacterial (A) and fungal (C) communities, and bacterial (B) and fungal (D) rarefaction curves for all samples at a 97% OTU sequence similarity threshold.

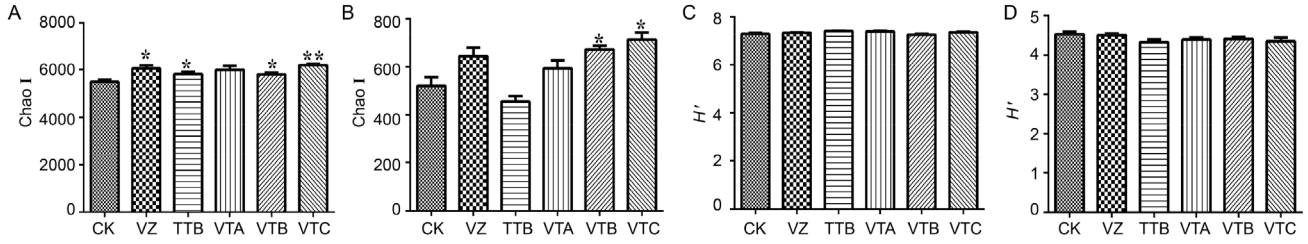

Fig. 7. Bacterial and fungal diversity in rhizosphere soil of biofertilizer application and CK groups. (A) and (B) show the Chao I values for the bacterial and fungal community, respectively, (C) and (D) show the *H'* values for the bacterial and fungal community, respectively.

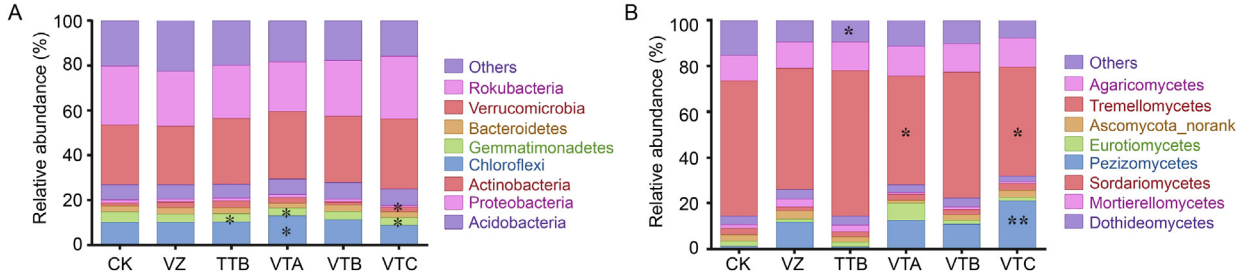

Fig. 8. Taxonomic classification at the phylum level of bacterial (A) and class level of fungal (B) reads retrieved from biofertilizer application and CK group. The bar marked "Others" represents the relative abundance of all phyla or classes not specifically listed.

excessive application of fertilizers, herbicides, pesticides, etc. have given rise to public concern. Biofertilizers have recently been widely studied and applied as potential substitutes for chemical fertilizers due to their strong promotion of plant growth, disease control, soil improvement, etc (Alori & Babalola, 2018). The present study clearly showed that all five biofertilizers (VZ, TTB, VTA, VTB and VTC) significantly improved the underground biomass. The impact of biofertilizers on underground biomass is considered to

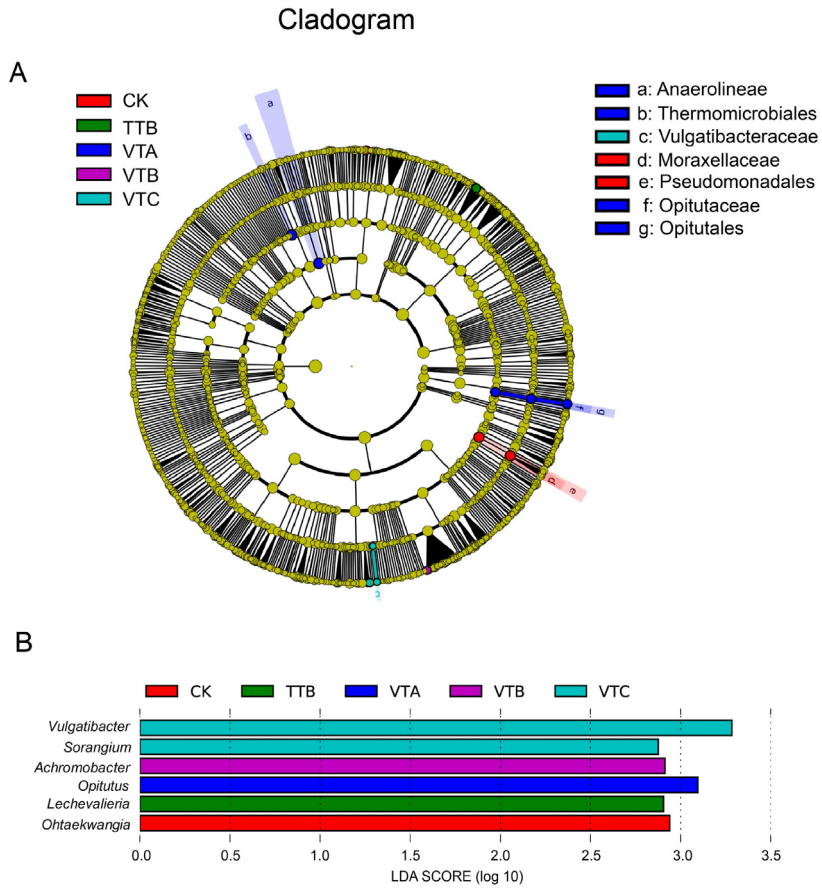

Fig. 9. Cladogram showing enriched bacterial taxa that were sensitive to different treatments (A) (phylum, class, order, and family, respectively). Linear discriminant analysis effect size (LEfSe) showing bacterial taxa that were significantly different between biofertilizer applications and CK groups (B) (genus level). Significant differences are defined at $P < 0.05$ and an LDA score > 2.0.

be an important indicator because the underground parts (roots and rhizome) are the medicinal parts of *S. miltiorrhiza*. In addition, aboveground biomass was also significantly enhanced by the application of the VZ and VTA biofertilizer treatments. Thus, it can be concluded that the biofertilizers tested in the present research had a significantly positive impact on the growth of *S. miltiorrhiza*.

The possible explanation is that the beneficial microorganisms contained in the tested biofertilizers, such as *Bacillus, Anabaena* and *Chlorella pyrenoidosa,* possess a good ability to promote plant growth. Aligning with findings from this article, the above beneficial microorganisms were previously reported to exert growth-promoting effects on plants, such as wheat, tomatoes and *Panax notoginseng* (Kong et al., 2016; Zuo et al., 2015). Besides, soil microorganisms play a key role in agricultural soil productivity, plant growth, plant health and soil-borne disease control. In this study, the significant changes in RAs of some bacterial and fungal genera were revealed through high-throughput sequencing. In the meanwhile, an increasing trend in bacterial and fungal Chao1 diversity indexes was identified in rhizosphere soils amended with biofertilizers. This was consistent with previous studies indicating that biofertilizer application increased Chao I values in the rhizospheres of other crops, such as watermelon and banana (Fu et al., 2017; Ling et al., 2014). Among those notably enriched genera in biofertilizer treatments, many of them are beneficial microorganisms, which possess the capacity to promote plant growth (*Penicillium* and *Achromobacter*), metabolite accumulation (*Beauveria* and *Phoma*), and heavy metal biosorption (*Achromobacter* and *Beauveria*), degrade organic pollutants (*Penicillium*, *Achromobacter* and *Paraphoma*), control plant diseases (*Sorangium, Phlebiopsis, Beauveria* and *Plectosphaerella*) and so on. In particular, because several microorganisms belonging to the genus *Penicillium* are characterized as plant growth promoters, *Penicillium* plays an important role in agriculture. *Penicillium* sp. can be exploited for countless applications, including agricultural, biotechnological and pharmaceutical applications (Toghueo & Boyom, 2020). Plant growth-promoting endophytes (PGPE), *Penicillium chrysogenum* and *Penicillium crustosum*, are indole acetic acid (IAA) producers that exhibit variable capacity for phosphate solubilization and play a vital role in improving plant growth (Hassan, 2017).

4.2. Biofertilizers exhibited a good ability to improve S. miltiorrhiza quality

The contents of bioactive constituents and heavy metals are important indexes for evaluation of the quality of SMRR. Cryptotanshinone, tanshinone I, tanshinone II_A and salvianolic acid B are the main active ingredients in SMRR and exhibit significant pharmacological activities, such as anticancer, anti-inflammatory, and antioxidant activities as well as effects on the cardiovascular system (Jiang et al., 2019; Shi et al., 2019). The present study clearly showed that the application of the TTB and VTB biofertilizer treatments significantly increased the contents of tanshinones (including cryptotanshinone, tanshinone II_A and total tanshinone) and salvianolic acid B (which only occurred in the TTB treatment) in SMRR. The close relationship between microorganisms and the accumulation of active ingredients in medicinal plants has been reported by many previous studies. For example, a recent study

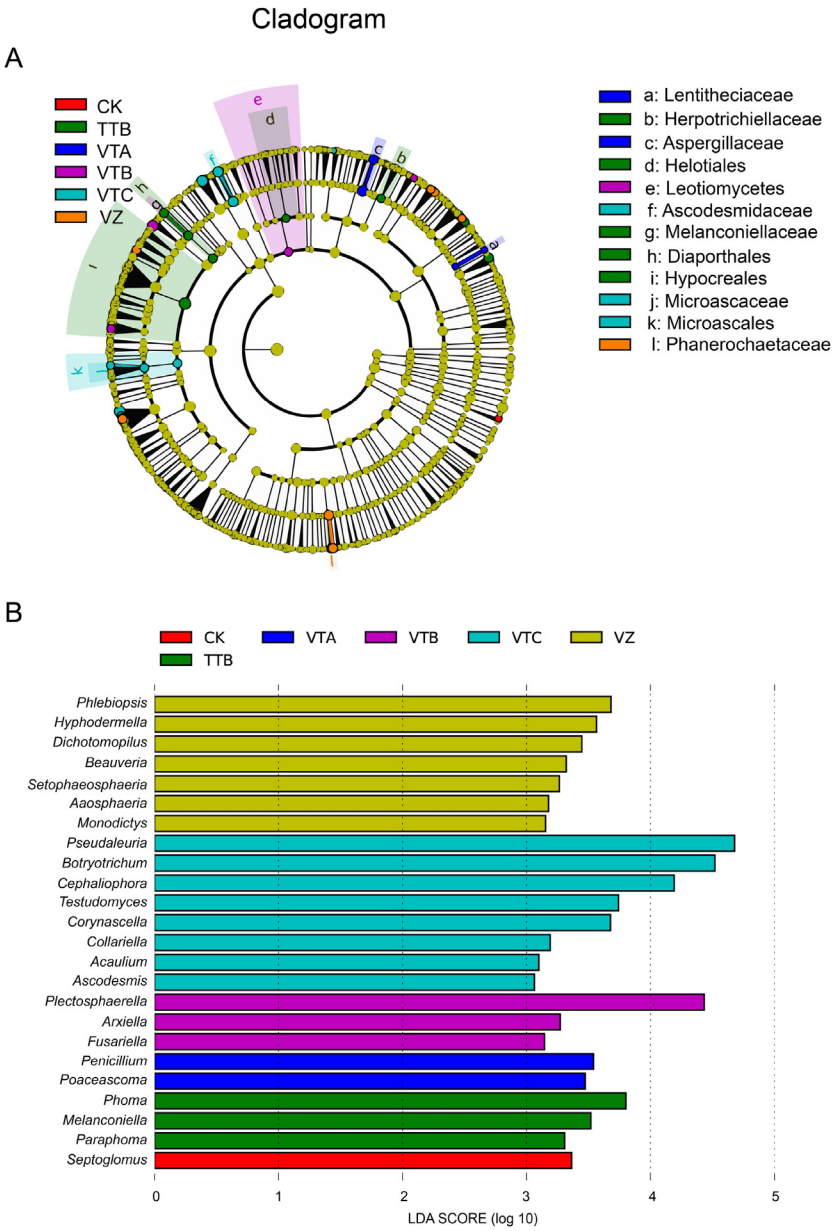

Fig. 10. Cladogram showing enriched fungal taxa that were sensitive to different treatments (A) (phylum, class, order, and family, respectively). Linear discriminant analysis effect size (LEfSe) showing fungal taxa that were significantly different between biofertilizer applications and CK groups (B) (genus level). Significant differences are defined at $P < 0.05$ and an LDA score > 3.0.

demonstrated that a strain from the genus *Agrobacterium* that was very close to *Agrobacterium rhizogenes* showed a high capacity to produce the rare ginsenosides Rg_3 and Rh_2 (Yan et al., 2019). The present study showed that the RAs of microorganisms, such as *Beauveria* and *Phoma*, which are associated with metabolite accumulation in medicinal plants, were notably increased. Inoculation of *Beauveria bassiana* could improve the yield of secondary metabolites in the cultivated medicinal plant *Allium schoenoprasum* L (Espinoza et al., 2019). In this study, the RA of *Phoma* was significantly increased in the TTB treatment, while the salvianolic acid B content in the SMRR also significantly increased. Surprisingly, previous research has clearly demonstrated that a strain from the genus *Phoma*, *Phoma glomerata* D14, can produce salvianolic acid C (Li et al., 2016). In light of these findings, it can be concluded that the significant changes in rhizosphere microorganisms associated with the accumulation of metabolites are a possible explanation for the improvement of the yield of active chemical constituents in SMRR.

The negative impacts of heavy metals on human health have been reported worldwide. Thus, the measurement of heavy metal contents in herbs is necessary. The present study clearly indicated that the Pb contents in the SMRR showed a decreasing trend under the biofertilizer treatments. Results showed in this study that the contents of Pb all decreased in five treatments compared to the CK. In particular, the Pb contents were significantly decreased by 46.03% and 37.58% through VTC and TTB application, respectively. One possible reason could be that, in VTC and TTB treatment, the amount of the bacteria and fungus which were capable of removing the heavy metals increased. For example, *Rhodococcus*, which was known to degrade different polycyclic aromatic hydrocarbons into simpler non-hazardous metabolites (Goswami et al., 2017), was increased 5.86 times in VTC and 9.86 times in TTB respec-

tively. Another possible mechanism could be that, as LEfSe analysis revealed, the amendment of biofertilizers enriched the rhizosphere soil with soil remediation microorganisms, which exhibit good ability in heavy metal biosorption, such as *Achromobacter* (VTB) and *Beauveria*(VZ). *Achromobacter* sp. TERI-IASST N exhibits considerable biosorption of Zn (430 mg/L) and Pb (30 mg/L) (Subudhi et al., 2014). Arsenic-resistant bacteria from the genus *Achromobacter* contain arsenic (As) resistance genes and can grow in the presence of high As concentrations (over 100 mmol/L arsenate and 10 mmol/L As) (Cavalca et al., 2010). In addition, strains from the genus *Beauveria,* such as *Beauveria bassiana,* show biosorption activity for various heavy metals, such as Zn, Cu, Cd, Cr, Pb and Ni. Different functional groups on the cell surface of *Beauveria bassiana* are involved in the biosorption of different metals (Gola et al., 2016). *Beauveria bassiana* is an efficient biosorbent for Pb (II) and Cd(II) from aqueous metal solutions, with maximum adsorption capacities of (83.33 ± 0.85) and (46.27 ± 0.12) mg/g for Pb and Cd, respectively (Hussein, Hassan, & Joo, 2011). High removal (58%−75%) of heavy metals (Cu, Ni, Cd, Zn, Cr and Pb) was observed in the aqueous solution during the growth of *B. bassiana* (Gola, Malik, Namburath, & Ahammad, 2018). Therefore, biofertilizers showed a potential ability to improve the quality of SMRR by reducing its heavy metal contents.

4.3. Biofertilizers exhibited a good ability to improve soil fertility

The strong capacities of biofertilizers for regulating soil biological properties and restoring soil fertility have been reported for a long time. Bacillus, as one of the widely applied components in microbial agents, can take part in nitrogen fixation, solubilization and mineralization of phosphorus and other nutrients directly or indirectly (Saxena, Kumar, Chakdar, Anuroopa, & Bagyaraj, 2020). A previous study showed *Bacillus subtilis* biofertilizer could reduce NH_3 volatilization and increase the abundance of functional genes and ammonia-oxidizing bacteria (Sun et al., 2020). Microalgae and cyanobacteria, which are also ubiquitous in soil, have received increasingly attention in terms of maintaining soil fertility and soil health. The effect of soil fertility improvement by using microalgae and cyanobacteria has been discovered widely on eight different types of soil (Abinandan, Subashchandrabose, Venkateswarlu, & Megharaj, 2019). It is clear from the results of the soil physicochemical properties in this study that biofertilizer amendment cause an increasing trend in soil fertility, especially the AN content. The findings in this study are in agreement with those of previous work that demonstrated that inoculation with biofertilizer (containing beneficial microorganisms) significantly increased the AN content of soil (Mohammed, Jaiswal, Sowley, Ahiabor, & Dakora, 2018; Tang et al., 2020). This implies that biofertilizers contribute to improving soil quality.

Surprisingly, statistics in this study showed all the highest soil OM, TN, AN and AK contents were determined in soil treated with VTA. Compared to other treatments, VTA was the only one which had no significantly impact on bacterial Chao I, which meant under VTA treatment the bacterial population quantity had indistinctively changed compared to CK. Importantly, VTA significantly increased the RA of Chloroflexi by 29.46%. Researches showed many of Chloroflexi members are related to the soil nitrogen cycle. Chloroflexi in the early growth stage of rice contributed 5.33% to the SON content variation(Jing et al., 2021). Besides, the RA of *Opitutus* in VTA is 3.26 times higher than CK. *Opitutus* owned to the microorganisms decomposing polysaccharides and reducing nitrates to nitrites (Tanikawa et al., 2018). Recently research suggested that *Opitutus* was closely related to the carbon utilization of plants (Hünninghaus et al., 2019). In light of these findings, possibly, it is the increasing relative abundances of beneficial microorganism that make a significant contribution to the increasing content of rhizosphere soil nutritions.

4.4. Biofertilizers had positive influence on rhizosphere bacteria and fungi involved in plant disease biocontrol

The experiments carried on in this study were based on healthy and 2-year continuous cropping soil. In the analysis of the changes of microbial community composition in rhizosphere, it was unexpectedly found that the abundance of microorganisms with antagonistic effect on diseases increased. Root-knot nematodes (*Meloidogyne* spp.) are among the most serious plant pathogenic nematodes that harm medicinal plants in China, especially *M. incognita, M. arenaria, M. javanica* and *M. hapla*. The disease caused by root-knot nematodes is one of the main diseases affecting the roots of *S. miltiorrhiza* and poses a strong threat to the quality and yield of *S. miltiorrhiza. M. incognita, M. arenaria* and *M. javanica* are the main root-knot nematodes that harm *S. miltiorrhiza* in Shandong Province, China (Gao, 2018). Biological control of these nematodes using fungi is the most effective control strategy. In this study, the RA of the genus *Plectosphaerella* in *S. miltiorrhiza* rhizosphere soil was found to be significantly increased after treatment with biofertilizer (VTB). Interestingly, numerous studies have reported the strong ability of *Plectosphaerella* species against plant parasitic nematodes. *Plectosphaerella cucumerina,* a nematophagous fungus, exhibits great ability for the biocontrol of potato cyst nematode (PCN) and is considered a potential biological agent against PCN (Atkins et al., 2003; Jacobs et al., 2003). *Plectosphaerella plurivor* is an effective antagonist for the plant parasitic nematode *Nacobbus aberrans,* which is an endoparasite causing severe losses to a wide range of crops (Sosa, Rosso, Salusso, Etcheverry, & Passone, 2018). The preliminary investigation showed that there were almost no root-knot nematode diseases found in the experimental fields involved in this study. Thus, no evaluation was conducted of the impacts of biofertilizer on the incidence of root-knot nematode disease.

Additionally, among the specific microbial genera with statistically enriched RAs, four other genera (*Lechevalieria*, *Sorangium, Phlebiopsis* and *Beauveria*) have been reported to possess antagonistic abilities against plant pathogens. Commercial isolates of *Beauveria bassiana* can help prevent larvae from boring into vines and can kill adults of *Xylotrechus arvicola* (an important pest in vineyards); these isolates are considered effective biocontrol agents for *Xylotrechus arvicola* (Rodríguez-González et al., 2018). The secondary metabolite of *Sorangium cellulosum* Soce26, soraphen, has inhibitory activity against numerous phytopathogenic fungi (Gerth et al., 1994). In addition, *Sorangium cellulosum* KYC 3262 was selected as a biological agent for the control of anthracnose on hot pepper caused by the pathogen *Colletotrichum acutatum*, with a control efficiency up to 89% (Yun, 2014). Isolates from the genus *Lechevalieria* show great antagonistic activity towards phytopathogenic strains such as *Fusarium oxysporum, Phytophthora cinnamomi, Pythium debaryanum*, *Sclerotinia sclerotiorum* and *Thanatephorus cucumeris* (Cuesta et al., 2012). Moreover, strain *Phlebiopsis gigantean* is a good biocontrol fungus for root rot fungi. *O*-orsellinaldehyde, an important compound produced by *Phlebiopsis gigantean*, has antifungal activity against the root rot fungi *Heterobasidion occidentale* and *Fusarium oxysporum* (Kälvö et al., 2018). Given that these genera were enriched under the treatments with biofertilizers and their potential in the biocontrol of plant disease, it can be implied that the application of tested biofertilizers contributed to improving the resistance of *S. miltiorrhiza* to plant diseases such as root-knot nematode and root rot. More researches are required to fully elucidate the relationships between biofertilizers and disease incidence, and to explore the

potential of microbial agents in alleviating continuous cropping obstacles of *S. miltiorrhiza*.

5. Conclusion

According to the results of this study, commercial biofertilizers tested in this study significantly promoted biomass accumulation, especially in the underground medicinal parts of *S. miltiorrhiza*, and notably improved the quality of SMRR by increasing bioactive compound accumulation and reducing Pb uptake. The soil microbial diversity (Chao I) showed a significant increasing trend, and the rhizosphere microbial communities and soil fertility improved after biofertilizer application. The findings in this research provide vital information for guiding biofertilizer application in the cultivation of medicinal plants and contribute to the sustainable production of *S. miltiorrhiza*.

Author contributions

JPH designed and supervised this study. XMW, PC and XJB conducted the experimental work. XJB and ZZ drafted the manuscript. All authors contributed to the writing of the manuscript and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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