MULTI-SPECIES *BACILLUS* INOCULANT AND ITS EFFECT ON POTATO GROWTH AND CONTROL OF POTATO (*SOLANUM TUBEROSUM* L.) BLACKLEG DISEASE

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Abstract. Potato blackleg disease (PBD) is a common bacterial disease of potato, causing serious losses in potato (*Solanum tuberosum* L.) production. Using a probiotic biofertilizer that improves the structure of rhizosphere microbiota may be an effective way to prevent the disease. To develop a mixed *Bacillus*-based inoculant that would control PBD, active strains resistant to PBD were screened by the plates, and the fertilizer effect of the multi-species *Bacillus* inoculant on potato was tested in field experiment; the abundance of rhizosphere bacteria, actinomycetes, ammonifying bacteria, and nitrogen-fixing bacteria, and enzyme activities in potato leaves and rhizosphere were analyzed in the pot experiment. Our results showed that *Bacillus licheniformis* exhibited the most antagonistic effect on the PBD pathogen, followed by *Bacillus pumilus* and *Bacillus megaterium*. The highest yield of potato tubers was 2.05 kg/plant with the application of the multi-species *Bacillus* alternated with Gexi Tianzhuang fertilizer. The amendment also effectively improved the activity of defense enzymes in potato rhizosphere and leaves, and significantly changed the composition of potato rhizosphere microbiota. These results showed the multi-species *Bacillus* inoculant could effectively improve potato yield and had the potential to resist PBD.
Keywords: *compound agent, probiotic bio-fertilizer, microbial fertilizer, rhizosphere microbiota, soil*

Introduction

Potato (*Solanum tuberosum* L.) is one of the main crops worldwide (Bera et al., 2015). Soil-borne bacterial diseases (SBD) cause serious reductions in the yield of potato (Mao et al., 2019,2020). Potato blackleg disease (PBD) caused by *Pectobacterium atroseptica* is one of the most common SBD; it is transmitted mainly through the diseased potato seeds and soil. PBD occurs in all growth stages of potato. The infected plants grow slowly, the leaves turn yellow, wither, and fall off, and the stem base is brown to black soft rot, and extends upward (Cheng, 2020), which eventually leads to plant lodging, seeding shortage and loss, and in serious cases, the yield is reduced by 30 - 50% (Czajkowski et al., 2011; Mao et al., 2019). It propagates rapidly and is difficult to control, which seriously affects potato production (Mao et al., 2020). Although potato varieties resistant to PBD have been cultured, and thiazolidone, streptomycin sulfate, and other chemical agents were used to control PBD, the effect is not ideal.

Microbial fertilizers can improve the utilization of chemical fertilizers, reduce the occurrence of crop diseases, and decrease the use of pesticides, improving the agricultural ecology (Gharib et al., 2008; Ortíz-Castro et al., 2008; Mahdi et al., 2010; Haneef et al., 2014; Fan, 2017). *Bacillus* species are widely used in microbial fertilizers, biological control and environmental protection due to their advantages of high stress resistance and production of a variety of beneficial metabolites (Ongena et al., 2004; Liu, 2015). *Bacillus* can antagonize many common plant pathogens and effectively inhibit their growth (Ongena et al., 2004; Chen et al., 2007; Hu et al., 2019). Additionally, the high stress resistance of

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Bacillus makes it advantageous in the production and transport of microbial fertilizers, and survival in the harsh farmland environments (Ongena et al., 2004; Li et al., 2008).

To develop a multi-species *Bacillus* fertilizer to effectively prevent PBD and enhance growth, the activities and yields of defense enzymes and the dynamics of microbial communities in potato rhizosphere were characterized by pot and field experiments in this study. This study provides an important technical guidance for the ecological control of PBD.

Materials and Methods

Source of bacterial strains

Bacillus subtilis, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus mucilaginosus*, and *Bacillus amyloliquefaciens*, were isolated from the rhizosphere of different plants in the nursery and vegetable fields of Huizhou University, China. Pathogenic *Pectobacterium atroseptica* MB001 was isolated from Jiuhua Potato Base located in Tieyong Town, Huizhou City, China. The strains were grown on nutrient agar (NA) medium. *B. mucilaginosus* was inoculated into a silicate liquid medium (Huankai Bio-Science, China). Other strains were inoculated into the lysogeny broth (LB) liquid medium (Huankai Bio-Science, China), and were cultured at 28 °C with 120 r/min for 18 h.

In vitro experiments

Antagonism of Bacillus strains to the PBD pathogen

The bacterial plates of *P. atroseptica* MB001 were prepared by gradient dilution. The pathogenic strain was cultured at 28 °C for 24 h, and the optical density (OD) of culture suspensions reached 1.0. After 100 μL of the suspension was evenly spread on LB solid culture medium, filter paper pieces soaked with 5 μL of *Bacillus* suspensions were placed on the medium. The antagonistic effect of *Bacillus* strains to the pathogenic strain was judged based on the size of the bacteriostatic halo zone. Each treatment contained three replicates.

Determination of nitrogen fixation, phosphorus release and potassium release by Bacillus strains

Each *Bacillus* strain was cultured in organic phosphorus medium, inorganic phosphorus medium, silicate bacterial fermentation medium, and nitrogen-free medium in three replicates. The effect of phosphorus and potassium release and nitrogen fixation was judged from the growth of each *Bacillus* strain on the plates (Song, 2015).

Field experiments

Fertilizer effect of multi-species Bacillus strains

The multi-species *Bacillus* (FH) inoculant was a liquid microbial preparation composed of equal proportions of the six *Bacillus* species, with a live bacterial content of 1×10^8 CFU/mL. The tested potato variety was Holland No. 7. The fertilizer effect of the multi-species *Bacillus*, Gexi Tianzhuang fertilizer (GT; Tanghua Shiye, China), and LvShuo No. 1 fertilizer (LS; LvShuo Bio-Tech, China) was characterized in the field experiment by comparing the average yield per potato plant. The GT was a water-soluble fertilizer. The

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ratio of nitrogen (N), phosphorus (P) and potassium (K) in the GT was 1:1:1. N, P_2O_5 and K_2O contents were greater than or equal to 50% w/w; and the total content of cuprum (Cu), ferrum (Fe), manganese (Mn), zinc (Zn), boron (B), and molybdenum (Mo) was 0.3%-3.0% w/w. The LS was an organic fertilizer with spores of various microbes, and contained fulvic acid, alginic acid, mixed microbes (*Bacillus belesii*, *Bacillus subtilis*, and *Bacillus licheniformis*), soybean powder, and trace elements. The effective viable bacteria content is 5×10^8 CFU/mL.

The field experiment was conducted in the Experimental Base of Huizhou University (114.692 E, 23.067 N; altitude is 30 m). It is located in subtropical monsoon climate. The experimental field area was approximately 300 m^3. The experiment adopted a randomized grouping design, with 5 treatment groups and 1 control group, and each group had 3 repetitions. The area of each repetition was approximately 50.0 m^2. Potatoes were planted on ridges, with a length of 15 m, a width of 1.2 m and a plant spacing of 0.3 m. Approximately 100 plants were planted in each repetition. The plant density was 2 plants/m^2. The potatoes were sowed on November 23, 2017. When sowing, the seed potatoes were cut longitudinally, and each potato block had bud eyes. Then the potato blocks were sowed to ridges with 5 cm depth. After sowing, the ridges were covered by 5 cm of straw and soil, and then covered with black plastic film to keep warm, moisturize, and prevent weeds. On or about January 3, 2018, potato seedlings emerged successively. After emergence, the seedlings were pulled out of the plastic film manually, and the potato field was irrigated for 3 times. When the potato plants grown to 6 - 8 leaves (on January 13, 2018), apply corresponding fertilizers to each group according to the fertilization scheme shown in *Table 1*. The FH fertilizer was applied at 20 mL to each plant. Other fertilizers were applied according to the manufacturer's instructions. The rhizosphere soil pH was 5.81 ± 0.09. Content of organic matter, alkali-hydrolysable nitrogen, available phosphorus, and available potassium in the soil were 118.98 ± 1.50 g/kg, 130.22 ± 7.02 mg/kg, 31.02 ± 1.41 mg/kg, and 66.99 ± 10.07 mg/kg, respectively. These soil chemical indices were determined as described previously (Mao et al., 2020). Pathogenic *P. atroseptica* MB001 culture medium (2×10^7 CFU/mL) was sprayed on potato plants with a watering can for three times on January 29, February 1, and February 4, 2018, with an average of about 3 mL per plant each time. Routine field management and observation of potato plants were carried out after February 4, 2018 (Vashisht et al., 2015). The potatoes were harvested on March 15, 2018 (*Fig. 1*), and the average yield was determined by weighing tubers of 6 potato plants in each of three replicates. Moreover, the infection rate of each group was counted.

Table 1. *The experiment design of fertilizer effect of multi-species Bacillus inoculant*

Treatment	First day	Third day	Fifth day	Seventh day	Nineth day
GT	GT	GT	GT	GT	GT
GL	GT	LS	GT	LS	LS
FH	CW	MSB	CW	MSB	MSB
GF	GT	MSB	GT	MSB	MSB
LS	CW	LS	CW	LS	LS
BC	CW	CW	CW	CW	CW

GT fertilizer was diluted to 300 times with clear water to irrigate the root of potato; LS fertilizer was diluted to 100 times with clear water to irrigate the root of potato; The compound Bacillus was diluted to 200 times with clear water to irrigate the root of potato. MSB, multi-species Bacillus; CW, clean water; GT, GT fertilizer; GL, GT fertilizer and LvShuo No. 1 fertilizer; FH, multi-species Bacillus inoculant; GF, GT fertilizer and multi-species Bacillus inoculant; LS, LvShuo No. 1 fertilizer; BC, Blank control

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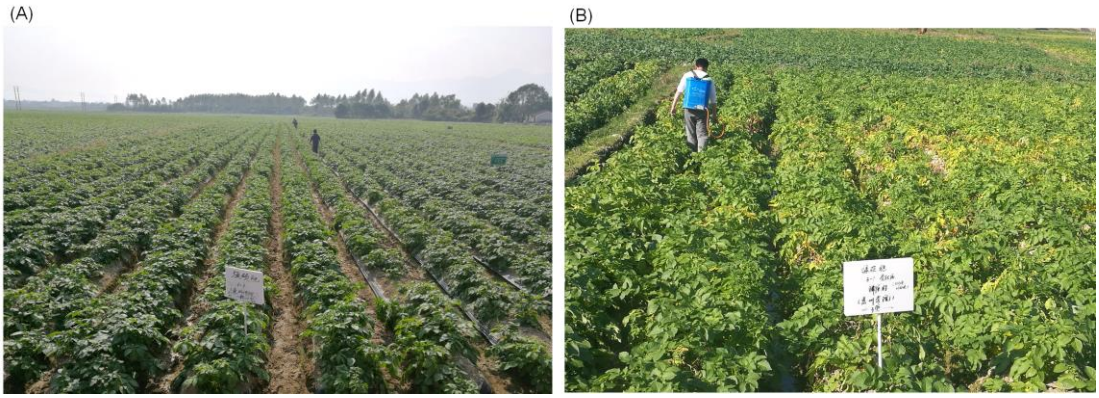

Figure 1. *Photos of field experiment. (A) photo during fertilization; (B) pre-harvest photo*

Determination of enzyme activities in potato rhizosphere soil and leaves

After the last fertilization of potato at the seeding stage, three plants were randomly selected from each group to measure the urease and saccharase activities in the rhizosphere soil of potato at the third day, sixth day, nineth day, and 12th day after fertilization. The activities of peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), polyphenol oxidase (PPO), and phenylalanine ammonialyase (PAL) in potato leaves were measured at the sixth day after fertilization according to the previous description (Lin et al., 2010; Yang, 2014).

Rhizosphere microbiome composition

The total DNA of rhizosphere microbiota was extracted from three parallel soil samples before (PecQ) and after (PecH) infection with *P. atroseptica* MB001 as well as from the non-infected control using the improved CTAB method (Ni et al., 2017) and was purified as our previously described (Mao et al., 2019). The 16S rDNA V4 - V5 hypervariable region was amplified using the 515F and 909R primers according to the published reports (Huang et al., 2018). Polymerase chain reaction products were purified and pooled together at equal molar amounts from each sample as previously described (Ni et al., 2019), and then sequenced using a MiSeq system (Illumina, USA) at Guangdong Meilikang Bioscience, Ltd., China.

The raw reads were merged using FLASH 1.2.8 software to get merged sequences (Magoc and Salzberg, 2011), and the low-quality merged sequences and chimeric sequences were filtered out as previously descripted before further analysis (Ni et al., 2019). The high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% identity using UPARSE (Edgar, 2013). The phylogenetic taxon of each OTU was assigned using the Ribosomal Database Project classifier (Wang et al., 2007) with greengene gg_13_8_otus dataset.

Data analysis

The data were showed as the mean ± standard error. One-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc test was conducted using R 3.5.1 (Dixon, 2003). Correspondence analysis (CA), principal component analysis (PCA) and non-parametric multivariate analysis of variance (PERMANOVA) (Anderson, 2001) were conducted using the vegan package of R 3.5.1 (Dixon, 2003). The Kruskal-Wallis test was conducted using

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the Statistical Analysis of Metagenomic Profiles software. Box plots were drawn using the ggpubr R package. $P < 0.05$ was considered significantly different.

Results and Discussion

In vitro experiments

Antagonistic effect of Bacillus against P. atroseptica

Previous studies showed that *Bacillus* inhibited the growth of pathogenic bacteria and fungi in soil to prevent soil borne diseases of plants (Cai et al., 2012; Patel et al., 2015; Gond et al., 2015). We found in present study that the antagonistic effects of various *Bacillus* species to *P. atroseptica* MB001 were *B. licheniformis* > *B. pumilus* > *B. megaterium* > *B. subtilis*, with the diameters of their bacteriostatic halos being 14.32 ± 0.53 mm, 13.72 ± 0.12 mm, 11.60 ± 0.23 mm, and 2.28 ± 0.10 mm, respectively (One-way ANOVA, $p < 0.001$; *Fig. 2*). *B. amyloliquefaciens* and *B. mucilaginosus* were not antagonistic to *P. atroseptica* MB001 (*Fig. 2*).

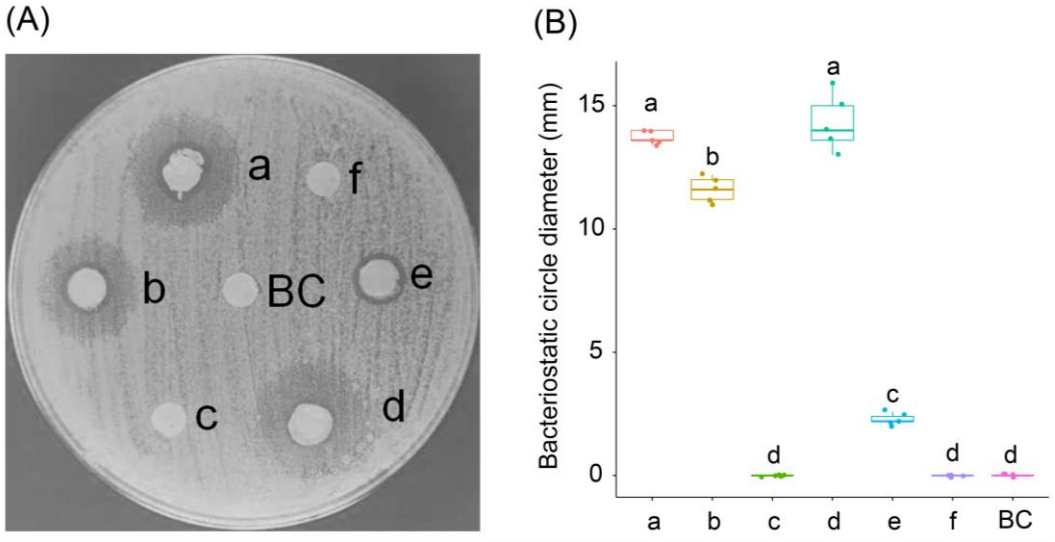

Figure 2. *Inhibition of Pectobacterium atroseptica by Bacillus pumilus (a), Bacillus megaterium (b), Bacillus amyloliquefaciens (c), Bacillus licheniformis (d), Bacillus subtilis (e), Bacillus mucilaginosus (f), and blank control (BC). Different letters above the boxplots indicate significant differences*

Nitrogen fixation and phosphorus and potassium release by Bacillus strains

Probiotics are used extensively in crop pest control and for improving yield because of their safety and environmental friendliness (Berendsen et al., 2012; Patel et al., 2015; Marcano et al., 2016). *Bacillus* is used widely in developing biological fertilizers because it can fix nitrogen, and solubilize unavailable forms of phosphorus and potassium to make them available (Glick, 2012; Patel et al., 2015; Fan, 2017; Prabhukarthikeyan et al., 2018). Our results showed that *B. licheniformis* and *B. mucilaginosus* could grow on the four types of selective media, which showed that they had the capacity to fix nitrogen and dissolve phosphorus and dissolve potassium. *B. licheniformis* and *B. mucilaginosus* were the only species with the capacity to dissolve potassium. *B. subtilis* could not use organic

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phosphorus, fix nitrogen or dissolve potassium, but had a strong capacity to use inorganic phosphorus. These results implied that the multi-species *Bacillus* amendment could not only prevent and control PBD, but also promoted the growth of potato (*Table 2*).

Table 2. *Growth of different Bacillus strains on nitrogen free medium, inorganic phosphorus medium, organophosphorus medium, and silicate bacterial fermentation medium*

Bacillus strains	Nitrogen free medium	Inorganic phosphorus medium	Organophosphorus medium	Silicate bacterial fermentation medium
B. lincheniformis	+	+	+	+
B. subtilis	-	+	-	-
B. pumilus	+	+	+	-
B. megaterium	+	+	+	-
B. mucilaginosus	+	+	+	+
B. amyloliquefaciens	+	+	+	-

"+" indicates the strain could grow on the medium; "-" indicates the strain could not grow on the medium

Field experiments

Effects of multi-species Bacillus inoculant on potato yield, rhizosphere soil and leaf enzyme activities

The potato yields of the treatment groups were significantly higher (one-way ANOVA, $F = 112420$, $p < 0.001$; *Fig. 3A*). The GF treatment had the highest yield, followed by the GL treatment (*Fig. 3A*). The infection rate of the GF treatment was the lowest, followed by the FH and GL treatments. The infection rate of BC was the highest (one-way ANOVA, $p < 0.001$; *Fig. 3B*).

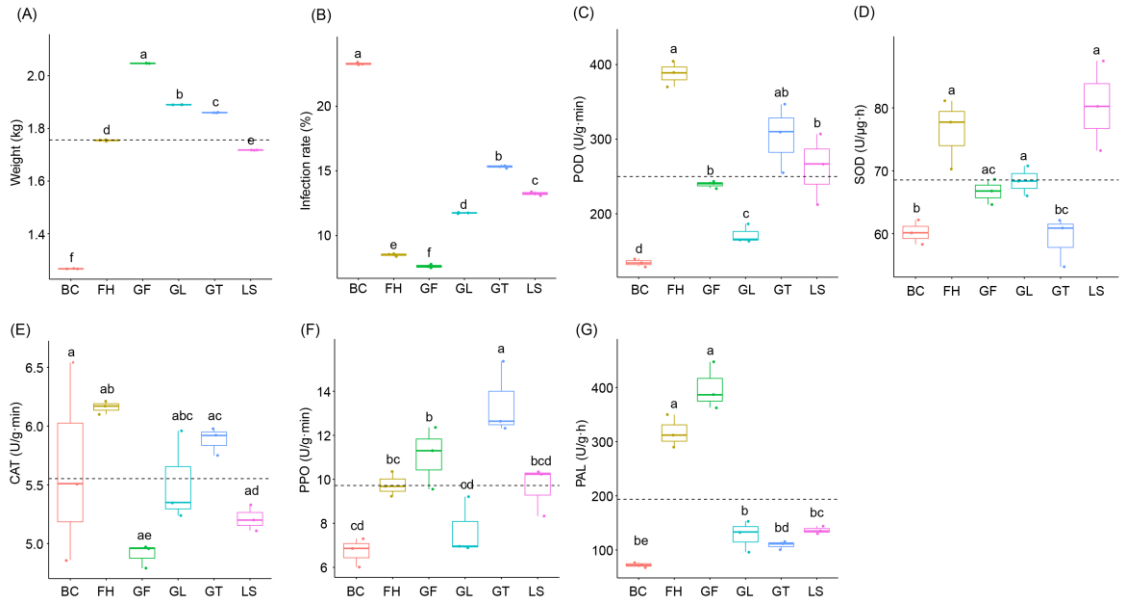

Figure 3. *Average yield per plant (A) and activities of peroxidase (B), superoxide dismutase (C), catalase (D), polyphenol oxidase (E), and phenylalanine ammonialyase (F) in potato leaves. GT, GT fertilizer; GL, GT fertilizer and LvShuo No. 1 fertilizer; FH, Compound Bacillus; GF, GT fertilizer and Compound Bacillus; LS, LvShuo No. 1 fertilizer; BC, Blank control. Different lower-case letters above the box diagrams show the significant difference among the treatments*

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B. amyloliquefaciens was reported to induce defense gene expression, including pathogenesis related protein 1 and pathogenesis related protein 4, which act against fungal pathogens (Gond et al., 2015). Thuricin 17 and bacthuricin F4 purified from *Bacillus* strains induced defense-related enzymes in soybean leaves (Jung et al., 2011). The activities of POD, SOD, PPO, and PAL in potato leaves in the FH and LS treatments were significantly increased compared with the control group in present study. The potato leaf activities of POD and SOD in the GL treatment, and those of POD, PPO and PAL in the GT treatment, were also increased significantly (*Fig. 3C-3G*). According to the results of enzyme activities of potato leaves, the FH treatment improved them to a greater extent than the other treatments.

Sucrase can hydrolyze sucrose into glucose and fructose that are easy to be absorbed and utilized in soil, and can increase soluble nutrients in soil, which is one of the important indicators characterizing the biochemical activity in the rhizosphere soil (Li et al., 2012). Soil urease is the key enzyme of nitrogen transformation in soil, and urease activity is often used to characterize the nitrogen status of soil and evaluate soil fertility (Kang et al., 2017). In this study, urease and saccharase activities of potato rhizosphere soil in each treatment were significantly higher, except for urease activity in the FH treatment on day 12. However, the changes in the urease and saccharase activities differed among the treatments (*Fig. 4*). In the GL treatment, the urease activity reached the peak on the sixth day after fertilization, those in the GF and GT treatments reached the peak on the ninth day after fertilization, whereas those in the LS and FH continued to decrease from the third day after fertilization (*Fig. 4A*). The saccharase activity in the GL treatment reached the peak value on the 6th day after fertilization, that in the GT reached the peak value on the 9th day after fertilization, and other treatments maintained relatively stable saccharase activity (*Fig. 4B*).

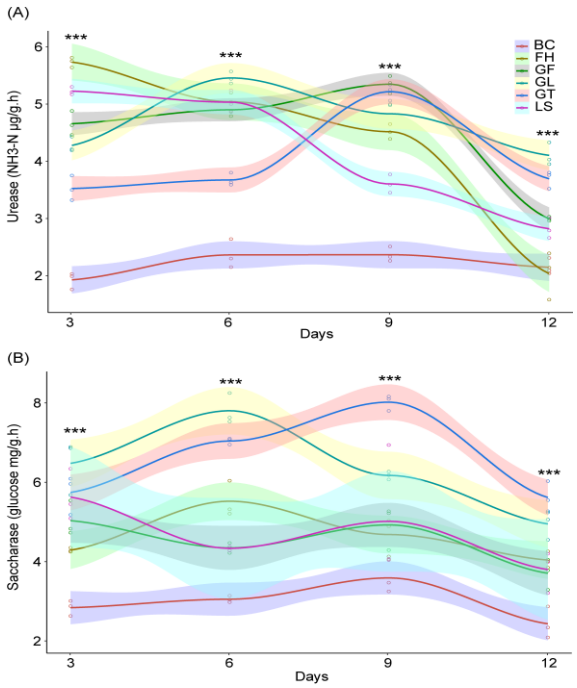

Figure 4. *Changes in urease (A) and saccharase (B) activities in the potato rhizosphere soil. GT, GT fertilizer; GL, GT fertilizer and LvShuo No. 1 fertilizer; FH, Compound Bacillus; GF, GT fertilizer and Compound Bacillus; LS, LvShuo No. 1 fertilizer; BC, Blank control. ***, $p < 0.001$*

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Changes in bacterial abundance and composition in the rhizosphere soil

Rhizosphere microbiota is vital in plant disease resistance (Mao et al., 2019, 2020). However, no significant difference was detected in bacteria, actinomycetes, ammonifying bacteria, and nitrogen-fixing bacteria abundances in the rhizosphere soil during the experiment (one-way ANOVA, $p > 0.05$; *Fig. 5*).

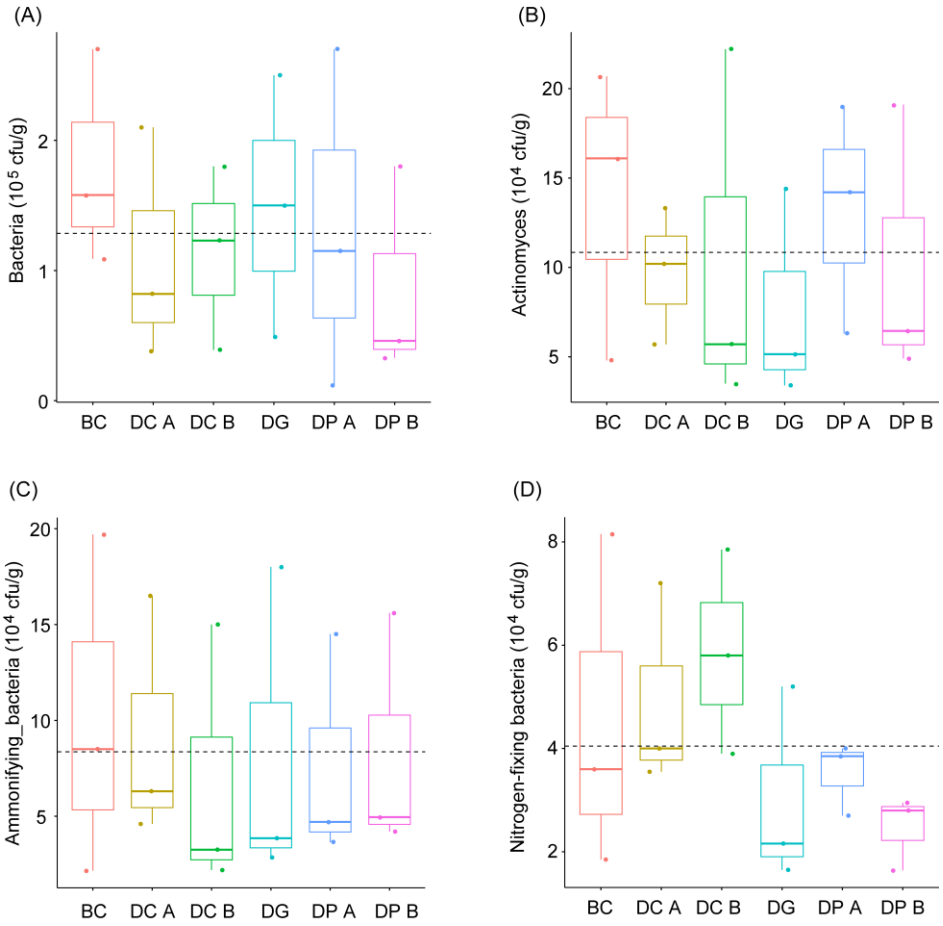

Figure 5. *Amount changes of microorganisms in potato rhizosphere. DC, disease cure group; DG, disease group; DP, disease prevention group; BC, blank control*

Total of 402,392 (44,710 ± 2,713) high-quality sequences were obtained from the nine rhizosphere samples. Finally, 33,004 sequences were randomly resampled from each sample for subsequent analyses. These sequences were classified into 15,619 OTUs. The Shannon index (one-way ANOVA, $F = 32.63$, $p < 0.001$; *Fig. 6A*) and Chao1 coverage (one-way ANOVA, $F = 8.025$, $p = 0.02$; *Fig. 6B*) of rhizosphere microbiota were significantly higher in the treatments. The bacteria were classified into 56 phyla. However, only 11 phyla dominated the microbiomes (*Fig. 6C*). Proteobacteria was significantly reduced in the control than PecQ and PecH, whereas Actinobacteria in BC was significantly enhanced (*Fig. 6D*).

CA and PERMANOVA based on the compositions of all genera and dominant genera in the rhizosphere microbiome showed that there were significant differences in the rhizosphere soil microbiota between the treatments and the control (PERMANOVA,

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F = 3.52, p = 0.015 for all genera, and F = 4.73, p = 0.025 for dominant genera; *Fig. 7A* and *7B*). The LefSe and heatmap results showed that *Carnobacterium* was significantly enriched in the multi-species *Bacillus*-amended rhizosphere soil microbiome before infection with *P. atroseptica* MB001, whereas *Mesorhizobium*, *Aquicella* and *A17* were significantly enriched after the infection (*Fig. 7C* and *7D*). The bacteria with altered abundances were related to plant diseases (Young et al., 2014; Town et al., 2016; Wang et al., 2018). However, the effects of the change in the rhizosphere microbiome on the occurrence of PBD needs further experimental verification.

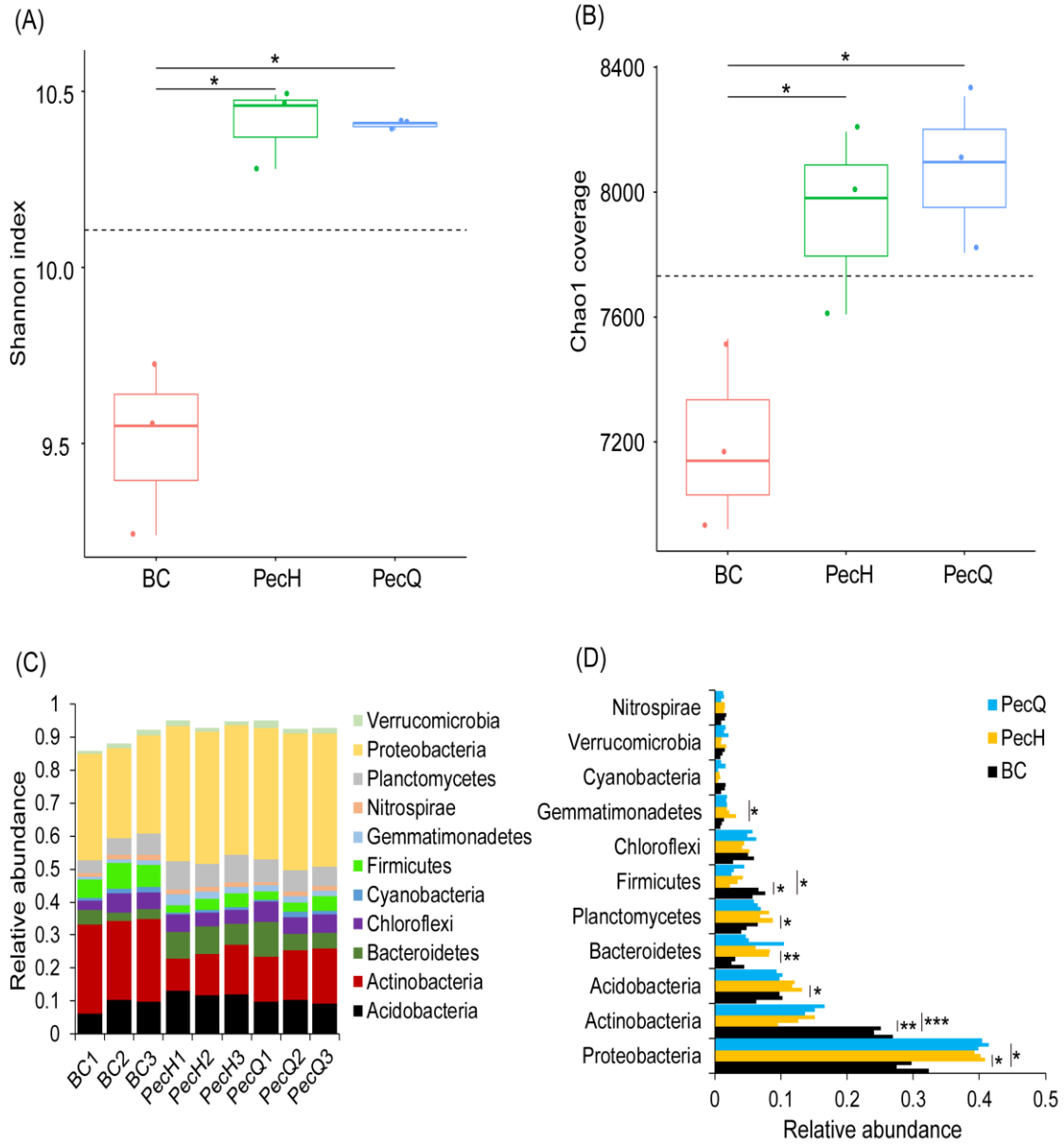

Figure 6. *The Shannon index (A), Chao1 coverage (B), dominant phylum compositions (C), and dominant phylum differences in the rhizosphere microbiome among different treatments (D). BC, blank control; PecH, the samples before infection with Pectobacterium sp. MB001; PecQ, the samples after infection with Pectobacterium sp. MB001. * p < 0.05, ** p < 0.01, *** p < 0.001*

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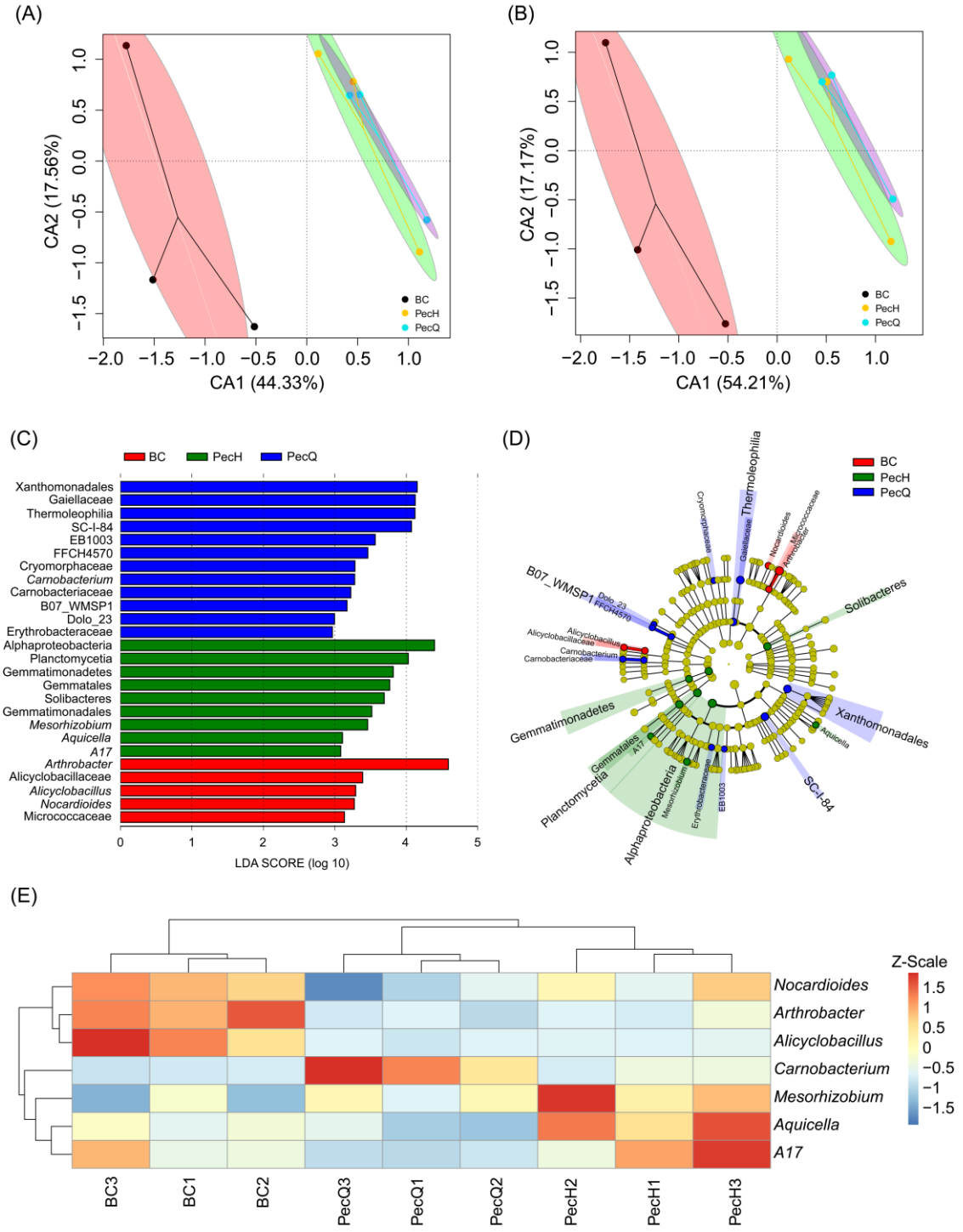

Figure 7. *Changes in microbial composition in the potato rhizosphere. (A), CA profile for all the genera in the potato rhizosphere microbiome; (B), CA profile for the dominant genera in the potato rhizosphere microbiome; (C), LEfSe results; (D), Cladogram profile of LEfSe results; (E), Heatmap of the significantly different genera among different treatments. BC, blank control; PecH, the samples before infection with Pectobacterium sp. MB001; PecQ, the samples after infection with Pectobacterium sp*

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Conclusion

We constructed a multi-species *Bacillus* inoculant and showed that it increased potato yield and the potential PBD resistance, and also changed the composition of the potato rhizosphere microbiome. A larger area of field experiment, the impact of large-scale production and storage, and the molecular mechanism of preventing PBD of the multi-species *Bacillus* inoculant on its function need to be further studied in the future.

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