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Green Fluorescent Protein-Tagged *Bacillus axarquiensis* TUBP1 Reduced Cotton Verticillium Wilt Incidence by Altering Soil Rhizosphere Microbial Communities

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Abstract

Verticillium wilt is a major disease of cotton that considerably decreases yield and crop quality. Soil microbial communities play an important role in plant health. Therefore, biocontrol bacteria that regulate microbial communities in rhizosphere soil can improve plant resistance to pathogens. Previously, the antagonistic strain *Bacillus axarquiensis* TUBP1 was screened and found to act against *Verticillium dahliae* with 43% biocontrol effect in cotton fields. We studied the effect of *Bacillus axarquiensis* TUBP1 with a green fluorescent protein (GFP) gene marker on the microbial community structure of cotton rhizosphere soil and cotton yield and quality. Cotton Verticillium wilt incidence, soil biochemical properties, and soil bacterial and fungal communities were analyzed. Results showed that bacterial and fungal abundance in cotton rhizosphere soil was temporarily changed after applying *B. axarquiensis* TUBP-315GFP. However, *Bacillus* significantly increased, whereas *V. dahliae* significantly decreased. The incidence of cotton Verticillium wilt after treatment with *B. axarquiensis* TUBP-315GFP was significantly lower and cotton production increased by 40.6%. Our findings indicated that the application of *B. axarquiensis* TUBP-315GFP can change microbial community structure of cotton rhizosphere soil, leading to a reduction in the incidence of cotton Verticillium wilt and increasing cotton yield.

Introduction

Cotton Verticillium wilt, caused by *Verticillium dahliae* Kleb, is a major soil-borne disease, which seriously reduces cotton yield and quality in cotton-producing areas globally [1]. The disease is difficult to control because the pathogen forms microsclerotia in the soil that can survive for long periods, and fungicides fail to eliminate the fungus once it has entered the xylem of host plants [2]. Some control measures have been attempted, including improved agricultural management practices and the integrated use of resistant cultivars and biocontrol agents. The use of biological agents to control Verticillium wilt in cotton has attracted much attention [3, 4]. Biocontrol strains play a beneficial role in plant growth either directly or indirectly under disease stress, which includes antibiosis, nutrient competition, and systemic resistance induction. *Bacillus* strains, as spore-forming bacteria that are more easily stored, are the best candidates for controlling many plant diseases, and some important biocontrol agents, including *B. subtilis* strain GB03, *B. pumilus* GB34, *B. licheniformis* SB3086, *B. subtilis* GB122, *B. amyloliquefaciens* GB99, *B. amyloliquefaciens* ZM9, *B. subtilis* N11 [5], and *B. amyloliquefaciens* RWL-1 [6] have been reported.

The application of biocontrol agents can also alter microbial community structure in rhizosphere soil, which can improve the disease resistance of plants, and this has become a focal point in the use of biocontrol agents. The structure of microbial communities in the rhizosphere soil of ginseng was altered after applying *B. subtilis* 50-1 [7]. The relative abundance of *Fusarium oxysporum* and the mortality of ginseng replantation significantly decreased. After the application of biocontrol bacteria, the mortality of ginseng after different seedling years was significantly reduced.

In our previous work, we isolated a strain of TUBP1 that exhibited antagonistic activity against *V. dahliae*

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resulting in a 43% biocontrol effect in cotton fields [8]. The experimental results showed perturbation of the plasma membrane of the spores and the hyphae of *V. dahliae* when they were treated with the TUBP1 protein, which induced mitochondrial damage in *V. dahliae* leading to apoptosis [9]. Then, we successfully constructed *B. axarquiensis* TUBP1 with a green fluorescent protein (GFP) signature, which didn't change the bioactivity in vitro. *B. axarquiensis* TUBP1 was able to colonize cotton plants and the rhizosphere soil [10]. However, little is known about how microbial communities, soil biochemical properties, and cotton Verticillium wilt incidence respond to the inoculation of rhizosphere soil with *B. axarquiensis* TUBP-315GFP. In the present study, we aimed to determine whether, (i) microbial community composition shifted in cotton rhizosphere soil, (ii) Verticillium wilt and *B. axarquiensis* TUBP-315GFP were correlated, (iii) soil biochemical properties and cotton quality index responded *B. axarquiensis* TUBP-315GFP inoculation in cotton rhizosphere soil.

Materials and Methods

Test Strain

Bacillus axarquiensis TUBP-315GFP were provided by the Key Laboratory of Biological Conservation and Utilization, Tarim University. And the fermentation supernatant of *B. axarquiensis* TUBP-315GFP was obtained after it was fermented at 37 °C and 180 rpm/min for 48 h in Luria–Bertani medium (LB, Invitrogen, Carlsbad, USA).

Overview of Test Site and Material

The cotton variety tested in the present study was Xinluzhong 70, which was also provided by the Key Laboratory of Biological Conservation and Utilization, Tarim University. The experiment was carried out in the experimental field of the 12th regiment farm (40°32′ N, 81°17′ E; altitude 1011 m) in Alar City, Xinjiang Province, China from March to October of 2018–2019, sandy soil in the experimental field, growing cotton all the year round. The basic chemical properties of the 0–20-cm layer of arable soil in this area is pH 7.4, organic matter 8.09 g/kg, available nitrogen (AN) 39.02 mg/kg, available phosphorus (AP) 27.83 mg/kg, and available potassium (AK) 92.63 mg/kg. The average temperature is 20 °C during the day and 4 °C at night, annual sunshine hours are 2900 h, frost-free period is 205–219 days, and annual average rainfall is 50 mm.

Experimental Design and Sampling

Three treatments and one control were set up at the test site. Three different concentrations of *B. axarquiensis* TUBP-315GFP fermentation broth (10^6, 10^8, or 10^{10} CFU/mL) were applied during the four growth periods of cotton (referred to as T1.1, T1.2, T1.3, T2.1, T2.2, T2.3, T3.1, T3.2, T3.3, T4.1, T4.2, and T4.3, respectively), and the control group was given the same volume of Luria–Bertani medium (referred to as CK1, CK2, CK3, and CK4).

Each treatment was 60 m^2, and each treatment was repeated three times (contained ten lines with pianting spacing of 60 cm). 0–20-cm root irrigation inoculated with *B. axarquiensis* TUBP-315GFP. At each treatment stage, 500 g rhizosphere soil was extracted from the same depth and divided into two samples. One 250 g sample was put into an aseptic bag and quickly put into a dry ice box and stored at − 80 °C. A total of 48 soil samples were obtained for the four treatments at four growth stages and subjected to high-throughput sequencing. The other 250 g sample was stored at 4 °C for analysis of the physical and chemical properties of the rhizosphere soil.

Effects of B. axarquiensis TUBP-315GFP on Enzyme Activities and Chemical Properties of Cotton Rhizosphere Soil

The physical and chemical properties of the soil were determined by the following methods. Total nitrogen (TN) was estimated using the Kjeldahl method [11]. Organic matter (OM) was determined using the potassium bichromate titrimetric method [12]. AN was determined using the alkali diffusion method [13]. AP was extracted with 0.5 M $NaHCO_3$, and then the content in solution was calculated using molybdenum antimony colorimetric determination [14]. Soil AK was determined with the fame photometric method [15]. Following which for each soil sample the 1:5 soil–water extract was prepared [16, 17], determined using a DDS-11A digital conductivity instrument electrical conductivity (TS).

Soil enzymatic activity reagents were bought from the Suzhou Keming Biotechnology Co., Ltd, Su Zhou, China [14]. Alcalase protease activity (ALPT) was determined using casein as the substrate [18]. Alkaline phosphatase activity (AKP) was determined using phenolphthalein phosphate as the substrate [19]. Urease activity (UE) was determined using the urea-phenol red method [20]. Polyphenol oxidase (PPO) can oxidize monophenol and diphenol to produce quinone under aerobic conditions to determine the activity of PPO [21]. Sucrase activity (SC) was determined using the colorimetric method [20].

Catalase (CAT) can decompose hydrogen peroxide, so that the absorbance (A_{240}) of the reaction solution decreases with the reaction time. CAT activity can be measured by measuring the rate of change in absorbance [22].

Soil DNA Extraction and High-Throughput Sequencing

Total microbial DNA in the rhizosphere soil was extracted by the combined SDS-CTAB method [23] with a soil DNA kit (Shanghai Shenggong Co.). Briefly, total microbial DNA of 5 g rhizosphere soil was crude extracted by the SDS-CTAB method, and then, 0.25 g of the crude extract was used for DNA extraction, according to the manufacturer's protocol from the Ezup column soil DNA extraction kit of Shanghai Shenggong. Finally, the quality of the total DNA of the extracted soil microorganisms was detected by gel electrophoresis. The qualified genomic DNA was sent to Beijing Nuohe Zhiyuan Bioinformation Technology Co., Ltd. for amplification of the V3–V4 and ITS regions of 16S rDNA and high-throughput sequencing using the Illumina MiSeq 2500 sequencing platform.

Data Analysis and Processing

UPARSE (v7.0.1001, http://www.drive5.com/uparse/) [24] software was used to cluster all effective tags of all samples. By default, 97% identity was used to cluster the sequences into operational taxonomic units (OTUs). Then, representative sequences of OTUs were selected. According to the algorithm principle, the sequences with the highest frequency in OTUs were selected as representative sequences of OTUs. Species annotation was carried out for OTUs representative sequences, and species annotation analysis was carried out with mothur software and SSUrRNA [25] database of SilVA [26] (threshold value was 0.8–1). To obtain the taxonomic information, we used the mothur software package to divide the 16S rDNA sequences into OTUs with a threshold of 97% and constructed a dilution curve [27]. The dilution curve and species accumulation box chart were used to analyze species richness and determine the rationality of sampling. The alpha diversity was estimated by calculating the Simpson and Shannon indexes in the QIIME (v1.7.0) software [28]. The chao index is often used to quantitatively estimate the abundance of microbial flora. The Shannon index reflects the diversity of the samples, and the higher the Shannon index, the higher the diversity of the community. Beta diversity was estimated as the microbial community composition of different samples. The similarity clustering tree was constructed by QIIME software. Principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) were conducted in R (Version 2.15.3), and can be used to assess the species composition differences among communities in different habitats, and to determine the differences among samples to reveal the microbial diversity of the rhizosphere soil.

Influence of *B. axarquiensis* TUBP-315GFP on Cotton Yield and Quality

Cotton yield after *B. axarquiensis* TUBP-315GFP application was determined by hand harvesting the four central rows in each treatment on October, 2018. Then, we calculated the yield per acre of cotton, and determined the quality of cotton based on the following indicators: length, strength, and micronaire value of cotton fiber [29].

Statistical Analysis

All results of the experiments are presented as the mean ± standard deviation of three independent experiments. The single factor analysis of variance (ANOVA) was performed to test the mean value of multiple groups of data, based on Tukey's multiple range tests use SPSS statistics (version 18.0) software. $P < 0.05$ was considered to be significant, and $P < 0.01$ was considered to be very significant. Metastats and distance-based redundancy analysis (db-RDA) were conducted in R (Version 2.15.3). Metastats reflects significant differences in the communities between groups. RDA reflects the influence of environmental factors on the composition of different sample communities.

Results

Enhancement of Yield and Quality of Cotton Through Inoculation with *B. axarquiensis* TUBP-315GFP

The incidence of cotton Verticillium wilt was detected, and the results are shown in Fig. 1. Compared with CK, the incidence of cotton Verticillium wilt was significantly lower after cotton root was inoculated with *B. axarquiensis* TUBP-315GFP at the budding, flowering, and bolling stages ($P < 0.05$). However, there were no significant differences in the incidence of cotton Verticillium wilt between T2 and T3.

The yield and quality of cotton were evidently changed after cotton rhizosphere soil was inoculated with *B. axarquiensis* TUBP-315GFP (Table 1). The cotton yield in the *B. axarquiensis* TUBP-315GFP-treated group significantly increased compared with that of CK, of which the yield of T2 increased by 40.6% ($P < 0.05$). However, cotton yield did not significantly change in the rhizosphere soil in T3, which indicated that cotton yield was closely related to the application concentration of *B. axarquiensis* TUBP-315GFP. Furthermore, biocontrol agents have been

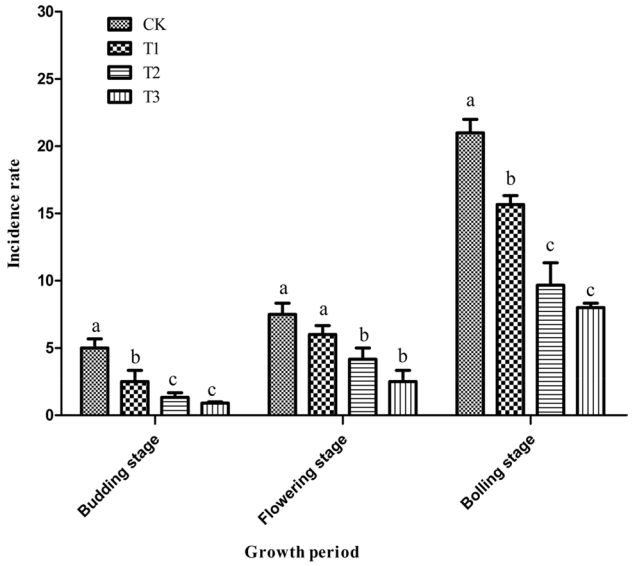


Fig. 1 Effects of *Bacillus axarquiensis* TUBP-315GFP on the incidence of Verticillium wilt in cotton. TUBP1 changed the incidence of cotton Verticillium wilt (the symptoms of cotton Verticillium wilt were mainly reflected after the flower bud stage). GFP, green fluorescent protein. The inoculation concentration of T1 was 10^6 CFU/mL. The inoculation concentration of T2 was 10^8 CFU/mL. The inoculation concentration of T3 was 10^{10} CFU/mL. Different letters (a, b, c) indicate statistically significant differences ($P<0.05$) between inoculated and control plants according to *t*-test. Error bars represent SD ($n=3$)

reported to improve crop quality [30]. Thus, eight cotton quality indexes FL (fiber length), UI (uniformity index), STR (fiber strength), ELO (fiber elongation), MIC (micronaire), REF (reflectivity), FY (fiber yellowness), and SHI (short fiber index) were evaluated after cotton rhizosphere soil was inoculated with different concentrations of *B. axarquiensis* TUBP-315GFP (Table 1). Among them, LE, UI, STR, and SHI significantly changed in the rhizosphere soil in T2 compared with that in CK ($P<0.05$). However, ELO, MIC, REY, and FY were not evidently changed compared with CK. Therefore, cotton yield and some quality indexes improved when cotton rhizosphere soil was inoculated with *B. axarquiensis* TUBP-315GFP. All these results indicate that cotton Verticillium wilt decreased, while cotton yield was increased, and some cotton quality index were also significantly changed after cotton rhizosphere soil was inoculated with *B. axarquiensis* TUBP-315GFP.

Soil Enzyme Activity Response to *B. axarquiensis* TUBP-315GFP

In addition to the qualitative and quantitative changes induced by the microbial inoculants on the rhizosphere microbial community, there were also changes in the functioning of the system, as evaluated by soil enzyme activity (Table 2). The activities of PPO, ALPT, SC, UE, and AKP all increased to some extent compared with those in CK after the application of *B. axarquiensis* TUBP-315GFP. Both UE and SC activity significantly increased with the advancement of cotton growth stage and peaked during the bolling stage (The change of CAT is not significant). The enzyme activities of UE and SC increased significantly with the advance of cotton growth period, among them, the activity of UE in budding stage increased significantly by 18.08%, bolling stage SC enzyme increased by 36.01%. The soil enzyme activity changed in rhizosphere soil inoculated with *B. axarquiensis* TUBP-315GFP, the activities of PPO, ALPT, SC, UE, and AKP showed a clear upward trend, and the activity of UE and SC was also closely related to the growth period of cotton. After the application of bacterial agent, the possible mechanism is that the diversity of microbial community has changed after the action of bacterial agent, and the increase of the number of microorganisms and the increase of growth rate have promoted the enzyme activity to the greatest extent.

Table 1 Quality and yield of cotton were changed after rhizosphere soil inoculate with *B. axarquiensis* TUBP1-GFP

	FL (mm)	UI (%)	STR (g/tex)	ELO (%)	MIC (μg/inch)	REF (%)	FY (%)	SHI (%)	YIE (g)
CK	28.55 ± 0.84^{b}	81.6 ± 0.53^{b}	25.32 ± 1.82^{c}	7.86 ± 0.54^{a}	3.75 ± 0.29^{a}	77.33 ± 1.26^{a}	8.23 ± 0.12^{a}	14.96 ± 0.59^{a}	392.47 ± 3.48^{c}
T1	29.07 ± 0.58^{b}	83.2 ± 0.63^{ab}	27.49 ± 0.81^{b}	7.63 ± 0.74^{a}	4.47 ± 0.47^{a}	75.8 ± 0.64^{a}	8.33 ± 0.09^{a}	12.16 ± 1.79^{b}	516.88 ± 3.18^{b}
T2	30.71 ± 0.34^{a}	84.2 ± 0.49^{a}	29.50 ± 1.13^{a}	6.83 ± 0.32^{a}	3.92 ± 0.38^{a}	77.66 ± 0.26^{a}	8.43 ± 0.12^{a}	10.6 ± 0.89^{c}	552.03 ± 6.06^{a}
T3	28.81 ± 0.23^{b}	83.13 ± 1.01^{ab}	26.92 ± 1.24^{c}	8.03 ± 0.81^{a}	3.80 ± 0.18^{a}	77.86 ± 0.18^{a}	8.36 ± 0.21^{a}	13.43 ± 4.12^{a}	396.16 ± 5.39^{c}

Cotton yield and 8 traits of cotton fiber

FL fiber length, *UI* uniformity index, *STR* fiber strength, *ELO* fiber elongation, *MIC* micronaire, *REF* reflectivity, *FY* fiber yellowness, *SHI* short fiber index, *YIE* yield

[a–c]The same letter in the same row means no significant difference, and different letters indicate significant differences, $P<0.05$. The statistical analysis was determined by Tukey's Studentized Range (HSD) test: $\alpha=0.05$, $n=3$

Table 2 Changes in soil enzyme activity

Enzyme	Seedling stage				Budding stage			
	Ck1	T1.1	T1.2	T1.3	Ck2	T2.1	T2.2	T2.3
PPO (mg/d/g)	36.75 ± 9.2b	34.68 ± 8.4 a	38.24 ± 10.0a	36.32 ± 9.2b	33.88 ± 11.9c	36.53 ± 12.1b	35.73 ± 9.9b	45.81 ± 6.5a
CAT (μmol/d/g)	41.54 ± 1.7a	42.35 ± 1.3a	43.06 ± 0.9a	41.72 ± 1.0a	49.98 ± 0.31a	48.45 ± 2.1b	47.02 ± 43.9c	46.87 ± 2.4c
ALPT (mg/d/g)	16.47 ± 3.9a	15.82 ± 2.7b	16.17 ± 4.3a	16.79 ± 3.5a	13.38 ± 6.3d	20.60 ± 12.0c	28.16 ± 7.5a	27.01 ± 5.9b
SC (mg/d/g)	43.24 ± 7.1a	41.35 ± 4.3c	42.67 ± 8.9b	42.73 ± 5.1b	74.03 ± 11.0b	76.75 ± 4.9a	77.85 ± 4.6a	77.90 ± 3.5a
UE (μg/d/g)	76.13 ± 11a	77.48 ± 8.6a	73.16 ± 10.4b	76.39 ± 11.2a	164.48 ± 7.0c	187.65 ± 11.9b	185.91 ± 12.5b	194.21 ± 5.7a
AKP (μmol/d/g)	8.06 ± 1.7c	8.26 ± 1.2b	7.98 ± 1.5c	8.49 ± 2.4a	9.72 ± 0.7c	11.61 ± 3.2a	10.89 ± 1.6b	8.56 ± 0.3d
Enzyme	Flowering stage				Bolling stage			
	Ck3	T3.1	T3.2	T3.3	Ck4	T4.1	T4.2	T4.3
PPO (mg/d/g)	32.9 ± 13.7b	33.94 ± 12.7b	43.89 ± 7.9a	43.38 ± 11.1a	43.15 ± 11.0c	45.23 ± 3.8b	49.76 ± 9.8a	49.17 ± 11.3a
CAT (μmol/d/g)	47.43 ± 1.6ab	48.35 ± 1.5a	42.72 ± 2.9c	48.95 ± 2.1a	49.98 ± 1.4a	47.78 ± 1.0b	45.33 ± 0.5d	46.86 ± 1.4c
ALPT (mg/d/g)	30.78 ± 4.9c	42.81 ± 8.9a	35.22 ± 7.8b	34.44 ± 3.3b	25.05 ± 1.6c	33.07 ± 7.5a	25.32 ± 3.1c	29.86 ± 11.4b
SC (mg/d/g)	79.15 ± 2.1c	83.28 ± 1.3b	89.08 ± 6.7a	80.64 ± 8.0c	81.02 ± 6.1d	107.23 ± 5.1b	110.20 ± 6.7a	89.61 ± 4.2c
UE (μg/d/g)	166.23 ± 7.8c	190.72 ± 9.9b	192.90 ± 1.5b	203.40 ± 9.2a	202.96 ± 4.4c	205.59 ± 3.0c	213.02 ± 6.4b	222.64 ± 9.4a
AKP (μmol/d/g)	9.08 ± 0.7b	12.08 ± 1.5a	9.05 ± 0.3b	7.26 ± 05c	7.94 ± 3.5c	10.27 ± 1.1a	8.90 ± 1.5b	9.87 ± 7.0a

Activity of six enzymes in cotton rhizosphere soil

ALPT alcalase protease, *AKP* alkaline phosphatase, *UE* urease, *PPO* Polyphenol oxidase, *SC* sucrase, *CAT* catalase

a–d: The same letter on each line means no significant difference, while different letters mean significant difference, $P < 0.05$. The statistical analysis was determined by Tukey's Studentized Range (HSD) test: $\alpha = 0.05$, $n = 3$

Soil Property Response to *B. axarquiensis* TUBP-315GFP

Bacillus axarquiensis TUBP-315GFP treatment affected the soil properties (Table 3). In the presence of *B. axarquiensis* TUBP-315GFP, soil TN, AP, and AK were significantly higher than those in CK during the period of T1–T4 ($P < 0.05$). Besides, NN and TS content did not differ between the treatment groups and CK. The soil with the same concentration of *B. axarquiensis* TUBP-315GFP, flowering stage has the highest content of TN and AK, the content of AP is the highest in bolling stage. Therefore *B. axarquiensis* TUBP-315GFP increased the contents of TN, AK, and AP in soil, and improved the original soil environment.

Microbial Diversity Response to *B. axarquiensis* TUBP-315GFP

All collected samples were analyzed using IonS5^{TM}XL platform Novogene Bioinformatics Technology Co., Ltd (Beijing, China), and the sequencing results are shown

Table 3 Effect of *B.axarquiensis* TUBP-GFP on physicochemical properties of soil samples

	Seedling stage				Budding stage			
	Ck1	T1.1	T1.2	T1.3	Ck2	T2.1	T2.2	T2.3
NN mg/kg	2.03 ± 0.2a	2.03 ± 0.2a	2.03 ± 0.1a	2.03 ± 0.1a	2.05 ± 0.2a	2.03 ± .10a	2.03 ± 0.2a	2.03 ± 0.1a
TN mg/kg	0.92 ± 0.3b	1.02 ± 0.2a	1.02 ± 0.1a	1.02 ± 0.1a	0.92 ± 0.1c	1.12 ± 0.1b	1.45 ± 0.2a	1.44 ± 0.1a
AP mg/kg	0.18 ± 0.1a	0.18 ± 0.2a	0.18 ± 0.1a	0.18 ± 0.2a	0.16 ± 0.05d	0.24 ± 0.01c	0.30 ± 0.04a	0.28 ± 0.01b
AK mg/kg	46.90 ± 4.24d	51.56 ± 2.16c	58.56 ± 1.11b	63.56 ± 1.16a	60.70 ± 4.17d	78.26 ± 3.39c	85.20 ± 1.61a	82.53 ± 1.55b
TS us/cm	313.5 ± 57.28a	300.00 ± 12.66b	261.33 ± 16.76c	261.33 ± 12.66c	690.33 ± 24.09c	717.66 ± 65.26b	725.66 ± 29.05a	610.00 ± 27.40d
	Flowering stage				Bolling stage			
	Ck3	T3.1	T3.2	T3.3	Ck4	T4.1	T4.2	T4.3
NN mg/kg	2.04 ± 0.1a	2.02 ± 0.1a	2.04 ± 0.2a	2.01 ± 0.1a	2.07 ± 0.1a	2.08 ± 0.2a	2.02 ± 0.1b	2.07 ± 0.1a
TN mg/kg	0.90 ± 0.1c	1.20 ± 0.1b	1.50 ± 0.2a	1.50 ± 0.1a	0.83 ± 0.1c	1.21 ± 0.1b	1.34 ± 0.1a	1.31 ± 0.3a
AP mg/kg	0.19 ± 0.03c	0.29 ± 0.01b	0.46 ± 0.04a	0.33 ± 0.08b	0.28 ± 0.15c	0.34 ± 0.01b	0.47 ± 0.02a	0.46 ± 0.07a
AK mg/kg	77.00 ± 2.02c	90.83 ± 4.37b	99.13 ± 1.04a	88.40 ± 8.28b	80.26 ± 1.95c	88.50 ± 3.76b	93.63 ± 7.70a	88.06 ± 8.51b
TS us/cm	611.00 ± 37.04d	1148.33 ± 112.11a	658.66 ± 15.02c	973.00 ± 119.55b	1058.66 ± 36.07b	949.00 ± 19.97c	922.66 ± 29.28d	1350.00 ± 141.46a

TN total nitrogen, *NN* nitrate nitrogen, *AP* available phosphorus, *AK* available potassium, *TS* soil conductivity, *T1-T4* four growth periods of cotton, *T1.1-T4.3* Three different concentrations of TUBP-315GFP fermentation broth (10^6, 10^8, or 10^{10} CFU/mL)

a–d: The same letter on each line means no significant difference, while different letters mean significant difference, $P < 0.05$. The statistical analysis was determined by Tukey's Studentized Range (HSD) test: $\alpha = 0.05$, $n = 3$

Table 4 Analysis on the difference of microbial community in cotton rhizosphere soil

	Each sample reads	Effective reads	97%		
			Effective quality control (%)	OTU	Note to genus
Bacterial	79,097	74,351	94.02	5877	2058
Fungus	82,560	78,975	95.72	4582	1146

Soil DNA sequencing results include valid sequences and species annotation information. OTUs clustering analysis with 97% similarity by default. Effective reads represents the percentage of the number of Clean reads to the number of Raw reads

in Table 4. After cutting and filtering the reads, 79,097 bacterial reads were obtained on average for each sample, and 74,351 effective reads were obtained on average after quality control; the effective rate of quality control was 94.02%. At 97% identity, the sequences were clustered into 5877 OTUs. Then, the OTU sequences were annotated against the Silva132 database (27 January 2019). A total of 2058 (35.02%) OTUs were annotated at generic level.

The average number of fungal reads per sample was 82,560, and the average number of effective reads was 78,975. The effective rate of quality control was 95.72%. With 97% identity, the sequences were clustered into 4582 OTUs. Then, the OTU sequences were annotated against the UNITE database. A total of 1146 (25.01%) OTUs were annotated at generic level.

The Chao values represent richness, and the Shannon diversity index indicates both richness and evenness. For both of these indices, higher values indicate higher diversity. The α-diversity of soil microbial communities slightly increased in the rhizosphere at the flowering and bolling stages for all samples (Fig. 2A–D). However, there was no significant difference between the treatment groups and CK at the flowering and bolling stages. In addition, the diversity index of the treatment groups was higher than that in CK based on the ANOVA at early stages, implying that diversity was higher in the treatment groups. The Chao 1 and Shannon values in the treatment groups with rhizosphere soil inoculated with *B. axarquiensis* TUBP-315GFP were apparently higher than those in CK at the budding stage, which indicated that *B. axarquiensis* TUBP-315GFP inoculants affected the diversity of microbial communities at early stages after inoculation.

Multi-Sample Comparative Analysis

PCA and NMDS were applied to evaluate microbial community structure in rhizosphere soil treated with *B. axarquiensis* TUBP-315GFP (Fig. 3). Bacterial community structure is shown in Fig. 3A; the contribution rate of the first (PC1) and second (PC2) principal components was 11.52% and

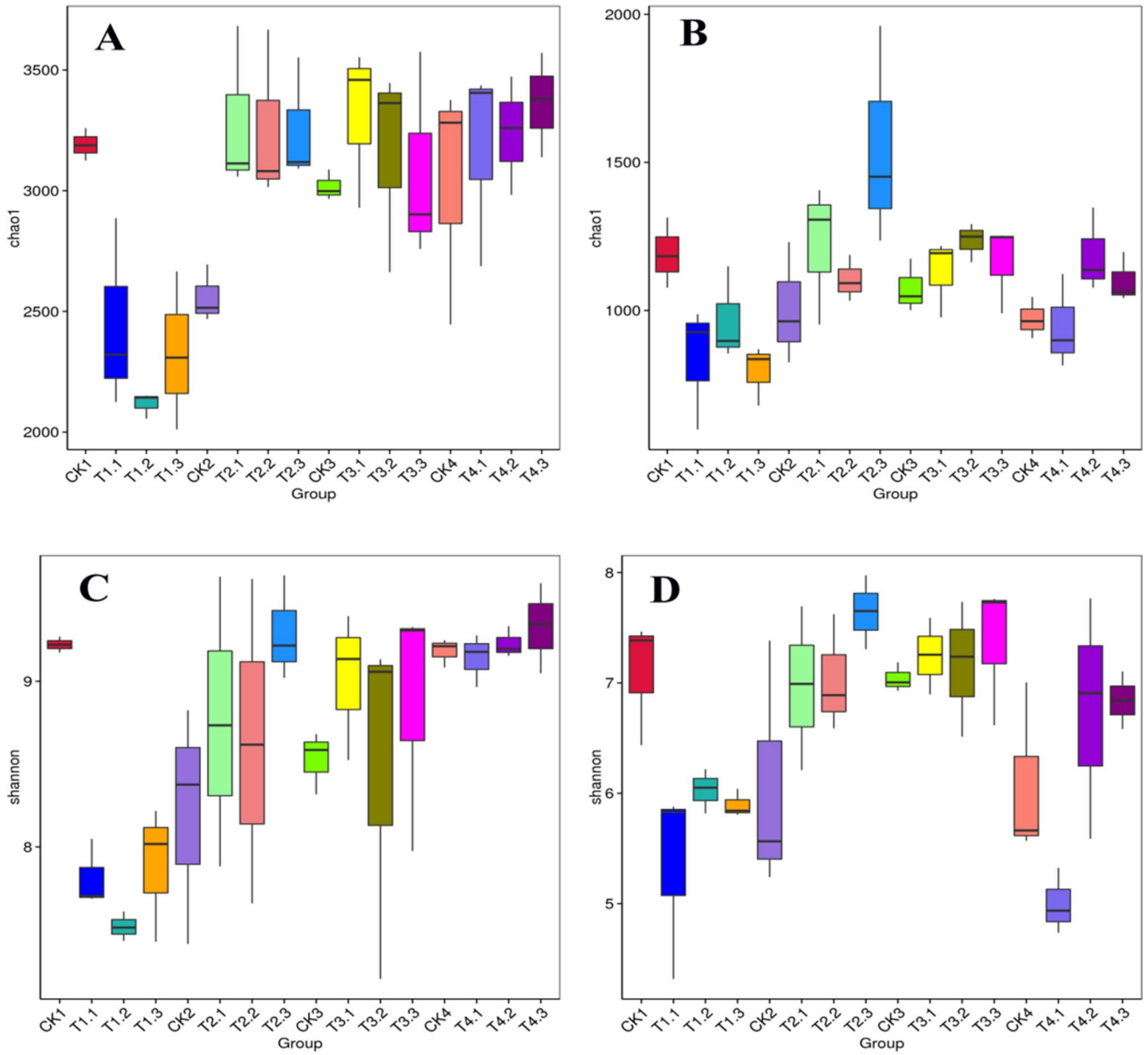


Fig. 2 Diversity indices in different growth stages and treatments in the rhizosphere soil of cotton. **A**, **B** Chao 1 index of cotton rhizosphere soil for bacteria and fungi, respectively; **C**, **D** Shannon index of cotton rhizosphere soil for bacteria and fungi, respectively. Differences are $P < 0.05$ as measured by *T*-test

7.46%, respectively. Fungal community structure is shown in Fig. 3B; the contribution rate of the PC1 and PC2 based on PCA was 7.54% and 5.18%, respectively. The application of *B. axarquiensis* TUBP-315GFP inoculants affected the composition of indigenous rhizosphere microbial communities at the seedling stage, whereas no major effect was evident at the flowering and bolling stages. However, the bacterial and fungal community showed a clear temporal shift in the different groups. The NMDS results were similar to those of PCoA, as shown in Fig. 3C–D, indicating that bacterial and fungal community structure differed between the seedling stage and the other three growing stages. Besides, at the budding stage, bacterial and fungal community structure showed significant differences between CK and the treatment groups. However, microbial community structure was not evidently changed between CK and the treatment groups at the flowering and bolling stages after applying *B. axarquiensis* TUBP-315GFP.

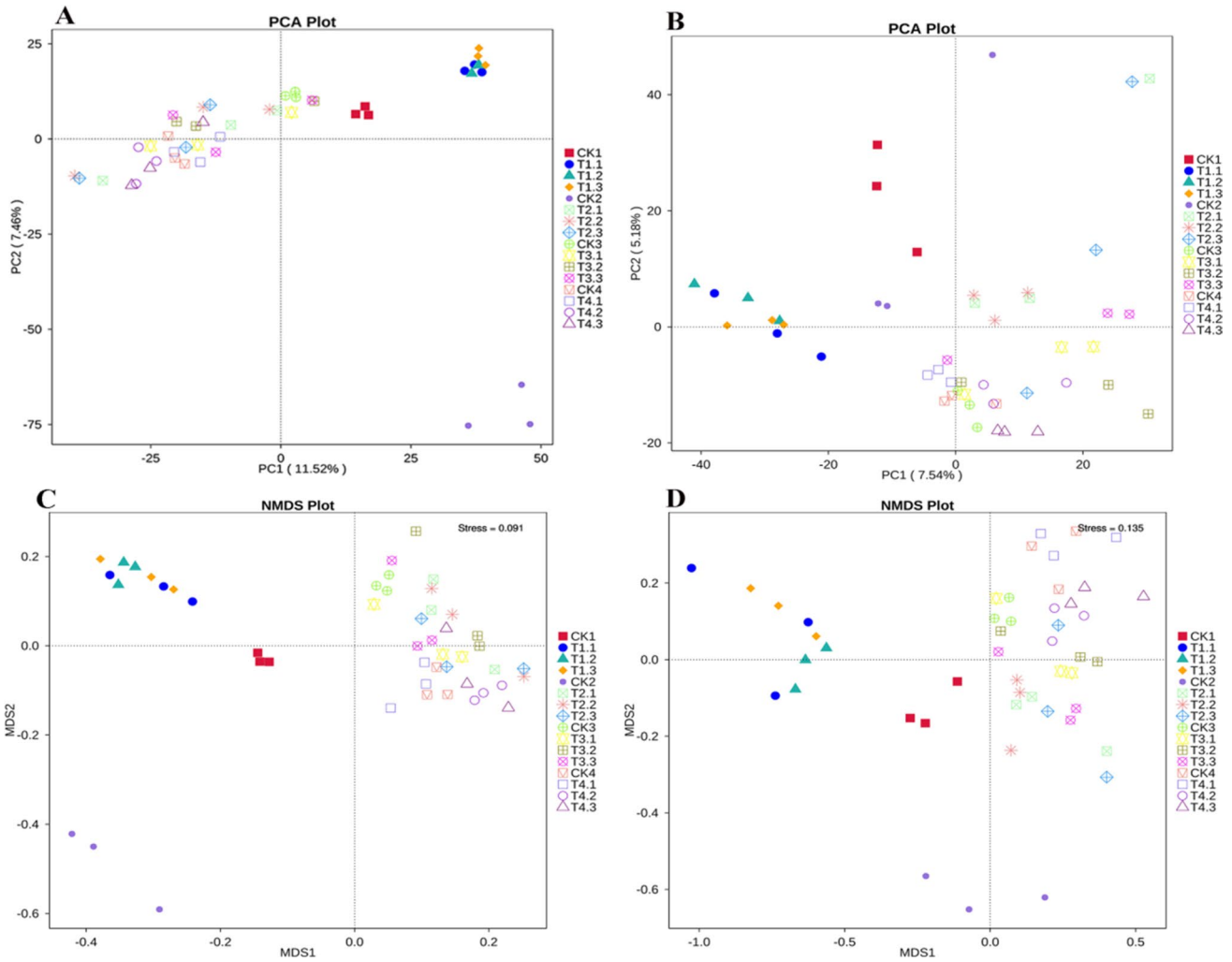


Fig. 3 Effect of *Bacillus axarquiensis* TUBP-315GFP application on the rhizosphere bacterial and fungal community in cotton rhizosphere soil. **A**, **B** Bacterial and fungal PCA analysis of cotton rhizosphere soil, respectively. **C**, **D** Bacterial and fungal *NMDS* analysis of cotton rhizosphere soil, respectively. The differences of diversity index between groups were analyzed by R software, and Tukey test and wilcox test were used. *GFP* green fluorescent protein, *PCA* principle components analysis, *NMDS* non-metric multi-dimensional scaling

Bacterial Composition Response to *B. axarquiensis* TUBP-315GFP

Metastats analysis was performed to evaluate the rhizosphere bacterial community that influenced the composition of microbial communities, and the results indicated that *Proteobacteria*, *Acidobacteria*, *Latescibacteria*, and *Gemmatimonadetes*, were the major taxa shaping the rhizosphere community (Supplementary Fig. S1, A).

However, bacterial diversity significantly shifted after inoculation with *B. axarquiensis* TUBP1-315GFP at the seedling and budding stages. The most abundant bacterial genera were *Arthrobacter*, *Streptococcus*, *Lysobacter*, *Sphingomonas*, *Pseudomonas*, *Bacillus*, *Bifidobacterium*, and *Pontibacter*. Among them, *Pontibacter* and *Streptococcus* were significantly decreased, whereas *Bauldia*, *Acidobacteria*, *Haliangium*, *Pseudarthrobacter*, *Arthrobacter*, *Actinomarinales*, *Sphingomonas*, *Lysobacter*, and *Bacillus* significantly increased ($P < 0.01$) (Fig. 4A). The content of *Bacillus*, as the main biocontrol bacterium, in the soil indicates the incidence of cotton Verticillium wilt. In the budding stage, *Bacillus* was the dominant genus in the treatment groups. Figure 4B shows that the content of *B. axarquiensis* TUBP-315GFP in the rhizosphere soil increased with the change in cotton growth period. In particular, the content of *B. axarquiensis* TUBP-315GFP in the treatment groups was significantly higher than that in CK at the budding, flowering, and bolling stages. In addition to the seedling stage, however, there was almost no significant difference in the content of *B. axarquiensis* TUBP-315GFP in the soil between the seedling and CK groups.

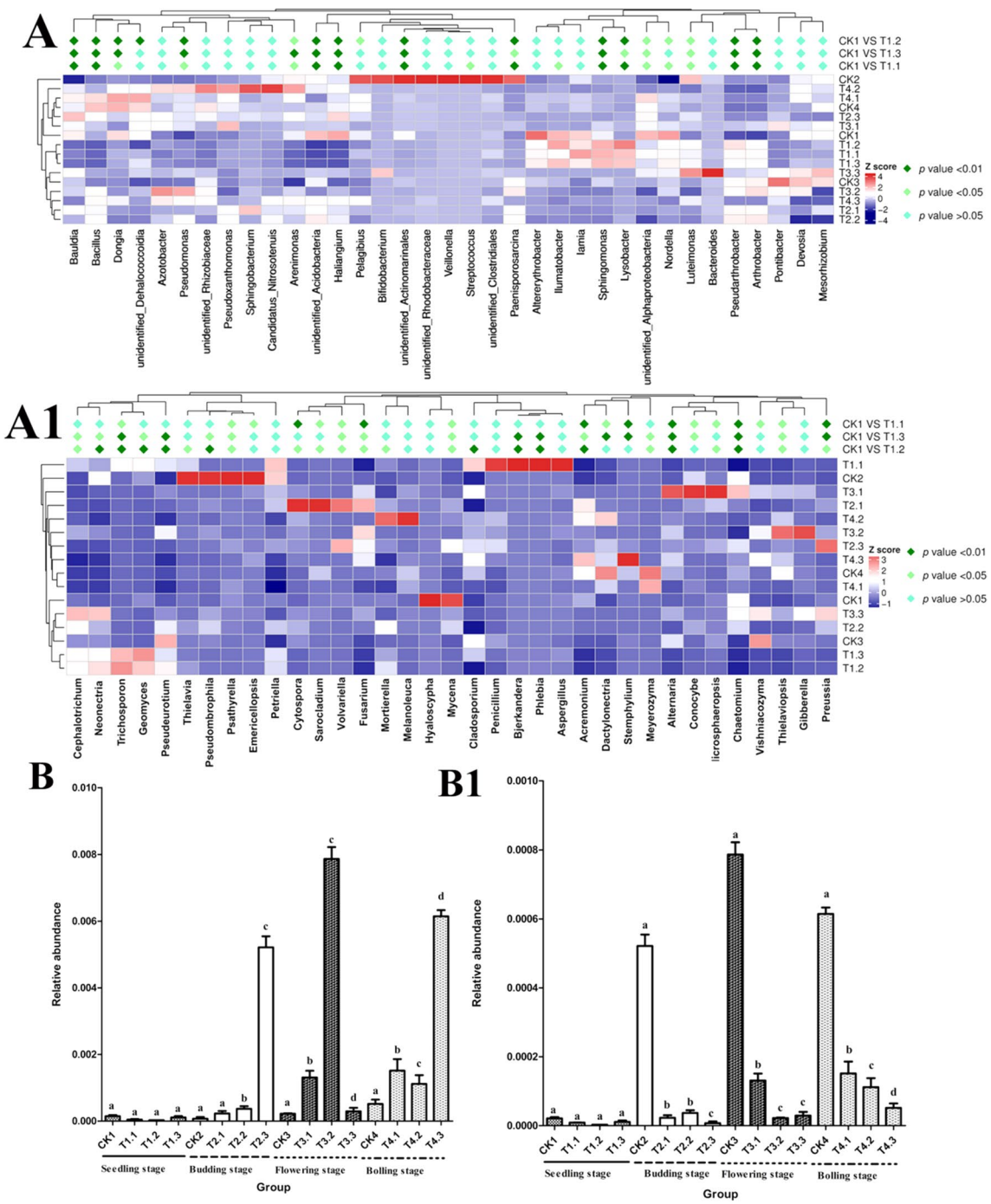


Fig. 4 Changes in main species of bacteria and fungi in cotton rhizosphere soil. **A** Top-10 species of cotton rhizosphere soil bacteria, **B** *Bacillus* changes in cotton rhizosphere soil, **A1** top-10 species of cotton rhizosphere soil fungi, **B1** change in *Verticillium dahliae* in cotton rhizosphere soil. **A**, **A1** The bacterial and fungal richness and diversity indices (Abundance-based) were estimated using Mothur (version v.1.30.1). **B**, **B1** Different letters (a, b, c) indicate statistically significant differences ($P < 0.05$) between inoculated and control plants according to t-test. Error bars represent SD ($n = 3$)

Fungal Composition Response to *B. axarquiensis* TUBP-315GFP

Meta Stat analysis was performed to evaluate the variation in rhizosphere fungal community influenced by *B. axarquiensis* TUBP1 inoculants. *Ascomycota*, *Mortierellomycota*, and *Basidiomycota* were the major taxa shaping the rhizosphere community between the CK and treatment groups, and there

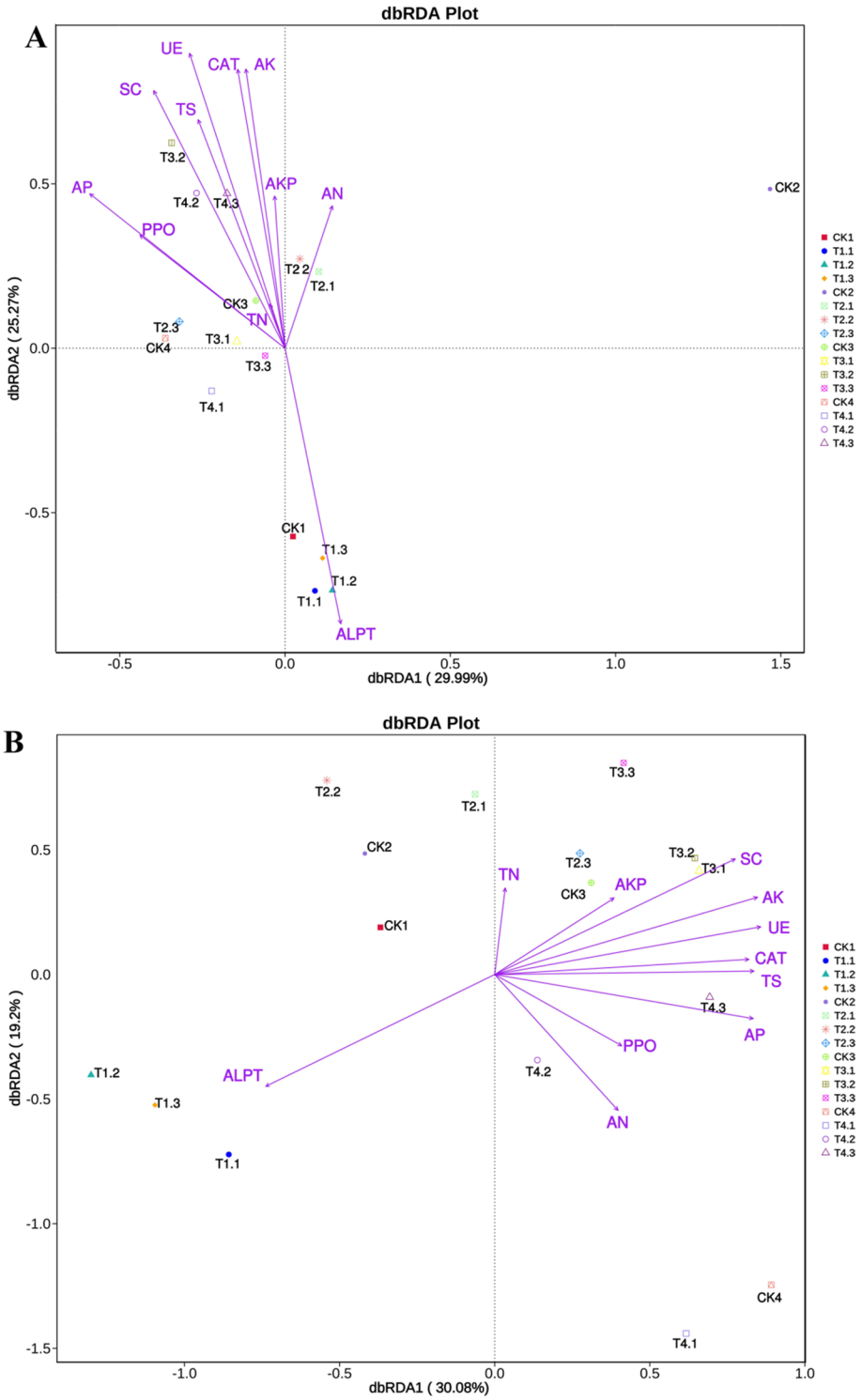


Fig. 5 Effects of TUBP-315GFP application on cotton rhizosphere soil and environmental factors. RDA, the influence of environmental factors on the composition of different sample communities. **A** dbRDA analysis of bacteria and environmental factors, **B** dbRDA analysis of fungi and environmental factors. Redundancy analysis (RDA) was employed to examine effects of TUBP-315GFP application on cotton rhizosphere soil and environmental factors using the vegan package of R software (Version 3.0.2). *GFP* green fluorescent protein

were no evident differences between them (Supplementary Fig. S1, A1). However, from the top-12 phyla, in contrast with those in CK, *Mortierellomycota*, *Rozellomycota*, and *Basidiomycota* were higher in the budding, flowering, and bolling stages of the treatment groups. The most abundant fungal genera in the rhizosphere soil were *Chaetomium*, *Preussia*, *Stemphylium*, *Trichosporon*, and *Pseudeurotium* ($P < 0.05$) (Fig. 4A1). The dominant species in the treatment groups were *Papiliotrema flavescens*, *Acremonium*, and *Cladosporium chasmanthicola* at the seedling, budding, and flowering stages, respectively.

Verticillium dahliae was the main fungal pathogen, and its content in the rhizosphere soil was detected by the dilution-plate method. Figure 4B1 shows that the content of *V. dahliae* decreased after inoculation with *B. axarquiensis* TUBP-315GFP at the budding, flowering, and bolling stages compared with CK.

Correlation Between Soil and Environmental Factors

It can be seen from Fig. 5A that, based on the analysis of dbRDA, the contribution rate of PC1 (dbrda1) and PC2 (dbrda2) was 29.99% and 25.27%, respectively, and ALPT was negatively correlated with other environmental factors. For the first axis, the more important environmental factors were AP, AN, and PPO, whereas on the second axis, the important environmental factors were AKP, CAT, AK, and ALPT. In the three growth stages after the seedling stage, soil bacterial diversity in the T2.2, T3.2, and T4.2 treatment groups was most affected by environmental factors.

As can be seen from Fig. 5B, based on dbRDA analysis, the contribution rate of PC1 (dbRDA1) and PC2 (dbRDA2) was 30.08% and 19.2%, respectively. Similar to bacteria, ALPT was negatively correlated with other environmental factors for fungi. For the first axis, the more important environmental factors were TS, CAT, UE, and AP, whereas for the second axis, the important environmental factors were TN and AN. Soil fungal diversity was most closely related to ALPT in the seedling stage. The fungal diversity in the budding and flowering stages was greatly affected by environmental factors.

For bacteria, based on dbRDA analysis, the contribution rate of PC1 (dbRDA1) and PC2 (dbRDA2) was 49.26% and 35.79%, respectively (Fig. 6A). T3 and T4 were more closely related to the relative abundance of *Bacillus* (BRA), whereas CK2 and CK3 in CK were more closely related to the relative abundance of *V. dahliae* (VARA). *Clostridium butyricum*, *Rhodovulum* sp., *Pontibacter populi*, and *Bifidobacterium pseudocatenulatum* were more closely related to VARA, and *Bacillus halotolerans* and *Rhizobium helanshanense* were more closely related to BRA. The application of biocontrol bacteria *B. axarquiensis* TUBP1 not only changed the abundance of *V. dahliae* and *Bacillus* in the soil but also affected the abundance of bacterial populations of other species in the soil (Fig. 6A). For fungi, based on dbRDA analysis, the contribution rate of PC1 (dbRDA1) and PC2 (dbRDA2) was 62.88% and 25.11%, respectively (Fig. 6B). Similar to bacteria, CK2 and CK3 were more closely related to VARA, and T2, T3, and T4 were more closely related to BRA. *Alternaria alternata* and *Thielaviopsis basicola* were more related to BRA, while *Cladosporium limoniforme* and *C. chasmanthicola* were more related to VARA. It showed that the application of the biocontrol bacteria TUBP1 also changed the population abundance of other fungi in the soil (Fig. 6B).

Discussion

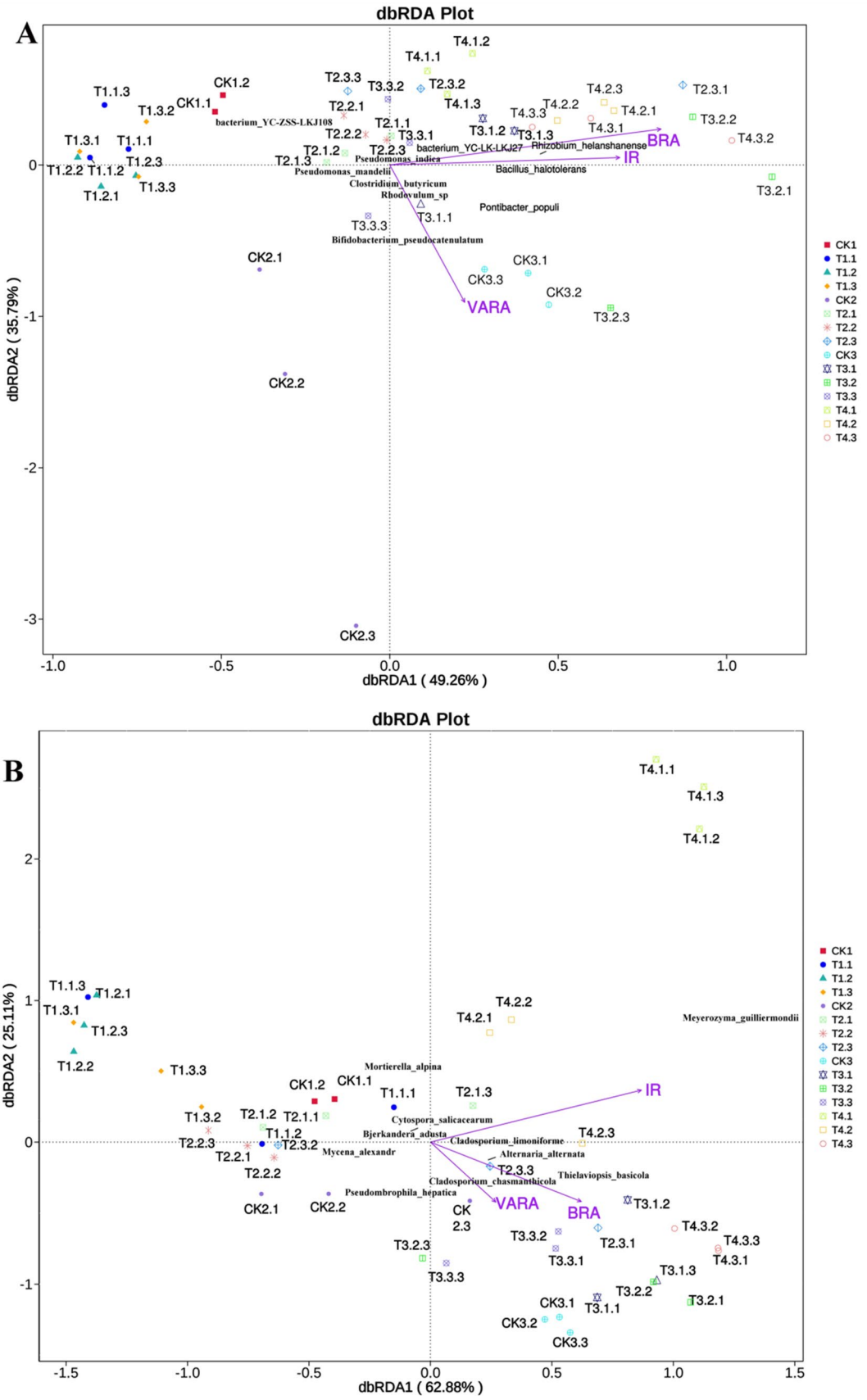
Understanding the biocontrol mechanisms of *B. axarquiensis* TUBP1 against cotton Verticillium wilt under field conditions will improve its biological control effect. Previous studies reported this bacterial control agent as a potential candidate against *V. dahliae* via peptide-T-inducing mitochondrial damage and mitochondria-mediated apoptotic cell death [9]. In the cotton field, *B. axarquiensis* TUBP1 exhibited efficient biocontrol effects against the Verticillium wilt pathogen. The GFP-tagged *B. axarquiensis* TUBP1 was obtained by electrotransformation, and it colonized different parts of the cotton plants from the rhizosphere soil (data unpublished). In the present study, we evaluated its efficiency in control of Verticillium wilt and its effects on the cotton rhizosphere microbial community in a field trial. The application of *B. axarquiensis* TUBP-315GFP biocontrol agents can significantly reduce the incidence of cotton Verticillium wilt and increase cotton yield. FL, UI, STR, and SHI were also significantly changed when cotton plants were treated with *B. axarquiensis* TUBP-315GFP at 10^8 CFU/mL (Table 1). *Bacillus* strains are potent biological control agents against diseases of plants and crops, including tomatoes [31], peppers [32], and tobacco [33].

Bacillus spp. are also considered as bioorganic fertilizers because they promote the growth and quality of crops. Besides, crop yield and quality have a positive relationship between some soil properties, including alkaloid content, pH, nitrate K, and total soluble salt [34]. Our results are largely consistent with those of these previous studies in that TN, AP, and AK in the treatment groups were significantly higher than in CK (Table 3). These factors could have contributed to the increased yield and quality of cotton.

Based on Spearman's rank correlation coefficient, *Bacillus* were strongly and positively associated with TS, AP, UE, and CAT. In contrast, negative associations between cotton Verticillium wilt and the relative abundance of *Sporichthya*, *Achromobacter*, *Burkholderia*, *Comamonas*, *Ramlibacter*, and *Pontibacter* were observed.

The soil microbial community structure is closely related to plant health status and is shaped by, e.g., biocontrol bacteria inoculants, crop rotation, fertilization, and tillage. Some studies reported that *Paenibacillus polymyxa* CP-S316 evidently changed the rhizosphere microbial community [35].

It is important to understand whether applications of *B. axarquiensis* TUBP-315GFP can change the composition of the indigenous populations of soil microorganisms. In the present study, we discovered that the cotton soil rhizosphere microbial community was temporarily changed at the seedling and budding stages after *B. axarquiensis* TUBP-315GFP inoculation. These results are in agreement with those of previous studies that reported some biocontrol agents


A
dbRDA Plot
dbRDA2 (35.79%)
dbRDA1 (49.26%)
BRA
IR
VARA
bacterium_YC-ZSS-LKJ108
bacterium_YC-LK-LKJ27
Rhizobium_helanshanense
Pseudomonas_indica
Bacillus_halotolerans
Pseudomonas_mandelii
Clostridium_butyricum
Rhodovulum_sp
Pontibacter_populi
Bifidobacterium_pseudocatenulatum
CK1
T1.1
T1.2
T1.3
CK2
T2.1
T2.2
T2.3
CK3
T3.1
T3.2
T3.3
T4.1
T4.2
T4.3
B
dbRDA Plot
dbRDA2 (25.11%)
dbRDA1 (62.88%)
IR
VARA
BRA
Meyerozyma_guilliermondii
Mortierella_alpina
Cytospora_salicacearum
Bjerkandera_adusta
Cladosporium_limoniforme
Alternaria_alternata
Thielaviopsis_basicola
Cladosporium_chasmanthicola
Mycena_alexandr
Pseudombrophila_hepatica

◂**Fig. 6** Effects of biological factors on bacteria and fungi in cotton rhizosphere soil. RDA, the influence of environmental factors on the composition of different sample communities. **A** dbRDA analysis of bacteria and biological factors, **B** dbRDA analysis of fungi and biological factors. Redundancy analysis (RDA) was employed to examine effects of biological factors on bacteria and fungi in cotton rhizosphere soil using the vegan package of R software (Version 3.0.2)

affecting and sometimes destroying the original microflora but only temporarily [36–40].

Notably, *Bacillus* significantly increased, whereas *V. dahliae* significantly decreased over the whole growing stage. Besides, the diversity of bacterial species evidently changed at the seeding and budding stages in *B. axarquiensis* TUBP-315GFP-treated groups. Some beneficial microbes, including *Bauldia*, *Acidobacteria*, *Haliangium*, *Pseudarthrobacter*, *Arthrobacter*, *Actinomarinales*, *Sphingomonas*, *Lysobacter*, and *Bacillus*, significantly increased in the treatment groups, indicating that the biocontrol inoculant *B. axarquiensis* TUBP-315GFP may promote the growth of cotton and improve the quality.

Plants growing in soil develop close associations with soil microorganisms, which inhabit the areas around, on, and inside their roots [41]. In the study of gramineae plant and soybean, the rhizosphere microbial community changes for a whole life cycle, and the trend is that the soil-derived microbial communities gradually differentiates from some specific microbial communities [42, 43]. Chaparro et al. [44] showed that rhizosphere bacterial communities at the seedling stage of Arabidopsis thaliana were distinct from vegetative, bolting, and flowering stages. Our experimental results showed that in the four growth periods of cotton, the species diversity of rhizosphere soil microorganisms was significantly different due to the difference of growth period, the species richness of bacteria in seeding stage soil is the most uniform, and the species diversity of bacteria increases gradually with the passage of growth cycle. Bacillus strain is an effective biocontrol agent for plant diseases [6, 45], and the degree of colonization and proliferation on the host plant affects the biocontrol effect [46].

Conclusion

The application of *B. axarquiensis* TUBP-315GFP inoculants affected the composition of cotton rhizosphere microbial communities at the seedling and budding stages, but no major effects on the microbial communities were observed at the flowering and bolling stages. Especially, *Bacillus* significantly increased, while *V. dahliae* significantly decreased. The incidence of cotton Verticillium wilt after treatment with *B. axarquiensis* TUBP-315GFP was significantly lower, and cotton production increased by 40.6%. With 10^8 CFU/mL of *B. axarquiensis* TUBP-315GFP in the soil, the influence on microbial diversity was the most significant, and the application of *B. axarquiensis* TUBP-315GFP effectively reduced the incidence of cotton Verticillium wilt and increased cotton yield. These results provide a reference for future research direction on biofertilizers. The present

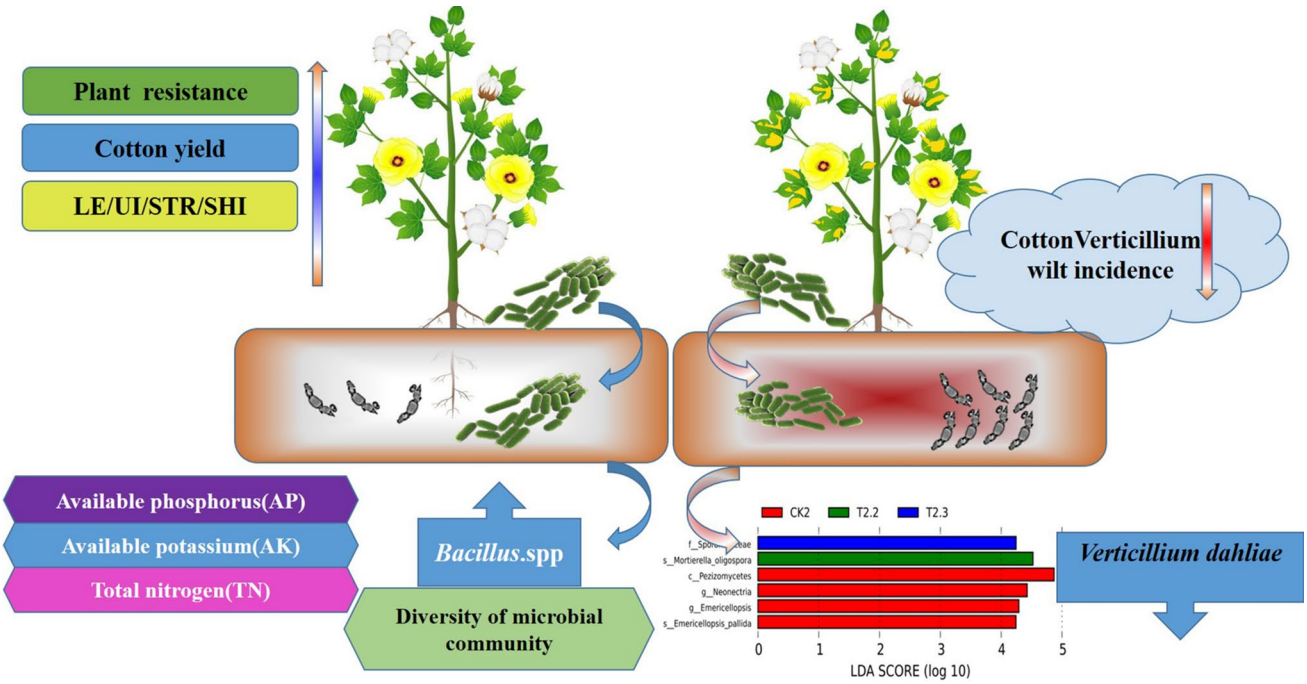


Fig. 7 Outline *Bacillus axarquiensis* TUBP1 reduced cotton Verticillium wilt incidence by altering soil rhizosphere microbial communities

study indicated that the application of *B. axarquiensis* TUBP-315GFP can change the microbial community composition of the cotton rhizosphere soil, thereby reducing the incidence of cotton Verticillium wilt and increasing cotton yield. In the future, compost fermented with *B. axarquiensis* TUBP-315GFP and the detection of beneficial bacteria may be employed to improve the biocontrol of cotton Verticillium wilt disease (Fig. 7).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-021-02618-2.

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Declarations

Conflict of interest All authors declare that they have no conflicts of interest.

Research Involving Human and Animal Participants This article does not contain any studies with human participants or animals performed by any of the authors.

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