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Effect of Rhizobacterium *Rhodopseudomonas palustris* Inoculation on *Stevia rebaudiana* Plant Growth and Soil Microbial Community

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ABSTRACT

There is an increasing concern that the continuous use of chemical fertilizers might lead to harmful effects on soil ecosystem. Accordingly, a biocompatible approach involving inoculation of beneficial microorganisms is presented to promote plant growth and simultaneously minimize the negative effect of chemical fertilizers. In this study, *Rhodopseudomonas palustris*, a plant growth-promoting rhizobacterium (PGPR), was inoculated into both fertilized and unfertilized soils to assess its influence on *Stevia rebaudiana* plant growth and microbial community in rhizosphere soils in a 122-d field experiment. Soil enzyme assays (dehydrogenase, urease, invertase, and phosphomonoesterase), real-time quantitative polymerase chain reaction (RT-qPCR), and a high-throughput sequencing technique were employed to determine the microbial activity and characterize the bacterial community. Results showed that the *R. palustris* inoculation did not significantly influence *Stevia* yields and root biomass in either the fertilized or unfertilized soil. Chemical fertilization had strong negative effects on soil bacterial community properties, especially on dehydrogenase and urease activities. However, *R. palustris* inoculation counteracted the effect of chemical fertilizer on dehydrogenase and urease activities, and increased the abundances of some bacterial lineages (including Bacteroidia, Nitrospirae, Planctomycetacia, Myxococcales, and Legionellales). In contrast, inoculation into the unfertilized soil did not significantly change the soil enzyme activities or the soil bacterial community structure. For both the fertilized and unfertilized soils, *R. palustris* inoculation decreased the relative abundances of some bacterial lineages possessing photosynthetic ability, such as Cyanobacteria, Rhodobacter, Sphingomonadales, and Burkholderiales. Taken together, our observations stress the potential utilization of *R. palustris* as PGPR in agriculture, which might further ameliorate the soil microbial properties in the long run.

Key Words: bacterial community structure, beneficial microorganism, chemical fertilization, plant growth-promoting rhizobacterium, soil enzyme activity

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INTRODUCTION

An ever-increasing global population demands the application of efficient strategies to maintain high agricultural productivity. As a result, soil fertilization, especially chemical fertilization, is commonly applied. Since the 1950s, chemical fertilizers have played a major role in increasing crop yields (Francioli *et al.*, 2016). However, their successive and inappropriate application might be harmful to agriculture and may threaten environmental sustainability owing to runoff loss and leaching of phosphorus (P) and nitrogen (N) (Wong *et al.*, 2014) and deterioration of soil physical and biochemical properties (Hartmann *et al.*, 2015). Accordingly, more sustainable approaches are required. Owing to the physiological functions of plant growth-promoting rhizobacteria (PGPR), which include N fixation, P solubilization, and bioactive compound secretion (Nain *et al.*, 2010; Trabelsi *et al.*, 2011), it seems plausible to use PGPR as a substitute for, or additive to, chemical fertilizers. Diverse inoculants have been proven to stimulate plant growth. For example, inoculation with rhizobia increased corn yields by 13.2%–26.6% (Spolaor *et al.*, 2016). Atmospheric N fixers (*e.g.*, *Azospirillum*) directly contribute to plant N nutrition in soil, which is of significance for non-legume species and decreases N fertilizer usage without compromise in crop yield (Ferreira *et al.*, 2013).

Rhodopseudomonas palustris is a species of purple non-sulphur bacteria ubiquitously distributed in soil and wet-field ecosystems. It can utilize a variety of sub-

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stances as carbon (C) and energy sources (Hunter *et al.*, 2009) owing to its surprising metabolic versatility, and it is capable of photoautotrophy, photoheterotrophy and/or chemoheterotrophy under anaerobic-light or microaerobic-light conditions (Xu *et al.*, 2013a). It is one of the few diazotrophs that have alternative nitrogenases, and it excretes phytohormones to ameliorate plant growth (Kim *et al.*, 2004; Kantachote *et al.*, 2005; Kantha *et al.*, 2015). Previous greenhouse and field assessments have demonstrated the positive effects of *R. palustris* on the plant height and root length of rice (Kantha *et al.*, 2015; Kantachote *et al.*, 2016), the growth of *Stevia* (Wu *et al.*, 2013), the dry weight of tomato shoots (Lee*et al.*, 2008), and the development of cabbage seedlings (Wong *et al.*, 2014). Those outcomes suggest that *R. palustris* could be used as a beneficial inoculant in agriculture.

However, most of the existing studies focus on plant responses to *R. palustris* inoculation, with less emphasis on its effect on soil properties, especially soil microorganisms. As indispensable components of soil, microorganisms play important roles in soil biogeochemical processes such as organic matter decomposition and nutrient cycling. They are sensitive to environmental disturbances and therefore may often serve as an indicator of soil quality. A previous study showed that the soil bacterial community is not profoundly shifted with the inoculation of *Rhodopseudomonas* spp., as assessed by denaturing gradient gel electrophoresis (DGGE) (Lee *et al.*, 2008). This phenomenon could be partially explained by the low resolution of DGGE, whose gel bands typically represent numerically-dominant taxa, and which cannot detect community shifts at a fine scale. Currently, advances in high-throughput sequencing techniques have provided the means for enormous sequence datasets to be generated, allowing for the exploration of microbial consortiums at both the community and taxon levels. With the aid of high-throughput sequencing, more exhaustive information can be gathered in regard to the properties of soil microbes.

Stevia rebaudiana (Bertoni) is an economically important plant in huge demand in the pharmaceutical and food industries owing to its low calorie content and high sweetness (Xu *et al.*, 2016). During the process of sweetener extraction from *Stevia* leaves, a large quantity of wastewater, abundant in low-molecular-mass compounds, is produced. This wastewater, from the perspective of sustainability, might be a suitable substrate for the cultivation of beneficial microorganisms. Indeed, our earlier assay has proven the feasibility of using such wastewater as culture media for *R. palustris* and, more importantly, that *R. palustris* excretes more phytohormones (*e.g.*, indole-3-acetic acid) when cultivated in such wastewater than it does in chemical media (Xu *et al.*, 2013a). Consequently, we are inspired to further evaluate the effect of *R. palustris* inoculation on *Stevia* plant growth and rhizosphere properties.

In this study, we hypothesized that in both fertilized and unfertilized soils, the inoculation of *R. palustris* could: i) promote *Stevia* growth; ii) enhance soil enzyme activities; and iii) change the abundances of some key guilds in the bacterial community that relate to C and N cycling in soil. To test our hypotheses, a field experiment was carried out at an upland site of eastern China, where *Stevia* plants had been cropped for 3 years with chemical fertilizers. The yield of *Stevia* leaves, the soil enzyme activities (dehydrogenase, invertase, urease, and phosphomonoesterase), and the bacterial composition were determined. These results are expected to increase our knowledge of microbial feedback resulting from *R. palustris* inoculation in soil.

MATERIALS AND METHODS

Site description

The field experiment was carried out in an upland at Zhucheng City (36°40′ N, 119°34′ E), Shandong Province, China. This region has an average annual precipitation of 750 mm and a mean annual temperature of 12 °C. The soil of the study site, a lime concretion black soil, had physicochemical properties in the surface layer (0–15 cm) as follows: 12.8 g kg^{-1} of organic matter, 7.4 g kg^{-1} of total N (TN), 37.9 mg kg^{-1} of available P, 365.1 mg kg^{-1} of available potassium (K), and pH 7.40 (soil:water = 1:2.5).

Preparation of bacterial suspensions

Rhodopseudomonas palustris used in this study was isolated in our laboratory. For *R. palustris* enrichment, a chemical synthetic medium was used, comprising (per liter) 4 g D,L-malic acid, 1 g NH_4Cl, 7.5 mL phosphate buffer (0.2 mol L^{-1}, pH 7.0), 0.2 g $MgSO_4\cdot 7H_2O$, 0.075 g $CaCl_2\cdot 2H_2O$, 0.012 g $FeSO_4\cdot 7H_2O$, 0.04 g ethylenediaminetetraacetic acid (EDTA), 1 mL trace element solution (g L^{-1}, $MnSO_4\cdot 4H_2O$ 2.1, H_3BO_3 2.8, $Cu(NO_3)_2\cdot 7H_2O$ 0.04, $ZnSO_4\cdot 7H_2O$ 0.24, and $Na_2MoO_4\cdot 2H_2O$ 0.75), and 1 mL vitamin solution (g L^{-1}, nicotinic acid 10, vitamin B_1 5, and biotin 0.1) (Xu *et al.*, 2013a). Subsequently, the bacteria were cultured at 28 ± 4 °C under a 60-W incandescent lamp at a distance of 25 cm. The *R. palustris* cells were first obtained by centrifugation (10 000 × g for 15 min), then re-suspended in sterile water and adjusted to 10^7

cells mL^{-1}, and determined with a spectrophotometer at 660 nm. The *R. palustris* suspensions were then used in the field experiment.

Experimental design and soil sampling

The experiment adopted a split-plot design with three replications. The main plots were either chemical fertilization or no-fertilizer control, denoted as CF and CK, respectively. In the chemical fertilization, N, P, and K fertilizers were applied in the form of urea (108 kg N ha^{-1}), superphosphate (60 kg P_2O_5 ha^{-1}), and potassium sulfate (130 kg K_2O ha^{-1}), respectively. The subplots were *R. palustris* treatments in the CF plots and CK plots, denoted as CF+Rp and CK+Rp, respectively. On the 60th, 67th, 74th, and 81st d after *Stevia* seedlings were transplanted, the *R. palustris* suspensions (10^7 cells mL^{-1}, 100 mL) were irrigated into the ditch around each plant (*ca.* 5 cm from the main stem) in CF+Rp and CK+Rp, while the equivalent volumes of water were irrigated in CF and CK. There were a total of 20 *Stevia* plants receiving *R. palustris* inoculation in each subplot of CF+Rp and CK+Rp.

After 122-d growth, the *Stevia* plants were harvested, and the weights of the fresh roots and the oven-dried leaves of each plant were determined. Meanwhile, samples of rhizosphere soil were collected. In each subplot, six plants were randomly selected, and the soil adhering to the roots was removed by shaking gently. After debris and plant material were removed, the soils from the roots of the six plants were mixed to form one composite sample and sieved (< 2 mm). Each sample was then divided into two portions: one kept at 4 °C for analysis of soil enzyme activities and physicochemical properties and the other stored at −80 °C for DNA extraction.

Soil characteristic analysis and soil enzyme activity assays

Soil pH was measured using a soil-to-water ratio of 1:2.5 (weight/volume) with a glass electrode. Soil total organic C (TOC) and TN were determined with an elemental analyzer (Elementar VarioEL III, Germany). The soil enzyme activity assays used were described in our previous study (Xu *et al.*, 2013b). In short, soil dehydrogenase activity was determined by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to 2,3,5-triphenyl formazan (TPF), urease activity using the indophenol blue colorimetric method, invertase activity using the 3,5-dinitrosalicylic acid method, and phosphomonoesterase activity based on *p*-nitrophenol release after cleavage of disodium phenyl phosphate.

DNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

DNA was extracted from 0.5 g soil samples using a Fast DNA® SPIN Kit for soil (MP Biomedicals, USA), according to the manufacturer's instructions. The abundances of bacterial 16S rRNA gene fragments were quantified by RT-qPCR using the primer set 519F/907R (519F: 5′-CAGCMGCCGCGGTAATWC-3′; 907R: 5′-CCGTCAATTCMTTTRAGTTT-3′) (Biddle *et al.*, 2008). Standard curves were obtained using 10-fold serial dilutions of the *Escherichia coli*-derived vector plasmid pMD18-T (TaKaRa, China) containing a cloned target gene, from 10^2 to 10^8 gene copies $\mu\mathrm{L}^{-1}$. The reactions were performed in a C1000™ thermal cycler equipped with a CFX96™ real-time system (Bio-Rad, USA). The 25-μL reaction mixture contained 12.5 μL of SYBR® Premix Ex Taq™ (TaKaRa, China), primer sets (0.5 μmol L^{-1} each), 200 ng bovine serum albumin (BSA) $\mu\mathrm{L}^{-1}$, and 1.0 μL template containing approximately 2–9 ng DNA. Sterilized water instead of soil DNA extract was used as the negative control. The qPCR program was: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s (plate reading). The specificity of the amplification products was assessed by melting curve analysis, and the sizes of the amplified fragments were checked on a 1.5% agarose gel. The RT-qPCR was performed in triplicate for each sample, and amplification efficiencies of 97.4%–104% with R^2 values of 0.990–0.997 were obtained.

Bacterial 16S rRNA gene PCR and high-throughput sequencing

Soil bacterial 16S rRNA genes were amplified using the universal primer set 519F/907R (Biddle *et al.*, 2008). To perform sequencing with the MiSeq sequencing system (Illumina Inc., USA), primers were tagged with unique 5-bp barcodes for each replicate DNA sample. The PCR reactions were carried out using a 50-μL reaction mixture containing each deoxynucleoside triphosphate at a concentration of 1.25 μmol L^{-1}, 2 μL (15 μmol L^{-1}) forward and reverse primers, 2 units (U) of Taq DNA polymerase (TaKaRa, China), and 1 μL (50 ng) of microbial genomic DNA as a template. The thermal cycling was as follows: 35 cycles of 95 °C for 45 s, 58 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. Negative controls using sterilized water instead of soil DNA were included to check for primer or sample DNA contamination. Each DNA sample was amplified in three technical replicates. Reaction products were then pooled, purified using a QIAquick PCR Purification Kit (QIAGEN, Germany), and quantified with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA). The bar-coded PCR products from all samples were normalized in equimolar amounts, prepared using a TruSeq^{TM} DNA Sample Prep LT Kit (Illumina, USA), and sequenced using a MiSeq Reagent Kit V2 (Illumina, USA) on a MiSeq platform following the manufacturer's protocols.

Processing of the high-throughput sequencing data

After sequencing was completed, 16S rRNA gene data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline using default parameters unless otherwise stated (Caporaso *et al.*, 2010). Reads shorter that 200 bp or with quality scores below 20 were discarded, and the remaining reads binned according to their unique 5-bp barcodes. Operational taxonomic units (OTUs) were generated following the UPARSE pipeline, using USEARCH with a sequence similarity cut-off of 97% (Edgar, 2017); chimeric sequences were also removed. The OTUs were taxonomically classified against a subset of the Silva 119 database (http://www.arb-silva.de/download/archive/qiime/) using PyNAST.

There were 512 409 16S rRNA gene sequences that passed QIIME quality filtering. We obtained 13 200–66 675 sequences per sample for all the soil samples. Sequence read number was normalized by random subsampling to 13 000 reads per sample for both alpha-diversity and beta-diversity analysis.

Data calculation and statistical analysis

For the high-throughput data, phylogenetic diversity (PD) and Chao1 indices were calculated after equalization at a depth of 13 000 reads using QIIME. Nonmetric multidimensional scaling (nMDS) analyses were performed on the Bray-Curtis dissimilarity for the bacterial community data (OTU level) using the vegan package in R program version 3.3.3 (R Development Core Team, 2017). Analysis of similarities (ANOSIM) (Clarke, 1993) was performed to quantitatively compare the community differences between different groups, based on Bray-Curtis dissimilarity. The number of permutation tests was set at 999.

To understand which specific microbial populations were affected by *R. palustris* inoculation, the response ratio was calculated based on the relative abundances of classes or proteobacterial orders (with proportions larger than 0.1%), following the statistical method of Luo *et al.* (2006). If the 95% confidence interval (CI) range of a specific bacterial group is higher than zero (not including zero), it can be concluded that it responds positively and significantly. Alternatively, negative 95% CI equates to a negative and significant response.

Plant yields, root biomass, soil enzyme activities, gene copy number, and the relative abundances of bacterial taxa between samples were tested for normal distribution using the Shapiro-Wilk test. Homogeneity of variances was checked with Levene's test. If the data were not normally distributed, variables were log-transformed as required to normalize the distributions prior to statistical analysis (Qin *et al.*, 2014). Following this, the influence of *R. palustris* inoculation and chemical fertilization on the stated parameters were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's test, using SPSS software version 19.0 (IBM Corporation, USA), and P values of 5% or less were considered statistically significant.

RESULTS

Soil physicochemical properties and Stevia plant growth

For soil pH, TOC, and TN, no significant differences were found between any of the treatments ($P > 0.05$) (Table I). Compared to CK, CK+Rp slightly increased the dry weight of *Stevia* leaves by 3.2%, but the difference was not statistically significant ($P > 0.05$). A similar trend was found for CF+Rp, with an increase of 4.2% as compared to CF ($P > 0.05$). By contrast, CF significantly increased the leaf yield by 29.1% in comparison with CK ($P < 0.05$). In terms of root biomass, no significant differences were detected between any of the treatments ($P > 0.05$).

Soil enzyme activities

For soil enzyme activities, however, it was noticed that CF significantly decreased the activities of dehydrogenase and urease, by 82.1% and 16.7%, respectively, when compared to CK ($P < 0.05$) (Table II). When *R. palustris* was inoculated into fertilized soil (CF+Rp), however, these same parameters were nearly comparable to those in unfertilized soil with or without inoculation (CK and CK+Rp). Invertase activity was found to be significantly lower in both CF and CF+Rp ($P < 0.05$). No significant differences in phosphomonoesterase activity were observed between any of the treatments. In contrast, *R. palustris* inoculation into unfertilized soil (CK+Rp) did not significantly change any of the measured enzyme activities ($P > 0.05$).

Soil bacterial 16S rRNA gene copy number and diversity

The RT-qPCR results showed that the average copy numbers of bacterial 16S rRNA genes varied from 3.10×10^{10} to 3.69×10^{10} g^{-1} soil dry weight (Table III). No significant differences were observed between the treatments ($P > 0.05$). In terms of soil PD and Chao1 indices, CF and CF+Rp significantly decreased these two parameters compared to CK and CK+Rp ($P < 0.05$). In contrast, *R. palustris* inoculation did not significantly change these two indices in comparison with those without inoculation, a result which differed from the patterns exhibited in the soil enzyme assays.

Shifts in soil bacterial taxonomic distribution

After aligning the OTUs with the Silva database, a total of 21 phyla were classified. Across the 12 samples, the dominant phyla were Proteobacteria (24.9%–38.2%), Acidobacteria (13.9%–18.5%), Actinobacteria (13.5%–17.1%), Chloroflexi (5.5%–6.4%), Firmicutes

TABLE I

Soil physicochemical properties and *Stevia rebaudiana* plant growth in different treatments

Treatment[a)]	pH	Total organic carbon	Total nitrogen	Dry weight of *Stevia* leaves	Fresh biomass of *Stevia* roots
		g kg^{-1}		kg $plot^{-1}$	
CK	5.74 ± 0.15[b)]a[c)]	8.42 ± 0.32a	7.41 ± 0.03a	4.92 ± 0.07c	15.12 ± 1.05a
CK+Rp	5.44 ± 0.22a	8.95 ± 0.41a	7.42 ± 0.04a	5.13 ± 0.21bc	16.12 ± 0.72a
CF	5.62 ± 0.09a	8.53 ± 0.38a	7.43 ± 0.02a	6.32 ± 0.14ab	16.53 ± 1.54a
CF+Rp	5.52 ± 0.12a	8.62 ± 0.35a	7.42 ± 0.02a	6.48 ± 0.19a	15.87 ± 0.96a

[a)] CK = no-fertilizer control; CK+Rp = CK plus *Rhodopseudomonas palustris* inoculation; CF = chemical fertilization; CF+Rp = CF plus *R. palustris* inoculation.
[b)] Mean ± standard deviation ($n = 3$).
[c)] Mean values in a column followed by different letters are significantly different at $P < 0.05$ (Tukey's test after two-way analysis of variance).

TABLE II

Soil enzyme activities in different treatments

Treatment[a)]	Dehydrogenase activity	Urease activity	Invertase activity	Phosphomonoesterase activity
	μg TFP[b)] g^{-1} soil d^{-1}	mg NH_4-N g^{-1} soil d^{-1}	mg glucose g^{-1} soil d^{-1}	mg *p*-nitrophenol g^{-1} soil d^{-1}
CK	9.89 ± 1.79[c)]a[d)]	0.48 ± 0.02a	10.14 ± 2.08ab	2.46 ± 0.19a
CK+Rp	13.85 ± 3.74a	0.50 ± 0.03a	13.65 ± 2.83a	2.62 ± 0.43a
CF	5.74 ± 2.77b	0.42 ± 0.03b	7.89 ± 0.81b	2.49 ± 0.12a
CF+Rp	10.45 ± 2.55a	0.49 ± 0.01a	8.48 ± 1.45b	2.48 ± 0.31a

[a)] CK = no-fertilizer control; CK+Rp = CK plus *Rhodopseudomonas palustris* inoculation; CF = chemical fertilization; CF+Rp = CF plus *R. palustris* inoculation.
[b)] Triphenyl formazan.
[c)] Mean ± standard deviation ($n = 3$).
[d)] Mean values in a column followed by different letters are significantly different at $P < 0.05$ (Tukey's test after two-way analysis of variance).

TABLE III

Soil bacterial 16S rRNA gene abundance determined by real-time quantitative polymerase chain reaction and the biodiversity indices (Chao1 and phylogenetic diversity) calculated from high-throughput sequencing under different treatments

Treatment[a)]	16S rRNA gene copy number	Chao1	Phylogenetic diversity
	$\times 10^{10}$ g^{-1} soil dry weight		
CK	3.22 ± 0.13[b)]a[c)]	28 297.6 ± 1 530.6a	546.4 ± 8.9a
CK+Rp	3.35 ± 0.21a	31 360.2 ± 4 496.8a	532.4 ± 37.8a
CF	3.69 ± 0.16a	23 229.0 ± 2 302.9b	463.5 ± 17.8b
CF+Rp	3.10 ± 0.52a	24 585.6 ± 1 277.8b	478.1 ± 7.9b

[a)] CK = no-fertilizer control; CK+Rp = CK plus *Rhodopseudomonas palustris* inoculation; CF = chemical fertilization; CF+Rp = CF plus *R. palustris* inoculation.
[b)] Mean ± standard deviation ($n = 3$).
[c)] Mean values in a column followed by different letters are significantly different at $P < 0.05$ (Tukey's test after two-way analysis of variance).

(4.6%–6.5%), Bacteroidetes (4.5%–6.5%), Planctomycetes (2.6%–4.3%), Nitrospirae (1.7%–2.5%), Cyanobacteria (1.1%–8.8%), and Gemmatimonadetes (0.9%–1.5%) (Fig. 1). Several phyla, including Armatimonadetes, Chlorobi, and Elusimicrobia, were identified as minor groups with relative abundances under 0.47% (Fig. 1). At the phylum level, no significant differences were observed for the dominant phyla between the treatments ($P > 0.05$).

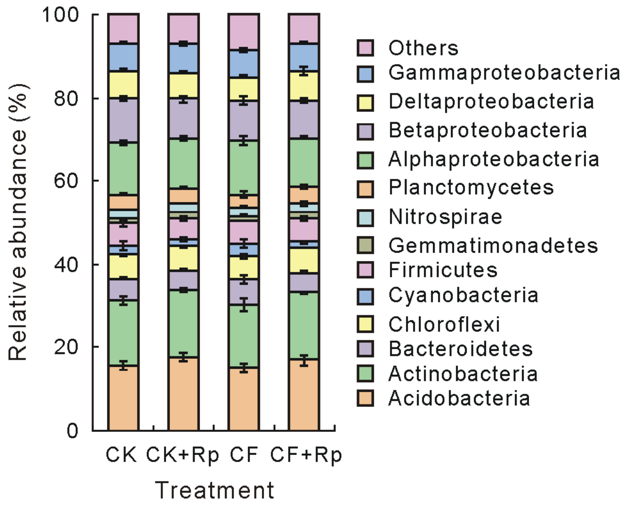

Fig. 1 Stacked column chart of relative abundances of the dominant bacterial phyla derived from 16S rRNA genes in soil of different treatments. CK = no-fertilizer control; CK+Rp = CK plus *Rhodopseudomonas palustris* inoculation; CF = chemical fertilization; CF+Rp = CF plus *R. palustris* inoculation.

The nMDS plot (stress = 0.084) (Fig. 2), illustrating the effect of *R. palustris* inoculation on bacterial community structure, demonstrated the separation of CF from the other treatments along the horizontal axis. In addition, a clear separation was found between CF+Rp and CF, whereas no obvious difference was detected between CK+Rp and CK. The test of community dissimilarity (Bray-Curtis) also showed that bacterial communities in CF were significantly different from those in the other treatments (ANOSIM $R = 0.4123$, $P = 0.012$), indicating a shift in bacterial community composition. In contrast, *R. palustris* inoculation did not significantly influence the community structure, as indicated by the ANOSIM results (ANOSIM $R = 0.1241$, $P = 0.141$).

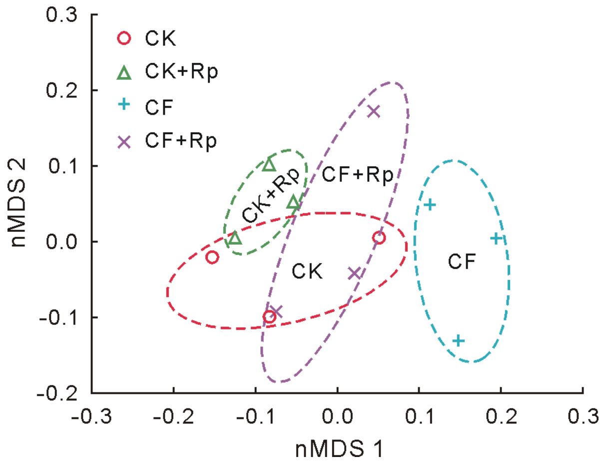

Fig. 2 Non-metric multidimensional scaling (nMDS) using Bray-Curtis dissimilarity matrix across all soil samples, illustrating the effect of *Rhodopseudomonas palustris* inoculation on bacterial community structure in soil of different treatments. CK = no-fertilizer control; CK+Rp = CK plus *Rhodopseudomonas palustris* inoculation; CF = chemical fertilization; CF+Rp = CF plus *R. palustris* inoculation.

In order to compare the pairwise variations in soil bacterial community, the lineages with relatively high abundances (larger than 0.1% at the level of class or Proteobacterial order) were selected and used to calculate the response ratios between CK and CK+Rp and between CF and CF+Rp (Fig. 3). Among the 38 selected bacterial lineages, 15 lineages responded positively and 16 lineages responded negatively to CK+Rp; correspondingly, the responsive lineages to CF+Rp were 15 and 15, respectively. In both the fertilized and unfertilized soils, some bacterial groups were stimulated by *R. palustris* inoculation, including Gemmatimonadetes and Chloroflexia, while some others were negatively influenced, *e.g.*, Cyanobacteria, Rhodobacterales, Sphingomonadales, and Burkholderiales. Some lineages, such as Bacteroidia, Nitrospirae, Planctomycetacia, Myxococcales, and Legionellales, responded positively to CF+Rp, but negatively or neutrally to CK+Rp. Moreover, although lots of the lineages exhibited similar responses to CF as compared to CK (Fig. 4a) and to CF+Rp as compared to CK (Fig. 4b), some lineages demonstrated divergent responses. For example, Nitrospira, Planctomycetacia, Myxococcales and Thermoleophilia responded negatively to CF, but positively or neutrally to CF+Rp. It is noteworthy that the genus *Rhodopseudomonas* (from the order Rhizobiales) was not significantly influenced by *R. palustris* inoculation either with or without fertilization.

DISCUSSION

Absence of change in Stevia plant with R. palustris inoculation

With or without oxygen, *R. palustris* can grow and use light, inorganic, or organic compounds for energy. It can also excrete auxin and cytokinin, which benefit plant development (Nunkaew *et al.*, 2014). However, in this study the inoculation of *R. palustris* resulted in minor, insignificant increases in the dry weight of *Ste-*

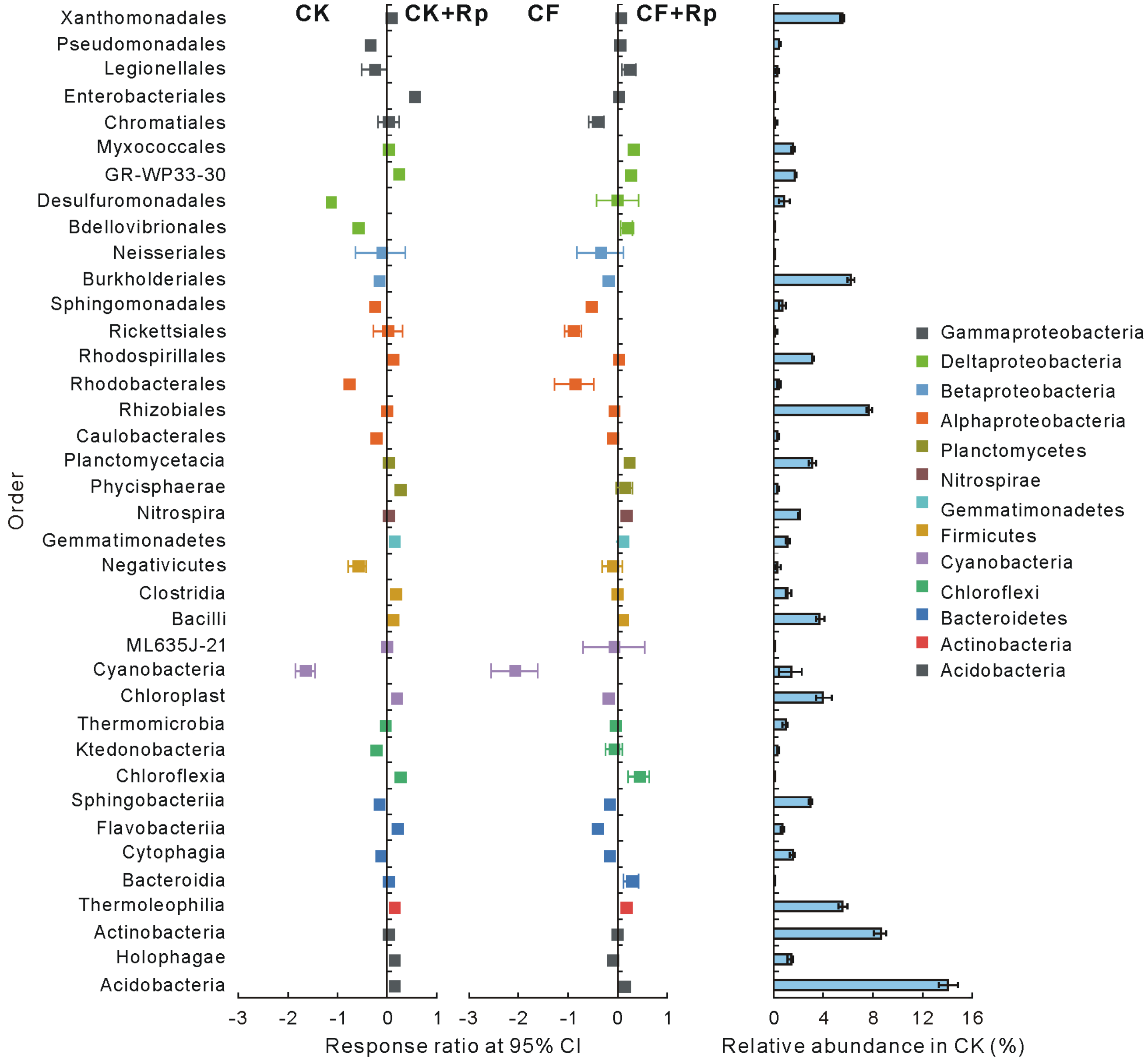

Fig. 3 Changes of the dominant orders in soil bacterial community between CK+Rp and CK and between CF+Rp and CF detected using the response ratio method at 95% confidence interval (CI), as well as their relative abundance in CK, where CK = no-fertilizer control, CK+Rp = CK plus *Rhodopseudomonas palustris* inoculation, CF = chemical fertilization, CF+Rp = CF plus *R. palustris* inoculation.

via leaves and fresh root biomass, regardless of whether the soils were fertilized or not (Table I). This result did not support our hypothesis that *R. palustris* inoculation might promote *Stevia* growth. In a study of Lee *et al.* (2008), a high increase (up to 34.6%) in tomato shoot length was observed after weekly inoculation of 400 mL *Rhodopseudomonas* sp. suspension (10^7 cells mL^{-1}) for each plant in a pot experiment, a dose nearly 10-fold higher than that of the current study. In another field investigation (Kantachote *et al.*, 2016), *R. palustris* strains (TK103, TN114, and PP803) significantly stimulated rice growth in the fertilized treatment, but had a marginal effect on the unfertilized one. In this regard, inoculation rate and soil management strategy are important factors influencing the outcome of *R. palustris* inoculation, and further study is warranted to investigate its effect at varying inoculation rates or under different environmental conditions.

Changes in soil enzyme activities

Soil enzyme activities can serve as indicators of soil quality. Dehydrogenase activity indicates the overall soil microbial activity, while invertase, urease, and phosphomonoesterase activities are closely related to

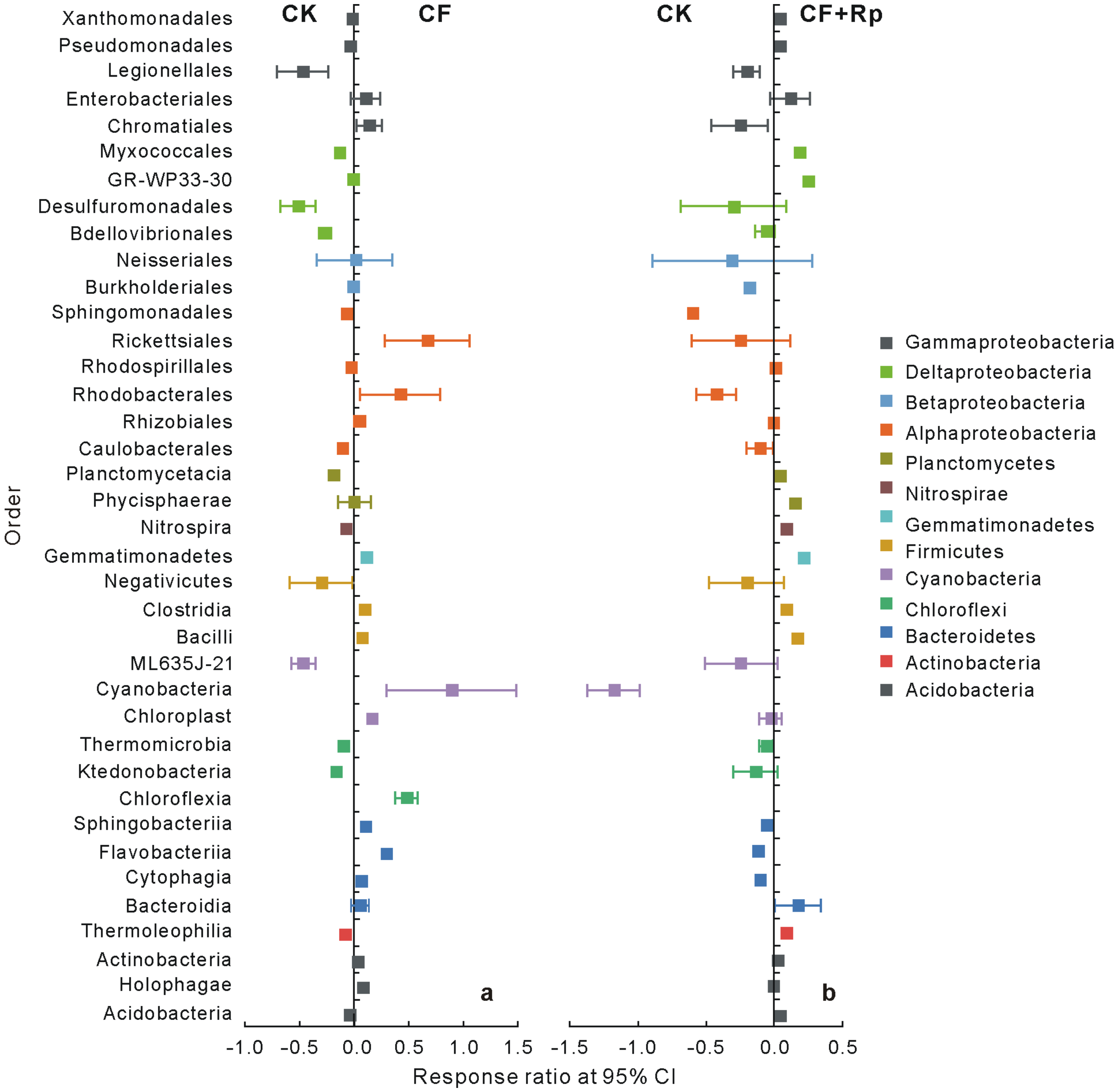

Fig. 4 Changes of the dominant order in soil bacterial community between the chemical fertilization treatment (CF) and the no-fertilizer control (CK) (a) and those between the chemical fertilization plus *Rhodopseudomonas palustris* inoculation treatment (CF+Rp) and CK (b), detected using the response ratio method at 95% confidence interval (CI).

the C, N, and P cycling in soil (Nannipieri *et al.*, 2003). In this study, CF significantly decreased the activities of urease and dehydrogenase compared to CK ($P < 0.05$) (Table II). When *R. palustris* was inoculated into fertilized soil (CF+Rp), these parameters were significantly higher than those without inoculation (CF) ($P < 0.05$), and reached the levels found in unfertilized soil without inoculation (CK). This feature implied that *R. palustris* inoculation could counteract the overall negative effect that chemical fertilization had on microbial activity (dehydrogenase) and the N cycle. However, this was not always true, as a similar trend was not observed for invertase activity. Based on these results, we might infer that *R. palustris* inoculation might not improve all the enzyme activities in fertilized soil, but does counteract the negative effect of chemical fertilization.

Shifts in bacterial community with R. palustris inoculation

In CF, the Chao1 and PD indices decreased significantly compared to those in CK (Table III). Nevertheless, in contrast to the trends observed in soil enzyme activities, the Chao1 and PD indices were not significantly influenced by *R. palustris* inoculation in either the fertilized or unfertilized soil. This implied that *R.*

palustris inoculation did not help to restore the soil bacterial richness and diversity negatively affected by chemical fertilizer application.

Taxonomic analysis revealed that *R. palustris* inoculation changed the bacterial community structure (Fig. 3). Some bacterial lineages, *e.g.*, Chloroflexia and Bacilli (Firmicutes), were stimulated by *R. palustris* inoculation with or without fertilization. These bacterial lineages are involved in C cycling in soil. For example, Bacilli can degrade recalcitrant C sources, such as cellulose and chitin (Shange *et al.*, 2012; Sapp *et al.*, 2015), and produce a vast array of antimicrobial and xenobiotic compounds (Owen *et al.*, 2015; Zhou *et al.*, 2015). Chloroflexi are likely specialized in the degradation of ancient organic matter in soil (Fierer *et al.*, 2007). By pairwise comparison of the bacterial lineages between CF and CF+Rp and between CK and CK+Rp (Fig. 3), some taxa, including Nitrospirae, Legionellales, and Myxococcales, were found to respond positively to CF+Rp, but neutrally or negatively to CK+Rp. Nitrospirae are known to participate in nitrification (Baker *et al.*, 2013) and stimulate N transformation and cycling (Eo and Park, 2016). Legionellales are involved in ammonium assimilation and are associated with higher plant yields (Vishnivetskaya *et al.*, 2013). Myxococcales act as micro-predators in the soil microbial food web and play important roles in soil C sequestration (Li *et al.*, 2017). As such, the elevated proportions of these taxa indicated that soil biochemical processes, such as nitrification and the turnover of organic matter, might be recovering from the effect of chemical fertilizer application. This was also coherent with the increased dehydrogenase and urease activities in CF+Rp, confirming our hypothesis that *R. palustris* inoculation could enhance soil enzyme activities and change some key guilds in the soil bacterial community. It was noteworthy that some bacterial lineages, *e.g.*, Nitrospirae, Planctomycetacia, Myxococcales and Thermoleophilia, were negatively influenced by CF, but responded neutrally or positively to CF+Rp (Fig. 4). This pattern implied that different microbial processes are induced by chemical fertilizer application and *R. palustris* inoculation, with the latter potentially counteracting the effect of the former.

In both the fertilized and unfertilized treatments, some key bacterial guilds in soil, such as Cyanobacteria, *Rhodobacter*, Sphingomonadales (Alphaproteobacteria), and Burkholderiales (Betaproteobacteria), exhibited negative responses to *R. palustris* inoculation (Fig. 3). Most of these bacterial lineages possess photosynthetic abilities. For example, some strains from the Sphingomonadales are classified as phototrophs owing to the presence of chlorophyll α (Abbasian *et al.*, 2015). Burkholderiales and *R. palustris* both belong to the purple non-sulfur bacteria because of their inability to use reduced sulfur compounds as electron donors (Hallenbeck, 2017). Also, most of the phototrophic Cyanobacteria require nutrients specifically related to the fixation of C or N (Nain *et al.*, 2010). Owing to the limited availability of some nutrients (such as N) for metabolism in phototrophs (Idi *et al.*, 2015), we suppose that competition might exist between those bacterial lineages in this study, subsequently leading to the suppression on them. An example for this is the inhibition of nitrifying bacteria (*e.g.*, Nitrospirae) in the presence of Cyanobacteria (Choi *et al.*, 2010).

It should be noted that the relative abundance of *Rhodopseudomonas* was not significantly different between any of the treatments (Fig. 3). One possible explanation was that our soil samples were collected on the 40th d after the final inoculation, which was a lengthy period for *R. palustris* to maintain a high level of activity, especially in the upland soil. Therefore, further investigation based on time-dependent series would be helpful in understanding the influence of *R. palustris* inoculation on soil microorganisms.

CONCLUSIONS

In this study, the *R. palustris* inoculation did not influence *Stevia* yields and root biomass in either the fertilized or unfertilized soil. The *R. palustris* inoculation to the fertilized soil elevated the activities of dehydrogenase and urease to the levels detected in the no-fertilizer control, suggesting the resilience of soil properties. In the soil bacterial community, although some lineages were consistently influenced by *R. palustris* inoculation in both the fertilized soil and no-fertilizer control, there were some lineages responding to *R. palustris* inoculation to a greater extent in the fertilized soil than in the no-fertilizer control. The changes in microbial activity and bacterial community structure suggested that *R. palustris* inoculation assists soil community recovery from the negative effects of chemical fertilization.

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