New Phytologist

Bio-organic soil amendment promotes the suppression of *Ralstonia solanacearum* by inducing changes in the functionality and composition of rhizosphere bacterial communities

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Summary

- Stimulating the development of soil suppressiveness against certain pathogens represents a sustainable solution toward reducing pesticide use in agriculture. However, understanding the dynamics of suppressiveness and the mechanisms leading to pathogen control remain largely elusive.
- Here, we investigated the mechanisms used by the rhizosphere microbiome induces bacterial wilt disease suppression in a long-term field experiment where continuous application of bio-organic fertilizers (BFs) triggered disease suppressiveness when compared to chemical fertilizer application. We further demonstrated in a glasshouse experiment that the suppressiveness of the rhizosphere bacterial communities was triggered mainly by changes in community composition rather than only by the abundance of the introduced biocontrol strain.
- Metagenomics approaches revealed that members of the families Sphingomonadaceae and Xanthomonadaceae with the ability to produce secondary metabolites were enriched in the BF plant rhizosphere but only upon pathogen invasion. We experimentally validated this observation by inoculating bacterial isolates belonging to the families Sphingomonadaceae and Xanthomonadaceae into conducive soil, which led to a significant reduction in pathogen abundance and increase in nonribosomal peptide synthetase gene abundance.
- We conclude that priming of the soil microbiome with BF amendment fostered reactive bacterial communities in the rhizosphere of tomato plants in response to biotic disturbance.

Introduction

The soil microbiome is a silent ally for various ecosystem services, including the protection of plants against abiotic and biotic stresses (Bender *et al.*, 2016). Suppressive soils offer microbiome-mediated protection for crop plants against soil-borne pathogen infections (Schroth & Hancock, 1982; Mendes *et al.*, 2011; Cha *et al.*, 2016), contribute to reduced chemical pesticide application and promote sustainable agriculture. The mechanisms associated with disease suppression in soils include general suppression via competitive interactions of the overall soil microbiome and/or specific suppression owing to direct antagonism and competition with specific group(s) of microorganisms such as *Pseudomonas*, *Flavobacterial* and *Streptomyces*, which act against a specific pathogen (Raaijmakers & Mazzola, 2016; Kwak *et al.*, 2018).

Several agricultural practices are currently used to induce soil suppressiveness, such as soil tillage, organic amendments and antagonistic agent application (Hoitink & Boehm, 1999; Peters *et al.*, 2003; Haas & Défago, 2005; Kinkel *et al.*, 2011). Among these practices, disease suppression induced by organic amendments and biocontrol agents has been widely explored and shown to induce suppression through both general and specific soil microbiome driven mechanisms (Raaijmakers *et al.*, 2009; Bonanomi *et al.*, 2010). For instance, bio-organic fertilizer (BF), which is a combination of bio-control agents and mature compost, has been indicated to control Fusarium wilt (Shen *et al.*, 2015), Verticillium wilt (Lang *et al.*, 2012) and bacterial wilt (Ding *et al.*, 2013). These combinations of organic and biological amendments modify the soil microbiome, thus promoting general soil suppressiveness against soil-borne plant diseases

rather than direct pathogen inhibition (Fu *et al.*, 2017; Xiong *et al.*, 2017a; Tao *et al.*, 2020). Interestingly, the suppressiveness induced by BF can be transmitted to conducive soil, thus highlighting the potential of this approach (Xiong *et al.*, 2017b). Although many reports have identified the soil suppression mechanism in response to long-term crop monoculture (Mendes *et al.*, 2011; Chapelle *et al.*, 2016; Carrión *et al.*, 2019) or short-term agricultural practices (Deng *et al.*, 2021), the mechanism that promotes soil suppressiveness by long-term soil microbiome manipulation via BF application is unknown. Understanding the changes induced by BF on the microbiome in the bulk soil or in the soil in close association with the plant, i.e. the rhizosphere, represents a key step in developing general approaches to promote disease suppression.

The rhizosphere is the infection court where pathogens establish a parasitic relationship with plants. The rhizosphere microbiome is assembled through dispersal, drift and speciation from the bulk microbiome and selection by plants (Dini-Andreote & Raaijmakers, 2018). Although bulk soils are reservoirs of the microbiome, the success of a pathogen is influenced by the rhizosphere microflora (Raaijmakers *et al.*, 2009; Berendsen *et al.*, 2012). *In situ* studies tracking the abundance of pathogens, such as *Ralstonia solanacearum*, in the rhizosphere of suppressive soil showed that the abundance of invasive pathogens is often lower than that in conducive soil (Wei *et al.*, 2011). This low abundance is associated with higher microbiome diversity (Garbeva *et al.*, 2004; Hu *et al.*, 2016), beneficial microbes (Kwak *et al.*, 2018), and stronger microbial interactions in the suppressive rhizosphere (Mendes *et al.*, 2018). However, the development of the rhizosphere microbiome over time as well as the functional changes of the rhizosphere microbiome in response to practices that induce disease suppression remain less clear. Unraveling the compositional and functional dynamics of the rhizosphere microbiome is relevant in the context of understanding the (disease-induced) recruitment of beneficial microbes and functions associated with plant health in response to invasion by bacterial pathogens.

The purpose of this study was to assess the mechanisms through which the composition and functionality of the resident bacterial communities associated with the rhizosphere of tomato plants, grown under conducive or suppressive soil conditions, impact a single model pathogen, *R. solanacearum*. We first investigated the soil microbiome of a field with a low incidence of bacteria-induced tomato wilt disease, which was induced by the continuous application of BF and compared this microbiome with those from fields that received organic fertilizer (OF) and chemical fertilizer (CF) applications. Based on these results we hypothesized that long-term application of BF will induce soil suppressiveness through microbiome manipulation, but that suppression occurs via mechanisms that are associated with the rhizosphere rather than the bulk soil bacterial community. To address this hypothesis, we started by disentangling the effect of soil nutrients from the microbiome because pathogen suppressiveness can be influenced by soil properties (Duffy *et al.*, 1997). We performed a microcosm experiment in which tomato plants were grown in pots with artificial soils harboring the microbiomes from BF and CF. Once root colonization by the BF or CF microbiome had taken place, the seedlings were transferred to new pots with germ-free substrate and subsequently inoculated with or without *R. solanacearum*. We then followed the development of pathogens as well as shifts in the composition, diversity and function of bacterial communities associated with the tomato rhizosphere soil in response to pathogen inoculation. This approach allowed us to identify potential key players in rhizosphere suppressiveness, which were then isolated and further tested *in vivo* to confirm their activity. Overall, we show that the dynamics of the rhizosphere microbiome, and associated mechanisms drive disease suppression in the rhizosphere manipulated by the application of BF.

Materials and Methods

Description of the long-term bio-organic amendment field experiment description

The long-term field trial was performed in Hengxi town in Nanjing, Jiangsu Province (32°02′N, 118°50′E) which has a subtropical monsoon climate with average annual temperature and precipitation of 15.4°C and 1106 mm, respectively. The continuous trial started in March 2013 and lasted for two seasons each year. Three treatments of the field experiment were selected for this study: CF: 120 kg ha^{-1} nitrogen (N), 180 kg ha^{-1} phosphorus (P) and 120 kg ha^{-1} potassium (K) mineral fertilizers were applied to the soil in this season; OF: 7500 kg ha^{-1} organic fertilizer (1.75% N, 0.82% P and 1.42% K in 2018) was applied; BF: 7500 kg ha^{-1} bio-organic fertilizer (1.85% N, 0.80% P and 1.46% K in 2018) was applied. In addition, the nutrient differences among fertilizers applied to each treatment were compensated using mineral fertilizers as necessary. Each treatment had three independent replicate plots (long × wide = 2 m × 4 m), and each replicate contained 40 tomato plants (*Solanum lycopersicum*). The tomato variety used in this experiment was a kind of commercial cultivar 'Shi Ji Hong Guan', which is one of the early ripening tomato cultivars with round, large, red fruit. The BF was produced by inoculating 5% (v/DW) *Bacillus amyloliquefaciens* T-5 (Tan *et al.*, 2013), which was isolated in our laboratory, into an organic mixture of rapeseed meal and chicken manure (DW/DW = 1 : 4) compost and then fermented for 7 d. The OF was produced using an identical process to the BF but without bacterial inoculation. On the 40th day after transplanting, the plant height and stem diameter (taken at the oldest leaf position) of eight random plants from each plot were recorded. A bioassay for disease incidence was performed during the harvest season based on observations of typical wilt symptoms, including necrosis and leaf drooping. The disease incidence in this study was calculated by counting the number of tomato plants with bacterial wilt among the total number of tomato plants in each plot. One value was obtained from each plot; thus, each treatment had three disease incidence replicates.

Soil samples from these treatments were collected during the harvest season in June 2018 (Fig. 1a). For bulk soil sample collection, soil cores at depths of 0–15 cm from five points (*c.* 500 g

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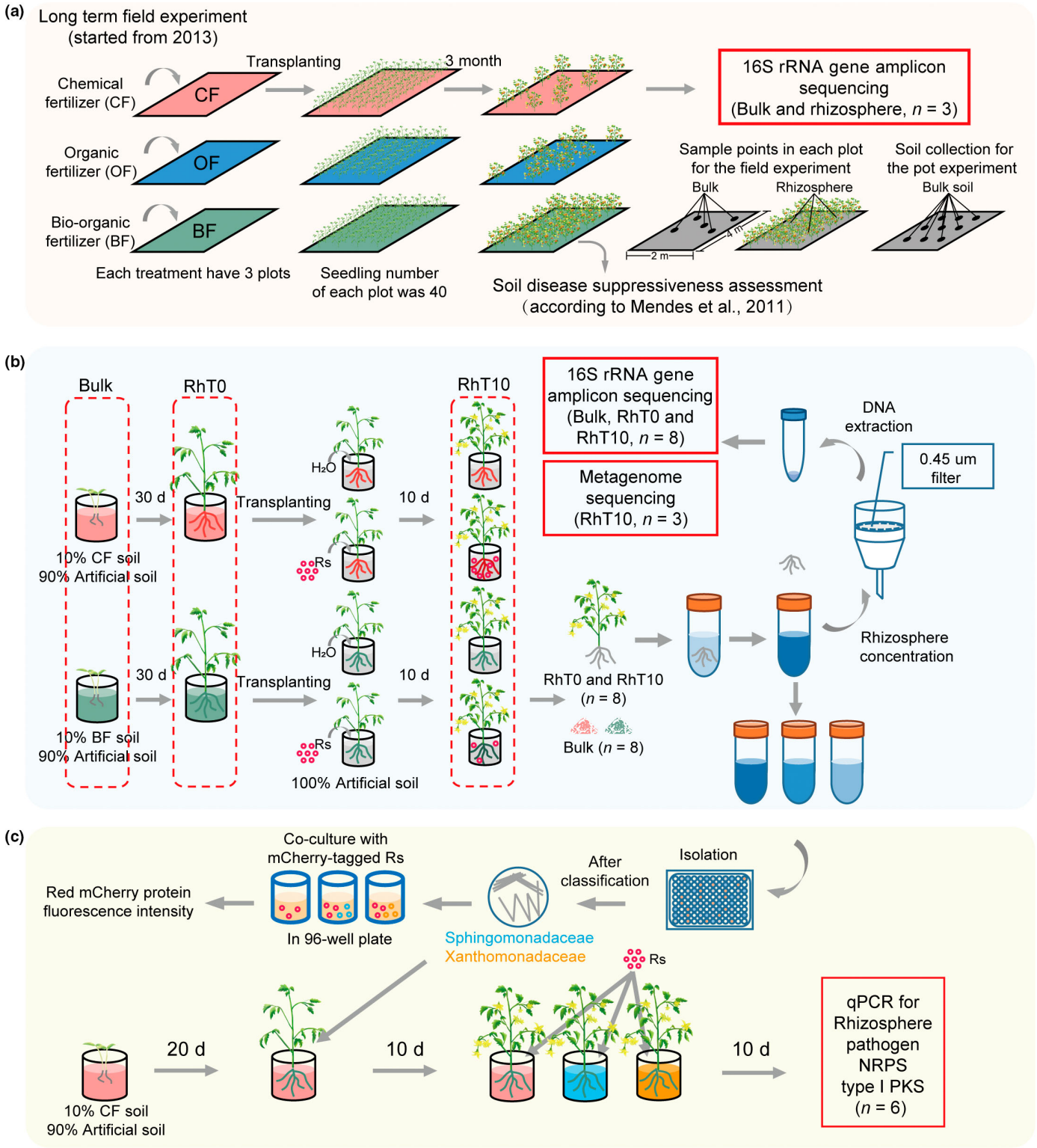

Fig. 1 Experimental flow for this study. (a) Long-term field experiment starting in 2013. Bulk and rhizosphere soils were sampled in June 2018 for this study; (b) procedure for the pot experiment. Because the organic fertilizer (OF) amendment did not show a significantly lower disease incidence than the chemical fertilizer (CF) treatment, the soil with long-term bio-organic fertilizer (BF) application and CF were selected for the pot experiment; (c) isolation of rhizosphere bacteria and verification of their suppressiveness. The isolate suppression of Sphingomonadaceae and Xanthomonadaceae was tested through a coculture (*in vitro*) and a reinoculation pot (*in vivo*) experiment. Rs, abbreviation of *Ralstonia solanacearum*. *n*, number of replicates per treatment.

from each point) were collected after removing the plants in each plot to form a composite sample as one replicate during the harvest season (Fig. 1a). All bulk soil samples from each replicate were subsequently individually mixed and sifted through a 2-mm sieve to homogenize the soil. Samples were stored at −80°C for DNA extraction. For the rhizosphere samples, three tomato roots were collected from the points of each plot and vigorously shaken to remove excess soil (Fig. 1a). Then, the soil adhering to the roots (rhizosphere soil) was collected by sterile phosphate-buffered saline (PBS). The rhizosphere soil samples were obtained after centrifugation at 10 000 ***g*** for 10 min, and stored at −80°C for soil DNA extraction. Thus, three bulk and three rhizosphere soil samples were collected for each treatment.

Bioassay to assess the disease suppressiveness of soils

Because the OF treatment did not show a significant bacterial wilt suppression effect compared with the CF treatment, soils from the BF and CF treatments were selected for the subsequent microcosm experiment to explore the potential suppression mechanism. First, we assessed the disease suppressiveness of BF soils according to a previously described protocol (Mendes *et al.*, 2011) (Fig. 1). In brief, soils from the CF and BF treatments in the field were collected. Five treatments were designed as follows: BF, soil from the BF treatment; CF, soil from the CF treatment; CF + SBF, mix of 90% CF soil and 10% 121°C autoclaved BF soil (w/w); CF + 50BF mix of 90% CF soil and 10% 50°C heat-treated (1 h) BF soil (w/w); and CF + BF, mix of 90% CF soil and 10% BF soil (w/w). Tomato seeds were surface sterilized by ethanol (75%) and bleach (3%), and subsequently germinated in culture dishes. A single germinated seed was transferred to a sterilized 200 ml pot containing 200 g of soil. For each soil treatment, three replicates were used in a completely randomized experimental design, and each replicate contained 10 pots. Seedlings were grown in a growth room under 16 h : 8 h, light : dark conditions at 28°C for 30 d. Pots were watered with half-strength Hoagland every 3 d and with deionized water when needed. Then, all pots were inoculated with *R. solanacearum* QLRs-1115 (Wei *et al.*, 2011) at a concentration of 10^6 cells ml^{-1} artificial soil for each pot. Finally, the seedlings were grown to record the disease incidence.

Ralstonia solanacearum invasion pot experiment description

Subsequently, a microcosm pot experiment was carried out to focus on the dynamics of the soil microbiome in the rhizosphere microbiome under pathogen invasion. Soil cores at depths of 0–20 cm from nine points in each plot of CF and BF were collected to form the soil inoculum. Tomato seeds were surface sterilized by ethanol (75%) and bleach (3%), and subsequently germinated in culture dishes that contained sterile absorbent paper and deionized water. We assumed that the seed endophytic community was negligible compared to the soil community as previously shown (Lundberg *et al.*, 2012; Tkacz *et al.*, 2015). The tomato variety used in this and all subsequent pot experiments was a kind of commercial cultivar 'Hezuo 903', which is also a kind of early ripening tomato cultivar with round, large, red fruit. The artificial soil in this study was well mixed vermiculite and quartz sand (horticultural grade; v/v, 2 : 1). All artificial soil was autoclaved twice at 121°C for 60 min. BF and CF soil inocula were added to the sterile artificial soil and mixed well (v/v, 1 : 9) as growth substrates (Fig. 1b). Afterward, a single germinated seed was transferred to a sterilized 200 ml pot containing 200 ml of the growth substrates, and 108 pots were allotted to each type of artificial soil. Seedlings were grown in a growth room under 16 h : 8 h, light : dark conditions at 28°C for 30 d. The pots were watered with half-strength Hoagland every 3 d and with deionized water when needed. Then, the seedlings that contained different rhizospheres were transplanted to new sterile pots containing sterile artificial soil. Among these pots, half were inoculated with *R. solanacearum* QLRs-1115 at a concentration of 10^6 cells ml^{-1} artificial soil for each pot. Finally, the seedlings in new pots were grown for 10 d (Fig. 1b).

Growth substrates containing each type of soil at the initial time, were collected into 2 ml sterilized microcentrifuge tubes and used as the 'Bulk' samples, which contained two treatments: Bulk_BF (sterile artificial soil mixed with BF soil inoculum) and Bulk_CF (sterile artificial soil mixed with CF soil inoculum). Rhizosphere samples before transplanting and *R. solanacearum* inoculants were defined as 'RhT0', which contained two treatments: RhT0_BF (rhizosphere derived from the BF soil microbiome) and RhT0_CF (rhizosphere derived from the CF soil microbiome). Ten days after pathogen inoculation, the samples were defined as 'RhT10', which contained four treatments: RhT10_BF (BF rhizosphere without *R. solanacearum* inoculation), RhT10_BF + Rs (BF rhizosphere with *R. solanacearum* inoculation), RhT10_CF (CF rhizosphere without *R. solanacearum* inoculation) and RhT10_CF + Rs (CF rhizosphere with *R. solanacearum* inoculation). Each treatment at each time point consisted of eight independent replicates (only healthy plants were sampled). The roots of each replicate from RhT0 and RhT10 were collected from each plot and vigorously shaken to remove excess soil. The rhizosphere soils from 3 g of roots from each sample were thoroughly washed in 30 ml sterile PBS, dried on sterilized 0.45 micron membrane filters, and transferred into sterilized 5 ml tubes. The samples were subsequently frozen in liquid nitrogen and stored at −80°C until the soil DNA was extracted (Fig. 1b).

DNA extraction and quantitative polymerase chain reaction (qPCR) assay

Field soil samples and Bulk, RhT0 and RhT10 samples from the pot experiments were chosen for subsequent DNA extraction using PowerSoil Soil DNA Isolation Kits (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's protocol. The concentration and quality of the DNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, NC, USA). The abundance of *R. solanacearum* was quantified by quantitative polymerase chain reaction (qPCR) with specific primers (forward, 5′-GAA CGC CAA CGG TGC

GAA CT-3′; reverse, 5′-GGC GGC CTT CAG GGA GGT C-3′) to target the fliC gene that codes the flagellum subunit (Schönfeld *et al.*, 2003). The abundance of *Bacillus* was quantified by qPCR with specific primers (forward, 5′-ATG TTA GCG GCG GAC GGG TGA G-3′; reverse, 5′-AAG TTC CCC AGT TTC CAA TGA CC-3′) (Mori *et al.*, 2004). The qPCR analysis was performed with a qTOWER 2.2 system from Analytik Jena (Jena, Germany) using SYBR green I fluorescent dye detection in 20 µl volumes, which contained 2 µl of template, 10 µl of SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan), and 0.4 µl of both forward and reverse primers (10 µM each) in 96-well plates. All qPCRs were performed using the standard temperature profile (Hu *et al.*, 2016). Each sample was analyzed in three replicates, and the results were expressed as $\log_{10}$ values (target copy number per gram soil in the Bulk treatments and per plant root in the RhT0 and RhT10 treatments).

Illumina MiSeq sequencing

The DNA extracted from the soil was used to amplify and sequence the bacterial 16S rRNA gene which targeted the variable V4–V5 region (forward primer, 515F-5′-GTG CCA GCM GCC GCG GTA A-3′; reverse primer 907R-5′-CCG TCA ATT CMT TTR AGT TT-3′). Amplicons were sequenced based on the Illumina MiSeq platform at Personal Biotechnology Co. Ltd, Shanghai, China. The operational taxonomic unit (OTU) table was built using the UPARSE pipeline (Edgar, 2013). Briefly, reads were truncated at 200 bp and quality-filtered using a maximum expected error of 0.5. After discarding replicates and singletons, we assigned the remaining reads to OTUs with a threshold of 97% identity. For the field experiment, this step generated a 16S rRNA OTU table of 12 samples × 4464 OTUs (309 241 reads). The number of high-quality sequences per sample was 9752–36 572. For the pot experiments, this step generated a 16S rRNA OTU table of 64 samples × 6354 OTUs (1541 951 reads). The number of high-quality sequences per sample was 16 356–36 189. Finally, bacterial representative sequences were searched through the RDP classifier (Wang *et al.*, 2007) with a confidence threshold of 80%. To obtain an equivalent sequencing depth for further bacterial community analysis, each sample was rarefied to the smallest sample size (9752 for field experiments and 16 356 sequences for pot experiments) in R through the package GUNIFRAC (Function: Rarefy).

Bioinformatics analysis of 16S rRNA gene profiling

Alpha diversity indicators such as richness (Sobs), diversity (Shannon) and phylogenetic diversity (PD) were determined for each sample. The richness and diversity of rarefied OTUs were calculated using the VEGAN (function: diversity) package and the phylogenetic diversity was calculated by the PICANTE (function: pd) package in R (v.3.5.1 for Windows). The weighted and unweighted UniFrac distances between treatments were calculated using the R package GUNIFRAC and presented based on a principal coordinate analysis (PCoA) through the GGPLOT2 package to visualize the differences in microbial community composition. Differences in community structure between treatments were tested using permutational multivariate analysis of variance (PERMANOVA), which was performed using the R package VEGAN (function: adonis) with 9999 permutations. A random forest model was calculated by the R package RANDOMFOREST to select important bacteria that correlated with the pathogen density. The relative abundance of the microbiome between inoculated and uninoculated rhizospheres as determined by DESEQ2 (function: DESeq) package through the Wald significance tests and presented in a volcano diagram through the GGPLOT2 package. For other statistical analyses, two-sample Mann–Whitney *U*-tests, Spearman correlations and Pearson correlations were calculated in IBM SPSS 23.0 (Armonk, NY, USA).

To evaluate the effects of the prior microbiome specificity and *R. solanacearum* inoculation, four networks were individually constructed for each treatment of RhT10. The OTU tables for each dataset were restricted to rarefied OTUs that were present in at least six samples and comprised ≥ 14 reads (relative abundance ≥ 0.01% in each treatment). For each table, Spearman correlation scores were calculated in the MENA online pipeline (http://ieg4.rccc.ou.edu/mena/).

Metagenome analysis

Three random DNA samples from each treatment of RhT10 (3 × 4 = 12 samples) were sequenced on the Illumina HiSeq 2500 platform using the PE150 sequencing strategy, with an average of 10 Gb per sample. All raw data were trimmed by the TRIMMOMATIC program (LEADING:3, TRAILING:3, SLIDINGWINDOW:5:20, MINLEN:50), which generated data containing clean reads that were subsequently aligned against the tomato genome to remove host-associated sequence contamination. The MEGAHIT program (https://github.com/voutcn/megahit, *k*-min: 35, *k*-max: 95 and *k*-step: 20) was used to assemble the scaftigs (≥ 500 bp); then, the Prodigal program (https://github.com/hyattpd/Prodigal) was used to predict the open reading frame (ORF) (≥ 90 bp). The gene catalog (unigenes) was obtained after clustering through the LINCLUST program (-e 0.001--min-seq-id 0.9 -c 0.80), and the longest sequence was used as the representative sequence for each cluster (Steinegger & Söding, 2018). The gene depth and relative abundance were calculated with the BBMAP program by comparing clean reads with the gene catalog. Finally, unigenes were annotated by BLASTP (v.2.2.31+, http://blast.ncbi.nlm.nih.gov/Blast.cgi) in the National Center for Biotechnology Information (NCBI) for taxonomy (*e*-value ≤ 0.0001) and DIAMOND in Kyoto Encyclopedia of Genes and Genomes (KEGG) and EGGNOG for function (*e*-value ≤ 0.0001). Furthermore, contigs > 5 kb were processed with ANTISMASH v.3.0 with default parameters (Weber *et al.*, 2015) to analyze the secondary metabolism ability.

Isolation and identification of culturable rhizosphere bacterial isolates

High-throughput isolation and identification of the isolates were performed according to previously described protocols (Bai

et al., 2015; Zhang *et al.*, 2019, 2021). In brief, tomato roots from 'RhT10' were washed in PBS on a shaking platform for 30 min at 170 rpm. For limiting dilution, homogenized rhizosphere soils were sedimented for 15 min and the supernatant was empirically diluted, distributed and cultivated in 96-well microliter plates that had 200 ml tryptic soy broth (TSB) medium in each well. After 1 wk of incubation, 3299 wells were turbid and the isolates were subsequently stored in 30% glycerol at −80°C. Then, a two-step barcoded PCR protocol in combination with Illumina HiSeq was adopted to define the sequences of bacterial 16S rRNA genes of rhizosphere bacteria. After annotating the sequences of the last step, cultivated bacteria were clustered into OTUs with > 99% 16S rRNA gene similarity. Representative isolates of each unique OTU identified from cultivated bacteria were purified by tryptic soy agar (TSA) medium before using individual colonies for validation by Sanger sequencing with 27F and 1492R primers and stored in 30% glycerol at −80°C. Afterward, the sequences were clustered into OTUs with > 99% 16S rRNA gene similarity and submitted to the RDP database for taxonomic identification. Finally, 163 isolates were obtained in this step. The phylogenetic analysis of the 16S rRNA gene sequences was performed using MEGA v.7.0.26 and visualized with iTOL (Fig. 1c).

Disease suppression of responsive isolates and consortia

Sphingomonadaceae (*Sphingomonas*) and Xanthomonadaceae (*Luteimonas*, *Lysobacter*, *Pseudoxanthomonas*, and *Stenotrophomonas*) isolates were selected to test the metagenomics indications. Two isolates (*Rhizobium* sp. and *Devosia* sp., which were response taxa in CF) were selected as negative controls, and *B. amyloliquefaciens* T-5 was used as a positive control. *In vitro* pathogen suppression was compared through coculture assays consisting of mCherry-tagged *R. solanacearum* and one of the targeted strains. Briefly, 20 µl of nutrient broth (NB) medium with *R. solanacearum* (density was adjusted to 10^6 cells ml^{-1}) and each targeted strain (density was adjusted to 10^6 cells ml^{-1}) was added to 140 µl of fresh nutrient broth medium in 96-well plates. Each treatment was replicated six times. After 24 h of growth at 30°C, the *R. solanacearum* density was measured as the red mCherry protein fluorescence intensity (excitation: 587 nm, emission: 610 nm) with a SpectraMax M5 plate reader (Fig. 1c).

Tomato seedlings were grown in 200-ml pots with *c.* 200 ml of growth substrates (described earlier) containing 10% CF soils. Twenty days after planting, the seedlings were inoculated with potential biocontrol agents. Isolates were grown in 100 ml of TSB for 2 d at 28°C on a rotary shaker at 170 rpm. The liquid culture of each isolate was centrifuged, washed and suspended in sterile 10 mM magnesium sulfate ($MgSO_4$) solution. Single isolate and microbial consortia suspensions were inoculated into pots with an initial density of 10^7 cells ml^{-1} growth substrates, and 10 mM $MgSO_4$ solution inoculation pots were used as a control. Each treatment contained 30 plants. For the microbial consortia suspension, all isolates at a cell density of 10^8 cells ml^{-1} were mixed in a 1 : 1 ratio. Approximately 20 ml of the suspension was inoculated on the growth medium surface of each pot on average. After 10 d, the pots were inoculated with *R. solanacearum* QLRs-1115 cells, and the final concentration of each pot was 10^6 cells ml^{-1} substrate. Rhizosphere samples were collected 10 d after the pathogen inoculation. All the treatments had six replicates. The abundances of the pathogen, nonribosomal peptide synthetase (NRPS; A3F, 5′-GCS TAC SYS ATS TAC ACS TCS GG-3′; A7R, 5′-SAS GTC VCC SGT SCG GTA-3′) (Ayuso-Sacido & Genilloud, 2005) and type-I modular polyketide synthase (PKS; degKS2F, 5′-GCI ATG GAY CCI CAR CAR MG IVT-3′; degKS2R, 5′-GTI CCI GTI CCR TGI SCY TCI AC-3′) (Schirmer *et al.*, 2005) were determined through qPCR (Fig. 1c). These two primer pairs amplify a variety of biosynthetic gene clusters (BGCs) belonging to NRPS and type-I PKS. Quantitative PCRs for NRPS and PKS were performed using the temperature profiles provided in Zhao *et al.* (2018) and Charlop-Powers *et al.* (2014), respectively (Fig. 1c).

Results

Disease control trait in the field experiment via long-term bio-organic amendment

After 11 seasons of continuous fertilization (Fig. 1a), the treatment with long-term application of BF had a significantly lower tomato bacterial wilt disease incidence than the inorganic treatment (CF). Although the long-term application of OF also resulted in a lower disease incidence than CF, the difference was not significant (Fig. 2a). BF and OF did not show any significant plant growth promoting effect relative to CF (Supporting Information Fig. S1a,b). The abundance of *R. solanacearum* in the rhizosphere showed similar trends as the disease incidence (Fig. 2b), although the pathogen quantity in the bulk soil among the treatments did not show significant differences. The abundance of *Bacillus* in the rhizosphere showed a negative correlation trend, albeit not a significant trend, with the pathogen density (Fig. S1c, d). The sequencing of the bacterial communities associated with bulk and rhizosphere soils revealed no significant difference in diversity among all treatments (Fig. 2c), although the bacterial composition among treatments was significantly different (Fig. 2d). Further random forest analysis showed that Xanthomonadaceae, Sphingomonadaceae, Thermomicrobiaceae, Sphingobacteriaceae, and Ignavibacteriaceae were the top five families that potentially contributed to the pathogen abundance in the rhizosphere (Fig. 2e).

Community shift of rhizosphere bacteria in the pot experiment under pathogen invasion

After assessing the disease suppressiveness of BF soil, our results showed that suppressiveness was driven by its microbiome and could be transfered to conducive soil (Fig. 3). To understand the process by which rhizosphere bacteria suppress *R. solanacearum* invasion, we directly inoculated *R. solanacearum* into the tomato rhizosphere and subsequently sequenced the rhizosphere bacterial community (Fig. 1b). Prior to invasion, we observed no significant differences in *R. solanacearum* abundance in the bulk soil

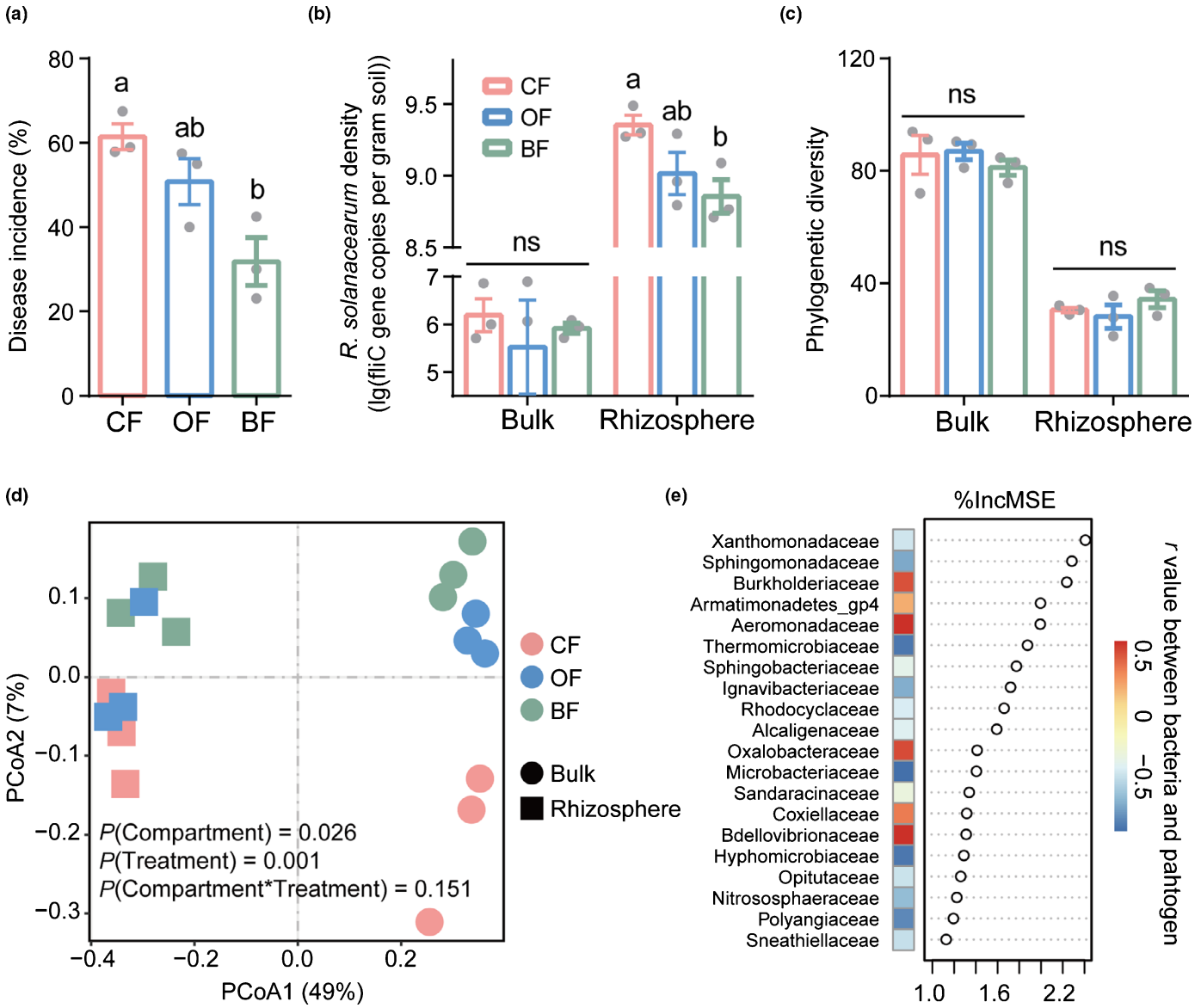

Fig. 2 Results of the long-term field experiment. (a) Disease incidence of bacterial wilt disease (mean ± SE); (b) abundance of *Ralstonia solanacearum* in bulk and rhizosphere soil (mean ± SE); (c) phylogenetic diversity of the bacterial community (mean ± SE); (d) principal coordinate analysis (PCoA) of the bacterial community based on weighted UniFrac distance (*P* values were obtained from the PERMANOVA); (e) 20 most important bacteria at the family level that correlated with the pathogen density based on the random forest model. The heatmap shows the *r* value between bacteria and *R. solanacearum* in the rhizosphere based on Spearman correlation. Chemical fertilizer (CF): soil with the long-term application of chemical fertilizer; organic fertilizer (OF): soil with the long-term application of organic fertilizer; bio-organic fertilizer (BF): soil with the long-term application of bio-organic fertilizer. Gray dot represents value of each replicate. Different letters indicate significant differences among the four treatments as defined by Tukey's test ($P \leq 0.05$). 'ns' indicates there was no significant difference among treatments as defined by ANOVA ($P > 0.05$). '%IncMSE' indicates the percentage increase in the mean square error. A larger %IncMSE value corresponds to greater influence of the characteristic variable on the target.

and rhizosphere between the CF and BF treatments. Similar to the field trial, *R. solanacearum* invasion showed significant differences in disease incidence between the CF and BF treatments (Fig. 4a). As expected, invasion increased the *R. solanacearum* abundance in the tomato rhizosphere of both the BF and CF communities; however, the abundance in the BF was significantly lower than that in the CF, indicating suppressiveness. Interestingly, although no obvious difference in *R. solanacearum* abundance was detected in the tomato rhizosphere between the CF and BF treatments before pathogen inoculation, the BF rhizosphere had a lower abundance of *R. solanacearum* than the CF rhizosphere after 10 d of growth (Fig. 4b). For *Bacillus*, pathogen invasion significantly increased its abundance in the rhizosphere. Although no significant difference between BF and CF was observed, higher *Bacillus* densities were observed in BF (Fig. S2b, c). The bacterial PD did not show a significant difference in the bulk and rhizosphere soils between the CF and BF treatments before invasion, but inoculation only increased the diversity in

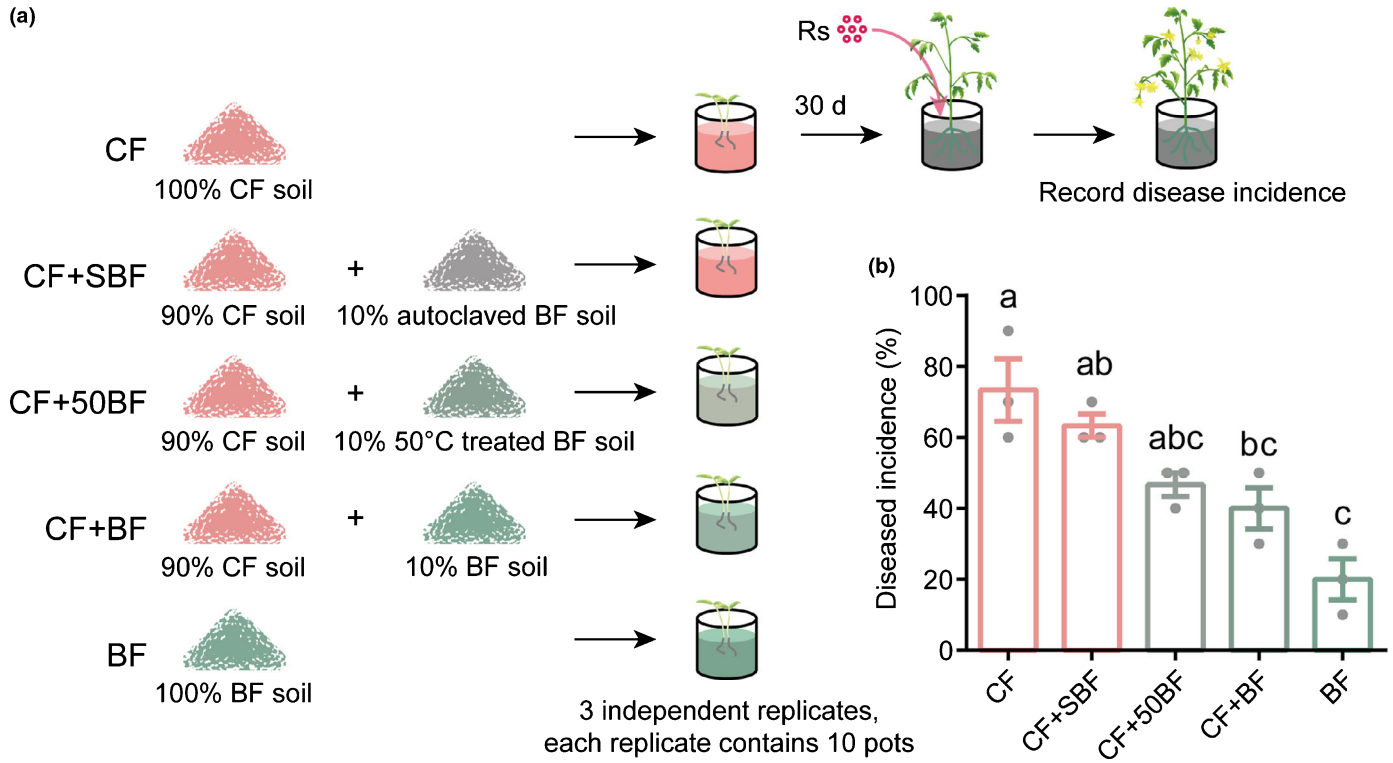

Fig. 3 Assessment of disease suppressiveness from bio-organic fertilizer (BF) soils. (a) Experimental flow for assessing the disease suppressiveness of soils; (b) disease incidence (mean ± SE) of seedlings in BF soil (BF), chemical fertilizer (CF) soil, CF soil amended with 10% (w/w) BF soil (CF + BF) or BF soil heat-treated at 50°C (CF + 50BF) or by autoclave (CF + SBF). Gray dot represents value of each replicate. Different letters indicate significant differences among the four treatments as defined by Tukey's test ($P \leq 0.05$).

the BF. Although the rhizosphere PD responded with pathogen invasion, on the 10th day after *R. solanacearum* inoculation, we observed no significant difference between BF and CF inoculated with *R. solanacearum* (Fig. 4c).

PCoA based on weighted and unweighted UniFrac distances showed that the largest variations in microbial community composition were observed with time along the first PCoA axis (Figs S3a, S4b). Then, the variations among community origins were observed along the second PCoA axis based on unweighted UniFrac distances (Fig. S3a). To evaluate the effect of invasion, we focused on the 10th day after *R. solanacearum* inoculation, which revealed that both community origin and inoculation affected tomato rhizosphere bacterial composition (Figs 4d, S3c). However, the distance shift under pathogen inoculation in the BF rhizosphere bacterial communities was greater than that in the CF rhizosphere bacterial communities, and significant responses were only observed in the BF rhizosphere (Tables S1, S2). Moreover, the response of the community was negatively correlated with rhizosphere pathogen survival (Table S3). Volcano plots showed a greater number of taxa with abundance changes under inoculation conditions in the BF rhizosphere than in the CF rhizosphere (Fig. 4e). In addition, the network analysis showed that *R. solanacearum* invasion increased the number of nodes and edges in both the BF and CF rhizosphere bacterial communities; however, greater changes were observed in the BF community (Fig. 4f). Further taxonomic analysis showed that the response of most OTUs was enriched in BF + Rs instead of CF + Rs under pathogen inoculation (Fig. S4a). Although the relative abundances of these taxa were similar between BF and CF before *R. solanacearum* inoculation, they sharply increased after invasion in BF only (Fig. S4b). In accordance with the results from the field experiment, genera associated with the families Xanthomonadaceae and Sphingomonadaceae were identified as responsive taxa (Table S4). Random forest analysis also indicated that these two families were potentially important in suppressing *R. solanacearum* in the rhizosphere (Fig. 4g).

Functional response of rhizosphere bacteria under *R. solanacearum* invasion

To reveal how the shifts in microbial composition reflected functional changes, we sequenced the whole metagenomes of several samples (Fig. 1b). The genes retrieved from metagenome data were annotated through the eggNOG and KEGG databases. Our results showed that the functional and community responses of BF were stronger than those of CF (Fig. S5), Thus corroborating the results based on 16S rRNA gene amplicon sequencing (Fig. 4). Specifically, the pathways in the rhizosphere of BF + Rs were distinct from those in the other treatments, which clustered together (Fig. 5a,b). The direction of the functional changes was similar in both the BF and CF rhizospheres under invasion, although the change scale was more drastic in BF than CF

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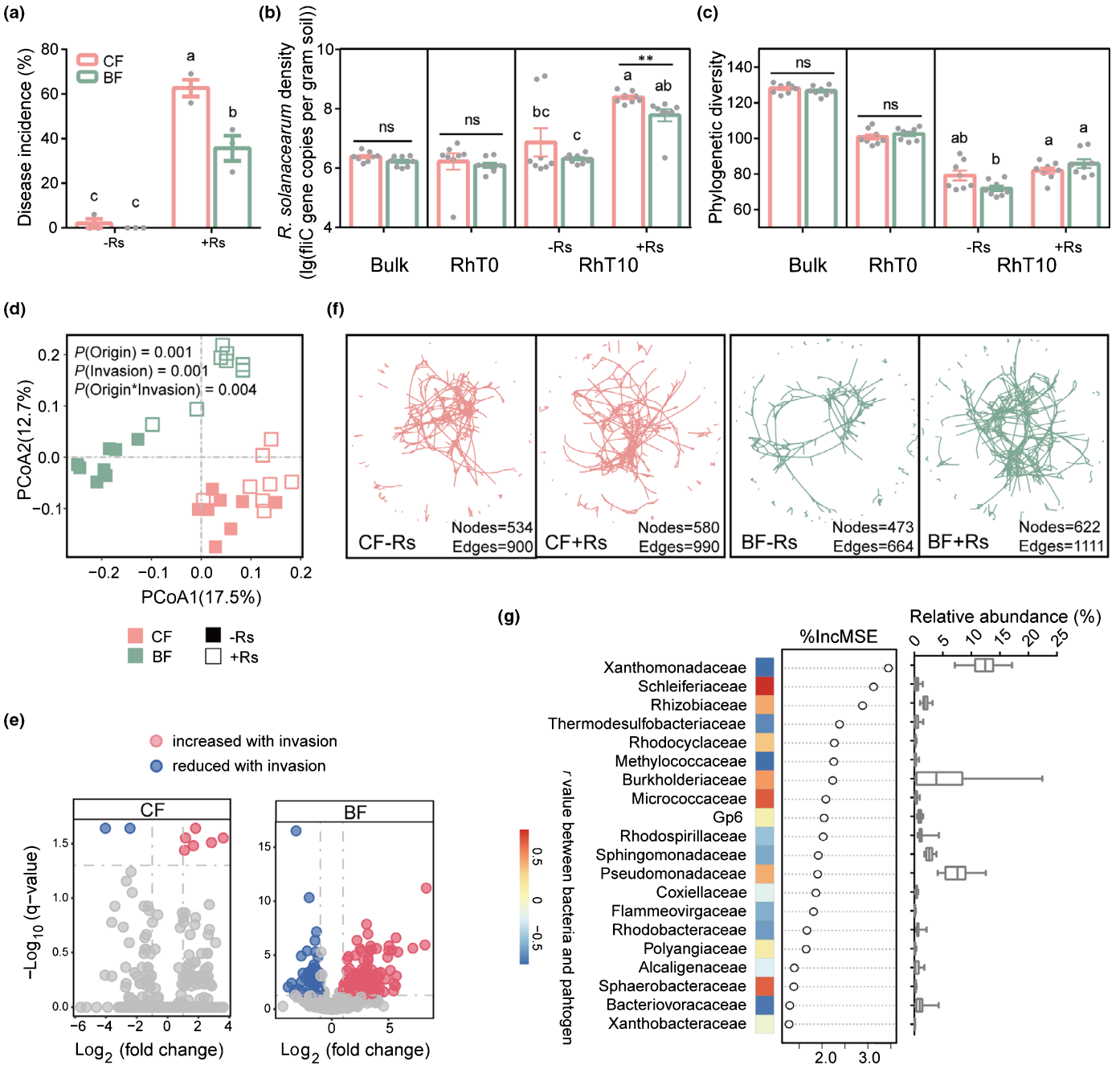

Fig. 4 Microbial community traits of rhizosphere bacteria under *Ralstonia solanacearum* invasion. (a) Bacterial wilt disease incidence at the 10th day after pathogen inoculation (mean ± SE); (b) abundance of *R. solanacearum* and (c) phylogenetic diversity of the bacterial community (mean ± SE); (d) principal coordinate analysis (PCoA) of the bacterial community in RhT10 based on the weighted UniFrac distance. The *P* values were from PERMANOVA, 'origin' represents the bacterial community from bio-organic fertilizer (BF) or chemical fertilizer (CF) soils, and 'invasion' represents whether the rhizosphere was inoculated with pathogen; (e) volcano diagram of responsive bacterial operational taxonomic unit (OTUs) in the BF and CF with *R. solanacearum* inoculation in RhT10; (f) network of the bacterial community in RhT10; (g) 20 most-important bacteria at the family level that correlated with the pathogen density based on the random forest model, and the box plot showed the relative abundance of the family in rhizosphere. Bulk: initial mixed material before planting; RhT0: rhizosphere before *R. solanacearum* inoculation; RhT10: rhizosphere after *R. solanacearum* inoculation. '–Rs' indicates that the rhizosphere microbiome was not inoculated with *R. solanacearum*; '+Rs' indicates that the rhizosphere microbiome was inoculated with *R. solanacearum*. Gray dot represents value of each replicate. The asterisks indicate significant differences (Mann–Whitney *U*-test, **, $P \leq 0.01$). 'ns' indicates there was no significant difference (Mann–Whitney *U*-test, $P > 0.05$). Different letters indicate significant differences among the four treatments as defined by Tukey's test ($P \leq 0.05$). '%IncMSE' indicates the percentage increase in the mean square error. A larger value of %IncMSE corresponds to a greater influence of the characteristic variable on the target. Box plot display the first and third quartile, with the horizontal bar at the median and whiskers showing the most extreme data point, which is no more than 1.5 times the interquartile range from the box.

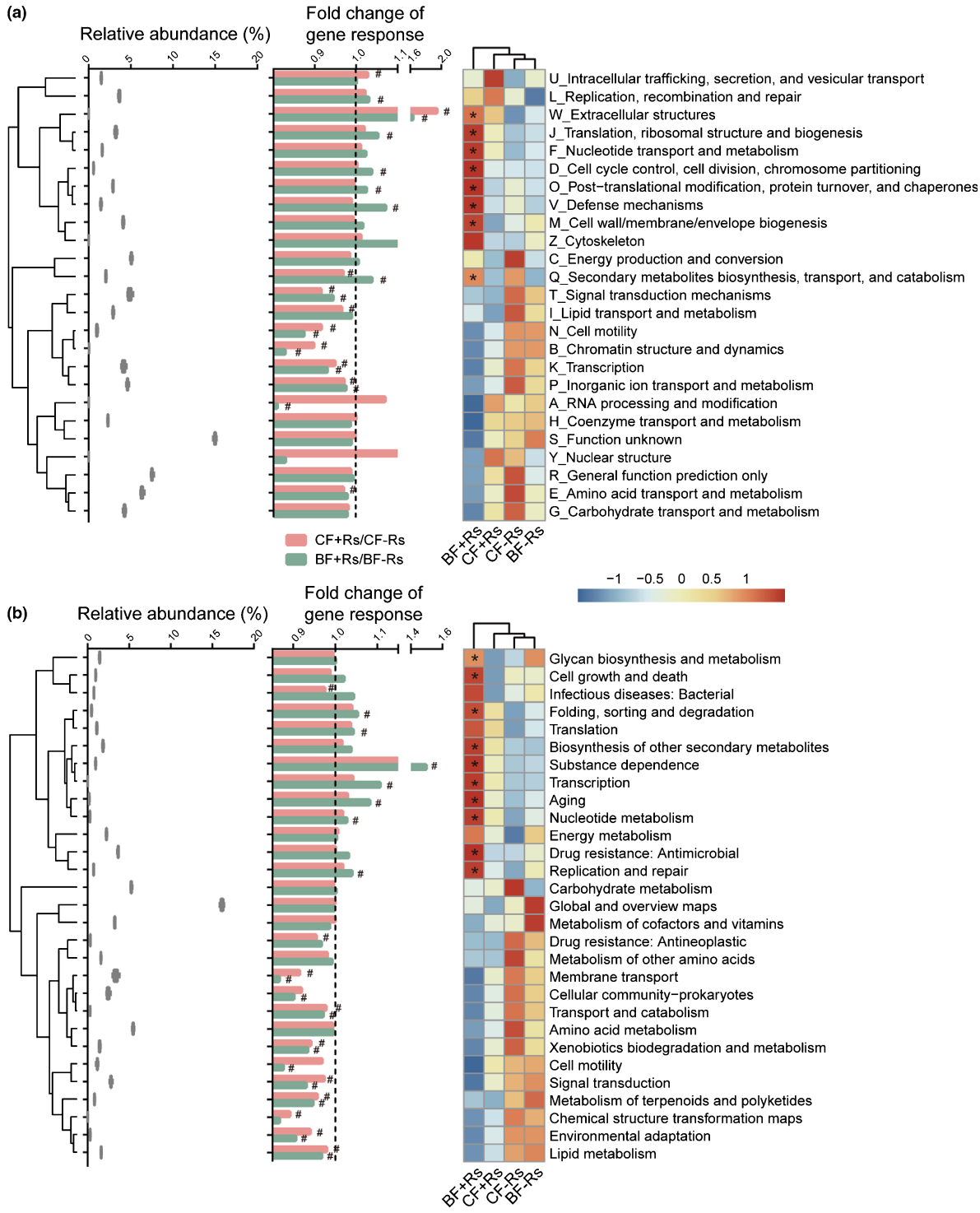

Fig. 5 Functional response of rhizosphere bacterial communities after 10 d of *Ralstonia solanacearum* invasion, which was determined by shotgun metagenome sequencing of chemical fertilizer (CF) and bio-organic fertilizer (BF) rhizosphere microbiomes, in the presence or absence of *R. solanacearum*. (a) Gene abundances based on the eggNOG database: relative abundance of the reads over all samples, in percentage; fold change in relation to the presence or absence of the pathogen; and heatmap of each pathway, which indicates their relative abundance according to the treatment; (b) gene abundances based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database: relative abundance of the reads over all samples, in percentage; fold change in relation to the presence or absence of the pathogen; and heatmap of each pathway, which indicates their relative abundance according to the treatment. The heatmap scale ranges from −1.5 to 1.5 (blue to red). CF − Rs, CF rhizosphere bacterial community without *R. solanacearum* invasion; BF − Rs, BF rhizosphere bacterial community without *R. solanacearum* invasion; CF + Rs, CF rhizosphere bacterial community with *R. solanacearum* invasion; BF + Rs, BF rhizosphere bacterial community with *R. solanacearum* invasion. Asterisk indicates a significant enrichment in the BF + Rs rhizosphere compared with that in CF + Rs (Mann–Whitney *U*-test, *, $P \leq 0.05$). The hash symbol indicates a significant change in the +Rs rhizosphere compared to its corresponding −Rs rhizosphere (Mann–Whitney *U*-test, #, $P \leq 0.05$).

(Fig. 5a,b). Among these pathways, eight (eggNOG) and 10 (KEGG) pathways were significantly enriched (Mann–Whitney *U*-test, $P \leq 0.05$) in the rhizosphere of BF + Rs compared with CF + Rs, including the post-translational modification, defense mechanisms and biosynthesis of secondary metabolites. A further secondary metabolite analysis (AntiSmash) also showed that NRPS, type-1 PKS and furan were significantly enriched in the rhizosphere under BF + Rs compared to CF + Rs (Fig. S6).

Moreover, similar to the indications described in the field experiment and 16S rRNA gene sequencing data of the pot experiment (Figs 2e, 3g; Table S4), the taxonomy analysis based on metagenome data showed that Sphingomonadaceae and Xanthomonadaceae were the most abundant responsive taxa in the BF rhizosphere after pathogen invasion (Fig. S7). Further analysis also showed that enriched functions such as secondary metabolite biosynthesis and defense mechanisms, were associated with the increases in Sphingomonadaceae, Xanthomonadaceae, Opitutaceae, and/or Bradyrhizobiaceae (Figs 6a, S8). Regarding the most abundant responsive taxa of Sphingomonadaceae and Xanthomonadaceae (Fig. 6b), the relative abundances of *Dyella*, *Luteibacer*, *Luteimonas*, *Lysobacter*, *Novosphingobium*, *Pseudoxanthomonas*, *Rhodanobacter*, *Rudaea*, *Sandarakinorhabdus*, *Sphingomonas*, *Stenotrophomonas* and *Xanthomonas* in the rhizosphere of BF + Rs were significantly (Mann–Whitney *U*-test, $P \leq 0.05$) higher than those in the other treatments (Fig. 6c), which indicates that the response of these taxa may contribute to the suppression function toward *R. solanacearum* invasion.

Pathogen suppressiveness of the responsive taxa

To verify the metagenomics analysis results, 163 different culturable strains were isolated from the seedling rhizosphere (Fig. S9). From those, a pool of potential functional isolates of Sphingomonadaceae and Xanthomonadaceae composed of seven *Sphingomonas* strains, one *Luteimonas* strain, three *Lysobacter* strains, three *Pseudoxanthomonas* strains and two *Stenotrophomonas* strains, as well as the negative control strains *Rhizobium* sp. and *Devosia* sp. and positive control strain *Bacillus* T-5, were tested *in vitro* and *in vivo* to determine their ability to suppress pathogens (Fig. 1c). The coculture results showed that most Xanthomonadaceae strains and *Bacillus* T-5 induced *R. solanacearum* suppression rather than Sphingomonadaceae strains, *Rhizobium* sp. and *Devosia* sp. (Fig. S10a). The *in vivo* results showed that three of seven *Sphingomonas* strains, all Xanthomonadaceae strains, *Bacillus* T-5 and the synthetic community significantly controlled bacterial wilt (Fig. 7a).

We further used qPCR targeting the NRPS and type-I PKS genes to quantify their potential role in disease suppression after the inoculation of bacterial isolates. We observed that the NRPS genes were enriched in the tomato rhizosphere after the inoculation of most isolates and the type-I PKS genes were also enriched in some *Sphingomonas*, *Pseudoxanthomonas* and *Stenotrophomonas* inoculation treatments (Fig. S11). Furthermore, the NRPS gene density, but not the type-I PKS gene density, was significantly negatively correlated with the abundance of pathogens (Fig. 7b).

Discussion

Intensive agricultural production systems are often associated with high disease incidence, which leads to a severe reduction in plant productivity. Improving natural soil suppressiveness is an important strategy to reduce disease incidence and pesticide use while maintaining plant health and agricultural production. While disentangling the mechanisms that drive suppression is important for promoting pathogen control, their identification is often blurred by confounding factors, such as soil properties. Moreover, these mechanisms are quite diverse, ranging from resource competition to antibiosis, which can act directly, such as by reducing the pathogen load, and indirectly, such as by influencing the rhizosphere microbiome. In this case, the plants may remain healthy despite the high pathogen load in the soil. In this study, we aimed to understand the mechanisms through which long-term fertilization with bio-organics leads to soil suppression against *R. solanacearum*. The results from the long-term field experiment showed that soil bacterial communities differ in response to fertilization, but that differences in pathogen density were observed only in the rhizosphere. By experimentally disentangling the effect of the rhizosphere from the bulk soil properties, we showed that microbiome manipulation due to long-term fertilization led to shifts in the composition and functionality of the bacterial communities associated with the tomato rhizosphere. Specifically, metagenomics approaches revealed that an enrichment in Sphingomonadaceae and Xanthomonadaceae as well as secondary metabolites contributed to the suppression ability toward *R. solanacearum* invasion in the rhizosphere of tomato plants. These responses were further validated by an experimental approach in which the inoculation of bacterial isolates belonging to Sphingomonadaceae and Xanthomonadaceae on conducive soil significantly reduced the pathogen abundance and increased the abundance of NRPS genes. Later, we decipher the mechanisms involved in the suppression of pathogenic bacteria by placing our results in light of the bacterial composition, function and dynamics.

Suppressiveness was achieved through rhizosphere microbiome dynamics in response to bio-organic fertilizer amendment

Continuous cropping and CF application often decrease the diversity and biomass of the soil microbiome (Dick, 1992; Wang *et al.*, 2018), thereby leading to the loss of soil function and increasing the incidence of soil-borne diseases (Mendes *et al.*, 2015). Many studies focused on counteracting disease incidence have reported that the application of OF can enhance general disease suppression by adding organic amendments with or without biocontrol agents (De Corato, 2020; Mawarda *et al.*, 2020). Organic amendments are known for their ability to alleviate environmental stress (Park *et al.*, 2011), induce specific taxa in the soil microbial community (Khodadad *et al.*, 2011) and broadly influence the soil microbiome (Hartmann *et al.*, 2015; Fu *et al.*, 2017). In this study, we showed that disease suppression induced by long-term BF could be transferred to

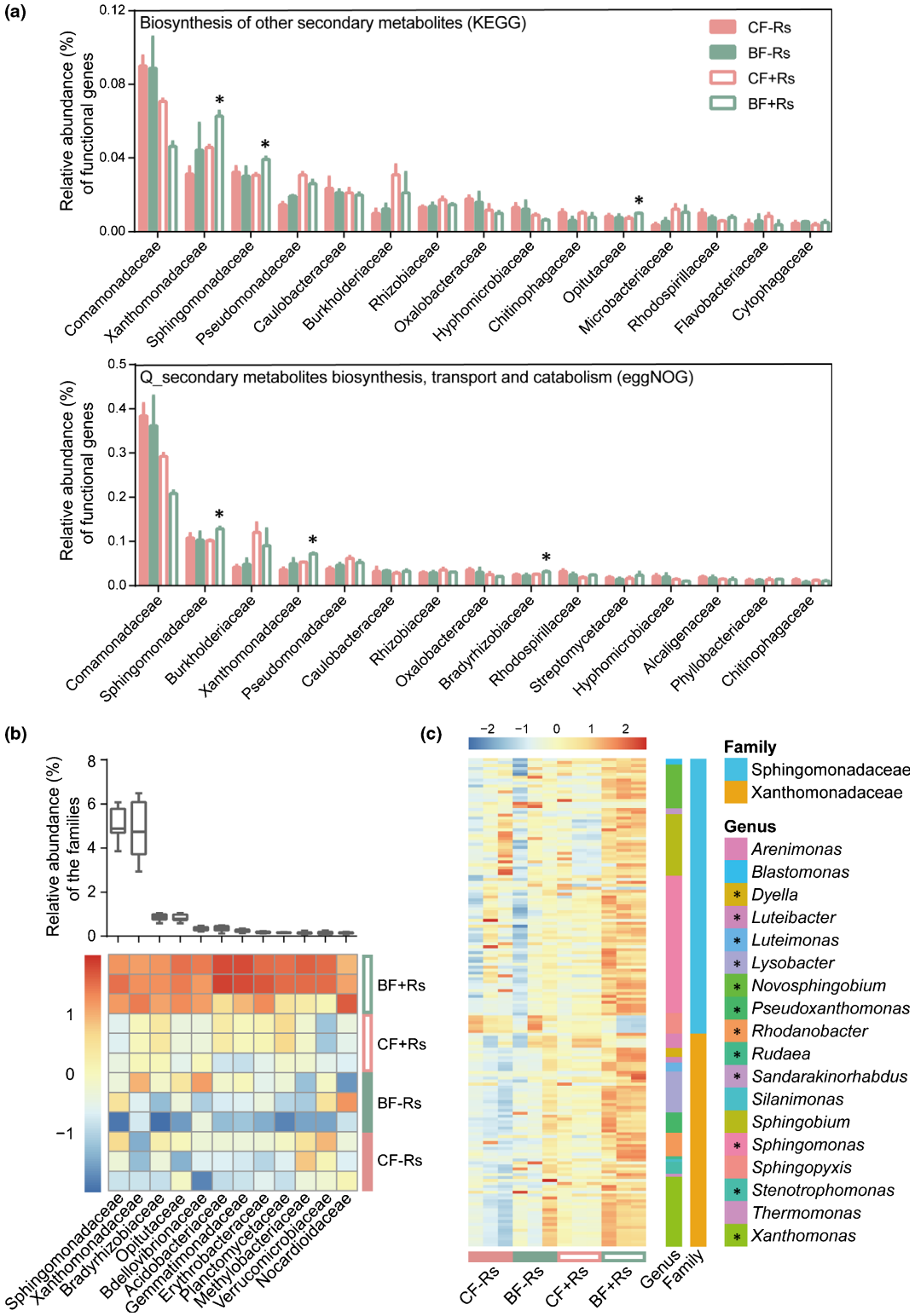

Fig. 6 Responsive rhizosphere bacterial families with *Ralstonia solanacearum* invasion based on metagenome sequencing. (a) Fifteen most-abundant bacteria that contributed to 'biosynthesis of other secondary metabolites' of Kyoto Encyclopedia of Genes and Genomes (KEGG) and 'Q_secondary metabolites biosynthesis, transport and catabolism' of eggNOG (mean ± SE). Only the family that was significantly enriched in bio-organic fertilizer (BF) + Rs was marked by an asterisk (*); (b) heatmap of the relative abundance for responsive rhizosphere bacterial families among all treatments. The box plot shows the relative abundance of the family in the rhizosphere; (c) heatmap of the relative abundance for species in Sphingomonadaceae and Xanthomonadaceae among all treatments. The heatmap scale ranges from −2 to 2 (blue to red). Asterisk indicates a significant increase in the BF + Rs rhizosphere (Mann–Whitney *U*-test, *, $P \leq 0.05$). Box plot display the first and third quartile, with the horizontal bar at the median and whiskers showing the most extreme data point, which is no more than 1.5 times the interquartile range from the box.

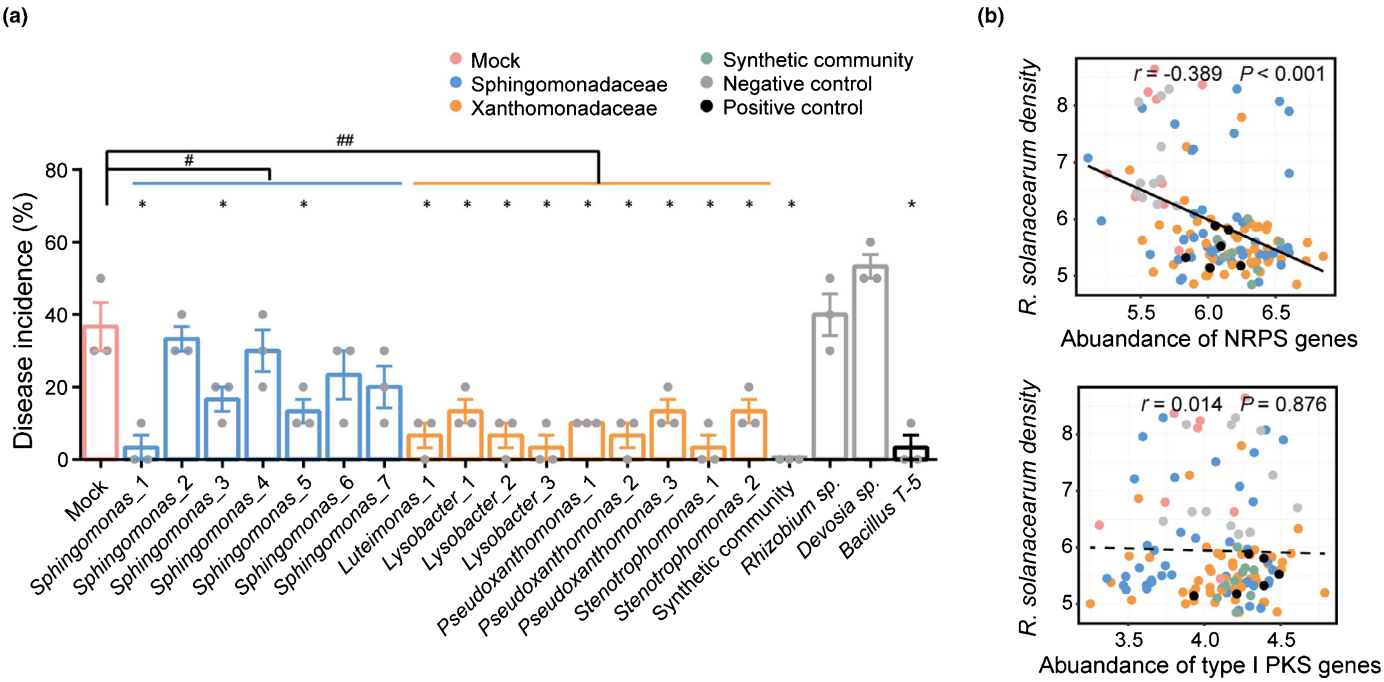

Fig. 7 Suppressiveness of isolates from Sphingomonadaceae, Xanthomonadaceae, negative controls (*Rhizobium* sp. and *Devosia* sp.) and positive control (*Bacillus amyloliquefaciens* T-5). (a) Bacterial wilt disease incidence on the 10th day after pathogen inoculation (mean ± SE). Gray dot represents value of each replicate; (b) linear regression between the abundances of nonribosomal peptide synthetase (NRPS) and type-I polyketide synthase (PKS) genes and the pathogen density in the rhizosphere. The *r* and *P* values were calculated through Spearman correlation. Asterisk indicates significant difference between the control and each isolate inoculation treatment (Mann–Whitney *U*-test, *, $P \leq 0.05$). The hash symbol indicates significant differences between the control and each family inoculation treatment (Mann–Whitney *U*-test, #, $P \leq 0.05$; ##, $P \leq 0.01$).

conducive soil and speculated that the long-term application of BF could suppress soil-borne disease through both general and specific suppression mechanisms.

Our results reveal that the continuous application of BF amended with *Bacillus* species but not OF alone can hinder disease development. Although the presence of the biocontrol agent was crucial for disease suppression, the presence of *Bacillus* in the rhizosphere of tomato plants was not significantly different among treatments under pathogen invasion (Figs S1, S2), although the genus *Bacillus* was enriched in the soils after continuous application of BF. Most of the microbiome in the organic amendment does not thrive in soil conditions due to chemical and biological constraints and disappears after 1 month (Lourenço *et al.*, 2018). Moreover, the presence of *Bacillus* in OF was reported to invoke soil suppression by inducing resident microbes, such as *Lysobacter* and *Pseudomonas* (Xiong *et al.*, 2017a; Tao *et al.*, 2020). Here, we show that the accumulation of potential suppressive taxa of the families Sphingomonadaceae and Xanthomonadaceae was promoted by both organic amendment and bio-control agent addition. Importantly, the inoculation of strains belonging to these families induced suppression in conducive soil, which validates their role in regulating disease development. Thus, suppressiveness of BF seems to be based on a combination of the inoculated bio-control agents as well as the promotion of beneficial microbial groups already residing in the soil.

Agricultural practices that promote soil biodiversity are expected to increase the stability of the soil microbiome and its resistance to pathogen invasion (Jurburg & Salles, 2015). For naturally or anthropogenically driven microbial invasions, the survival of an alien species is highly influenced by the resident community, namely, the so-called diversity-invasion effect, where higher diversity leads to lower invasion (Mallon *et al.*, 2015, Mallon *et al.*, 2018). Several studies have reported that a lower density of pathogens, whether bacterial (Wei *et al.*, 2015) or fungal (Fu *et al.*, 2017), is observed in suppressive rhizosphere microbial communities. We indeed observed a significant reduction in disease incidence and *R. solanacearum* density in response to the BF microbiome in both field trials and pot experiments. However, despite clear differences in the bacterial community composition, BF and CF did not lead to changes in the diversity of the rhizosphere bacterial communities for either experiment. Thus, it is unlikely that the suppression of *R. solanacearum* in response to BF treatment can be explained by the diversity of the native soil bacterial communities, both in the bulk and rhizosphere soils; rather, this effect is likely associated with community structure and the dynamics imposed by the treatment.

Functional mechanisms drive suppression

Among all enriched functions under pathogen invasion, microbial secondary metabolism is a rich source of low molecular weight bioactive compounds with various functions that facilitate the interaction of a microbe with its environment (Medema *et al.*, 2011; Seyedsayamdost, 2019). Two of the most concerning secondary metabolite (NRPS and PKS) genes were enriched

under pathogen invasion in the BF rhizosphere. These two biosynthetic enzymes are responsible for secondary metabolites, are assembled by acyl-coenzyme A or amino acid building blocks (Silakowski *et al.*, 2001), and exhibit a remarkable array of biological activities, such as inducing plant resistance, anti-microbial and anti-parasitic activities (Cane *et al.*, 1998; Cane & Walsh, 1999; Ma *et al.*, 2016; Zhao *et al.*, 2018). The overrepresentation of NRPS and PKS in BF + Rs suggests that the invasion of the rhizosphere by *R. solanacearum* triggered a suppressiveness enhancement. Furthermore, the enrichment of other potential auxiliary functions such as translation, post-translational modification, and protein turnover may potentially help NRPS and PKS expression (Marahiel, 2016). Thus, we speculate that the functional enrichment in the BF rhizosphere under pathogen invasion represented a suppression increase toward biotic stress.

The enrichment of Sphingomonadaceae and Xanthomonadaceae mainly contributed to the increase in functional genes involved in secondary metabolite biosynthesis under invasive conditions in the BF rhizosphere. A similar phenomenon was observed, where secondary metabolite biosynthesis genes were contributed by *Luteimonas*, *Novosphingobium*, *Xanthomonas* and *Stenotrophomonas*, which were enriched in the *Rehmannia glutinosa* rhizosphere under pathogen accumulation (Wu *et al.*, 2018). Among these two families, plant-colonizing *Lysobacter* and *Stenotrophomonas* showed direct pathogen inhibition ability, which is consistent with previous studies reporting antimicrobial abilities of these genera (Hayward *et al.*, 2010; Gómez Expósito *et al.*, 2015) through the secretion of secondary metabolites with antibiotic activity (Ryan *et al.*, 2009; Zhang *et al.*, 2014; Brescia *et al.*, 2020). Although plant-colonizing *Sphingomonas* did not promote *R. solanacearum* suppression directly, some of them showed strong *in vivo* bacterial wilt suppression, which was associated with NRPS gene enrichment. This suggests that *Sphingomonas* spp. might induce plant tolerance to bacterial wilt through secondary metabolite secretion (Asaf *et al.*, 2020). The taxa of these two families from suppressive soil have been widely reported to be induced by OF and BF, which potentially contribute to disease suppression (Wei *et al.*, 2011; Shen *et al.*, 2014; Xiong *et al.*, 2017a; Liu *et al.*, 2018). The key role of these taxa and associated functions in the suppression of *R. solanacearum* in response to BF application was further verified by *in vivo* experiments, where the presence of these strains induced suppressiveness in conducive soils, which was linked to the abundance of NPRS and PKS genes, albeit the latter at lower extent. Thus, we speculate that the enrichment of these functional genes led to higher *R. solanacearum* suppression.

Bio-organic fertilizer stimulates the responsiveness of rhizosphere bacterial communities upon invasion

Similar functions can be provided by varied taxonomic compositions due to their functional redundancy and overlap (Allison & Martiny, 2008; Strickland *et al.*, 2009; Reed & Martiny, 2013; Louca *et al.*, 2017). Similarly, although the two types of rhizosphere compositions in this study were assembled from different treatments, their functional compositions were similar in the absence of invasion. Upon biotic stress, however, rhizosphere functional changes tended to downregulate genes associated with nutrient metabolism and upregulate those associated with secondary metabolite biosynthesis (Fig. 5). Environmental stress and disturbance have been proven to cause dramatic and conserved shifts in the root microbiome (Ibekwe *et al.*, 2010; Bissett *et al.*, 2013; Rillig *et al.*, 2019; Sun *et al.*, 2021; Xu *et al.*, 2021). The changed community normally shows a composition and specific functional shifts to alleviate the corresponding stress (Doornbos *et al.*, 2012; Brown *et al.*, 2013; Lebeis *et al.*, 2015; Bernsdorff *et al.*, 2016; Xu *et al.*, 2021). These common response characteristics indicate that rhizosphere bacterial community succession under biotic stress tended to suppress pathogens. However, the functional genes were mainly increased in the BF rhizosphere under invasion, and the corresponding functional taxa of Xanthomonadaceae were relatively abundant in the BF rhizosphere without pathogens. Hence, the BF rhizosphere was colonized by more reactive bacterial taxa that were stimulated under disturbance. Our results indicate that the pre-assemblage of rhizosphere bacterial reservoirs recruited by plants plays an important role in regulating the ability of the rhizosphere to respond to various environmental disturbances. When pathogen invasion occurs, the community assembled from long-term inorganic practice soil is not sufficient to regulate the functional shifts to suppress the disturbance. However, when soils are cultivated in the form of long-term (bio-)organic amendments, a more reactive community will be assembled in the rhizosphere. Thus, we consider that the magnitude of the response reflects the community function ceiling toward environmental disturbance. Reactive rhizosphere bacterial communities can be fostered by soil primed with bio-organic practices in response to biotic disturbance.

Conclusion

Our study confirmed that the long-term application of BF yields suppressive rhizosphere soil bacterial communities and showed that suppressiveness is triggered by changes in the community composition of the rhizosphere soil rather than only the abundance of the introduced biocontrol strain. Specifically, our experimental design shows that members of the families Sphingomonadaceae and Xanthomonadaceae with the ability to produce secondary metabolites were enriched in the plant rhizosphere but only upon pathogen invasion. Thus, priming of the soil microbiome with BF amendment fostered reactive bacterial communities in the rhizosphere of tomato plants in response to biotic disturbances. The results of this study highlight the intricate and yet unknown dynamics of the rhizosphere microbiome, and ultimate associated mechanisms, driving disease suppression in the rhizosphere microbiome manipulated by application with BF. Importantly, we provide ecological support for the targeted manipulation of the microbiome in agricultural systems to promote soil suppressiveness.

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Competing interests

None declared.

Author contributions

RL, QS designed research; XD, NZ, YL, CZ, BQ performed research; XD, HL analyzed data; XD, RL, YB, JFS wrote the article.

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Data availability

The raw sequence data for the 16S rRNA gene and metagenomics of all samples were submitted to the NCBI Sequence Read Archive database (https://www.ncbi.nlm.nih.gov/) with the accession no. SRP314987 (field experiment) and SRP319842 (pot experiment). The scripts can be found on github (https://github.com/dxh7844/Manuscript-scripts/blob/eafb1a2d5b7d9fea00dd3d226300b4167f3e7200/Manuscript%20scripts_2022.2.10.txt).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Plant biomass and soil *Bacillus* density from field experiment.

Fig. S2 *Bacillus* density from tomato rhizosphere soil in pot experiment.

Fig. S3 PCoA of bacterial community in pot experiment.

Fig. S4 The dynamic relative abundance of potential key taxa among all compartment.

Fig. S5 Responsiveness of rhizosphere bacteria with *Ralstonia solanacearum* invasion based on metagenome data.

Fig. S6 Secondary metabolites response of rhizosphere bacterial community with *Ralstonia solanacearum* invasion based on ANTISMASH database.

Fig. S7 Heatmap of bacterial community based on family level that abundance was > 0.1%.

Fig. S8 Fifteen most abundant bacteria which contributed 'V_ defense mechanism' of eggNOG.

Fig. S9 Phylogenetic tree of culturable isolates from rhizosphere.

Fig. S10 The *in vivo* and *in vitro* pathogen suppression of the isolates.

Fig. S11 Functional gene abundance from isolates inoculation experiment.

Table S1 PERMANOVA for rhizosphere microbial community in 10th day after *Ralstonia solanacearum* inoculation.

Table S2 Unifrac distance among rhizosphere microbial community in 10th day after *Ralstonia solanacearum* inoculation.

Table S3 Correlation between change scale of community indexes and *Ralstonia solanacearum* abundance of RhT10.

Table S4 Relative abundance and classification details of responsive taxa under *Ralstonia solanacearum* inoculation.

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