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Plants recruit insecticidal bacteria to defend against herbivore attacks

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ABSTRACT

Pest feeding affects the rhizobacteria community. The rhizomicrobiota activates salicylic acid and jasmonic acid signaling pathways to help plants deal with pest infestation. However, whether plants can recruit special pesticidal microorganisms to deal with attack from herbivores is unclear. A system composed of peanuts and first-instar larvae of *Holotrichia parallela* were used to analyze whether peanuts truly enrich the insecticidal bacteria after feeding by larvae, and whether inoculation of the enriched bacteria promotes the resistance of plants to herbivore. In this study, high-throughput sequencing of 16 S rRNA gene amplicons was used to demonstrate that infestation of the subterranean pest *H. parallela* quickly changed the rhizosphere bacterial community structure within 24 h, and the abundance of Enterobacteriaceae, especially *Enterobacter*, was manifestly enriched. Root feeding induced rhizobacteria to form a more complex co-occurrence network than the control. Rhizosphere bacteria were isolated, and 4 isolates with high toxicity against *H. parallela* larvae were obtained by random forest analysis. In a back-inoculation experiment using a split-root system, green fluorescent protein (*GFP*)-labeled *Enterobacter* sp. IPPBiotE33 was observed to be enriched in uneaten peanut roots. Additionally, supplementation with IPPBiotE33 alleviated the adverse effects of *H. parallela* on peanuts. Our findings indicated that herbivore infestation could induce plants to assemble bacteria with specific larvicidal activity to address threats.

1. Introduction

As sessile organisms, plants have established sophisticated defense mechanisms in the evolutionary process to cope with various stresses. Compared with long-term continuous environmental stresses such as drought, low temperature, and salinity, biological stresses such as insect feeding and pathogen infection are often diverse, instantaneous, and sporadic. Therefore, plants respond quickly and diversely to such stresses. Insect feeding causes wounding in plants and physiological changes, like reduction in levels of Zn in shoots and inhibition of plant growth (Contreras-Cornejo et al., 2021). Herbivore elicitors contained in oral secretions are believed to induce plant defenses by activating phytohormone-mediated signaling pathways, including salicylic acid, jasmonic acid, and ethylene (Schmelz et al., 2009; Aljbory and Chen, 2018). Crosstalk between these pathways induces the indirect defense of plants to attract predators and parasitoids by releasing secondary metabolites, including terpenes, fatty acid derivatives, aromatic compounds, amino acid derivatives and phenolic compounds (Aljbory and Chen, 2018; Ali et al., 2011; Ayelo et al., 2021; McCormick et al., 2012; Schausberger et al., 2012; Wouters et al., 2016). Some metabolites, like benzoxazinoids and flavonoids affect the distribution of plant rhizosphere microorganisms (Rolfe et al., 2019; Cadot et al., 2021; Bag et al., 2022).

Plant root-associated microbes are numerous and complex, with 10^{11} microbial cells harbored per gram of root, 10–1000 times that of bulk soil (Berendsen et al., 2018; Egamberdieva et al., 2008; Miransari et al., 2011). This microbial community is utilized by plants to address biotic and abiotic stresses (Xun et al., 2021). Plants coexist with root-associated microbes and have evolved the ability to enrich and maintain beneficial microorganisms with related biological functions (Berendsen et al., 2018; Theis et al., 2016; Vorholt et al., 2017; Santos-Medellín et al., 2017) to protect plants from abiotic stresses, such as

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drought (Yuan et al., 2016) and high salinity (Kwak et al., 2018), and biotic stresses, such as plant pathogens (Berendsen et al., 2018; Pangesti et al., 2013). Root-associated microbes play a crucial role in the interplay between plants and insects, and some species or the whole community in native soil can trigger defense responses in plants to improve their performance against aboveground insects (Pineda et al., 2010, 2020; Hubbard et al., 2019; Friman et al., 2021). Even some species of rare soil microbes are involved in enhancing plant defense against pest herbivory (Lee et al., 2012). In addition to these indirect defenses induced by rhizobacteria, whether plants can directly recruit bacteria with specific insecticidal activity when they face threats from insect pests is still unclear.

Insect feeding also affect the rhizosphere microbial community. Phloem-sucking aphids and whiteflies feeding on pepper elicited defense responses against bacterial pathogens and changed the belowground microbiota in 1 to 2 weeks (Kong et al., 2016; Lee et al., 2012; Bezemer et al., 2013). Belowground herbivory also induced changes in fungal composition in ragwort (*Jacobaea vulgaris*) roots (Dematheis et al., 2012). Feeding of western corn rootworms caused shifts in the rhizosphere bacterial and fungal communities and induced an increase in the abundance of *Acinetobacter calcoaceticus* (Dematheis et al., 2012). Our previous study found that root feeding by *Holotrichia parallela* Motschulsky (Coleoptera: Scarabaeidae) larvae changed the rhizosphere bacterial community of peanut in 24 h in two independent repetitions of greenhouse experiments, and abundance of 67 genera, including *Pseudomonas* were increased (Geng et al., 2018). But the effects of larva-feeding on functions of rhizobacteria and effects of rhizobacteria-insect interactions on plants were unclear. Moreover, there are many insecticidal microorganism resources in the soil, such as *Bacillus thuringiensis* (Schnepf et al., 1998). We hypothesized that herbivore feeding may quickly change the rhizobacterial community structure and function and that plant roots may enrich specific species of bacteria with specific toxicity against insects to deal with these threats. In this study, a model consisting of peanuts, subterranean insect pests (larvae of *H. parallela*) and rhizosphere bacteria was utilized (Fig. S1) to test our hypothesis. High-throughput sequencing of 16 S rRNA gene amplicons, and metagenome sequencing, were used to analyze the effects of larval feeding on rhizobacterial community composition and function. We aimed to determine (1) whether microbial community analysis might help to find the bacterial strains with insecticidal activity, (2) whether peanuts truly enrich the insecticidal bacteria after feeding by larvae, and (3) whether inoculation of the enriched bacteria promotes the resistance of peanuts to the larvae of *H. parallela*.

2. Materials and methods

2.1. Experimental design of the field experiment

The field experiment was conducted at Langfang Experimental Station, IPP, CAAS (Langfang, Hebei, China). The soil type is the Cambisols soil. The annual average temperature of Langfang is 11.9 ℃, and the annual average precipitation is 554.9 mm. *Huayu22* peanuts were planted in a field approximately 0.067 ha in size. Forty-five stainless steel cages (35 cm high, 25 cm wide, and 45 cm long, 80 mesh) were buried at five points in the field to prevent the escape of *Holotrichia parallela* larvae (Fig. S1B and C). Three peanut seedlings were planted in each cage. Sixty days after germination, seedlings in cages were treated with root feeder *H. parallela* larvae (designated WG) or scissor injury (designated M), and nontreated seedlings were used as a control (designated Con) (Fig. S1A). Twelve 3rd-instar larvae starved for 2 days were inoculated in each cage (Fig. S1C) for WG treatment. At the same time, 10 root tips of 1 cm length were cut off from each seedling, and roots were buried again for mechanical wounding treatment. Ten to 12 peanuts were collected 1, 2 and 7 days after larvae feeding or scissor injury (Fig. S1A). Root samples without pest feeding were also collected as controls at each time point. A 3rd-instar larva was kept in each small stainless steel cage with soil and placed near the roots for 1 day, and soil samples surrounding the larva were collected (designated S, Fig. S1D).

Rhizosphere samples were collected as described in our previous study (Geng et al., 2018). The root segments were cut with sterile scissors and transferred to a sterile 50 mL plastic centrifuge tube containing 40 mL phosphate buffer saline (PBS, pH 7.4) buffer, soaked for 15 min and vibrated by using an IKA Vortex 2 vortex mixer (KA, German) at 2500 rpm for 15 s. The roots were removed, and the remaining suspension was centrifuged at 3200g for 15 min. The pellet was defined as the rhizosphere compartment and suspended in 1 mL PBS buffer.

2.2. DNA isolation and 16 S rRNA gene sequencing

Ten to 12 replicates were collected for each treatment (Fig. S1A). For each sample, 250 mg of soil was used to isolate genomic DNA. Genomic DNA was isolated from rhizosphere soils using a PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). The bacterial profile of these samples was determined by sequencing the hypervariable region of 16 S rRNA (V3 +V4 region, 469 bp) by Biomarker, Inc. (Beijing, China). The V3 +V4 region was amplified by primer 338 F (5′-ACTCCTACGGGAGGCAGCA-3′) and degenerate primer 806 R (5′-GGACTAC(A/C/T)VGGGT(A/T)TCTAAT-3′) (Mori et al., 2014) with barcodes using KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan). PCR products were purified with a Monarch DNA Gel Extraction Kit (NEB, Ipswich, MA). The library quality was assessed as described in our previous study (Geng et al., 2018) and sequenced on an Illumina HiSeq 2500 platform producing 250 bp paired-end reads. Over 50, 000 reads were obtained per sample. Data were deposited in the NCBI SRA database (accession no. SUB6975825). Raw tags were generated by merging paired-end reads using FLASH (version 1.2.11). Trimmomatic (version v0.33) (a 50-bp sliding window, minimum average quality score of 20) was used to preprocess raw tags (Bolger et al., 2014), and chimera sequences were determined and removed using UCHIME (version 8.1) (Edgar et al., 2011). Sequences with $\geq$ 97% similarity were grouped into the same OTUs by USEARCH (Version 10.0) (Edgar, 2013) and annotated using the Silva Database (Release132) based on the RDP classifier (version 2.2). Shannon, Simpson, ACE and Chao indices were calculated using Mothur (version 1.3.0) software. ANOSIM and principal coordinate analysis (PCoA) based on Bray–Curtis distance metrics were performed using R software (version 3.4.0). Welch's *t* test was used to compare the abundance differences between the treatments using STAMP (Parks et al., 2014). Random forest analysis was performed on BMKCloud (http://www.biocloud.net/) to analyze the OTU contributions to distinguish the larva-infested and uninfested samples. The networks were constructed from correlations of OTU abundance using a MENA pipeline (http://ieg4.rccc.ou.edu/mena/) (Zhou et al., 2010) and thresholds were set as 0.84. The graphics were generated using Gephi (Bastian et al., 2009).

2.3. Metagenomic sequencing

Three genomic DNA samples from each of the peanut rhizosphere samples that were *H. parallela*-treated and untreated for 24 h were mixed for metagenomic sequencing (5 WG-1d samples were mixed into one sample, and 4 Con-1d samples were mixed into one sample). One milligram of genomic DNA was used for library construction of each sample and was sequenced at Sinobiocore Biotechnology Co., Ltd. (Beijing, China). Libraries were sequenced using the Illumina NovaSeq 6000 System with 150-bp paired-end technology. Data were deposited in the NCBI SRA database (accession no. PRJNA610148). Base quality statistics of the raw FASTQ sequence were provided by FastQC (version 0.11.6) and filtered using Trimmomatic (version 0.36). Functional annotation was conducted by comparison of filtered reads to the annotated reads using FOCUS2 (version 0.1, https://github.com/metagenifocus2) based on the SEED annotation system. A library harboring insecticidal activity-related genes downloaded from the NCBI

nonredundant (NR) protein database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) was constructed, and insecticidal-related genes were identified with an E-value cutoff of 0.0001. DEseq2 was used to determine insecticidal-related genes with differential abundance.

2.4. Culture collection of rhizosphere bacteria and full-length 16 S rRNA sequencing

Peanut roots treated with *H. parallela* for 1, 2 and 7 days were collected and immersed in PBS buffer for 15 min and vibrated using an IKA Vortex 2 vortex mixer (KA, German) at 2500 rpm for 15 s. Cell suspensions were diluted 10^5-fold with PBS buffer, plated on six solidified growth media, trypic soy broth medium, tryptone yeast extract, yeast extract manitol medium, M408 medium, flour medium, and tap water yeast extract medium as described by Bai et al. (2015), and incubated for one week at 30 °C. Plates with 100–200 colony-forming units were selected. Each colony was transferred to a different well and cultured in 96-well plates supplemented with the corresponding liquid media. The 96-well plates were kept at 200 rpm at 30 °C for 1–4 days. No further purification was conducted to construct a community-based culture collection.

We used a two-step barcoded PCR protocol combined with PacBio RS II sequencing to define the 16 S rRNA genes (8th to 1492nd nucleotides) of rhizosphere bacteria as described by Armanhi et al. (2016) with minor changes in upstream and downstream primers (Fig. S2, Table S1). The PCR products were mixed in equal volumes, and 1 μg of the products was used for library construction and sequenced at Sinobiocore Biotechnology Co., Ltd. (Beijing, China) using the PacBio RS II platform (P6-C4 chemistry). Data were deposited in the NCBI SRA database (accession no. SUB8648428). After quality control process accomplished through SMRTPipe (https://github.com/bhatarchanas/smrtpipe), all circular consensus sequences (CCS) with less than 10 ×coverage were filtered out. A library with Upstream barcode and Downstream barcode (Table S1) combination was generated by in-house script and align with CCS reads' corresponding position by smith-waterman algorithm (https://github.com/lpp1985/lpp_Script/blob/master/go/src/lpp/SmithWaterman.go). After that only the alignments with less than 3-bp mismatches were retained for additional analysis.

2.5. Insecticidal activity assay

The bacteria were incubated on solidified LB medium at 30 °C for 12 h, collected and resuspended in 20 mL of sterilized water. The suspension was lysed by sonication for 15 min. Except for the preliminary screening of *H. parallela*, the single colony were used in the bioassay analysis.

H. parallela bioassays were conducted following the method described by Yu *et al.* (Yu et al., 2006) with some modifications. Carrots were cut into shreds, washed and dried naturally for approximately 1 h. Carrot shreds were soaked in a 50-mL centrifuge tube containing bacterial suspension for approximately 20 min, and the remaining suspension was evenly mixed in 120 g of sterilized soil (humidity of 18–20%) and divided into four 6-well culture plates. For preliminary screening, each well was inoculated with two 2-day-old larvae at 25 °C, and 144 larvae were used in 3 replicates for each strain. For calculating LC_{50} values, two 6-well culture plates were used with each well inoculated with one 2-day-old larvae, and 36 larvae were used in in 3 replicates for every bacterial concentration. Sterile water was used as the negative control. Larval mortality was scored after incubation for 7 days. Corrected mortality was calculated as follows:

$$M_c(\%) = \frac{(M_{obs} - M_0)}{(1 - M_0)} \times 100\%$$

M_c: Corrected mortality; M_{obs}: Observed mortality; M_0: Mortality in the control group.LC_{50} values and confidential limits were calculated using SPSS (version 26.0.0.0) according to Finney's probit analysis LC_{50} determination method (Finney, 1971).

A *Plutella xylostella* bioassay was conducted according to the method described by Tabashnik et al. (1987). A 9 cm qualitative filter paper was placed in a 9 cm petri dish and sprayed with distilled water. Chinese cabbage leaves were soaked in a 10 mL sample, dried and placed in the 9 cm petri dish. The second instar larvae were inoculated with a brush, 30 insects per dish, and cultured in an incubator at 25 °C. The concentration was 10^9 CFU/g with 3 replicates. After 48 h, the number of dead and living insects was investigated, and the mortality rate was calculated.

A *Spodoptera frugiperda* bioassay was performed according to the method described by Yang et al. (2022). First, 15 g artificial diet was placed in a sterile petri dish, and 3 mL of the sample solution was added, followed by stirring, and then evenly divided into a 24-well plate. Subsequently, the newly hatched larvae that were active and had a uniform size were picked up, inoculated into the 24-well plate and cultured under the conditions of 26 °C, RH (65 ± 5) %, and a light cycle of 16 L:8D. The concentration was 10^9 CFU/mL with 3 replicates. The mortality rate was calculated 7 days later.

To investigate the effects of bacterial strains with insecticidal activity on the performance of plants, 60 peanut seedlings were grown in pots (11 cm high and 13 cm in diameter) containing turfy soil (Honghua Company, China) and a vermiculite mixture (volume ratio 2:1) mixed with a bacteria strain IPPBiotE33 (showed toxicity against *H. parallela* larvae, isolated in this study) at a concentration of 10^9 CFU/g. Sixty peanut seedlings were grown in pots containing PBS buffer as a negative control. Seven days after germination, root and shoot length and fresh weight and dry weight of 30 seedlings without IPPBiotE33 or PBS buffer were measured as the initial values. For IPPBiotE33 and PBS treatment, 30 seedlings were inoculated with two 2-day-old *H. parallela* larvae in each pot, remaining 30 seedlings without larvae were used as control. Seven days after larval inoculation, changes in root and shoot length and fresh weight and dry weight were calculated by subtracting the initial values. The inhibitory effects on the growth of larvae were also investigated using the following equation:

$$IR = \frac{Wt - Wc}{Wc} \times 100$$

where IR is the inhibition rate of larval weight, Wt is the average weight of larvae in the group treated with IPPBiotE33, and Wc is the average weight of larvae in the group treated with PBS buffer.

2.6. Abundance analysis of IPPBiotE33 and IPPBiotC41 strains in peanut rhizosphere infested with H. parallela larvae

Strains IPPBiotE33 and IPPBiotC41 were transformed with plasmids harboring the *GFP* gene driven by a 132a promoter from *Enterobacter cloacae* (Wang et al., 2012). A vermiculite mixture and nutrient-enriched potting soil (volume ratio 1:2) were mixed with the *GFP*-IPPBiotE33 or *GFP*-IPPBiotC41 strain at a concentration of 10^5 CFU/g. Before planting, a wire mesh with an aperture of 1.70 mm was buried vertically in an acrylic tank to separate the roots into two parts (Fig. S3). A transparent acrylic tank was used to grow *Huayu22* peanuts (Geng et al., 2018) in a greenhouse at 28 °C, 60% RH, and a 14-h photoperiod for two weeks. Seven days after germination, 5 third-instar larvae starved for 48 h were inoculated per seedling (one side of the wire mesh). After 48 h, the peanut roots treated with larvae were cut off, and the remaining portions that had not been damaged by larvae were collected to prepare the rhizosphere compartments as described above (Geng et al., 2018). Selective agar plate counting and quantitative real-time PCR (qPCR) assays were used to quantify the densities of *GFP*-IPPBiotE33 or *GFP*-IPPBiotC41 using a 7500 Real-Time PCR System (Applied Biosystems). The genomic DNA was amplified with a SYBR Premix Dimer Eraser kit (TaKaRa, Dalian, China) and denatured at 95 °C for 3 min, followed by

40 cycles of 95 °C for 15 s and 60 °C for 32 s. The primers ActinF (5′-CAATGTTCCCGCCATGTAT −3′) and ActinR (5′- TTGTGCGAC-CACTTGCATA −3′) (Feng et al., 2016) were used to amplify the *Actin* gene. The primers GFPF (5′- TGGTGTTCAATGCTTTGCGAG −3′) and GFPR (5′- GCACGTGTCTTGTAGTTCCC −3′) were designed according to the *GFP* gene sequence (Table S2) and the standard curve indicated that amplification efficiency was 95.1% (Fig. S4). The abundance of strains with insecticidal activity was calculated by dividing the number of copies of the *GFP* gene by number of copies of the *Actin* gene.

2.7. Statistical analysis and data availability

Data on culturable bacteria and qPCR were analyzed using SPSS (version 19.0). For nonnormally distributed data, the rank sum test was used, and for normally distributed data, one-way ANOVA was used.

The raw sequence reads were deposited at the NCBI sequence reads archive (SRA). Accession no. SUB6975825: 16 S rRNA gene amplicon sequencing. Accession no. PRJNA610148: metagenomic sequencing. Accession no. SUB8648428: full-length 16 S rRNA sequencing of the culture collection of rhizosphere bacteria.

3. Results

3.1. Changes in the rhizosphere bacterial community structure and function caused by root-feeding pests

To investigate the effects of root-feeding pests on the rhizosphere bacterial community, root samples were collected 1, 2 and 7 days after *H. parallela* larval feeding in a field trial (Fig. S1). The total bacterial genome was isolated from the samples, and the 16 S rRNA amplicon for the V3 +V4 region was sequenced on the MiSeq Illumina platform. A total of 7359,896 reads were obtained. After filtering, 6370,896 clean tags were generated from 97 biological samples and classified into 3821 operational taxonomic units (OTUs) at a 97% sequence similarity level (2465 OTUs per sample, average length: 415 bp, Table S3). The Shannon–Wiener curve reached a plateau (Fig. S5), and the mean value of Good's coverage was 98.7 ± 0.2% (Table S3), indicating that most of the rhizosphere microbial community was detected by our analyses.

The data of 3 bulk soil samples were used to assess the validity of the OTUs (Bulgarelli et al., 2012). When the relative abundance of the OTUs was no less than 0.05%, the R^2 correlation reached 0.9 and was close to a

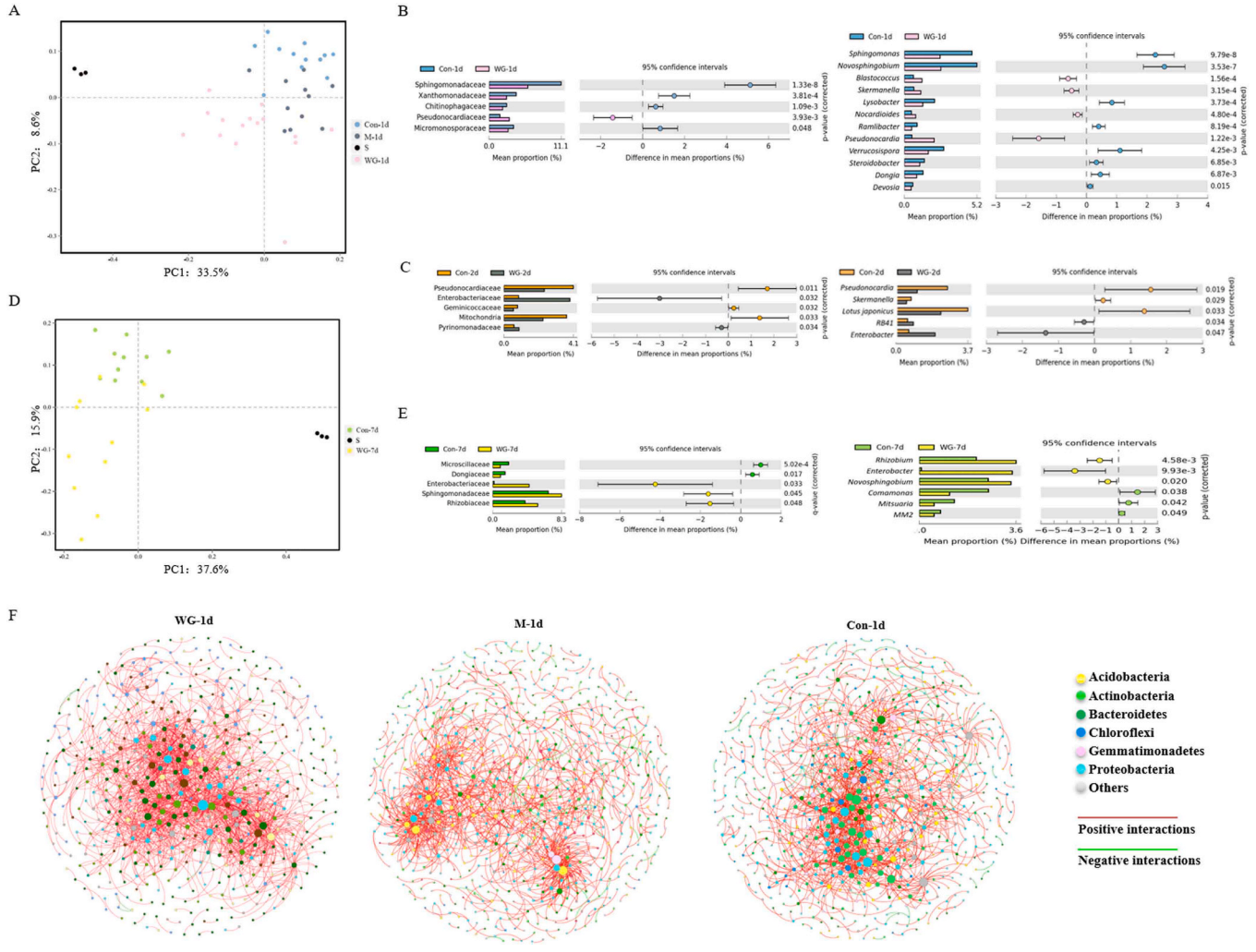


Fig. 1. *H. parallela* larval infestation affected the rhizosphere bacterial community structure of peanuts. PCoA based on Bray–Curtis distance revealed shifts in the rhizosphere bacterial community of peanuts infested with larvae after 1 d (A) and 7 d of treatment (D). The relative abundances of families and genera differed between larvae-infested and uninfested samples for the 1 d (B), 2 d (C) and 7 d (E) treatments (Student's *t* test, $p < 0.05$). Only the top 5 families and the genera with abundances greater than 0.5% are shown here. (F) Co-occurrence networks of rhizosphere microbiomes from larvae-infested (left), scissor-injured (middle) and untreated controls (right). The networks were constructed based on OTUs. Each node was marked at the phylum level, and the size of the node represented the degree calculated by Gephi. WG represents peanuts treated with white grubs (*H. parallela* larvae), M represents mechanical wounding treatment with scissor injury, Con represents the untreated control and S represents bulk soil surrounding the larvae.

horizontal line (Fig. S6). We determined that the OTUs with a relative abundance ≥ 0.05% were effective OTUs (Table S4). In total, 1379 OTUs were defined as effective OTUs, with an average of 1308 OTUs per sample (Table S3). The effective OTUs were used for the following analysis. No significant differences were observed in the alpha diversity between larva feeding treatment and negative control (Table S5). The similarity between the rhizobacterial community compositions of peanuts infested (white grub infestation, WG) and uninfested (control, Con) by larvae of *H. parallela* for 1, 2, and 7 days was analyzed using principal coordinate analysis (PCoA) and ANOSIM. The bulk soil samples (designated S) maintained with larvae showed a different bacterial profile from rhizosphere samples of peanuts (Fig. 1A). The rhizosphere bacterial community of peanut inoculated with larvae for one day significantly differed from the control plants (ANOSIM: $R = 0.352$, $P = 0.002$, Fig. 1A) and mechanically damaged plants by scissors (ANOSIM: $R = 0.297$, $P = 0.001$). Fifty-nine families and 87 genera contributed to the differences in the rhizobacterial community of peanut caused by pest feeding (Tables S6 and S7). The increase in Pseudonocardiaceae of WG-1d was driven by a subset of genera, predominantly *Pseudonocardia* (Fig. 1B). Among the top 5 different families and 12 genera between the WG-1d and Con-1d treatments, scissor wounding (designated M) only caused significant changes in the abundance of 1 family and 4 genera (Fig. S7A and B). For the two-day treatment, the relative abundance of 18 families and 22 genera changed significantly (Tables S8 and S9), but an overall shift in the rhizosphere bacterial community structure was not induced by larval infestation (ANOSIM: $R = 0.080$, $P = 0.111$). Among the top 5 families, Enterobacteriaceae was manifestly enriched in the WG-2d samples compared with the Con-2d samples, which was mainly caused by the increase in abundance of *Enterobacter* (Fig. 1C). An increase in Enterobacteriaceae and *Enterobacter* was not observed in the rhizosphere of peanuts with scissor wounding (Fig. S7C and D), indicating that the phenomenon was specifically caused by root feeding of larvae. For the seven-day treatment, clear differences in the rhizobacterial community between infested and uninfested peanuts were also observed (ANOSIM: $R = 0.357$, $P = 0.001$, Fig. 1D), and 23 families and 65 genera contributed to the difference (Tables S10 and S11). The relative abundance of Enterobacteriaceae (predominantly *Enterobacter*) was also significantly increased (Fig. 1E). Thus, *H. parallela* larval infestation affected the rhizosphere bacterial community compositions of peanuts and enriched the Enterobacteriaceae.

The co-occurrence network was analyzed to investigate the complexity of the rhizosphere microbiomes of peanuts infested or uninfested by larvae of *H. parallela*. The network modularities of the WG-1d, Con-1d and M-1d treatments were at the same level, and WG-1d exhibited a highly connected community with a higher number of edges (1795), greater connections per node (average degree = 7.342), and a shorter average path length (3.993) and diameter (10) than the Con-1d and M-1d treatments (Fig. 1F, Table S12). Although no significant differences were observed in the rhizobacterial community structure between WG-2d and Con-2d, the network of WG-2d was highly connected with more edges (2472), a higher degree (6.69), and a shorter average path length (4.916) and diameter (17) compared with WG-2d (Fig. S8A). For the seven-day treatment, the network of WG-7d harbored a shorter average path length (8.368) and a slightly smaller diameter (27) than that of WG-7d (Fig. S8B). Thus, root feeding induced rhizobacteria to form a more complex co-occurrence network than the control and mechanically damaged treatments.

Based on metagenome data, the function of the rhizosphere microbiota infested by larvae of *H. parallela* for 24 h was differentiated from that of the uninfested control (Fig. 2A). A higher abundance of sequences affiliated with electron donating reactions and fermentation was observed in the rhizobacterial community of peanut treated with larvae (Fig. 2B). We also used a self-built nucleotide sequence library to investigate the insecticidal function of the rhizosphere bacteria. In total, the abundance of 207 sequences associated with insecticidal activity increased, while the abundance of 113 sequences associated with insecticidal activity decreased (Table S13). The enriched genes associated with insecticidal activity were mainly annotated as *Pseudomonas*, *Serratia*, *Bacillus*, *Salmonella*, and *Rhodopila* (Fig. 2C). Overall, the infestation of subterranean insects for 24 h also caused a shift in rhizobacteria functions, especially insecticidal activity.

3.2. Bacterial culture collections from peanut roots infested by larvae of *H. parallela*

To analyze the function of the altered bacterial community, isolates were recovered from pooled roots (41 independent samples) fed on by larvae of *H. parallela* using community-based culture collection (Armanhi et al., 2016). In total, 4128 colonies picked from the primary plates, which may contain single or multiple bacterial strains, were obtained. We conducted two-step PCR amplification with barcoded primers (Fig. S2) and near-full-length 16 S rRNA gene amplicon sequencing on the PacBio platform to identify the taxonomic classification of colonies in each plate well (Armanhi et al., 2016). After quality filtering, 25,579 circular consensus sequences (CCSs) with coverage higher than 10 × were identified as usable CCSs. In total, 3148 of 4128 wells were recovered, and 85% of the wells contained more than 2 OTUs. The recovery rates of the top 100 OTUs from the aforementioned culture-independent bacterial community of the present study and our

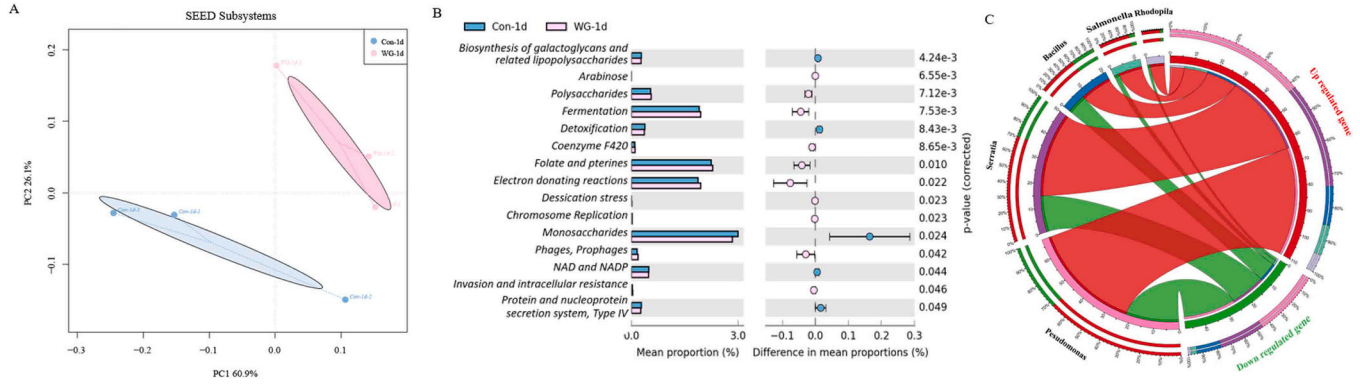


Fig. 2. *H. parallela* larval infestation affected rhizobacterial functions based on metagenome sequencing results. (A) Principal component analyses of the rhizobacterial function profiles of peanut based on the normalized SEED subsystems (level 2 classifications). (B) Differences in the abundance of functional sequences of the rhizosphere bacteria collected from larvae-infested and uninfested peanuts based on SEED databank level 2 using STAMP ANOVA. (C) Diagram representing the distribution of insecticidal-related genes with varying abundance at the genus level. Upregulated genes are marked in red, and downregulated genes are marked in green.

previous study were 31% and 29% (Fig. 3), respectively. The isolates recovered from the top 100 OTUs belonged to three phyla, Proteobacteria, Actinobacteria, and Firmicutes, and 13 families which represented 70% of the top 10 detectable bacterial families. Thus, the culture collection represented most rhizosphere bacterial families of peanut fed on by larvae of *H. parallela*.

3.3. Key strains determined by random forest analysis showed specific insecticidal activity against H. parallela larvae

Random forest analysis was performed to identify the key OTUs representing the bacterial community differences between larvae-infested and uninfested peanut roots at the three timepoints. The results showed that 11 of the top six OTUs at three timepoints belonged to Proteobacteria, and 5 of them were assigned to Enterobacteriaceae (Fig. 4). We found that 7 wells of bacterial culture collections containing isolates with the 16 S rRNA gene shared > 97% similarity with the top six OTUs (Table 1). Six out of 7 wells showed insecticidal activity against newly hatched *H.* parallela larvae (Table 1). Four single clones (corresponding to OTU30, OTU471, OTU6162, and OTU53) were isolated from the 6 wells. Based on the full 16 S rRNA sequences, they were assigned to *Enterobacter*, *Citrobacter*, and *Acinetobacter* (Table 1, Table S2). The 4 isolates possessed activity against *H. parallela* larvae with an LC_{50} of 2.86–9.49 × 10^7 colony-forming units/g (CFU/g) (Table 1, Table S14) but exhibited no insecticidal activity against *Plutella xylostella* and very little insecticidal activity against *Spodoptera exigua* even at the maximum concentration of 10^8 CFU/g (Table 1). Based on the key OTUs determined by random forest analysis, we obtained 4 new bacterial strains with specific insecticidal activity against *H.* parallela larvae.

3.4. H. parallela larvae infestation caused Enterobacter sp. IPPBiotE33 enrichment in uneaten roots

According to the results of 16 S rRNA gene sequencing, with the exception of OTU53, the abundance of OTU30, OTU471, and OTU6162, which belong to Enterobacteriaceae, significantly increased after 2 and 7 days of larval treatment (Fig. 5A), and no significant differences in the abundance of these OTUs were observed between the scissor injury treatments and uninfested control. To further analyze the abundance shift of the above bacterial isolates with insecticidal activity, the IPPBiotE33 (corresponding to OTU30) and IPPBiotC41 (corresponding to OTU471) strains were labeled with a green fluorescent protein gene (*GFP*). Peanut seedlings were planted in nutrient soil premixed with the *GFP*-IPPBiotE33 or *GFP*-IPPBiotC41 strain, and the roots were separated into two parts using a buried wire mesh, as shown in Fig. S3. To exclude the impact of the wound, 3rd instar *H. parallela* larvae were inoculated on one side of the mesh, and uneaten parts of roots were collected on the other side to analyze the abundance of strains IPPBiotE33 and IPPBiotC41 colonized on the root surface. Selective agar plate counting revealed that the density of the *GFP*-IPPBiotE33 strain attached to the uneaten root of larvae treatment significantly increased to 4.56 lg(CFU/g) in comparison with the uninfested control (Wilcoxon rank-sum test, $P = 0.011$, Fig. 5B), and the quantitative real-time PCR assay also showed that the abundance of *GFP*-IPPBiotE33 was augmented in the uneaten root of larvae treatment (ANOVA, $P < 0.001$, Fig. 5C). There was no significant change in the abundance of IPPBiotC41 belonged to *Citrobacter sp.* The above results indicated that uneaten peanut roots could assemble *Enterobacter sp.* strains when parts of the roots were attacked by subterranean pests.

3.5. Enterobacter sp. IPPBiotE33 alleviated the adverse effects of H. parallela larvae on peanuts

To further investigate whether rhizosphere bacteria help peanut seedlings deal with subterranean insect threats, *Enterobacter* sp. IPPBiotE33 was inoculated into the soil. The supplement of IPPBiotE33 had no clear effects on growth of peanut (Table 2). These plants grew well and were not seriously damaged by *H. parallela* larvae 7 days after treatment with the newly hatched larvae. The root and shoot length and biomass of peanuts were significantly higher than those of the control (Table 2). IPPBiotE33 showed an inhibitory effect on the growth of larvae with average weight of 0.014 g (Table 2) and the inhibition rate was 12.98%. Supplementation with bacterial isolates with insecticidal

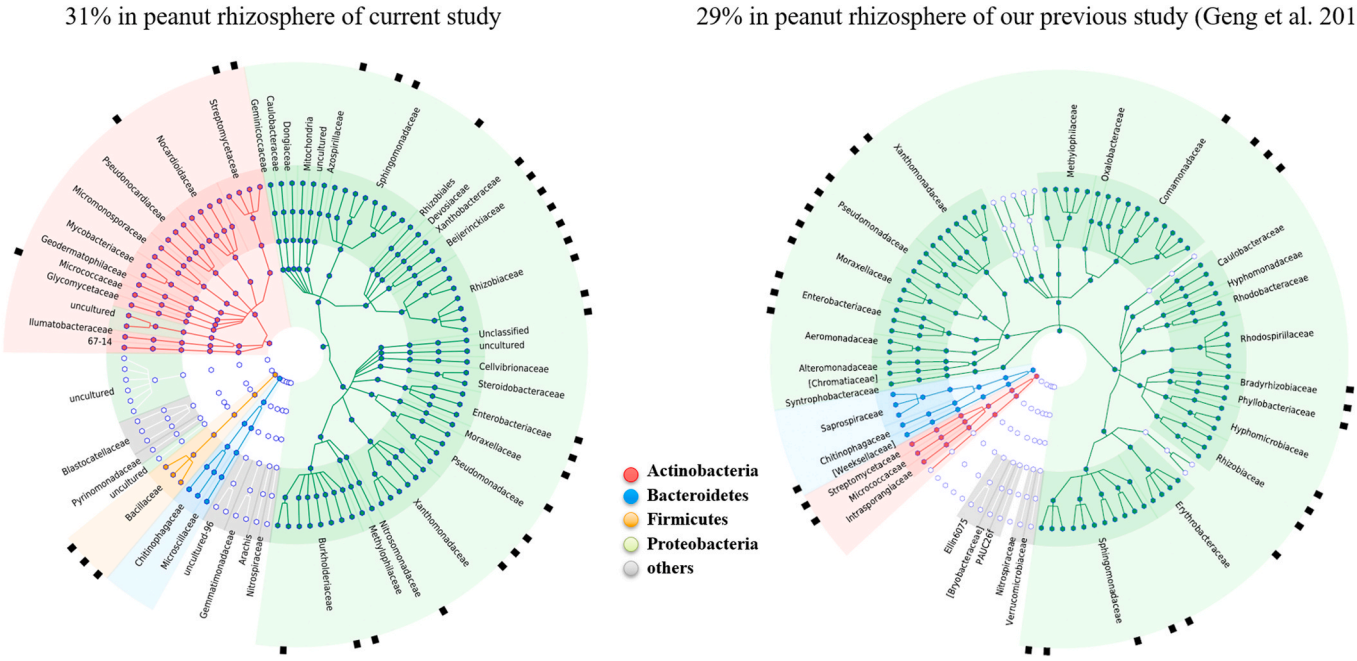


Fig. 3. Rhizosphere culture collection coverage of culture-independent bacterial communities. The inner rings display the taxonomic classification of the top 100 root-associated OTUs. Black squares of the outer circle indicate that at least one CCS of the culture collections shared ≥ 97% 16 S rRNA gene similarity with the OTUs.

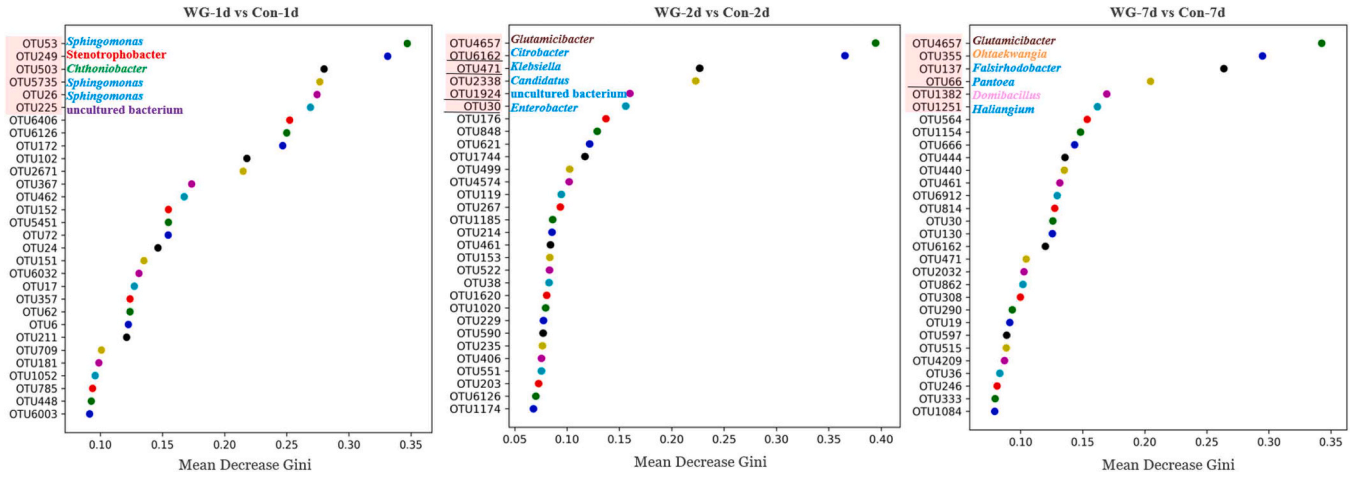


Fig. 4. Random forest analysis for the differentiation of larva-infested and uninfested samples. The ordinate displays OTUs sorted by importance, and the top six OTUs were labeled at the taxonomic level. The genera belonging to Proteobacteria are marked in blue; those belonging to Acidobacteria are marked in red, those belonging to Verrucomicrobia are marked in green, those belonging to Gemmatimonadetes are marked in purple, those belonging to Actinobacteria are marked in brown, those belonging to Bacteroidetes are marked in orange, and those belonging to Firmicutes are marked in pink. Underlined genera belong to Enterobacteriaceae. The Wilcoxon rank-sum test was used to test the difference in relative abundance between larvae-infested and uninfested samples; * represents $p < 0.05$, and * * represents $p < 0.001$.

Table 1
Bioassay analysis of isolated rhizosphere bacteria.

Key OTUs determined by random forest analysis	Well positions in the community-based culture collection (96-well plates)	Corrected mortality rate of the culture collection against *H. parallela* (Mean ±SD, %)[a]	Isolated single colony	16 S rRNA gene sequence similarity between key OTUs and single colonies	Genus level identification of single colonies (GenBank No., sequence similarity)	Insecticidal activity of the single colony		
						LC_{50} against *H. parallela*[b] (95% CL)	Corrected mortality rate (Mean±SD, %)	
							Plutella xylostella[c]	*Spodoptera frugiperda*[d]
OTU66	Plate4-H1	55.2 ± 2.1	——	——	——	——	——	——
OTU471	Plate32-C7	51.2 ± 8.7	IPPBiotC41	97.70%	*Citrobacter amalonaticus* (MN400093.1, 99.79%)	2.86 (1.26-7.08)	11.1 ± 6.7	18.8 ± 2.9
OTU53	Plate34-E8	74.1 ± 18.9	IPPBiotA42	76.10%	*Acinetobacter calcoaceticus* (MN911371.1, 100.00%)/*Acinetobacter pittii* (CP049806.1, 100.00%)	5.02 (2.15-13.61)	15.6 ± 5.1	25.0 ± 5.9
OTU6162	Plate32-B7	72.7 ± 1.4	IPPBiotC43	98.60%	*Citrobacter amalonaticus* (CP014015.2, 99.92%)	9.49 (3.43-37.99)	11.1 ± 5.8	29.2 ± 11.8
OTU4657	Plate38-D3	-2.7 ± 1.4	——	——	——	——	——	——
OTU30	Plate1-F7	40.4 ± 3.0	IPPBiotE33	97.40%	*Enterobacter xiangfangensis* (NR 126208.1, 99.23%)	7.12 (2.48-29.21)	8.9 ± 5.1	8.3 ± 11.8
OTU1924	Plate24-H10	54.8 ± 15.3	——	——	——	——	——	——

[a] The concentration of the bioassay was 10^8 CFU/g.
[b] 10^7 CFU/g. The bioassay analyses were repeated. The data of 2nd round experiment were shown in Table S13.
[c] The concentration of the bioassay was 10^9 CFU/mL.
[d] The concentration of the bioassay was 10^9 CFU/g.

activity decreased damage caused by insect pests.

4. Discussion

Some phloem-sucking pests and above- and underground chewing pests have been reported to affect rhizosphere microbial communities (Dematheis et al., 2012; Kong et al., 2016; Lee et al., 2012; Yang et al., 2011). Due to diverse effects on the rhizosphere microbiome caused by different pest species, plant species, and soil types, the influences of pest feeding on the whole microorganism community are not fully understood (Friman et al., 2021; Malacrinò et al., 2021; Tronson et al., 2022). In this study, no significant differences were found in the alpha diversity between groups, but shifts in the bacterial community were observed at 1 d and 7 d. In addition, larvae feeding caused the rhizobacteria to form a more complex co-occurrence network than the negative control, which might imply a prompt response of the bacterial community to herbivore invasion of plants, as reported by Mendes et al. (2018) and Wei et al. (2015) that the plant rhizosphere might form a more complex and highly connected network to cope with pathogen infection. Moreover, plants were treated with insects for seven days or even longer periods in previous studies analyzing the effects of pests on plant-associated microorganisms (Dematheis et al., 2012; Kong et al., 2016; Lee et al., 2012;

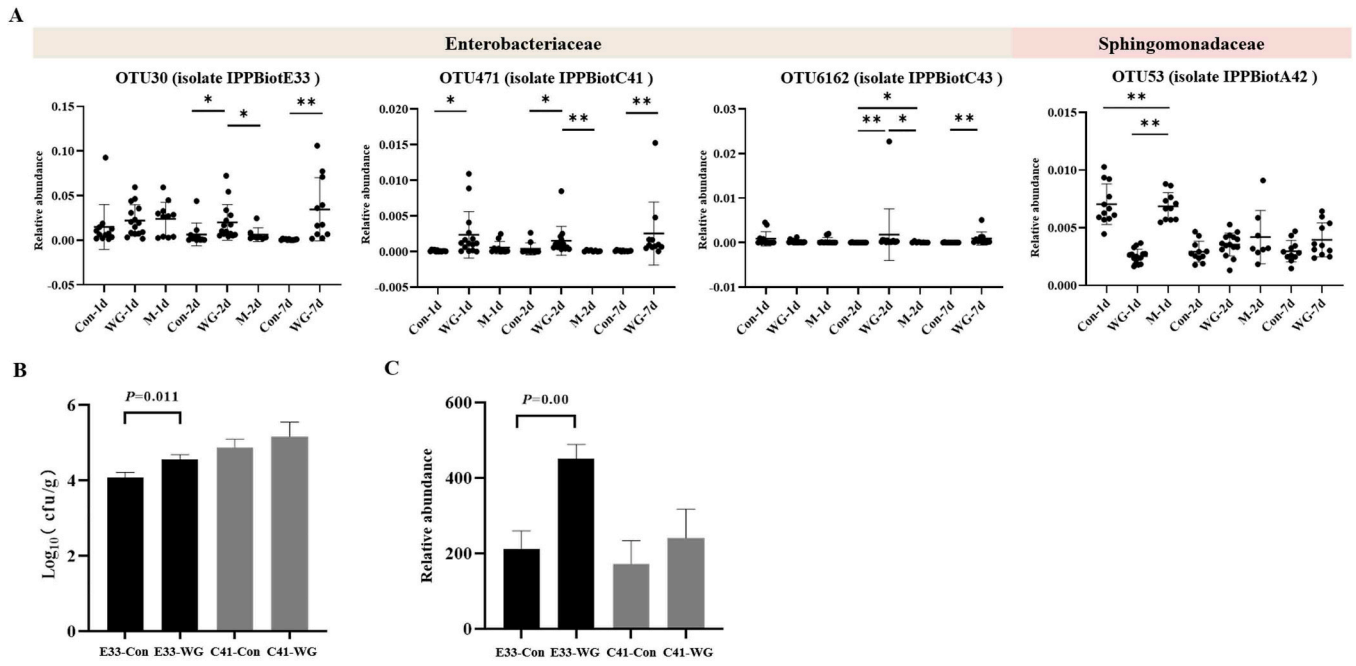


Fig. 5. Abundance shifts of bacteria with insecticidal activity. Dynamics of 4 key OTUs by MiSeq Illumina sequencing (A). Non-normally distributed data were compared by rank sum test. Plate counting (B) and qPCR (C) results showed that the population of IPPBiotE33 was enriched due to larval infestation. The relative abundance of qPCR was calculated by dividing the number of copies of the *GFP* gene by number of copies of the *Actin* gene. WG represents peanuts treated with white grubs (*H. parallela* larvae), M represents mechanical wounding treatment with scissor injury, and Con represents the untreated control.

Fig. S1. Design of the field experiment. (A) Peanut seedlings were treated with 3rd-instar larvae (WG) and scissors (M). Con represents the control without pest infestation. Root samples were collected 1 d, 2 d and 7 d after the treatments. (B) Schematic diagram of the positions of the stainless steel cages in the field. Different colors represent the treatments shown in (A). (C) A stainless steel cage was used to prevent the escape of larvae. (D) Small stainless steel cages were used to collect bulk soil, and each cage was filled with soil and one larva.

Fig. S2. A two-step PCR procedure was used to amplify the nearly full-length 16 S rRNA gene with position barcodes (Armanhi et al., 2016).

Fig. S3. Peanut seedlings were planted in an acrylic tank (A), and a wire mesh with an aperture of 1.70 mm was buried vertically to separate the roots into two parts (B). Larvae were inoculated on one side of the mesh, which prevented them from eating the roots on the other side (C). To eliminate the impact of the wound, the roots that were not eaten by the larvae were collected to analyze the densities of the IPPBiotE33 and IPPBiotC41 strains.

Fig. S4. The standard curve of *GFP* gene for qPCR analysis.

Fig. S5. The Shannon—Wiener curve of all samples.

Fig. S6. Scatter plot displaying the R2 correlation of the relative abundance of the OTUs. Three bulk soil samples were used, and Spearman's correlation coefficients were calculated using SPSS Statistics 24 (IBM, USA).

Fig. S7. The relative abundances of families and genera that differed between uninfested and scissor-injury samples at 1 d (A and B) and 2 d (C and D) (Student's *t* test, $p < 0.05$).

Fig. S8. Co-occurrence networks of rhizosphere microbiomes from larvae-infested (left) and untreated controls (right) for 2 d (A) and 7 d (B). The networks were constructed based on OTUs. Each node was marked at the phylum level, and the size of the node represented the degree calculated by Gephi. WG represents peanuts treated with white grubs (*H. parallela* larvae), Con represents the untreated control.

Fig. S9. Experimental flowchart for exploring new pesticidal bacteria that integrates 16 S rRNA gene sequencing and culturomics.

Fig. S10. Plants directly assemble pesticidal bacteria to address the threats of insect herbivores.

Yang et al., 2011). Therefore, how quickly pest-induced influences on rhizosphere microbes occur is unknown. Only a previous study (Geng et al., 2018) found that the rhizobacterial community rapidly responded to insect pest threats in 24 h in the greenhouse condition and the same phenomenon was observed by our present work in field trail. Whether the microbial community structure changes in a shorter period needs further study.

Enterobacteriaceae is a high-abundance family in plant rhizosphere. The abundance of *Escherichia/Shigella* of the family Enterobacteriaceae was increased in whitefly-infested pepper roots (Kong et al., 2016). Root feeding by *Delia radicum* also resulted in the appearance of two bacterial species of the family Enterobacteriaceae in the rhizosphere of cabbage plants, and conversely, shoot feeding did not induce the appearance of Enterobacteriaceae bacteria (Friman et al., 2021). Our previous results also found that the abundance of Enterobacteriaceae was enhanced after 24 h of feeding by *H. parallela* larvae in a greenhouse (Geng et al., 2018). In this study, root feeding for 2 d and 7 d by subterranean pests induced the enrichment of Enterobacteriaceae bacteria in peanut roots in a field trial based on the results of 16 S rRNA amplicon sequencing, and the increase of Enterobacteriaceae was not observed in mechanical wounding treatment and soil surrounding larvae. Moreover, nearly one-third of the top OTUs that were distinguished between the pest-affected and pest-unaffected rhizobacterial communities, as determined by random forest analysis, belonged to Enterobacteriaceae (Fig. 4). Enterobacteriaceae bacteria in the plant rhizosphere might respond to herbivore feeding or be attracted by new root exudate patterns caused by larva infestation.

Changes in the structure of the rhizosphere microbial community caused by insect pest feeding might lead to changes in their functions. However, research in this area is limited. Aboveground phloem-sucking pest feeding caused a bacterial community shift that induced plant defenses against bacterial pathogens in leaves and roots (Hannula et al., 2019; Yang et al., 2011). Only one report found that a mixed *Pseudomonas* spp. collected from plant roots fed on by whitefly exhibited a higher insecticidal activity against the greater wax moth than that collected from untreated plants (Kong et al., 2016). We found not only that genes with insecticidal activity were enriched in the roots treated with root-feeding herbivores (Fig. 2) but also that the abundance of

Table 2
The effects of IPPBiotE33 on the growth of peanut and *H. parallela* larvae.

Treatment Data (Mean±SD)	PBS buffer without larvae (n = 30)	PBS buffer with larvae (n = 30)	IPPBiotE33 without larvae (n = 30)	IPPBiotE33 with larvae (n = 30)
Changes in root length (cm)	3.79 ± 0.51[a]	0.56 ± 0.80[c]	3.83 ± 0.66[a]	3.37 ± 0.43[b]
Changes in shoot height (cm)	3.36 ± 0.66[ab]	0.42 ± 0.67[c]	3.46 ± 0.82[a]	3.00 ± 0.35[b]
Changes in fresh weight (g)	3.88 ± 0.94[a]	0.30 ± 0.14[b]	4.00 ± 1.37[a]	3.84 ± 0.45[a]
Changes in dry weight (g)	1.29 ± 0.40[a]	0.06 ± 0.18[b]	1.25 ± 0.38[a]	1.23 ± 0.21[a]
Changes in larval weight (g)	——	0.022 ± 0.005[a]	——	0.014 ± 0.002[b]

The Wilcoxon rank sum test showed a significant difference between the two treatments ($p < 0.001$).

bacteria with toxicity against subterranean insects was increased. In addition to the 16 S rRNA gene sequencing data, we also used IPPBiotE33 labeled with the *GFP* gene to confirm that peanut roots recruited bacteria with insecticidal activity in a split-root system. In addition, supplementation with IPPBiotE33 decreased the damage caused by *H. parallela* larvae. In summary, compared with only sequencing data, these results were more convincing for concluding that plants could assemble specific bacteria to address herbivore threats.

With the application of Bt pesticides and the widespread cultivation of Bt crops, insect resistance has been observed both in the laboratory and in the field (Tabashnik et al., 2013; Tabashnik et al., 2009). Therefore, it is urgent to explore new strains and genes with high virulence and no cross-resistance with commercially used genes. Although a large number of Bt strains and genes have been found, there are still certain limitations: conventional methods need to rely on large-scale bioassays, which are time-consuming and labor-intensive (Loguercio et al., 2001; Monnerat et al., 2007), or insecticidal genes can be predicted based on high-throughput sequencing and the existing insecticidal gene database, but it is difficult to obtain new bacterial strains and genes. In this study, we found that pest feeding induced peanut roots to enrich Enterobacteriaceae isolates with specific insecticidal activity against *H. parallela* larvae. Based on these findings, we established a new high-efficiency approach integrating microbiome analysis and culturomics to explore new insecticidal strain resources (Fig. S9).

Many Enterobacteriaceae strains exhibit insecticidal activity against insect pests. In this study, 4 isolates belonging to *Enterobacter*, *Citrobacter*, and *Acinetobacter* were obtained by blasting the 16 S rRNA gene of top six OTUs generated by random forest analysis (Fig. 4) among the CCSs data generated by PacBio sequencing. Some of the rhizobacteria are unculturable, and it is hard to isolate all of them. In order to obtain more bacterial strains, the isolates share 97% sequence similarity with top six OTUs were used for bioassay analysis for the following reasons: (a) OTUs are assigned at $\geq$ 97% similarity; (b) the strains with more than 97% nucleic acid similarity belong to the same genus; (c) V3 and V4 region of 16 S rRNA gene sequencing was performed on MiSeq Illumina platform and full length 16 S rRNA gene were sequenced on PacBio platform, and there might be sequencing errors. The 4 isolates showed high toxicity against *H. parallela* larvae. The results of 16 S rRNA high-throughput sequencing showed that in addition to the white grub infestation treatment, OTUs corresponding to the 4 isolates could also be detected in the control group (Fig. 5A). This indicated that the 4 isolates with insecticidal activity existed in the soil, and their abundance increased due to larva feeding, making them easier to isolate. *Enterobacter* sp. 532 was shown to be toxic against silkworms at a concentration of 4.54×10^8 CFU/larvae (Morishita et al., 2019). The metabolites of *Enterobacter gergoviae* and *Enterobacter cloacae* exhibited 97–100% mortalities against *Galleria mellonella* (Liao et al., 2017). Except for *Enterobacter* IPPBiotE33, no *Enterobacter* spp. have been reported to be larvicidal against coleopteran insects. Intestinal *Enterobacter* species with no toxicity accelerated poisoning in *Leptinotarsa decemlineata* infected with *B. thuringiensis* (Polenogova et al., 2021). *Citrobacter freundii* was found to affect the gut tissues of the potato beetle and increase larval susceptibility to *B. thuringiensis* (Polenogova et al., 2022). To our knowledge, *Acinetobacter* spp. are common insect gut bacteria (Spiteller et al., 2000), and our work is the first report showing their insecticidal activity. Further study is needed to determine whether the insecticidal substances of the 4 isolates are proteins, metabolites or other molecules.

5. Conclusion

The intricate interactions among plants, insect pests and plant-associated microorganisms are very important for ecosystems, and sustainable management of agricultural pests needs more in-depth and comprehensive studies on the interactions among them. In this study, we found that insect root feeding could cause changes in the rhizobacterial community structure and increase the abundance of genes related to insecticidal activity within the first 24 h of infestation. Moreover, plants could directly assemble pesticidal bacteria to address the threats of insect herbivores (Fig. S10). Microbiome analysis and culturomics provide us with a new way to explore bacterial resources for the biocontrol of pests (Fig. S9).

CRediT authorship contribution statement

Wenyue Xu: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Writing – original draft. **Xiaoxiao Sun:** Data curation, Formal analysis, Methodology, Resources, Software. **Liang Mi:** Data curation, Formal analysis, Investigation. **Kui Wang:** Formal analysis, Software. **Ziqiong Gu:** Formal analysis. **Meiling Wang:** Data curation, Formal analysis. **Changlong Shu:** Conceptualization, Methodology. **Xi Bai:** Formal analysis, Writing – review & editing. **Jie Zhang:** Supervision, Writing – review & editing. **Lili Geng:** Conceptualization, Data curation, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

The raw sequence reads were deposited at the NCBI sequence reads archive (SRA).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2023.127597.

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