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Enrichment of novel entomopathogenic *Pseudomonas* species enhances willow resistance to leaf beetles

Haitao Wang[1,2†], Fengjuan Zhang[2†], Yali Zhang[2], Mengnan Wang[2], Yiqiu Zhang[2] and Jiang Zhang[1,2*]

Abstract

Background Plants have evolved various defense mechanisms against insect herbivores, including the formation of physical barriers, the synthesis of toxic metabolites, and the activation of phytohormone responses. Although plant-associated microbiota influence plant growth and health, whether they play a role in plant defense against insect pests in natural ecosystems is unknown.

Results Here, we show that leaves of beetle-damaged weeping willow (*Salix babylonica*) trees are more resistant to the leaf beetle *Plagiodera versicolora* (Coleoptera) than those of undamaged leaves. Bacterial community transplantation experiments demonstrated that plant-associated microbiota from the beetle-damaged willow contribute to the resistance of the beetle-damaged willow to *P. versicolora*. Analysis of the composition and abundance of the microbiome revealed that *Pseudomonas* spp. is significantly enriched in the phyllosphere, roots, and rhizosphere soil of beetle-damaged willows relative to undamaged willows. From a total of 49 *Pseudomonas* strains isolated from willows and rhizosphere soil, we identified seven novel *Pseudomonas* strains that are toxic to *P. versicolora*. Moreover, re-inoculation of a synthetic microbial community (SynCom) with these *Pseudomonas* strains enhances willow resistance to *P. versicolora*.

Conclusions Collectively, our data reveal that willows can exploit specific entomopathogenic bacteria to enhance defense against *P. versicolora*, suggesting that there is a complex interplay among plants, insects, and plant-associated microbiota in natural ecosystems.

Keywords Willow, *Plagiodera versicolora*, *Pseudomonas*, Colonization, Defense

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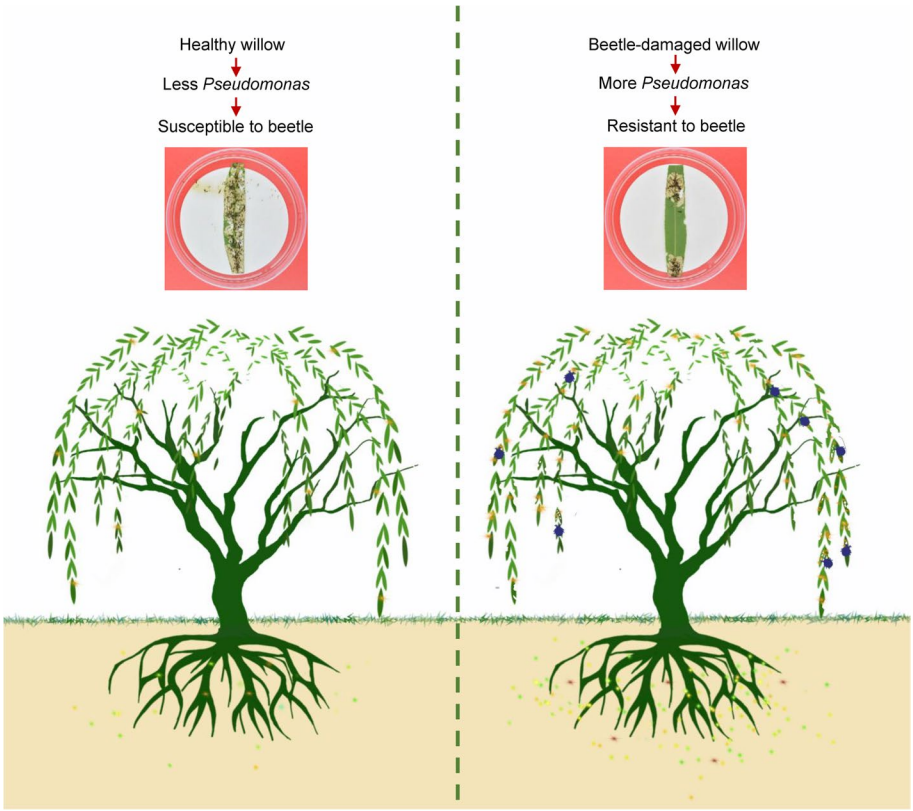
Graphical Abstract



Background

Plants face various environmental and biotic challenges during their lifetime. Among these challenges, insect herbivores represent a pervasive threat and are responsible for about 20% of the loss in total crop production worldwide annually [1]. Plants have evolved versatile defense strategies that help them withstand insect attacks, including the establishment of physical barriers, production of toxic metabolites, and activation of phytohormone responses [2].

Plants and their associated microbiomes have coevolved for more than 400 million years and have formed a "holobiont" consisting of the plant host and its symbionts [3]. Plant-associated microbiota are also referred to as the plant's second genome and play fundamental roles in plant growth and stress tolerance [4]. Accumulating evidence indicates that plants can recruit certain beneficial microbes to suppress the growth of soil-borne pathogens [5]. For example, the roots of pathogen-infected wheat [6, 7], sugar beet [8], and *Arabidopsis* [9] plants can attract groups of beneficial microbes that protect the next generation of plants. *Pseudomonas* spp. are bacteria that play an important role in promoting plant growth and pathogen resistance by producing various antimicrobial metabolites [10]. A greater richness of related *Pseudomonas* spp. has been demonstrated to be correlated with pathogen inhibition in plants and is required for plant health [11, 12]. Some strains of *Pseudomonas fluorescens* group, such as *P. protegens* and *P. chlororaphis*, also cause systemic infections and the eventual death of several Lepidopteran, Dipteran, Coleopteran, and Hemipteran insects [13–16]. However, whether plant-associated bacteria contribute to plant defenses against insects in natural ecosystems is unknown.

Plagiodera versicolora is one of the most damaging pest species to *Salicaceae* plants such as willow (*Salix*) and poplar (*Populus*). These insects are widely distributed across northern Africa, America, Europe, and Asia. Both *P. versicolora* larvae and adults feed on the leaves of willow and poplar, especially during the summer. During the winter season, adult *P. versicolora* individuals burrow into the soil to undergo overwintering, posing a continued threat to plants in the following year [17]. The life cycle of *P. versicolora* consists of several stages, including an

egg stage lasting 5–6 days, a larval stage lasting 14 days, a pupal stage lasting 4–5 days, and an adult stage that spans 3–4 weeks [18, 19]. Since Salicaceae plants are the natural hosts of *P. versicolora*, the *Salix-P. versicolora* association has been utilized as a model system to investigate the evolution adaptations of herbivorous insects in terms of their resource-utilization traits [20].

In this study, using the weeping willow (*Salix babylonica*)-*P. versicolora* association as a model system, we revealed that insect-damaged willows can enrich their microbiota in specific microbes that enhance their resistance against further damage by the insect pests. We demonstrate that insect-damaged willows are more resistant to *P. versicolora* than healthy willows in the field. We further identified certain novel *Pseudomonas* strains with insecticidal activities that are enriched in the microbiota of willows that were damaged by *P. versicolora*. Our results suggest a mechanism by which plants exploit specific entomopathogenic bacteria for defense against insect pests in nature.

Methods

Sampling

Samples were collected from two willow (*Salix babylonica*) field trials. Field site 1 (S1) is located at the Shahu Lake (30° 34′ 17″ N, 114° 20′ 4″ E), and field site 2 (S2) is located at the Nanhu lake (30° 28′ 47″ N, 114° 21′ 33″ E) in Wuhan, Hubei province, China (SI Appendix, Fig. S1A and 1B). The two sampling sites are located in a temperate climate zone with the same annual mean temperature of 22 °C and annual mean precipitation of 1343.5 mm. The leaves, roots, and rhizosphere soil of willows were sampled on August 11–12, 2021, as previously described [21]. The rhizosphere soil was defined as the soil particles adhering to the roots [21]. At each site, willows without beetle damage were classified as healthy (SI Appendix, Fig. S1C). Willows damaged by beetles were classified as sick (SI Appendix, Fig. S1D). The healthy leaves of healthy willows will be abbreviated as HL-^{HW}, the healthy leaves of sick willows as HL-^{SW}, and the sick leaves of sick willows as SL. Ten replicates from healthy and sick willows were collected from ten adjacent plots at each site. The distance between healthy and sick willows was about 0.5 km (SI Appendix, Fig. S1E). The samples were transported with dry ice and stored at − 80 °C until analysis.

Processing of samples

Willow leaves (three leaves per sample) were surface-sterilized with 75% (v/v) ethanol for 1 min and washed with sterile Millipore water three times [22]. Root samples were rinsed with sterile water to wash off the attached soil, surface-sterilized with 2.5% NaClO (w/v) for 5 min and subsequently with 70% (v/v) ethanol for 1 min, and washed with sterile Millipore water three times [23]. The treated plant samples (leaves and roots) were then chopped into small pieces using a sterile scalpel and homogenized with a sterile pestle under axenic conditions. Rhizosphere soil and homogenized plant samples were stored at − 80 °C prior to DNA extraction.

Preparation of axenic willow leaves

Preparation of axenic willow leaves was conducted as described previously [24–26]. Briefly, sick leaves (SL) and healthy leaves (HL) from sample sites were washed under running tap water for 3 min to remove all the dirt particles and impurities, and subsequently soaked in 75% alcohol for 1 min. After rinsed with sterile water three times, the leaves were immersed in 0.1% $HgCl_2$ for 5 min, and rinsed with sterile water three times. Removal of bacteria was confirmed by a colony-forming unit assay and PCR analysis using conserved primers for the 16S rRNA gene of bacteria (Fig. S2).

DNA extraction

Approximately 250 mg of rhizosphere soil was used for DNA extraction using a PowerSoil DNA Isolation Kit following the manufacturer's instructions (MoBio, Carlsbad, CA, USA). DNA extraction from willow leaves and roots (100 mg per sample) was performed using a MoBioPowerPlant Pro DNA Isolation Kit (Mo Bio Laboratories) according to the manufacturer's protocol.

Analysis of microbiota

The bacterial communities from all samples were determined based on their 16S rRNA amplicon profiles [22, 27] on an Illumina NovaSeq platform. Sequencing libraries from bacterial DNA were generated with a NEBNext® Ultra™ IIDNA Library Prep Kit (Cat No. E7645). Raw sequences were split according to their unique barcodes and trimmed off the adaptors and primer sequences using QIIME [28]. Paired-end reads were merged using FLASH (Version 1.2.11, http://ccb.jhu.edu/software/FLASH/). Quality filtering of the raw tags was performed using fastp software (version 0.20.0) to obtain high-quality clean tags, which were compared to the reference database (Silva database https://www.arbsilva.de/) to detect and remove chimeric sequences, yielding final effective tags [29]. Denoising was performed with DADA2 in QIIME2 software (Version QIIME2-202,006) to obtain initial amplicon sequence variants (ASVs). The sequences annotated as chloroplast were removed. ASVs with an abundance of < 5 reads were removed [30]. Microbial diversity and community composition were analyzed using vegan packages in R (version 3.5.3).

Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities was used to identify differences

between microbial communities. Compositional differences in NMDS between healthy and diseased samples were assessed using ANOSIM based on the Bray–Curtis distance (Table S3–8). Linear discriminant analysis (LDA) of effect size (LEfSe) was applied to the ASV table to identify differentially abundant bacterial taxa from the phyllosphere, root, and rhizosphere soil samples between beetle-damaged and healthy willows. Absolute LDA scores (> 2) were used to analyze statistical significance.

Genome sequencing and assembly

Samples were sequenced using multiplexed libraries on a Novaseq 6000 instrument to produce paired-end reads with lengths of 150 bp. For each sample, read quality was assessed with the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects_/fastqc/) and low-quality reads were removed by Quake [31]. All genome assemblies were performed with ABySS [32] using a k-mer size of 21. The best assembly for each strain, with the largest scaffold N50, was annotated by Prokka (v1.14.6) [33]. The final assembly genome information is shown in Table S9.

Phylogenetic analysis

Phylogenetic trees were reconstructed using the maximum likelihood method in MEGA version 7.0 [34]. Bootstrap support values were calculated from 1000 replicates. The phylogenetic tree was visualized with iTOL [35]. The 14 reference strains were as follows: *P. quercus* (MT036111), *P. coleopterorum* (KM888184), *P. koreensis* (AF468452), *P. neuropathica* (LR797591), *P. iridis* (LR797554), *P. piscium* (LR797558), *P. parafulva* (FNYJ01000011), *P. chlororaphis* (BBQB01000031), *P. alloputida* (LT718459), *P. flavescens* (FNDG01000047), *P. alcaligenes* (BATI01000076), *P. resinovorans* (Z76668), *P. aeruginosa* (BAMA01000316), and *P. psychrotolerans* (FMWB01000061). *Acetobacter ascendens* LMG 1590 (CP015164) was selected as an outgroup. The accession numbers of the 16S rRNA sequences are provided within the parentheses.

Quantification of *Pseudomonas* spp. colony-forming units, strain isolation, and identification

The density of *Pseudomonas* spp. from the phyllosphere, root, and rhizosphere soil samples was tested by cultivation-based methods. *Pseudomonas* colony-forming units (CFUs) were determined via serial dilutions. Three aliquots (100 µL) per dilution were spread onto CFC (cephaloridine fucidin cetrimide) medium designed for *Pseudomonas* selection [36]. The number of colonies was recorded after incubation at 28 °C for 3 days [12]. *Pseudomonas* isolates were purified, and genomic DNA from each *Pseudomonas* culture was extracted using a MiniBEST Bacteria Genomic DNA Extraction kit (TaKaRa, China) following the manufacturer's protocol. The 16S rRNA (1,466 bp) gene was amplified with primer pair 27F and 1492R (SI Appendix, Table S1) and sequenced. The 16S rRNA gene sequence of *Pseudomonas* was searched against the EzBioCloud Database [37], a well-curated database of 16S rRNA sequences and bacterial genomes.

Insect rearing

P. versicolora adults were collected from willow trees at Sha Lake Park in Wuhan, Hubei Province in China (30.35° N, 114.33 E). *P. versicolora* was regularly reared by feeding with detached fresh willow leaves at 28 °C and 60 ± 5% relative humidity under a 16-h light/8-h dark photoperiod in transparent plastic boxes (40 cm × 24 cm × 10 cm). Fresh willow leaves were replaced daily in transparent plastic boxes to serve as food for *P. versicolora* [38]. Newly laid eggs or hatched larvae were collected from willow leaves to conduct insect bioassays (at 28 °C, 60% ± 5% relative humidity, 16-h light/8-h dark photoperiod) [17]. They have been maintained since 2022. The colony is refreshed with wild-type stock every 3 months.

Insect bioassays on detached leaves

The leaves of beetle-damaged and healthy willows were fed to first-instar *P. versicolora* larvae ($n = 30$, divided into three experimental groups, with 10 larvae in each group). For each feeding experiment, synchronized larvae were selected, weighed individually, and divided into three groups (each group containing 10 larvae). To calculate the consumed leaf area, the leaves were photographed after feeding by *P. versicolora* larvae, and the consumed area was determined using ImageJ software (https://imagej.nih.gov/ij/). Pupation rate and eclosion rate were monitored as described [39]. The entire experiment was replicated three times.

(See figure on next page.)

Fig. 1 Feeding assays of *P. versicolora* larvae with leaves from beetle-damaged sick willows (SW) and healthy willows (HW). **A** Representative bioassay with detached sick leaves (SL) and healthy leaves (HL) from two sampling sites. Leaves were fed to first-instar *P. versicolora* larvae and replaced with fresh young leaves daily. The photographs were taken on day 3. Red arrows: the leaf sites damaged by beetle. **B** Leaf area consumed by first-instar *P. versicolora* larvae in **A**. **C**, **D** Pupation rate (**C**) and eclosion rate (**D**) of *P. versicolora* feeding with SL and HL from two sampling sites. **E** Larval weight after the indicated number of days of feeding on SL and HL from two sampling sites. S1, field site 1; S2, field site 2. HL-^{HW}: healthy leaves of healthy willows; HL-^{SW}: healthy leaves of sick willows; SL: sick leaves of sick willows. Significant differences between two groups were determined by Student's *t*-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

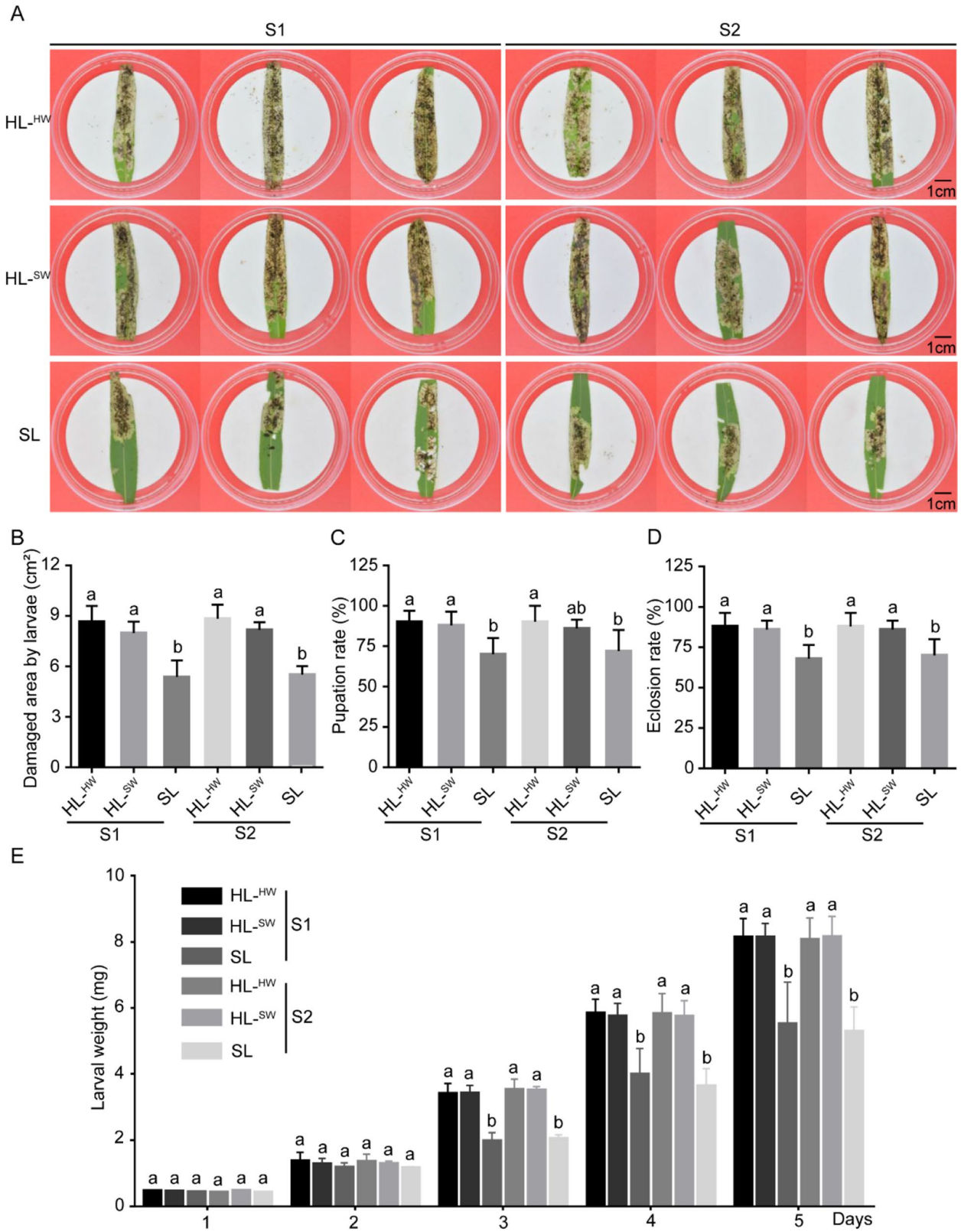


Fig. 1 (See legend on previous page.)

The pathogenicity test of *Pseudomonas* strains against insects employed the previously described methods of Vacheron et al. [14]. First-instar larvae were fed with fresh willow leaves (8 cm^2) that had been painted with 5 µL of 5.0×10^6 bacterial cells in sterile phosphate-buffered saline (PBS). Willow leaves painted with the equivalent volume of PBS solution were used as a negative control. Larvae were divided into three groups (for three biological replicates), and each group had 10 larvae per treatment. Willow leaves painted with an equivalent volume of PBS were used as negative control. Treated leaves were exchanged daily, and insect survival was recorded daily for seven consecutive days. The entire experiment was replicated three times.

Synthetic community of leaf *bacteria* (SynCom)

To generate SynCom, sick leaves (SL) and healthy leaves (HL) from sample sites were harvested (6 leaves were sampled from three individual plants), surface-sterilized with 75% ethanol for 1 min, and washed with sterile water three times. Leaves were ground in sterile water, and bacterial suspensions were diluted to 10^{-2} and plated on R2A plates for 4 days at 22 °C [22]. About 80 colonies from SL and HL were randomly picked to constitute SynCom-^{SL} or SynCom-^{HL}, respectively. SynCom-^{SL} or SynCom-^{HL} were prepared and the final OD_{600} was adjusted to 0.06 (~2×10^7 CFU/mL), and 10 µL of the SynCom-^{SL} or SynCom-^{HL} suspension was painted to each 1 cm^2 of willow leaf.

Introduction of *Pseudomonas* strains into willow plants

Willow plants were grown in a growth chamber under controlled conditions (25 °C under a 16-h light/8-h dark photoperiod) [40]. Bacterial re-introduction was conducted as described previously [41]. Briefly, *Pseudomonas* strains were incubated overnight at 28 °C in an orbital shake. Bacterial cells were collected by centrifugation at 4000 rpm for 5 min to remove the supernatant, washed with sterile PBS twice, resuspended in sterile PBS to a cell density of 10^8 cells/ml, and then inoculated onto the leaves of willow plants. SynCom-^{mix7} is a mixture of PSE-30, PSE-32, PSE-34, PSE-37, PSE-38, PSE-39, and PSE-49 (1×10^8 cells/ml for total SynCom-7 mixtures; 1.4×10^7 cells/ml for each strain), and used to inoculate leaves of willow plants. Willow plants treated with PBS served as control. The CFU of *Pseudomonas* strains on the leaves was determined by a serial-dilution method on the CFC plate [36].

Insect bioassays on whole plants

For bioassays with whole plants, first-instar *P. versicolora* larvae ($n=30$, divided into three experimental groups, with 10 larvae in each group) were allowed to feed on the healthy willow plants reintroduced with SynCom-^{mix7} or *Pseudomonas* strain PSE37/38. Larvae were divided into three groups, with 10 larvae in each group. Survival rates were recorded daily. The entire experiment was replicated three times.

Statistical analysis of data

Prior to statistical analysis, the normality of data distribution was assessed using the Shapiro–Wilk test ($P>0.05$). All statistical analyses were performed using SPSS version 19.0 software. Survival curves of first-instar *P. versicolora* larvae ($n=30$) were analyzed using the Kaplan–Meier method, and the differences in survival curves were determined using the log-rank test with a significance level at $P<0.05$. The damaged leaf area, pupation rate, eclosion rate, and larval weight were analyzed using one-way analysis of variance (ANOVA). Data comprising two groups were analyzed using a Student's *t*-test for unpaired comparisons, and data consisting of more than two groups were analyzed using one-way analysis of variance. Alpha diversity indices (e.g., Shannon index and Chao1 index) and beta-diversity were calculated using QIIME 2.

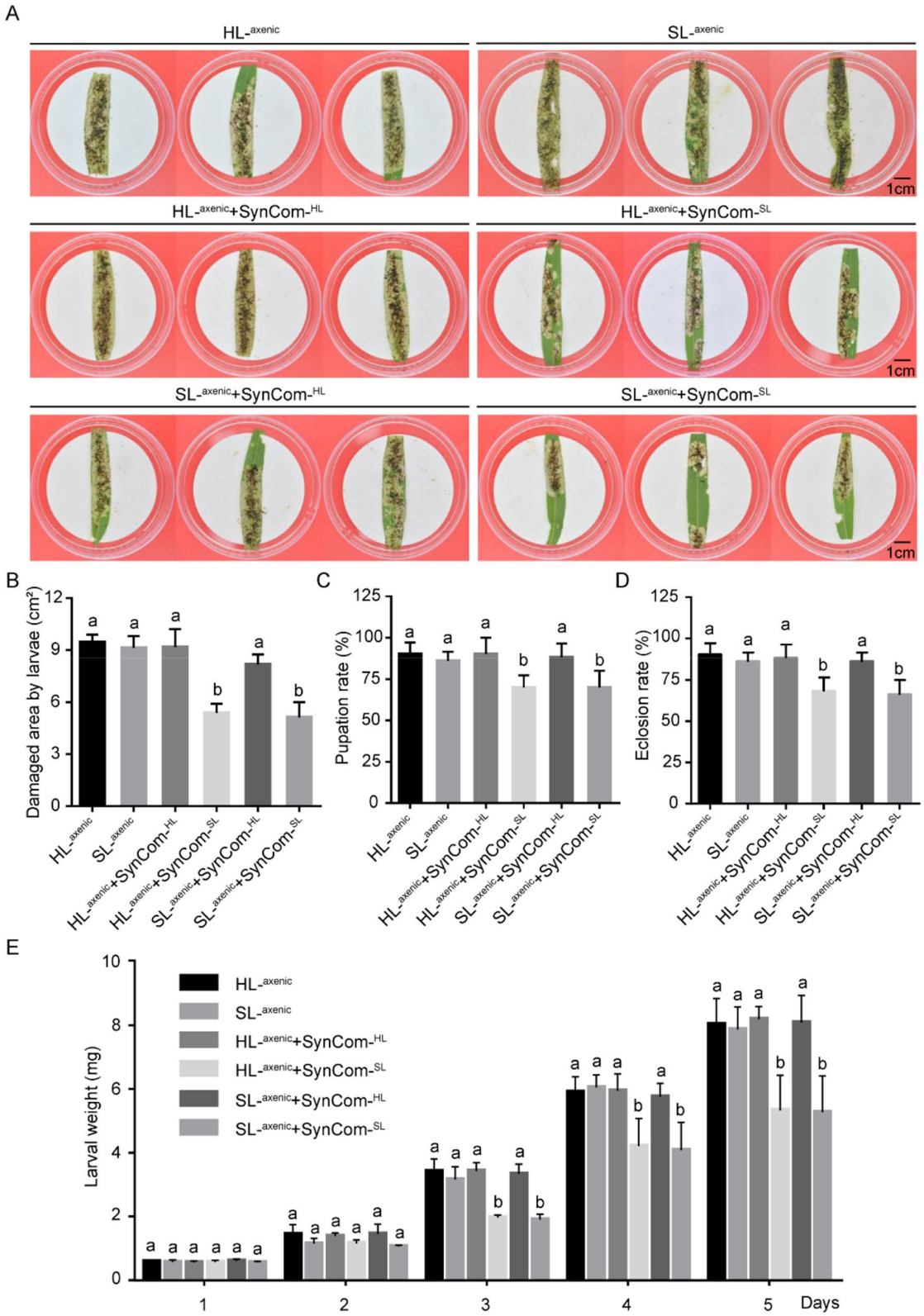
Results

Beetle-induced activation of plant defense against *P. versicolora*

To examine whether plants employ defensive tactics when repeatedly exposed to leaf beetles, we fed *P. versicolora* larvae with the sick leaves of insect-damaged sick willows (SL), the healthy leaves of healthy willows (HL-^{HW}), and sick willows (HL-^{SW}) that were sampled from two field sites in Wuhan, China (SI Appendix, Fig. S1). We observed that *P. versicolora* larvae cause less damage to SL than HL-^{HW} or HL-^{SW}, based on the area consumed by the larvae (Fig. 1A and B). Moreover, compared to that with HL, feeding with SL significantly reduced the growth of *P. versicolora* larvae from day 2 onward (Fig. 1E) and substantially delayed pupation (Fig. 1C) and eclosion rate (Fig. 1D). These results suggest that beetle damage can activate plant defenses against further damage by pests.

(See figure on next page.)

Fig. 2 Feeding assays of *P. versicolora* larvae with the detached axenic leaves that had reintroduced with SynCom-^{SL} or SynCom-^{HL}. **A** Representative bioassays with the detached axenic sick leaves (SL-^{axenic}) or healthy leaves (HL-^{axenic}) that had reintroduced with SynCom-^{SL} or SynCom-^{HL}. Leaves were fed to first-instar *P. versicolora* larvae and refreshed with the axenic leaves reintroduced with SynCom-^{SL} or SynCom-^{HL} daily. The photographs were taken on day 3. **B** Leaf area consumed by first-instar *P. versicolora* larvae in **A**. Pupation rate (**C**), eclosion rate (**D**), and larval weight (**E**) of *P. versicolora* feeding with the axenic leaves reintroduced with SynCom-^{SL} or SynCom-^{HL}



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Contribution of plant-associated microbiota in plant defense against *P. versicolora*

To assess the contribution of plant-associated microbiota in the resistance of the beetle-damaged willows to *P. versicolora*, we assembled two synthetic microbial communities from sick leaves (SynCom-^{SL}) and healthy leaves (SynCom-^{HL}) of sick and healthy willow plants, respectively, and introduced them onto the axenic sick or healthy leaves that had been in vitro sterilized (Fig. 2 and Fig. S3). Compared to the axenic healthy or sick leaves that were reintroduced with SynCom-^{HL}, reintroduction of SynCom-^{SL} significantly reduced the damage to willow leaves (Fig. 2A and B), and substantially delayed pupation (Fig. 2C), eclosion rate (Fig. 2D), and larval weight (Fig. 2E) of *P. versicolora*. These results demonstrated that plant-associated microbiota from the beetle-damaged willow contribute to the resistance of the beetle-damaged willow to *P. versicolora*.

Pseudomonas spp. is enriched in the phyllosphere of beetle-damaged willows

We next investigated the composition of the bacterial community within the phyllosphere of SL and HL by deep sequencing of amplified bacterial 16S rRNA sequences. Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity indicated that bacterial communities from SL and HL were significantly different in samples from both Shahu Lake and Nanhu Lake (Figs. 3A and 4A, B, ANOSIM, $P < 0.01$). We estimated alpha diversity based on the Shannon index and Simpson index and revealed a significant difference for the α-diversity indices between phyllosphere bacteria from SL and HL from Shahu Lake (Fig. 3B, C, $P < 0.01$).

Linear discriminant analysis of effect size (LefSe) showed that the genus *Pseudomonas* was significantly more abundant in the phyllosphere of SL compared to HL (Fig. 5A and B). Similarly, we thus isolated *Pseudomonas* from SL and HL; indeed, more *Pseudomonas* species were present in SL samples than in HL (Fig. 5C). These results suggest that *Pseudomonas* spp. is enriched in willow leaves damaged by beetles.

Enrichment of *Pseudomonas* in the root and rhizosphere soil samples of beetle-damaged willows

Several recent studies have indicated that infections with aboveground pathogens also alter the rhizosphere microbial community [13]. We thus tested whether leaf damage by beetles also influenced the bacterial

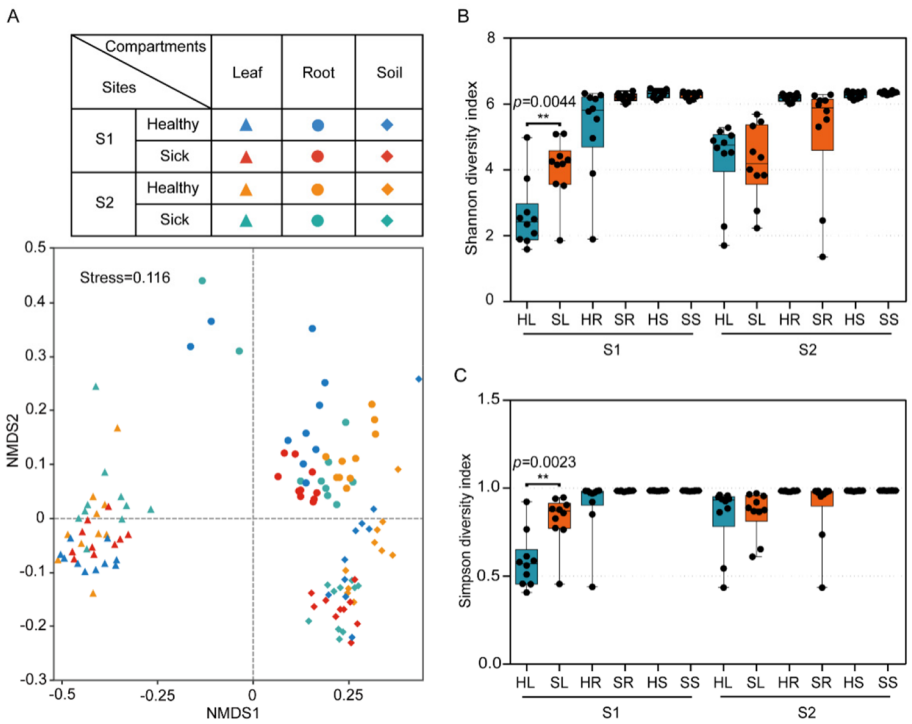

Fig. 3 Assembly of willow bacterial communities. **A** NMDS of bacterial communities in leaf, root, and rhizosphere soil of willow plants (3 sections). Solid and hollow represent two different sampling sites: Shahu Lake (S1) and Nanhu Lake (S2). Different symbols represent three different compartments: leaf (triangle), root (circle), soil (diamond). Different colors correspond to healthy (blue) and beetle-damaged sick (orange) willows, respectively. **B** Shannon diversity index of bacterial community in healthy and beetle-damaged sick willows. **C** Simpson diversity index of bacterial community in healthy and beetle-damaged sick willows

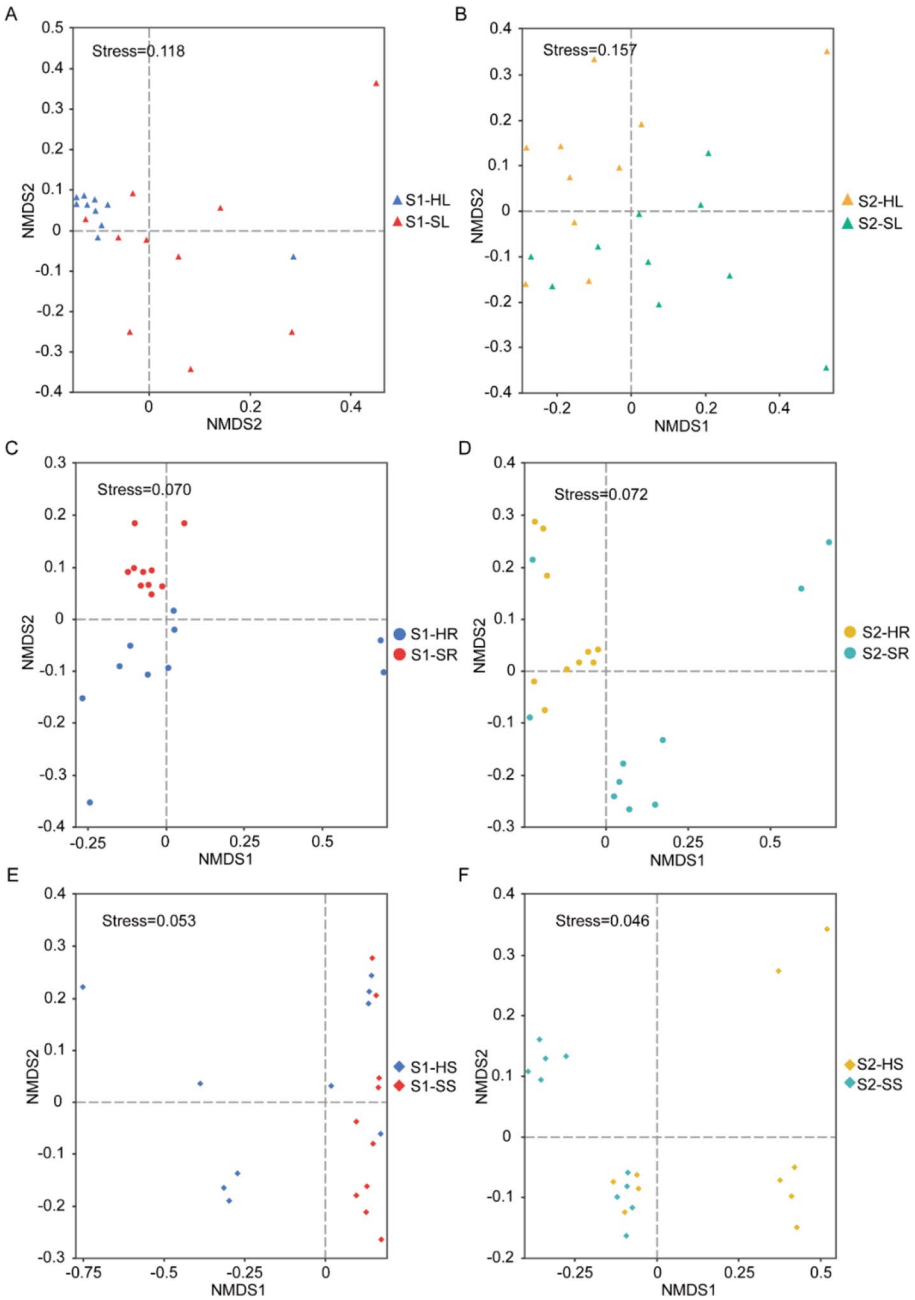

Fig. 4 Compositional differences in NMDS between healthy and diseased samples were assessed using ANOSIM based on the Bray–Curtis distance. NMDS plots depict the bacterial communities in leaves from Shahu Lake (**A**) and Nanhu Lake (**B**), roots from Shahu Lake (**C**) and Nanhu Lake (**D**), and rhizosphere soil from Shahu Lake (**E**) and Nanhu Lake (**F**). S1, field site 1 located in Shahu Lake; S2, field site 2 located in the Nanhu Lake

community in root and rhizosphere soil samples by 16S rRNA amplicon sequencing. NMDS based on Bray–Curtis dissimilarity revealed that bacterial communities from beetle-damaged sick root (SR) and healthy root (HR) were significantly different in samples from both Nanhu and Shahu Lake (Figs. 3A and 4C, D, ANOSIM, $P < 0.01$). Bacterial communities from beetle-damaged sick willows (SS) and healthy willows (HS) were significantly different in samples from both Nanhu and Shahu Lake (Figs. 3A and 4E, F, ANOSIM, $P < 0.05$). The alpha diversity of the bacterial community in the SR has no significant difference from that in the HR in terms of Shannon diversity index (Fig. 3B, C, $P > 0.05$). We observed no significant difference for α-diversity indices between rhizosphere soil bacteria from SS and those from HS (Fig. 3B, C, $P > 0.05$).

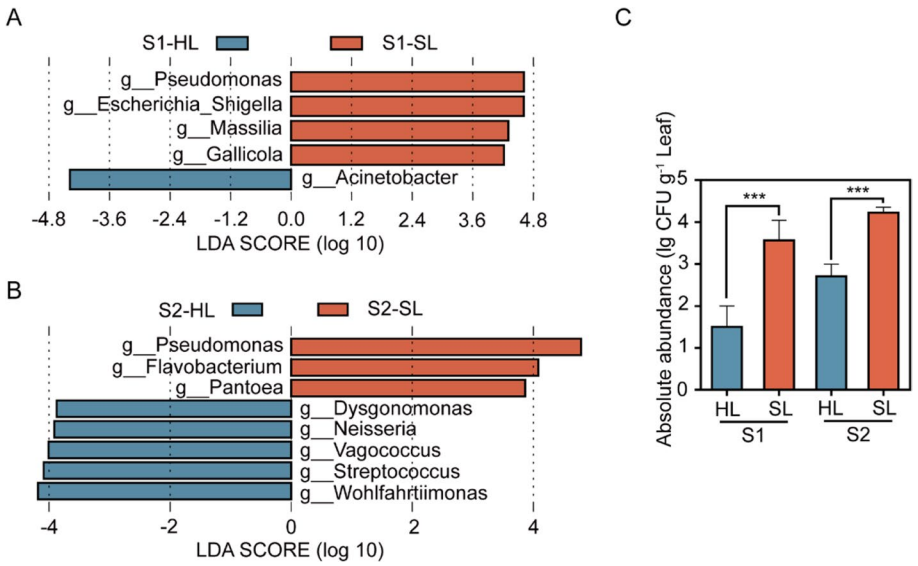

Fig. 5 *Pseudomonas* spp. is enriched in leaves from beetle-damaged sick willows. **A** and **B** Linear discriminant analysis (LDA) showing the enrichment patterns of bacteria in beetle-damaged sick leaves (SL) compared to healthy leaves (HL) in different sampling sites S1 (**A**) and S2 (**B**) at the genus (g) levels. **C** Number of *Pseudomonas* spp. isolated from SL and HL, in CFUs per gram leaf fresh weight. Significant differences between two groups were determined by Student's *t*-test. ***$P < 0.001$

LefSe showed that the genus *Pseudomonas*is was significantly more abundant in SR compared to HR samples (Fig. 6A and B). Similarly, *Pseudomonas* bacteria were significantly more abundant in SS compared to HS samples (Fig. 6C and D).

We next isolated *Pseudomonas* from root and rhizosphere soil samples collected from beetle-damaged and healthy willows. We determined that the abundance of *Pseudomonas* isolated from SR or SS samples is significantly higher than that from HR or HS samples (Fig. 6E and F). These results suggest that *Pseudomonas* spp. is also enriched in the roots and rhizosphere soil of beetle-damaged willows, similar to the phyllosphere results above.

The *Pseudomonas* enriched on beetle-damaged plants show insecticidal activity against beetles

We isolated and identified 49 *Pseudomonas* strains from the phyllosphere, root, and rhizosphere soil samples collected from beetle-damaged and healthy willows. Based on a phylogenetic analysis of these strains, we established that they represent distinct phylogenetic groups based on 16S rRNA gene sequences. Importantly, we detected nine *P. psychrotolerans* (Pp) strains specifically on samples collected from SL (Fig. 7).

To test whether isolated *P. psychrotolerans* strains have insecticidal activity, we allowed *P. versicolora* larvae to feed on healthy willow leaves that had been painted with a cell suspension of these strains. We scored the survival rate of larvae. Two *Pseudomonas* strains, *P. chlororaphis* PcS1-2 isolated from SS and PcR3-3 isolated from SR, were used as positive control. We discovered that, of the nine *P.psychrotolerans* strains tested, seven resulted in a higher mortality rate for *P. versicolora* larvae (Fig. 8A). Moreover, *P. versicolora* larvae caused less damage to willow leaves painted with these *P. psychrotolerans* strains compared to those painted with phosphate-buffered saline (PBS) as control (Fig. 8B and C). These results demonstrate that some of the *Pseudomonas* strains that are enriched in the beetle-damaged willows exhibit insecticidal activity against *P. versicolora*.

To identify potential genes associated with the observed insecticidal activities, we performed genome sequencing and assembly of *P. psychrotolerans* strains PSE37 or PSE38. Our analysis revealed the absence of genes encoding insecticidal proteins such as Fit toxin [42, 43], IPD072 [16], Chitinase C, and Phospholipase C [13], which are commonly present in *P. protegens* and *P. chlororaphis* [13], in *P. psychrotolerans* strains PSE37 or PSE38 genome (SI Appendix, Table S9).

Inoculation of a synthetic microbial community (SynCom) enhances willow resistance to beetles on whole plants

To measure the contribution of the enrichment of *Pseudomonas* strains in the beetle-damaged willows to the resistance to *P. versicolora*on whole plants, we reconstructed a synthetic microbial community (SynCom-^{mix7}) composed of seven *P. psychrotolerans* (Pp) strains isolated from SL. Re-inoculation of SynCom-^{mix7} or *Pseudomonas* strain PSE37/38 to the healthy

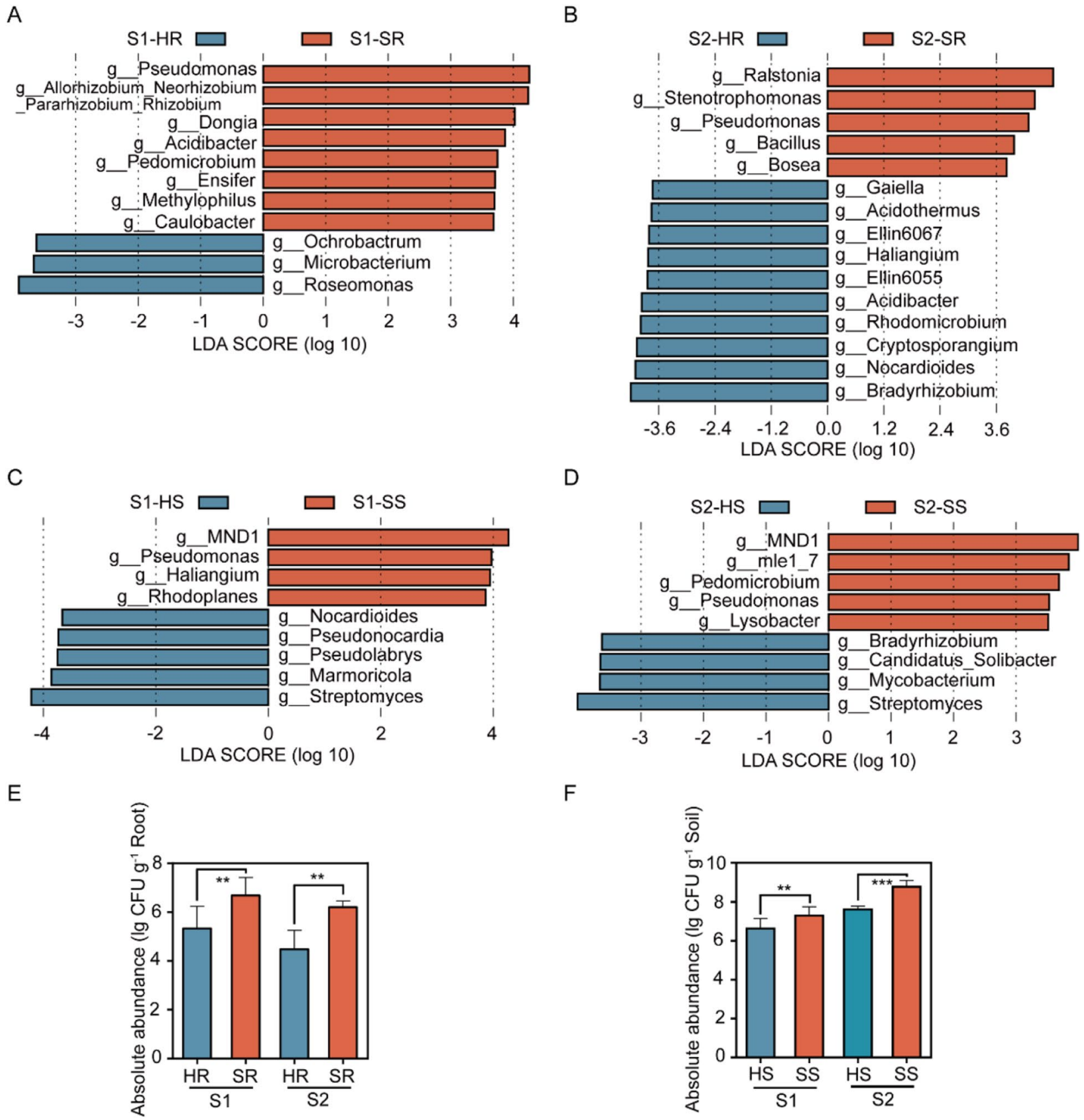

Fig. 6 *Pseudomonas*are enriched in roots and rhizosphere soils of beetle-damaged sick willows. **A** and **B** LDA showing the enrichment patterns of bacteria in SR compared to HR in different sampling sites S1 (**A**) and S2 (**B**) at the genus (g) levels. **C** and **D** LDA showing the enrichment patterns of bacteria in SS compared to HS in different sampling sites S1 (**C**) and S2 (**D**) at the genus (g) levels. **E** Number of *Pseudomonas* strains isolated from rhizosphere root samples from beetle-damaged or healthy willows. **F** Number of *Pseudomonas* strains isolated from rhizosphere soil samples from beetle-damaged or healthy willows. HR, root of heathy willow; SR, root of beetle-damaged sick willow; SS, rhizosphere soil of beetle-damaged sick willow. HS, rhizosphere soil of healthy willow. Significant differences between two groups were determined by Student's *t*-test. $^{**}P < 0.01$

willow brought out 56% mortality of *P. versicolora* larvae compared to those inoculated with PBS control (Fig. 9).

Discussion

Our data show that *Pseudomonas* strains become enriched in the microbiota of willows damaged by insect herbivores. We further determined that some of these enriched *Pseudomonas* strains have insecticidal

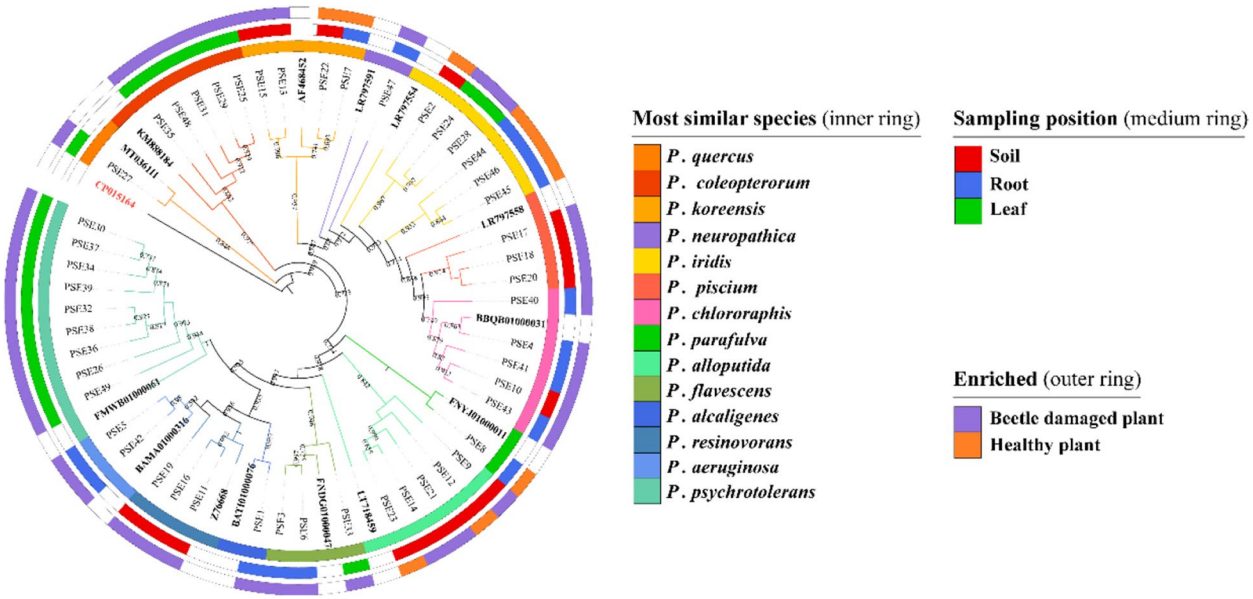

Fig. 7 Phylogenetic relationships among the *Pseudomonas* isolated from beetle-damaged and healthy willows. From the innermost to the outside circles: (1) species-level taxonomy of *Pseudomonas* spp.; (2) *Pseudomonas* spp. detected in the phyllosphere, root, or rhizosphere soil; (3) *Pseudomonas* spp. enriched in beetle-damaged willows or healthy willows. The phylogenetic tree was constructed based on bacterial 16S rRNA gene sequences using the maximum likelihood method. Fourteen reference sequences are marked in bold. *Acetobacter ascendens* LMG 1590 (CP015164) is chosen as the outgroup. The numbers at the nodes indicate the percentage levels of bootstrap support (%) based on analysis of 1000 re-sampled datasets (only values higher than 0.5 are shown)

activity, thus contributing to the protection of willows from further attacks by *P. versicolora*. We were able to reconstruct a synthetic microbial community (SynCom) of *Pseudomonas* that could enhance willow resistance to *P. versicolora*. It has been reported that SynComs have been reconstructed for the purpose of biocontrol. An example of this is the SynCom consisting of *Flavobacterium* and *Chitinophaga*, which has been shown to enhance sugar beet resistance to fungal root disease [8]. These findings provide new insights into the functional significance of insect damage-induced enrichment of *Pseudomonas* communities in willows. Consistent with this idea, plants inoculated with rhizobacterium decreased the growth of caterpillars after 4 days of feeding compared to non-inoculated control plants [44]. Similarly, recent studies showed that fungal infection of plant roots can lead to the assemblage of bacterial groups with disease-suppressive functions [8]. However, it should be noted that changes in plant nutrition, metabolite biosynthesis and secretion, and phytohormone contents can also significantly contribute to the plant resistance against insect pests. For example, introgression of the 7-epizingiberene biosynthetic pathway from wild tomato (*Solanum habrochaites*) to tomato (*Solanum lycopersicum*) cultivars produced plants that can synthesize this chemical that is toxic to spider mites while also making them less attractive to whiteflies [45].

We showed that the aboveground insect damage can also induce the assemblage of an enriched community of *Pseudomonas* in the roots and rhizosphere soil of willows (Fig. 3), suggesting that plant microbiome assembly and functions in the below and aboveground compartments under insect damage may be systemically linked. In agreement with this notion, several recent studies have also suggested that the pathogen infection of plants aboveground can induce the assemblage of a plant-beneficial bacterial consortium in the

(See figure on next page.)

Fig. 8 Insecticidal activity of *Pseudomonas* strains enriched on willows. **A** Mortality curves of *P. versicolora* in response to different isolated *Pseudomonas* strains. Kaplan–Meier survival curves of *P. versicolora* larvae ($n = 30$) after feeding with *Pseudomonas*-painted willow leaves. Feeding of PBS-painted willow leaves was used as negative control (CK). *P. chlororaphis* PcS1-2 or PcR3-3 that showed insecticidal activity toward *P. versicolora* was used as positive control. The survival curves were analyzed by the log-rank test. **B** Leaf area consumed by first-instar *P. versicolora* larvae in **A**. **C** Representative photographs of a bioassay with detached leaves from healthy willows painted with different isolated *Pseudomonas*. Leaves were fed to first-instar *P. versicolora* larvae and replaced with fresh young leaves daily. The photographs were taken after 3 days of feeding

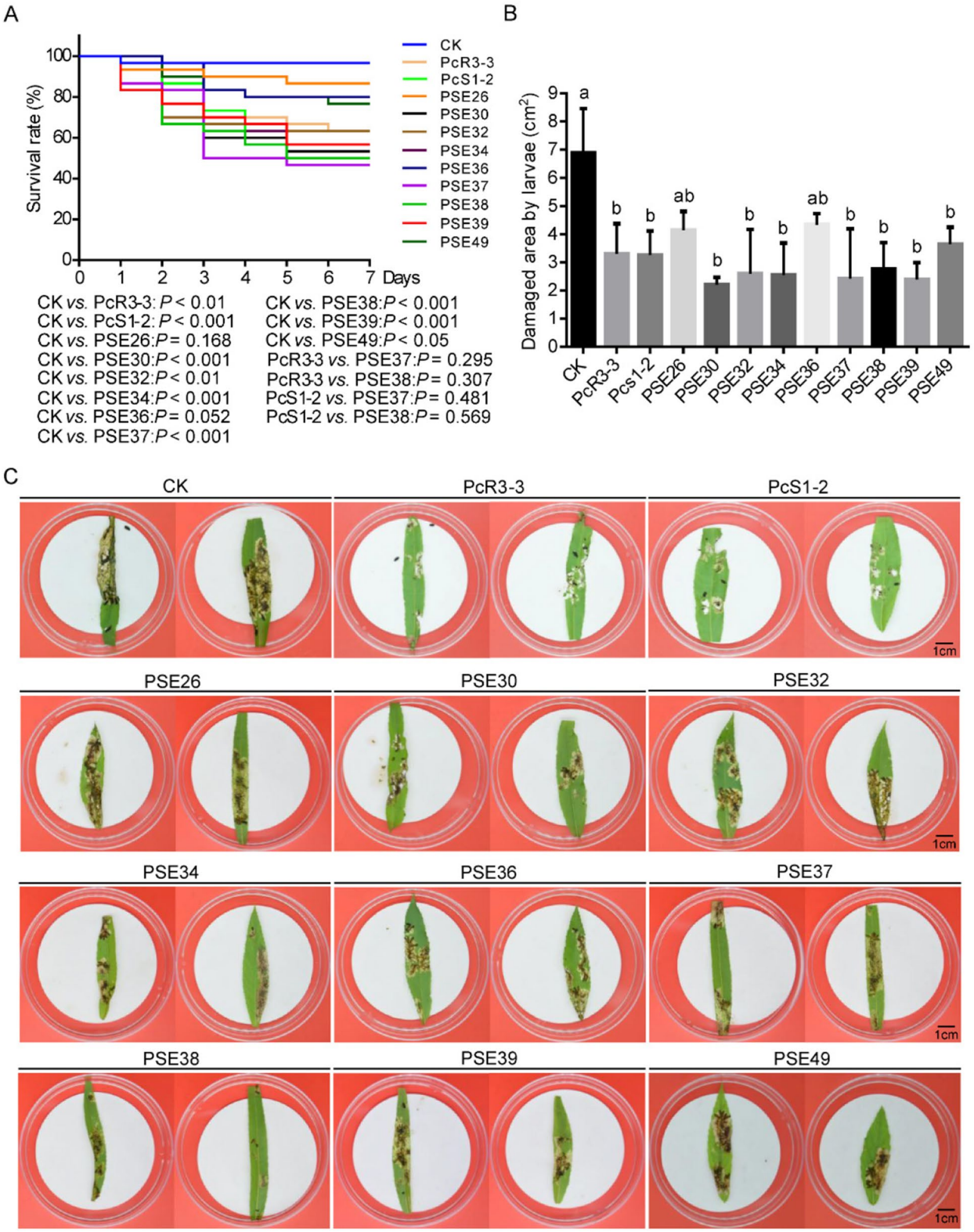

Fig. 8 (See legend on previous page.)

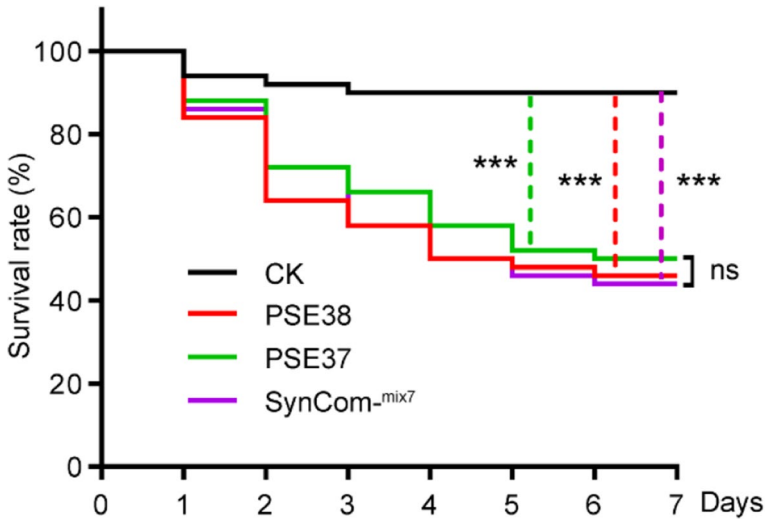

Fig. 9 Whole plant bioassays with SynCom-^{mix7} or *Pseudomonas* strain PSE37/38. Survival rates of *P. versicolora* larvae ($n = 30$) fed on the healthy willows introduced with SynCom-^{mix7}, *Pseudomonas* strain PSE37 and PSE38. Healthy willows treated with PBS solution (without bacteria) served as negative control (CK). The log-rank test was used to assess the significance of differences between two survival curves

root or soil [8, 46–48]. For example, Berendsen et al. [9] reported that upon foliar infection with the oomycete *Hyaloperonospora arabidopsidis*, three bacterial taxa are specifically enriched in the rhizosphere of *Arabidopsis* plants, induce systemic resistance against pathogens, and promote plant growth. It remains to be determined whether *Pseudomonas* spp. enriched in the rhizosphere soil also confer resistance against willow pathogens or increase the growth of willows.

In this study, we identified several novel *Pseudomonas* strains from the phyllosphere with insecticidal activities against *P. versicolora*. However, the underlying mechanism and the genes responsible for these insecticidal activities are unknown. For example, *P. psychrotolerans* strains PSE37 or PSE38 were shown to be toxic to *P. versicolora* but lack the genes encoding insecticidal proteins (SI Appendix, Table S9). Therefore, the novel insecticidal *Pseudomonas* identified in this study could provide new insecticidal genes. Although a group of insecticidal *Pseudomonas* strains was shown to be enriched in the phyllosphere of willows (Figs. 5 and 7), their origin in the phyllosphere is unclear. It has been suggested that the rhizosphere soil could be a major source of bacteria detected in the phyllosphere [49–52]. Whether insecticidal *Pseudomonas* strains can migrate from the rhizosphere soil to the phyllosphere needs to be investigated experimentally.

Conclusions

In summary, this study illuminates the complex interplay between plants, insects, and plant-associated microbiota in a natural system. By using willow, willow leaf beetle, and the associated microbiota as a model, we have uncovered a natural mechanism whereby upon damage by insect herbivory, plants can enrich their phyllosphere with insecticidal *Pseudomonas*to raise their defense against further damage by insect pests, providing new insight on plant defense against pests.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-024-01884-z.

Additional file 1. Fig. S1–S3 and Table S1–S8.

Authors' contributions

F. Z. and J.Z. conceived and designed the project; H.W., Y.Z., Y.Z., and M.W. conducted the experiments; H.W., F. Z. Y.Z. and J.Z. analyzed the data; F. Z. and J.Z. wrote the article with contributions from all other authors.

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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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