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Phenolic Acid-Degrading Consortia Increase *Fusarium* Wilt Disease Resistance of *Chrysanthemum*

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Abstract: Soil microbial community changes imposed by the cumulative effects of root-secreted phenolic acids (PAs) promote soil-borne pathogen establishment and invasion under monoculture systems, but the disease-suppressive soil often exhibits less soil-borne pathogens compared with the conducive soil. So far, it remains poorly understood whether soil disease suppressiveness is associated with the alleviated negative effects of PAs, involving microbial degradation. Here, the long-term monoculture particularly shaped the rhizosphere microbial community, for example by the enrichment of beneficial *Pseudomonas* species in the suppressive soil and thus enhanced disease-suppressive capacity, however this was not observed for the conducive soil. In vitro PA-degradation assays revealed that the antagonistic *Pseudomonas* species, together with the *Xanthomonas* and *Rhizobium* species, significantly increased the efficiency of PA degradation compared to single species, at least partially explaining how the suppressive soil accumulated lower PA levels than the conducive soil. Pot experiments further showed that this consortium harboring the antagonistic *Pseudomonas* species can not only lower PA accumulation in the 15-year conducive soils, but also confer stronger *Fusarium* wilt disease suppression compared with a single inoculum with the antagonistic bacteria. Our findings demonstrated that understanding microbial community functions, beyond the single direct antagonism, facilitated the construction of active consortia for preventing soil-borne pathogens under intensive monoculture.

Keywords: phenolic acid degradation; microbial consortium; antagonistic bacteria; disease suppression; indicator species

1. Introduction

Monoculture is the most common agricultural practice that repeatedly cultivated the same plants in soils without rotational cropping, due to limited arable lands and huge demand for foods and other economic plants. However, consecutive monoculture is not sustainable, as soil-borne pathogens rapidly emerge and cause serious yield losses [1,2]. Soil microbial community changes are responsible for pathogen establishment and invasion under monoculture systems [3,4]. Soil microbial diversity and composition are the essential components of soil heath [5]. Therefore, soil microbial community can protect plants from certain phytopathogens, which result in disease-suppressive soils [6,7].

Soil disease suppressiveness primarily relies on the mechanisms of nutrient competition and specific antagonism caused by soil micro flora [8]. This disease-control capability can be abolished by soil sterilization, and is transferred into the disease-conducive soil by mixing the disease-suppressive soil, indicating that the microbial community confers the property of the soil to prevent the build-up of pathogens and its invasion [9,10]. The phenomenon of disease-suppressive soil has been observed

for diverse soil-borne pathogens, such as *Gaeumannomyces graminis* [11], *Thielaviopsis basicola* [12], and *Rhizoctonia solani* [9]. In the suppressive soil, some indigenous microorganisms can restrain the growth of pathogens. By contrast, the conducive soil allows the rapid invasive spread of pathogens, and thus cannot offer protection against the pathogen [13]. In spite of being a vital component of soil quality, the mechanisms of soil disease suppressiveness remain elusive [12]. In some cases, disease suppression is an inherent property of soils that exhibits the compatibility with crop rotation, while the extent of disease suppression is mediated by crop rotation, involving changes of soil microbial activity [14]. Natural suppression is well documented for some fungal diseases, such as *Fusarium* wilt disease and black root rot caused by *T. basicola* [8,12]. In other cases, crop monoculture induces soil disease suppressiveness, as evidenced by a decline of disease incidence, following earlier disease outbreak. Induced disease suppression occurs in the wheat take-all disease, which is associated with the enrichment effects of *Pseudomonas* species [11].

Natural ecosystems display a distinct resistance to pathogen invasion, and soil disease suppression is closely related to microbial species diversity [15]. High microbial diversity and proper composition is in favor of soil health and plant growth [2]. Consecutive monoculture detrimentally impacts the composition of soil bacterial community and its function potentials, showing a marked decline in microbial diversity and beneficial species [16,17]. As for the conducive soil, the capability of soils to inhibit pathogens is gradually attenuated with continuous mono-cropping [2]. By contrast, the suppressive soil often maintains strong inhibition activity against pathogens under long-term monoculture, even though its microbial diversity declines [4]. However, the abundances of several specific species of microbial community are dramatically greater in the suppressive soil than in the conducive soil [14]. Some previous studies have attributed disease suppression to the *Pseudomonas* species that produce antifungal substances, such as 2,4-diacetylphloroglucinol and hydrogen cyanide [14,18]. More investigations have recently focused on the comparative analyses of the microbial community between the conducive and suppressive soils, and the identification of key indicators that participate in disease suppression [4,8]. In fact, unique soil situations lead to differential changes of microbial community, thereby contributing to variation in the ability of soils to suppress soil-borne pathogens [7,15,19,20].

Generally, phenolic acids (PAs) such as ferulic, *p*-coumaric and *p*-hydroxybenzoic acids are constantly secreted by plant roots and display allelochemical effects on plants. PAs can stimulate the growth of soil-borne pathogens and further promote the disease incidence of plants under monoculture systems [21–23]. PAs can promote the proliferation of the pathogens *Talaromyces helicus* and *Kosakonia sacchari* in the rhizosphere soil of *Radix pseudostellariae* under monoculture system [21]. In the rhizosphere of *Rehmannia glutinosa*, PAs have been shown to induce the mycelial growth and toxin production of soil-borne pathogen *Fusarium oxysporum*. PAs have also been found to inhibit beneficial microbiota in soil [22]. However, the accumulation of PAs in soils can be decomposed by various microorganisms, while the efficiency of PA decomposition largely relies on external conditions [23]. The microbial degradation of soil toxic contaminants such as 2,4-dichlorophenol and atrazine has been investigated extensively [24,25]. In many cases, a consortium participates in the biodegradation rather than the single species [26,27]. The structure and composition of microbial communities determine their function and the degradation processes, to a large extent [26,28,29]. However, bio augmentation practices readily fail by introducing single species or consortia to soils, due to complex interactions of biotic and abiotic factors in the soils, which impair the survival of inoculants and their degrading ability [30,31]. So far, it remains largely unclear whether the suppressive soil owns more efficient micro biota for degrading PAs than the conducive soil, and soil PA-degrading capacity was closely related to disease suppressiveness.

Chrysanthemum (*Chrysanthemum morifolium* cv. Chuju), a high-value medicinal plant, has been extensively planted in a mono-cropping pattern, although its yields are increasingly threatened by *Fusarium* wilt disease [32]. Here, the sequence-based approaches were conducted to investigate rhizosphere microbial community characters of both the conducive and suppressive soils of

Chrysanthemum. Our results indicated that long-term monoculture resulted in differential microbial community changes between the conducive and suppressive soils, which were likely related to soil disease suppressiveness. Through the prediction of indicator species, a model consortium was constructed on the basis of microbial antagonism and PA-degrading activity. The performances of alternative community assemblies predicted variations in the PA-degrading efficiency and were correlated with their degrading activities. Furthermore, an efficient PA-degrading consortium conferred stronger *Fusarium* wilt disease suppression as compared to single inoculum with antagonistic bacteria. This function-based assembly of optimized consortia provided an important strategy for overcoming *Fusarium* wilt disease under monoculture systems.

2. Materials and Methods

2.1. Soil Sampling

Two typical fields were continuously cropped with monoculture *Chrysanthemum* plants (*C. morifolium* cv. Chuju) for more than 15 years in Anhui province, China. Both two fields had similar soil properties and management regimes, but *Fusarium* wilt disease incidence significantly differed between the two *Chrysanthemum* plantations. The first site (conducive soil) was situated in the plantations of Shiji (32°17′ N, 118°06′ E), which had over 70% *Fusarium* disease incidence over the last three years. The second site (suppressive soil) located in the plantations of Jutai (32°13′ N, 118°23′ E), had less than 20% wilt disease incidence over the last three years. In the same regions of the two sites, the soils planted with *C. morifolium* cv. Chuju for 1 year were used for this study. No significant difference in disease incidence was observed between the 1-year conducive and suppressive soils. The two fields were 19 km apart at geography. For each site, 3 random subplots were chosen and 12 random cores (0–20 cm in depth) from each subplot were pooled to form one sample, leading to 3 samples per site.

2.2. Soil Disease Suppressiveness and Fusarium Wilt Incidence in Pot Assays

Soil samples were collected from the conducive and suppressive soils in the 1-year and 15-year monoculture for pot experiments. To examine whether soil disease suppressiveness was attributed to microbial community rather than soil physicochemical traits, soil disease-suppressive capability was evaluated as described by Mendes et al. [9] Four treatments were compared: 15-year conducive soil (Con15), 15-year suppressive soil (Sup15), Con15 amended with 10% (w/w) of Sup15, and Con15 mixed with 10% (w/w) of sterilized (autoclaving at 121 °C for 60 min) Sup15. The soils were poured into the pots, and three-week-old *Chrysanthemum* seedlings were planted in each pot. Treatments were replicated three times and each replicate contained 20 pots. These plants were cultured at 28 °C/25 °C (light/dark), and were recorded for the incidence of *Fusarium* wilt disease after three months. Disease incidence was calculated as the percentage of *Fusarium*-infected plants in total plants. To further verify that soil disease suppressiveness was attributable to induced or natural suppressiveness, disease incidence was calculated in the Con15 mixed with the Sup1 or Sup15 (w/w, 9:1).

2.3. Pyrosequencing and Quantification of Rhizomicrobial abundance

Soil DNA samples were isolated from 0.25 g of rhizosphere soils from each of three plants using a DNeasy® PowerSoil® DNA Isolation Kit (Mobio Laboratories Inc., Carlsbad, CA, USA), according to the manufacturer's instructions. DNA libraries were constructed by amplification of bacterial 16S rRNA (515F:'-GTGCCAGCMGCCGCGGTAA-3'; 907R: 5'-CCGTCAATTCMTTTRAGT TT-3') and fungal ITS regions (ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3'; ITS2: 5'-GCTGCG TTCTTCATCGATGC-3') as described by Guo et al. [33] Moreover, the sequences of Illumina Nextera adapters were ligated to the 5′ end of the universal primers for sequencing. The sequencing was carried out by the Magigene Biotechnology Co., Ltd. (Guangdong, China) on the Illumina MiSeq platform. Raw sequencing reads were deposited in the NCBI Sequence Read Archive (SRA) database, under accession number SRP188849 for bacteria and SRP188827 for fungi. All the raw reads were processed

for removing low quality sequences using the Quantitative Insights Into Microbial Ecology (QIME) software package, and operational taxonomic units (OTUs) were obtained according to the methods described by Guo et al. [33].

Quantitative real-time PCR (qPCR) was performed for quantifying bacterial, fungal, *Pseudomonas putida* and *F. oxysporum* abundances, using the SYBR® Premix ExTaq™ kit (Takara, Shiga, Japan). Overall, 25 μL of qPCR reaction mixture contained 0.5 μL of forward or reverse primers, 1 μL of soil DNA samples, 0.5 μL of ROX Reference Dye II (50 X), 12.5 μL of SYBR® Premix ExTaq™ and 10 μL of ddH_2O. qPCR reaction conditions were conducted in an ABI 7500 machine as follows: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, and then 40 s at 60 °C. Standard curves for quantifying the gene copy number of *P. putida* and *F. oxysporum* were obtained based on the previously reported methods, respectively [4,34]. The primers for *Xanthomonas* sp. NC8 (5′-CGTGCGTAGGTGGTG ATTTA-3′ and 5′-ATTCCGCTACCCTCTACCTC-3′) and *Rhizobium* sp. HU16 (5′-CATGTCCTCAT GG CCCTTAC-3′ and 5′-TGCAATCCGAACTGAGATGG-3′) were designed for qPCR analyses.

2.4. Detection of PA Content, Microbial degradation, Mcrobial inoculation and Colony counting

To extract soil PAs, 5 g of rhizosphere soil samples was incubated in a flask with 5 mL of 1 M NaOH for 16 h, and were then shaken at 220 rpm for 1 h. After centrifugation at 8000 g for 10 min, the supernatant was adjusted to pH 2.5 and incubated at room temperature for 2 h, and then were centrifuged at 8000 g for 15 min. Subsequently, the supernatant was filtered through 0.22 μm membrane and detected by high performance liquid chromatography (HPLC), as previously reported by Zhang et al. [23] To assess soil PA-degrading capability, 20 g of fresh soil samples were placed in a glass container containing 0.5 mg/g phenolic acid (ferulic, *p*-coumaric or *p*-hydroxybenzoic acid). The soils were incubated at 28 °C in dark for 50 d, and sterile soils were used as control. Soil samples were taken every 10 d for measuring the remaining PAs.

Different bacteria strains were isolated from rhizosphere soils of three-month-old *Chrysanthemum* plants cultured in the 15-year suppressive soils by the dilution plating method, and several isolates were identified by sequencing 16s rRNA genes, including *P. putida* A2 (Genbank No. MK645976), *Xanthomonas* sp. NC8 (Genbank No. MK645977), *Paenibacillus* sp. M7 (Genbank No. MK645979), *Stenotrophomonas* sp. YB9 (Genbank No. MK645981) and *Rhizobium* sp. HU16 (Genbank No. MK645993). These strains was stored in LB medium at −80 °C for the next experiments. For examining the ability of bacteria to degrade PA, 1 mL of bacterial strains at 5×10^6 CFUs was added into 100 mL of inorganic medium containing 0.9 g/L PA, and shaken at 150 rpm and incubated at 28°C, according to the method of Zhang et al. [23], with minor modifications. The supernatant of culture solutions was used for assessing the PA degradation, and the precipitate was resuspended in sterile water for measuring the values of OD_{600}. Moreover, the degradation of 0.9 g/L mixed PAs (1:1:1) performed by 16 consortia (random combination of A2 with NC8, M7, YB9 or HU16) were measured by HPLC.

To assess the growth performance of A2 in the consortia, the plasmid pJB861 was firstly transferred into this strain, and A2 harboring the pJB861 with a kanamycin resistance gene was then used to construct different combinations with other strains. As described above, the PA content in the supernatant of culture solutions was measured, and the precipitate was resuspended in sterile water and subjected to serial dilution. Serial dilutions from different combinations were spread on the Luria–Bertani (LB) medium, supplemented with 50 μg/mL of kanamycin and incubated at 28°C to enumerate A2 colonies. To perform soil inoculation, different suspensions of bacterial strains were harvested and diluted in sterile water to obtain indicated inoculum concentrations, and then the bacteria dilution solution was poured into the soils.

2.5. Effects of PAs on the Growth of F. oxysporum

PAs including ferulic, *p*-coumaric and *p*-hydroxybenzonic acids, or mixed PAs (ferulic, *p*-coumaric and *p*-hydroxybenzonic acids, 1:1:1), were prepared at concentrations of 0, 60, 180 and 540 μmol/L, respectively. *F. oxysporum* was inoculated onto the center of 9 cm Petri dishes, containing PDA

medium supplemented with different final concentrations of single PA or mixtures (0, 60, 180 and 540 μmol/L). Each treatment had ten replicates. After 1 week of incubation at 28 °C, the growth of *F. oxysporum* was measured. Spores of *F. oxysporum* was collected and then added into an 8-fold dilution of potato-sugar-agar (PSA) broth medium. After 1 week of culture with a shaker with 200 rpm at 28 °C, the spores were counted by a hem cytometer as described by Wu et al. [22]

2.6. Bioinformatics and Statistical analyses

The Chao1 richness and Shannon diversity of rhizosphere microbial community were estimated by the PAST software. Linear discriminant analysis (LDA) effect size (LEfSe) (http://huttenhower.sph.Harvard.edu/lefse/) analyses were applied to characterize the bacterial and fungal taxa significantly related to disease-conducive and -suppressive soils. The Kruskale–Wallis test was employed at $\alpha = 0.05$, and the logarithmic LDA score (>2) was used for discriminative features. Taxonomic cladograms illustrated significant differences between the conducive (Con15) and suppressive (Sup15) soils. An analysis of differential OTU abundance was carried out for selecting microbial indicator OTUs, and the Wald test was applied to determine significance (adjusted P value < 0.05) according to the method reported by Guo et al. [33] and these differential OTUs were then visualized by the Manhattan plots. Dominant OTUs (top 30 indicator OTUs based on relative abundance in the Sup15) were selected as indicator OTUs. Representative sequences of these indicator OTUs were used for the phylogenetic analyses through the neighbor-joining tree. The phylogenetic tree was drawn using iTOL (https://itol.emble.de). In addition, analyses of disease incidence, soil pH, richness, and diversity indices in soils were carried out by Tukey's HSD multiple range test ($p < 0.05$). Statistical analyses were performed in SPSS v16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Soil Characteristics and Fusarium Wilt Disease Incidence

Soil physiochemical characteristics in the two fields with one- or 15-year consecutive *Chrysanthemum* monoculture were examined (Table S1). The one-year suppressive soil (Jutai site) displayed higher OM, P and Fe content than the one-year conducive soil (Shiji site). Soil pH and EC was also higher in the 1-year conducive soil than the one-year suppressive soil. Moreover, the 15-year suppressive soil (Jutai site) exhibited higher OM, N, P and Fe content than the 15-year conducive soil (Shiji site). Similar results were also observed for the soil pH and EC. In pot experiments, three-week-old *Chrysanthemum* seedlings were transplanted into the soils, and *Fusarium* wilt incidence of *Chrysanthemum* was significantly lower in the suppressive soil after three months of culture (Figure 1a), showing only 17% disease incidence compared to about 68% disease incidence in the conducive soil (Figure 1b), indicating that the soils from the two fields differed distinctly in the suppressiveness against *F. oxysporum*.

To examine whether the capability of soil disease suppressiveness could be transferred, the 15-year conducive and suppressive soils were mixed in a ratio of 9:1. As shown in Figure 1b, transferring 10% of the suppressive soil (Sup15) into the conducive soil (Con15) markedly inhibited disease incidence, although the Con15 mixed with 10% (w/w) heat-treated suppressive soil (Sup15HT) shared similar disease incidence with the untreated conducive soil. We further assessed a microbial basis of disease suppression that resulted from the induction of crop monoculture or soil natural property. Pot experiments were designed in which the 1-year (Sup1) or 15-year (Sup15) suppressive soil was mixed with the Con15 in a ratio of 1:9, respectively. Then, three-week-old *Chrysanthemum* seedlings were transplanted into the soils and grown for three months. Transferring 10% of the Sup1 into the Con15 could not reduce *Fusarium* wilt incidence. However, transferring 10% of the Sup15 into the Con15 obviously suppressed disease outbreak. Consistently, lower *F. oxysporum* density occurred in the rhizosphere soils of Con15 mixed with the Sup15, than that with the Sup1 (Figure 1c). These data

indicated that long-term monoculture may shape specific microbiota in the suppressive soil against pathogen establishment.

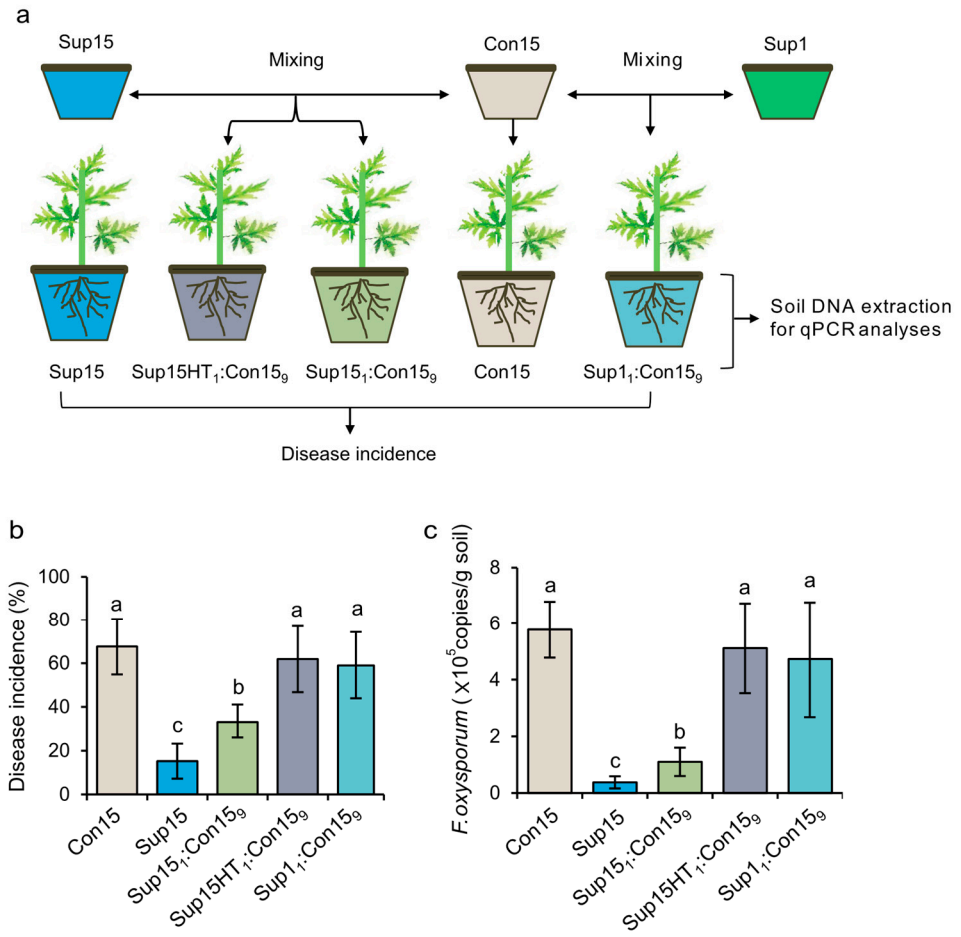

Figure 1. Disease incidence and *F. oxysporum* density of *Chrysanthemum* plants in the 15-year conducive soil (Con15), suppressive soil (Sup15), conducive soil mixed with 10% (w/w) of Sup15 ($Sup15_1$:$Con15_9$) or heat-treated Sup15 ($Sup15HT_1$:$Con15_9$), and Con15 amended with 10% (w/w) of one-year suppressive soil ($Sup1_1$:$Con15_9$). (**a**) Experimental scheme of pot designs for assessing soil suppressiveness to *Fusarium* pathogens. (**b**) Disease incidence in different treatments. (**c**) Quantification of *F. oxysporum* density in the rhizosphere soils by qPCR. Data are means ± SE. Different letters above the bars indicate statistically significant differences by Tukey's HSD test.

3.2. Rhizosphere Microbial Community Composition

Microbial community compositions in the *Chrysanthemum* rhizosphere were analyzed at the phylum level. As for bacterial communities, the conducive and suppressive soils shared the similar compositions with some major phyla, including *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Acidobacteria* and *Chloroflexi* (Figure 2a). The most dominant phylum was *Proteobacteria*, accounting for about 46%–55% of all bacterial sequences, following by *Actinobacteria*, *Acidobacteria*, *Firmicutes*, *Bacteroidetes* and *Chloroflexi*. Other minor phyla, such as *Cyanobacteria, Gemmatimonadetes* and *Patescibacteria* were identified, accounting for less than 7% of bacterial communities. However, there were a few differences among different soil samples with more *Proteobacteria* and *Firmicutes* in the suppressive soils, and the phylum *Proteobacteria* was most represented in the Sup15 compared with other soils. Concerning fungal communities, dominant OTUs mainly belonged to the two phyla *Ascomycota* and *Basidiomycota*, accounting for about 75% of the total sequences (Figure 2b). *Ascomycota* were more abundant in the suppressive soils than that in the conducive soils, but the opposite trend was observed for

Basidiomycota. The relative abundance of *Chytridiomycota*, *Glomeromycota* and *Zygomycota* was less than 1% of fungal communities.

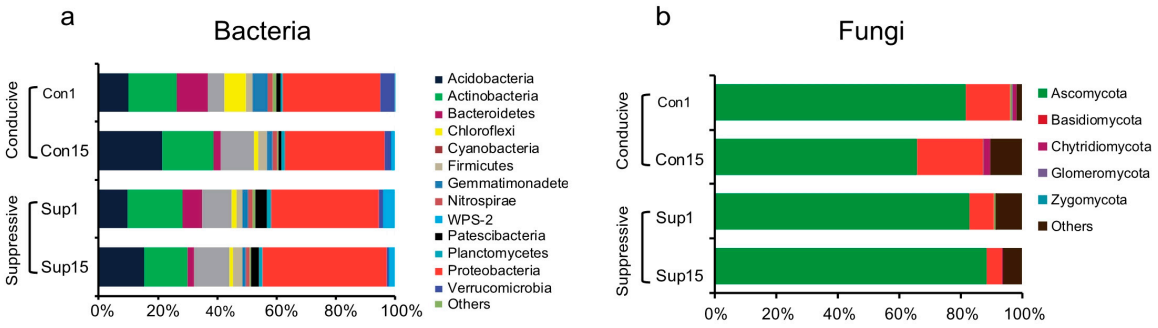

Figure 2. Comparative analyses of rhizosphere microbial communities among the conducive and suppressive soils at the phylum and operational taxonomic unit (OTU) levels. Taxonomic compositions of bacterial (**a**) and fungal (**b**) community under 1- or 15-year monoculture systems at the phylum level.

3.3. Rhizosphere Microbial Community Abundance and Diversity

The compositions of rhizosphere microbial community were compared among different soil samples by the qPCR and sequencing of 16s rRNA or ITS genes. As shown in Figure S1a,b, the highest bacterial and fungal abundance was present in the one-year conducive soil (Con1), and the microbial abundance was remarkably decreased in the 15-year conducive soil (Con15). A similar changing trend was observed for microbial diversity (Shannon index) and richness (Chao1 index) in the conducive soils, except for fungal diversity (Figure S1c–f). In contrast, the suppressive soils with 1-year or 15-year consecutive monoculture harbored lower microbial abundance, richness and diversity (Figure S1a–f). However, the bacterial (Figure S1a,d) and fungal (Figure S1b,f) abundance of the suppressive soils appeared to be less impacted by long-term monoculture compared with the conducive soils. Additionally, non-metric multi-dimensional analysis (NMDS) indicated that different soil samples were observably separated (Figure S2).

The differences of unique and shared OTUs were further investigated among different soil samples. Venn diagrams showed 802, 227, 190 and 117 unique bacterial OTUs in the Con1, Con15, Sup1 and Sup15, respectively, and 176, 99, 91 and 47 unique fungi OTUs were detected for these soils, respectively. Furthermore, 264 bacterial and 155 fungal OTUs were commonly shared in the conducive and suppressive soils (Figure 3a,b). As for bacteria, the relative abundance of *Acidobacteria* and *Bacteroidetes* in the unique OTUs was strikingly lower in the suppressive soils compared with the conducive soils (Figure 3c). However, the relative abundance of *Firmicutes* and *Patescibacteria* was relatively higher in the suppressive soils than the conducive soils. The majority of shared OTUs (average relative abundance > 5%) in these soils belonged to *Acidobacteria*, *Actinobacteria* and *Proteobacteria*, while the Sup15 had the highest *Proteobacteria* abundance and lowest *Actinobacteria* abundance, compared with the conducive soils (Figure 3d).

As for fungi, the majority of unique OTUs (average relative abundance > 5%) in these soils belonged to *Ascomycota* and *Basidiomycota*, and the Sup15 had the highest *Ascomycota* abundance (Figure 3e). In addition, long-term monoculture led to marked decreases in the relative abundance of shared OTUs belonging to *Ascomycota* in the conducive soils, but no marked changes were observed for the suppressive soils (Figure 3f). Hence, the conducive and suppressive soils shaped differential microbial communities under monoculture systems, as evidenced by the changes in both the specific (unique OTUs) and core microbiome (shared OTUs).

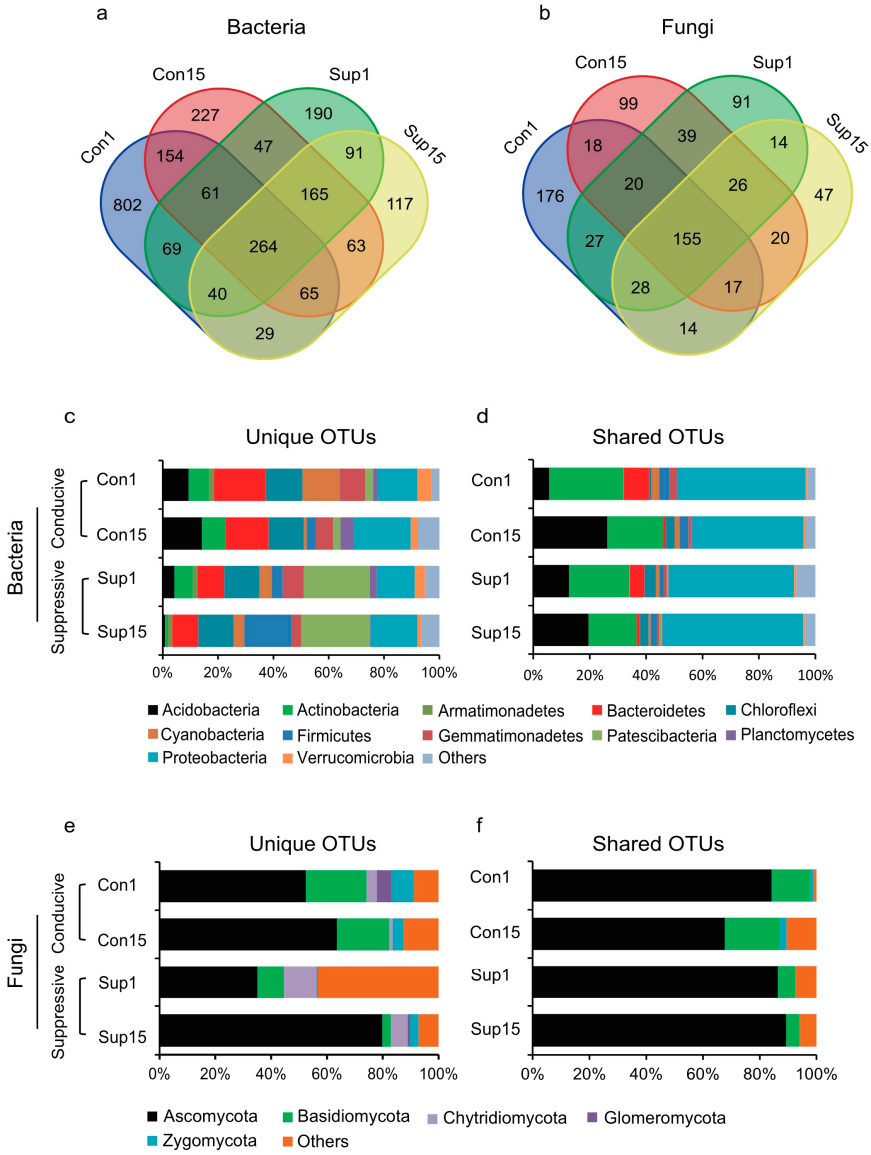

Figure 3. Shared and unique OTUs in both the conducive and suppressive soils under 1- or 15-year monoculture systems. Only the OTUs present in the triplicates of each soil sample were included. Venn diagram showing the number of shared and unique OTUs in the bacterial (**a**) and fungal (**b**) communities. Relative abundance of bacterial (**c**,**d**) and fungal (**e**,**f**) phyla in the unique and shared OTUs, respectively.

3.4. Bacterial Indicators of Disease Suppression to Fusarium Wilt Disease

LEfSe analyses were employed to explore the relationships of the top 30 abundant bacterial and fungal genera in both the 15-year suppressive and conducive soils. The bacterial genera, including *Pseudomonas*, *Rhizobium*, *Bryobacter*, *Occallatibacter*, *Pseudolabrys* and *Actinospica*, were more abundant in the suppressive soil (Figure 4). *Pseudomonas* was the most dominant bacterial phylum in the suppressive soil, accounting for 13.2% of the total sequences in the suppressive soil (only 0.1% in the conducive soil). For the fungi, 19 genera, out of the top 30 fungal genera, were more abundant in the conducive soils. The fungal genera, including *Mortierella*, *Ceratobasidium*, *Gliocladiopsis* and *Cylindrocladium*, were more abundant in the suppressive soil (Figure S3). *Fusarium* was the most dominant genus, comprising 9.3% of total fungal genera in the conducive soil (only 0.5% in suppressive soil).

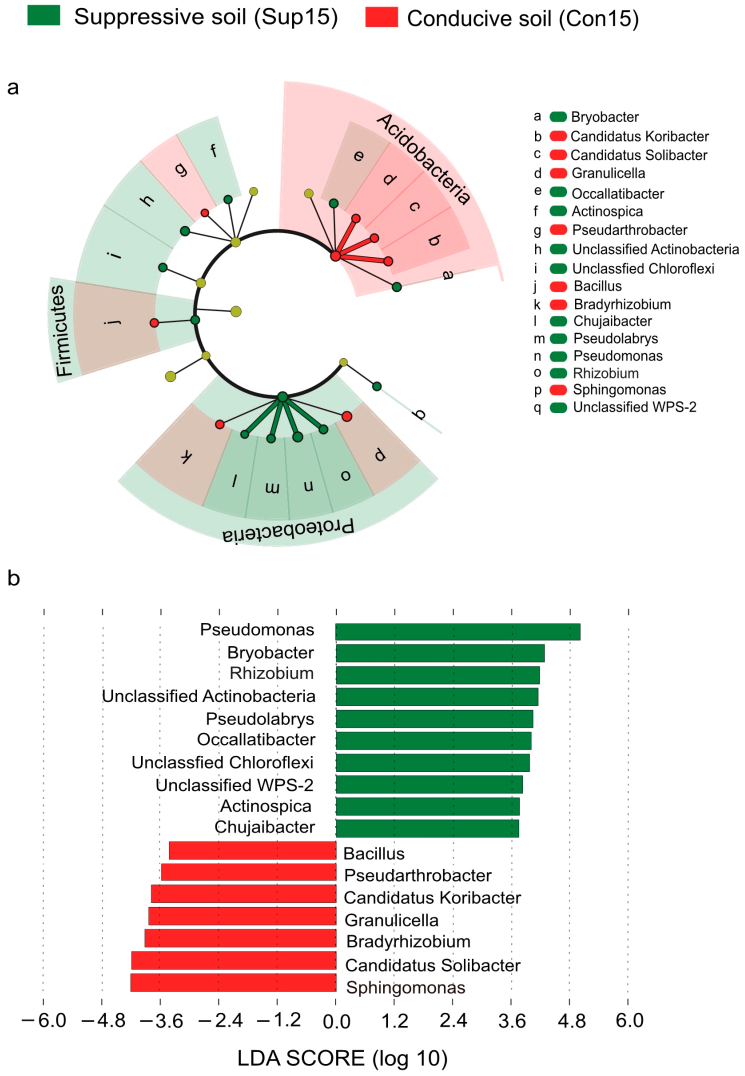

Figure 4. Linear discriminant analysis effect size (LEfSe) analyses of the most differentially abundant (top 30) taxa in the 15-year suppressive and conducive soils. (**a**) Taxonomic cladogram showing the phylogenetic distribution of bacterial lineages. (**b**) Histogram of the linear discriminant analysis (LDA) scores estimated for differential bacterial genera between the suppressive and conducive soils.

As shown in Figure 5a–d, a total of 98 and 145 depleted bacterial OTUs specifically occurred in the Sup15 compared with the Sup1 and Con15, and 28 depleted bacterial OTUs were commonly shared in the suppressive soil (Table S2). Moreover, a total of 53 and 92 enriched bacterial OTUs (compared with the Sup1 and Con15) specifically occurred in the Sup15, and 37 shared OTUs were defined as indicator OTUs that were highly abundant in the Sup15 (Table S3). These shared OTUs belonged to the phyla *Bacteroidetes*, *Patescibacteria*, *Chloroflexi*, *Actinobacteria*, *Firmicutes* and *Acidobacteria*, respectively (Figure 5e). Moreover, some bacteria strains were isolated from the 15-year suppressive soil, and were identified by sequencing 16s rRNA genes. Among these isolates, five bacterial strains, corresponding to the observed OTUs that were highly enriched in the Sup15, were selected for the next experiments, based on at least 97% similarity for 16S rRNA genes. One strain was identified as *P. putida* A2, corresponding to OTU2 that was the most abundant in the Sup15. Other strains were *Xanthomonas* sp. NC8 (OTU1881), *Paenibacillus* sp. M7 (OTU308), *Stenotrophomonas* sp. YB9 (OTU5), and *Rhizobium* sp. HU16 (OTU19), respectively. However, only *P. putida* A2 exhibited a strong inhibitory effect on the growth of *F. oxysporum* (Figure S4).

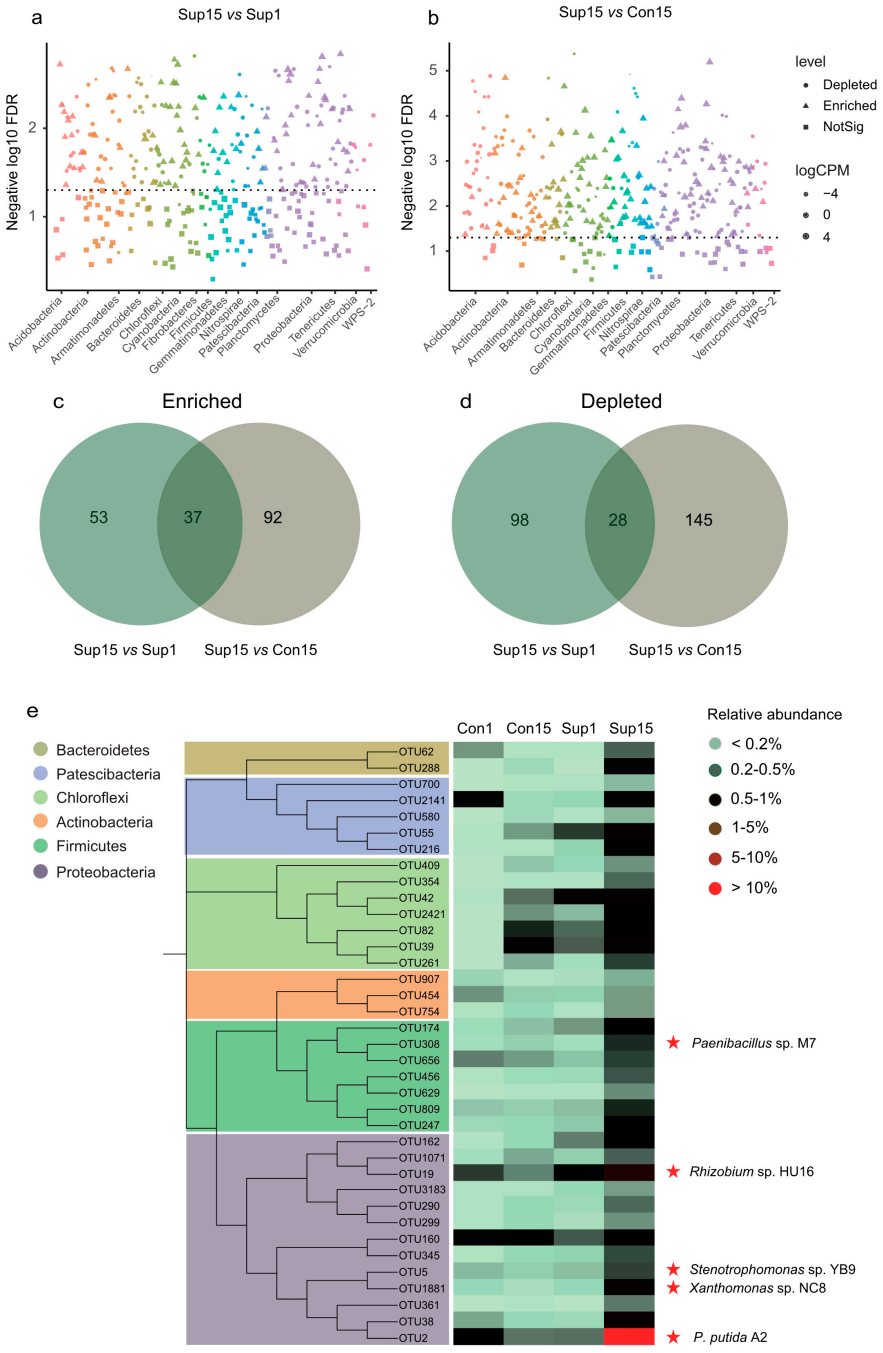

Figure 5. Analyses of differentially enriched or depleted OTUs in the 15-year suppressive soil (Sup15), compared with the 1-year suppressive soil (Sup1) or 15-year conducive soil (Con15), with an average abundance ≥ 0.1% and logFC ≥ 1.0. Manhattan plots showing differential OTUs in the Sup15 compared with Sup1 (**a**) and Con15 (**b**), respectively. Venn diagram showing the number of shared and unique OTUs in the enriched (**c**) and depleted (**d**) OTUs. Neighbor-joining analysis of the shared OTUs (left) among these enriched OTUs with relative abundance (right) in different soil samples, and stars (right) indicated five isolated strains corresponding to the shared OTUs that were highly abundant in the Sup15 (**e**).

3.5. Functional Selection of Consortia Associated with Soil Disease Suppressiveness

Considering that root-released PAs were the pivotal stimulators of *Fusarium* wilt incidence, we focused on the selection of potential combinations that could antagonize *F. oxysporum* and degrade PAs.

In this study, the content of PAs in soils was quantified by HPLC. The suppressive soil accumulated less ferulic, *p*-coumaric and *p*-hydroxybenzonic acids than the conducive soil under 15-year monoculture, while no significant difference was detected in the 1-year monoculture (Figure 6). Among the three PAs, ferulic acid displayed the greatest promoting effects on the growth of *F. oxysporum***.** By contrast, the growth-promoting effects of the mixed PAs were stronger than that of single PA (Figure S5a). Moreover, the mixed PAs markedly stimulated the sporulation of the fungal pathogen, and this effect was further enhanced, followed by the increased concentrations of the mixed PAs (Figure S5b). Additionally, degradation assays were performed for assessing the efficiency of soils to degrade PAs. As shown in Figure 6b–e, more rapid degradation of PAs occurred in the suppressive soils. By contrast, no or weak degradation was found in both the conducive and sterilized soils, eliminating the possible contribution of abiotic factors to the degradation of PAs. The content of PAs was notably lower in the suppressive soils than the conducive soils at 50 d of culture (Figure 6f). Thus, these results indicated that the suppressive soils may harbor more efficient PA-degrading consortia than the conducive soils.

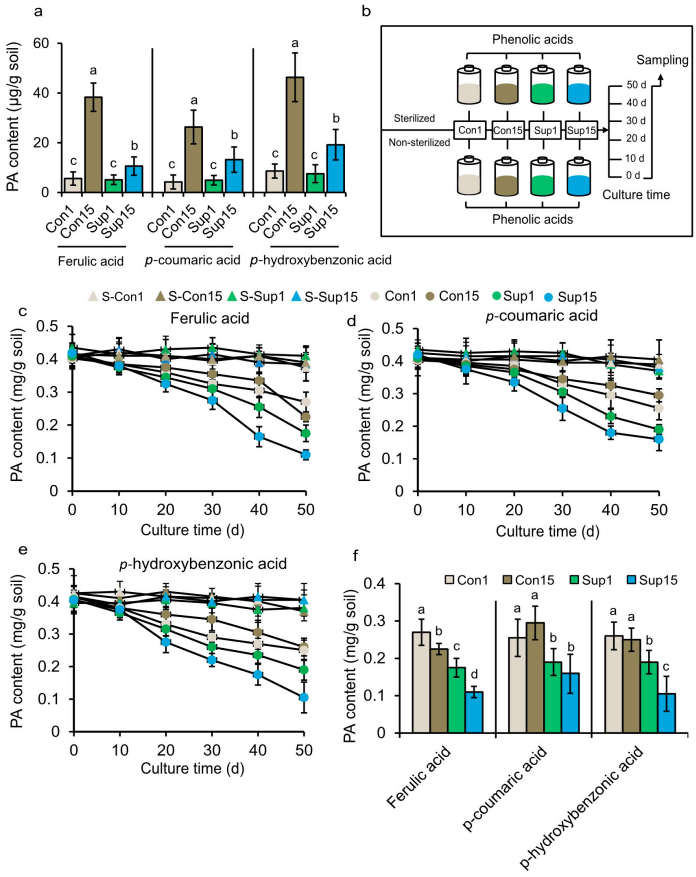

Figure 6. Analyses of the content of phenolic acids (PAs) and its degradation in both the conducive and suppressive soils. (**a**) The content of PAs in the soils. (**b**) Experimental scheme for evaluating the capacity of soils to degrade PAs. Different soil samples were amended with 0.5 mg/g PAs. During 50 d of culture, soil samples were harvested at different time points and used to measure the remaining PAs, including ferulic (**c**), *p*-coumaric (**d**) and *p*-hydroxybenzonic acids (**e**). At 50 d of culture, a fraction of the remaining PAs was calculated in both the conducive and suppressive soils (**f**). S-Con1, -Con15, -Sup1 and –Sup15: sterile soil samples. Con1, Con15, Sup1 and Sup15: non-sterile soil samples. Data are means ± SE. Different letters above the bars indicate statistically significant differences at $p < 0.05$ by Tukey's HSD test.

We further examined the capability of the above isolates to degrade PAs. Bacteria culture was inoculated into the medium containing 0.9 g/L ferulic, *p*-coumaric or *p*-hydroxybenzonic acid. During

36 h of culture, the content of PAs in the supernatant was measured. All five isolates could consume PAs in the medium, but varied considerably at the degrading levels (Figure S6). To compare the efficiency of different combinations to degrade the mixture of PAs, we constructed multi-species assemblies, representing all possible alliance that harbored *P. putida* A2, only an antagonistic bacterium (Figure 7a). The *Pseudomonas* species was used as a core point, allowing the prediction of positive or negative interactions of other species with *P. putida* A2. For each community, two core indexes of community performances were examined, including both the PA-degrading efficiency and *P. putida* A2 growth. As shown in Figure 7b, the performances of different species' assemblies were compared, and variations among different combinations were evaluated for the performance indexes. The control group, containing only *P. putida* A2, had the least performances, while multi-species assembly improved performances in some cases (Figure 7c,d). All top performing combinations shared three species: *P. putida* A2, *Xanthomonas* sp. NC8 and *Rhizobium* sp. HU16.

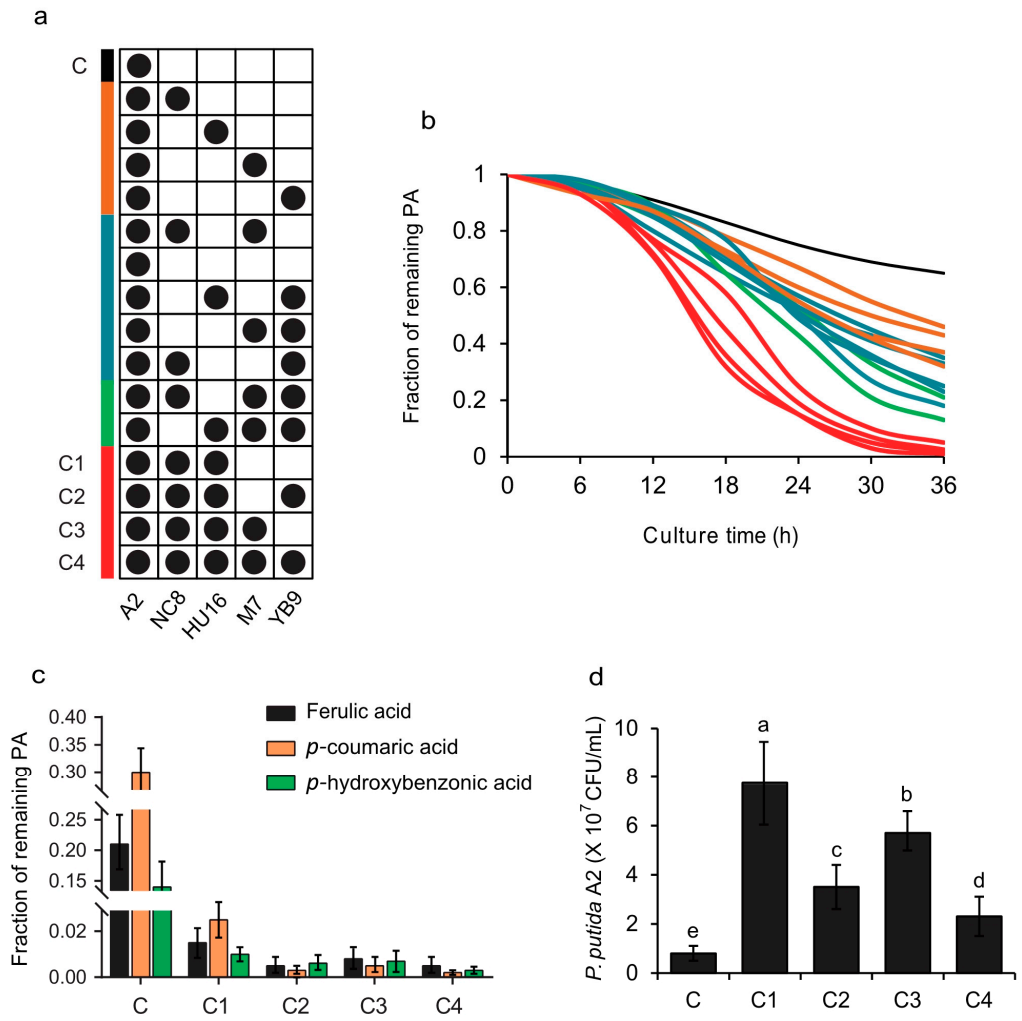

Figure 7. Assays of PA-degradation and *P. putida* A2 growth in different combinations. Line colors represented different combinations, as indexed in the right grid. PA-degradation performances of 16 combinations (**a**). In vitro PA degradation and A2 growth performance in inorganic media with 0.9 g/L mixed PAs (ferulic, *p*-coumaric and *p*-hydroxybenzonic acids, 1:1:1). During 36 h of culture, the remaining PAs was measured at different time points (**b**). At 36 h of culture, the remaining PAs (**c**) and A2 growth performance (**d**) were measured in the C-C4 combinations. C, A2 only; C1, combinations of A2, NC8 and HU16; C2, combinations of A2, NC8, HU16 and YB9; C3, combinations of A2, NC8, HU16 and M7; C4, combinations of A2, NC8, HU16, M7 and YB9. Data are means ± SE. Different letters above the bars indicate statistically significant differences at $p < 0.05$ by Tukey's HSD test.

3.6. Efficient Suppression of Fusarium Wilt Disease by a PA-degrading Consortium

We examined whether *P. putida* A2 could suppress *F. oxysporum*-induced wilt disease in *Chrysanthemum* plants. This strain, at 2×10^7 CFU/g of soil, was poured into the base of three-week-old seedlings grown in the 15-year conducive soils (Figure 8a). Three months later, the plants inoculated with *P. putida* A2 exhibited a significant decrease in disease incidence, compared with non-inoculated (control) plants. However, in the second and third planting (cycle) into the same pots, the suppressiveness of soils to *Fusarium* wilt was greatly weakened (Figure 8b). The dynamics of *P. putida* and *F. oxysporum* in the rhizosphere soils were tracked by qPCR of DNA copy numbers. The population level of *P. putida* in the soils rapidly decreased for three months and stabilized at 1.6×10^5 CFU/g soil. In the second cycle, the population level of *P. putida* declined to 2.5×10^4 CFU/g soil. In the third cycle, the population level of *P. putida* almost returned to the same level of control soils (Figure 8c). However, the opposite trend of population changes was observed for *F. oxysporum* (Figure 8d).

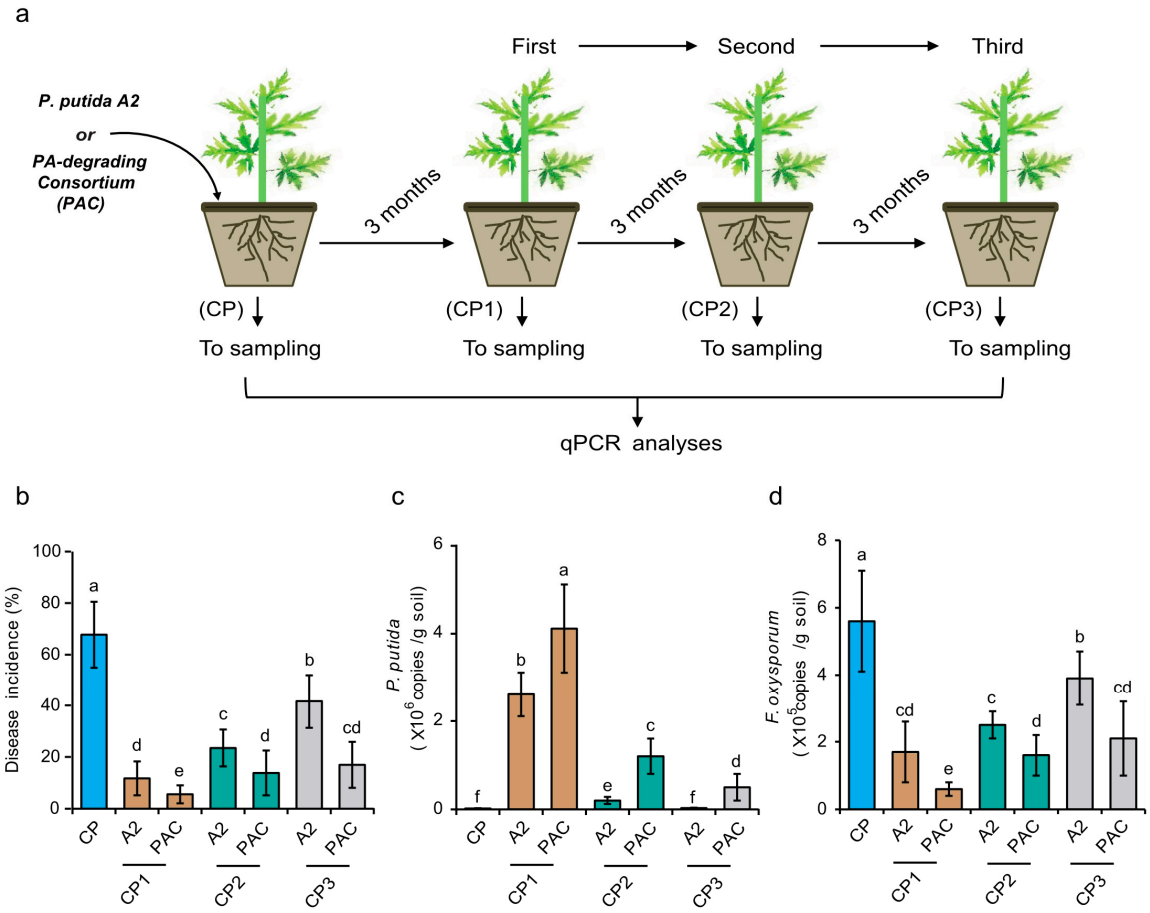

Figure 8. Effects of *P. putida* A2 or a PA-degrading consortium (PAC) on disease incidence in the 15-year conducive soils (Con15). (**a**) Experimental scheme for pot designs. *P. putida* A2 at 2×10^7 CFU/g or PAC harboring *P. putida* A2 (2×10^7 CFU/g), *Xanthomonas* sp. NC8 (5×10^6 CFU/g) and *Rhizobium* sp. HU16 (5×10^6 CFU/g) was poured into the base of three-week-old *Chrysanthemum* seedlings, grown in the 15-year conducive soils. After three months, three-week-old seedlings were subjected to the second and third successive plantings (cycles) into the same pots. Disease incidence (**b**), A2 growth (**c**), and *Fusarium* density (**d**) were determined in the rhizosphere soils. CP, consecutive planting (non-treated Con15); the A2- or PAC-treated soils were subjected to first (CP1), second (CP2) and third (CP3) three-month planting. Data are means ± SE. Different letters above the bars indicate statistically significant differences by Tukey's HSD test.

We further tested whether a PA-degrading consortium (PAC) conferred increased suppression of conducive soils to *Fusarium* wilt disease. The 15-year conducive soils were inoculated with a model consortium harboring *P. putida* A2 (2×10^7 CFU/g), *Xanthomonas* sp. NC8 (5×10^6 CFU/g) and *Rhizobium* sp. HU16 (5×10^6 CFU/g). After three months, the PAC-treated soils had lower levels of PAs than the A2-treated soils (Figure S7). As shown in Figure 8b, the PAC-treated soils also displayed lower disease incidence than the A2-treated soils. In the third cycle, the consortium-treated soils still displayed stronger disease suppressiveness than the A2-treated soils. Consistently, the PAC-treated rhizosphere soils had relatively higher density of A2, compared with the A2-treated soils (Figure 8c). It was also found that both NC8 and HU16 effectively colonized in the PAC-treated rhizosphere soils during three-cycle monoculture (Figure S8). Furthermore, the PAC-treated rhizosphere soils had a lower density of *F. oxysporum* than the A2-treated soils during three-cycle monoculture (Figure 8d).

4. Discussion

Fusarium wilt is the most wide-ranging fungal disease that leads to severe yield loss in many crops such as banana, vanilla and cucumber [2,33,35]. Dissecting characters of rhizosphere microbial communities is essential for understanding the microbial basis of disease-suppressive soil and improving plant fitness [36]. However, most researches on the disease-suppressive soil are confined to one microbial population, ignoring the remaining microbial communities [12]. In this study, the inoculation with *P. putida* A2 made the soil acquire disease suppressiveness, but this seemed not to be as durable as naturally suppressive soils. We further compared analyses of rhizosphere microbial community changes between the conducive and suppressive soils under long-term monoculture. Several indicator species were screened, which were associated with soil disease suppressiveness. Combined with conventional methods of bacterial isolation, a model PA-degrading consortium was constructed, conferring stronger suppressiveness to *Fusarium* wilt disease.

Bacterial diversity and abundance are pivotal for maintaining plant health by diverse tactics such as the prevention of pathogen invasion, promotion of plant growth, and activation of host immune responses [37–39]. The suppressive soil often harbors strikingly higher bacterial abundance and diversity than the conducive soil [15]. In contrast, our suppressive soil displayed lower bacterial diversity and abundance. These findings were in contrast with a recent study, showing that the diversity of bacterial communities was markedly greater in the suppressive soil of potato, compared with the conducive soil [36]. The lower diversity of bacterial communities in our suppressive soil may have resulted from lower pH conditions, which was similar to the observation that bacterial diversity was distinctly increased in higher pH soils [41,42]. Fungi are also an integral component of microbes in the soil ecosystem, which are important for soil functions and plant health [40]. Consecutive monoculture can shift the diversity and composition of a soil fungal community [17,41,42]. Xiong et al. [17] have shown that vanilla monoculture notably increased soil fungal diversity. Moreover, soil amendment with *p*-coumaric acid, a key stimulator of *Fusarium* wit disease incidence, increases the diversity of fungal community [41,42]. The fungal diversity in the conducive soils of *Chrysanthemum* was obviously increased under long-term monoculture. However, the opposite trend was observed for the suppressive soils. Additionally, the fungal richness in the suppressive soils was markedly lower than that in the conducive soils, but that was less impacted by long-term monoculture. Soil fungal richness has recently been shown to be positively associated with disease incidence and yield decline, indicating that higher fungal richness may be an important driver for *Fusarium* wilt disease incidence [2,43]. Additionally, soil characteristics are also important indicators of disease suppressiveness. Here, the levels of OM, available N, P and Fe were observably higher in the suppressive soil, compared with the conducive soil. In the potato monoculture system, the abundance of *Fusarium* pathogens has been found to be negatively related to soil OM [44]. Higher available P in soils is also positively correlated with less incidence of *Fusarium* wilt disease in the suppressive soil of banana [2]. Furthermore, a higher level of OM and nutrient elements in the soils may also be beneficial to plant growth and health, thereby increasing the capacity of plants to resist disease [2]. In this study, soil properties could not

effectively induce soil disease suppression, because pasteurization of the suppressive soils did not transfer soil suppressive capabilities. Therefore, the changes of microbial community in the suppressive soil primarily contributed to higher disease suppression.

Consecutive monoculture adversely impacts plant growth and pathogen defense in many crop plants, which are mainly attributable to root-released autotoxic substances, exacerbation of soil physiochemical properties, and microbial community changes [22,42,45]. The imbalance of soil microbial populations mediated by root exudates is responsible for disease incidence under monoculture systems [22]. In cucumber, root-released *p*-coumaric acid alters soil microbial communities and promotes the growth of *F. oxysporum* under continuously mono-cropping patterns [42]. In *Rehmannia glutinosa*, root-secreted PAs can stimulate the growth and toxin production of *F. oxysporum*, but inhibit the antagonistic bacteria *Pseudomonas* species [22]. Among the major components of root exudates, PAs are crucial for inducing the allelopathy and replanting disease, and are prone to modifying soil microbial community, rather than directly inducing autotoxic effects on the mono-cropping plants [1]. It was found here that the levels of PAs were remarkably higher in the conducive soil than in the suppressive soil under long-term monoculture. Concurrently, the abundance of *F. oxysporum* distinctly differed between the conducive and suppressive soils. This variation between both the soils may at least partially explain the larger discrepancy of disease incidence. This was in line with recent reports that the abundance of *F. oxysporum* was positively correlated to *Fusarium* wilt disease of vanilla and banana [2,4]. The differences we observed allowed us to speculate that certain key microbiota existed in the suppressive soil, impeding the growth and invasion of *F. oxysporum*. As expected, mixing a small quantity of the 15-year suppressive soil led to a relatively low disease incidence in the conducive soil, while transferring the one-year suppressive soil did not lower disease incidence. Hence, long-term monoculture shaped specific microbial communities to hinder the growth of pathogens.

The taxonomic compositions of rhizosphere microbial communities varied among the conducive and suppressive soils of *Chrysanthemum*. Compared with the conducive soils, *Proteobacteria* and *Firmutes* were more abundant in the suppressive soils, which were in concert with previous studies [12,46]. *Proteobacteria* is the most common phyla in different soil types, but varied with relative abundance [47]. Banana *Fusarium* wilt disease suppression is negatively related to *Proteobacteria in the* organic fertilizer-amended soils [48]. It is well documented that *Actinobacteria* is more abundant in the suppressive soils than the conducive soils [9,39,48]. However, no significant difference in the phyla was observed between our conducive and suppressive soils. Thus, soil disease suppressiveness may not have resulted primarily from the antagonistic effects of *Actinobacteria*. Moreover, *Ascomycota* and *Basidiomycota* were the two most prevalent fungal phyla, in agreement with some studies exploring the microflora of vanilla and peanut continuous-cropping soils [4,49]. Compared with the conducive soils, *Ascomycota* was the most abundant in the suppressive soils of *Chrysanthemum*. Similarly, some member of *Ascomycota* dominated the fungal community in the suppressive soils of vanilla under monoculture systems [4]. Through the analyses of LEfSe and indicator OTUs, some specific species may be tightly related to soil disease suppressiveness. *Pseudomonas* was one of the most dominant genus belonging to the phylum *Proteobacteria* in the 15-year suppressive soil. It has previously been indicated that several *Pseudomonas* species can produce antibiotics against various plant pathogens, which are considered as key indicators of soil disease suppressiveness [12,50]. Accordantly, the 15-year conducive soil inoculated with a *Pseudomonas* species (*P. putida* A2) displayed a significant decrease in disease incidence, although this acquired suppressiveness quickly declined during the following monoculture. Moreover, dynamic changes of *Pseudomonas* in the soils displayed a similar trend. Thus, the survival of soil inoculants in the plant rhizosphere was required for improving soil suppressiveness to soil-borne pathogen.

PAs can trigger disease burst in monoculture soils by inhibiting beneficial soil microbes and promoting pathogen growth [22,51]. In this study, the suppressive soil accumulated less PAs than the conducive soil under long-term monoculture. The results of PA degradation assays indicated that the mixture of PAs obviously inhibited the growth of *P. putida* A2, although this

strain could efficiently degrade single type of PAs, indicating that PA-enriched soils were unsuitable to the growth of the *Pseudomonas* species. Thus, construction of PA-degrading consortia was conducive to enhancing the colonization of *P. putida* A2 in the PA-enriched conducive soils. It is becoming clear that the manipulation of microbial communities is a prospective application for environmental and agricultural practices [52–54]. The functions of consortia, rather than single species, determine the efficiency of toxic substance degradation [26,27]. However, major obstacles of consortia construction are isolating all the community species and understanding the complex interactions of the microbiome [27]. Here, we tried to develop PA-degrading consortia that can suppress *Fusarium* pathogen by functional selection methods. Our results showed that long-term monoculture shifted the bacterial community, and the shift was associated with the suppressiveness of soils to *Fusarium* pathogens, as evidenced by the strong suppressiveness in the 15-year suppressive soil, not in the one-year suppressive soil. The antagonistic and PA-degrading species may be greatly abundant in the 15-year suppressive soil, but the abundance changes could not offer a direct justification for the functional alterations, which were related to interspecies interactions. We thus examined the performance (PA-degrading activity) and valid combinations of the selected isolates that were greatly abundant in the 15-year suppressive soil in different consortia. The increased efficiency of PA-degrading activity and growth performance of *P. putida* A2 may indicate mutual benefits related to species interactions. According to this, we designed bacterial combinations and validated the improved performances of selected combinations. The enhanced degradation of PAs was not only attributable to the performance of *P. putida* A2, but also the functions of other members in the community. Pot experiments further revealed that a model consortium harboring *P. putida* A2 markedly reduced PA levels in the 15-year conducive soils, with lower disease incidence and better *P. putida* growth during three-cycle monoculture.

5. Conclusions

Comparative analyses of rhizosphere microbial community characters between the conducive and suppressive soils of *Chrysanthemum* were conducted, to unravel potential microbiota associated with disease suppressiveness. Our findings demonstrated that the combination of *P. putida* with *Xanthomonas* and *Rhizobium* species greatly enhanced the efficiency of PA degradation. Furthermore, the PA-degrading consortium harboring the antagonistic *Pseudomonas* species conferred stronger soil suppressiveness to *Fusarium* wilt disease than the *Pseudomonas* species alone. Collectively, the functional combination of consortium can assist one to overcome the disease-suppressive instability of single inoculum with antagonistic bacteria in the *Chrysanthemum* monoculture soils.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/3/385/s1. Table S1 Soil physiochemical characteristics at two cultivated *Chrysanthemum* orchards that were disease-suppressive (Jutai) and -conducive (Shiji) to *Fusarium* wilt disease. Table S2 Differentially depleted OTUs in the 15-year suppressive soil (Sup15) compared with the Sup1 or Con15. Table S3 Differentially enriched OTUs in the Sup15 compared with the Sup1 or Con15. Figure S1 Rhizosphere microbial abundance and α-diversity in both the conducive and suppressive soils under 1- or 15-year monoculture systems. Bacterial (a) and fungal (b) abundance was quantified by qPCR. Microbial Shannon diversity (c, bacteria; e, fungi) and Chao1 richness (d, bacteria; f, fungi) were estimated in different soil samples. Data are means ± SE. Different letters above the bars indicate statistically significant differences at $p < 0.05$ by Tukey's HSD test. Figure S2 NMDS analyses of rhizosphere bacterial (a) and fungal (b) community. The matrix was generated based on the Bray–Curtis similarity algorithm. Con1 indicates 1-year conducive soil; Con15 indicates 15-year conducive soil; Sup1, indicates 1-year suppressive soil; Sup15, indicates 15-year suppressive soil. Figure S3 LEfSe analyses of the most differentially abundant (Top30) taxa in the 15-year suppressive and conducive soils. (a) Taxonomic cladogram showing the phylogenetic distribution of fungal lineages. (b) Histogram of the LDA scores estimated for differential fungal genera between the suppressive and conducive soils. Figure S4 Inhibitory effects of five isolated strains on *F. oxysporum*. 1×10^7 CFU/mL spore suspension of *F. oxysporum* was mixed in PDA media, and 2 μL of bacterial culture was dipped on the surface of the PDA media. Then, area of inhibition zone was calculated after 48 h of culture at 28 °C. Data are means ± SE. Different letters above the bars indicate statistically significant differences by Tukey's HSD test. Figure S5 Effects of PAs or mixed PAs on the mycelial growth (a) and sporulation (b) of *F. oxysporum*. Data are means ± SE. Different letters above the bars indicate statistically significant differences by Tukey's HSD test. Figure S6 Degradation of PAs in inorganic media by five bacterial strains. The media were amended with 0.9 g/L PAs. During 36 h of culture, fraction of the remaining ferulic (a), *p*-coumaric (b) and

p-hydroxybenzoic acids (c) in culture solutions was calculated. The growth performances of different strains were examined in the media containing ferulic (d), *p*-coumaric (e) and *p*-hydroxybenzoic acids (f). Figure S7 Analyses of the content of PAs in the 15-year conducive soils treated with *P. putida* A2 or a PA-degrading consortium (PAC). After three months of inoculation, soil samples were harvested for determining the remaining PAs, including ferulic, *p*-coumaric and *p*-hydroxybenzonic acids. Data are means ± SE. Different letters above the bars indicate statistically significant differences by Tukey's HSD test. Figure S8 Colonization of *Xanthomonas* sp. NC8 and *Rhizobium* sp. HU16 in the PA-degrading consortium (PAC)-treated soils. The 15-year conducive soils were inoculated with the PAC harboring *P. putida* A2 (2×10^7 CFU/g), *Xanthomonas* sp. NC8 (5×10^6 CFU/g) and *Rhizobium* sp. HU16 (5×10^6 CFU/g). After three months, three-week-old seedlings were subjected to the second and third successive plantings (cycles) into the same pots. The density of NC8 9 (a) and HU16 (b) in the rhizosphere soils was quantified by qPCR, respectively. CP, consecutive planting (non-treated Con15); the PAC-treated soils were subjected to first (CP1), second (CP2) and third (CP3) three-month planting. Data are means ± SE. Different letters above the bars indicate statistically significant differences by Tukey's HSD test.

Author Contributions: Designed experiments, L.Z. and J.W.; writing manuscript and editing, L.Z. and C.Z.; conducted experiments, C.Z.; conducted statistical analysis, Z.M. and C.Z.; review and editing, X.L. and Z.M.; writing—original draft and review, C.Z. and L.Z.; funding acquisition, L.Z. All authors have read and agreed to the published version of the manuscript.

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