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Rhizosphere bacteria assembly derived from fumigation and organic amendment triggers the direct and indirect suppression of tomato bacterial wilt disease

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ABSTRACT

With no efficient method widely recognized for controlling or preventing tomato bacterial wilt, a devastating disease caused by the soil-borne bacterium *Ralstonia solanacearum*, tomato production is severely hindered. Therefore, the objective was thus to provide a strategy based on fumigation using ammonium bicarbonate along with organic amendment to reduce disease severity, and the impact of this treatment on the soil microbiome as well as the underlying mechanism leading to disease suppression were evaluated using high-throughput sequencing. Results showed that this combined strategy effectively controls tomato bacterial wilt disease despite the high abundance of *R. solanacearum* in both the bulk and rhizosphere soil in all treatments. The treatment led to significant changes in the soil bacterial and fungal communities, and at harvest time, fumigation and organic amendment equally affected the variation in the rhizosphere microbiome. Further, a shift in the rhizosphere bacterial community acted as the key factor directly and indirectly suppressing *R. solanacearum* and controlling bacterial wilt disease. In addition, *Rhodanobacter*, *Terrimonas* and *Chitinophaga* in the rhizosphere were the potential taxa involved in disease suppression. Both fumigation and organic amendment contributed to disease suppression by decreasing the abundance of *R. solanacearum* and altering the bacterial composition in combination with the stimulation of key bacterial taxa.

1. Introduction

Continuous intensive cropping often causes drastic changes to the soil microbiome, leading to an unbalanced community represented by a loss of beneficial microbes and an increase in plant pathogens (Avis et al., 2008), which ultimately result in the occurrence of several soil-borne diseases. Tomato bacterial wilt caused by *Ralstonia solanacearum* (Smith), a widely distributed soil-borne plant pathogen, is one of the most lethal diseases in tomato plantations (Hayward, 1991). Although various strategies have been adopted to manage the disease, their success has so far been limited (Dalal et al., 1999; King et al., 2008; Pradhanang et al., 2003; Rivard et al., 2012), highlighting the urgent need to develop an efficient strategy to suppress the disease and maintain the tomato industry around the world.

The soil microbiota is the most vital component maintaining the

sustainability and functioning of agroecosystems (Altieri, 1999). On one hand, beneficial soil microbes improve plant growth by exerting profound impacts that increase mineral solubilization (Bever et al., 1997), modulate plant hormone production (Hayat et al., 2010) and suppress plant pathogens (Mendes et al., 2011). On the other hand, pathogenic microorganisms, which naturally dwell in the bulk soil, always reduce plant growth and fruit yield (Whipps, 1997). The rhizosphere is the playground and infection court where pathogens establish a parasitic relationship with plants (Raaijmakers et al., 2009). The pathogens need to grow saprophytically in the rhizosphere to reach their host or to achieve sufficient numbers on their host before they can infect the host tissue (Berendsen et al., 2012), and their invasion is affected by the rhizosphere, where this process takes place (Berg, 2009). Given that the composition of the rhizosphere microbiota is mainly influenced by the soil microbiota (Dey et al., 2012; Lundberg

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et al., 2012), strategies that promote the generation of a healthy rhizosphere microbiota to maintain sustainable cultivation are urgently needed.

Fumigation is the most direct approach for altering the soil microbiota and is a common practice for controlling soil-borne disease (Li et al., 2016). Many fumigants, e.g., methyl bromide, methyl iodide, chloropicrin, and 1,3-dichloropropene, have been widely used to control weeds, insects, nematodes and soil-borne pathogens (Hoshino and Matsumoto, 2007); however, these fumigants are harmful to high-value agricultural production systems because of their high toxicity to the environment and nearby residents (Chellemi et al., 2011), potentially also affecting the beneficial soil microbiota. Previous research in our laboratory showed that fumigation with ammonium bicarbonate significantly suppressed cucumber *Fusarium* wilt disease and altered the soil microbial composition (Li et al., 2016). Furthermore, several reports suggest that ammonia release through high-N amendments is efficacious for resisting pathogens and root-knot nematodes (Gilpatrick, 1969; Shiao et al., 1999; Stirling, 2018). Despite its potential, the suppression ability of a novel fumigant on tomato bacterial wilt in the field remains untested. Moreover, given that fumigation not only impacts soil pathogens but also reduces the abundance and richness of the resident soil microbiota (Elsas et al., 2012), the efficiency of a novel fumigation strategy in the field should be evaluated in the context of its impact on the soil microbial community and plant yield.

The use of organic amendments, such as farmyard manure composts, represents another well-known strategy to control different soil-borne diseases (Hoitink and Fahy, 1986). The mechanisms of disease suppression are associated with the amount of carbon in organic amendments, which stimulates microbial biomass and activity, and with the microbial community present in the organic amendments (Termorshuizen et al., 2006). Importantly, the positive effects of organic amendments on the soil microbiome may represent an alternative solution to counteract the potential deleterious effects associated with soil fumigation.

The objective was to use field experiments to evaluate the effects of a combined strategy based on fumigation and subsequent organic fertilizer application on the suppression of tomato bacterial wilt disease. We monitored the effects of the combined strategy as well as respective controls on the soil and rhizosphere microbiome by examining the response of the pathogen to treatment, the recovery of the soil microbiota after organic fertilizer application and the subsequently formation of disease suppressive rhizosphere microbiota. We hypothesized that the biocontrol efficiency of the combined strategy is due to the combination of the direct suppression of pathogen abundance (fumigation) and an indirect effect resulting from the shifts in soil microbial composition in response to both fumigation and fertilization.

2. Materials and methods

2.1. Site description

The field experiment was conducted in a plastic greenhouse in Hengxi town of Nanjing, Jiangsu province (32°02'N, 118°50'E) from March to July 2014. Soils of the areas in Nanjing was comprised of Stagnic Anthrosols developed from loess parent material with clay loam texture (Soil Survey Staff, 2014). This region has a subtropical monsoon climate with an average annual temperature and precipitation of 15.4 °C and 1106 mm, respectively. Bacterial wilt disease had already been a serious problem after many years of tomato (*Lycopersicon esculentum* Mill.) monoculture. To evaluate the control effect of the combined strategy, the experiment had a completely randomized block design with four replications that had the following treatments: 1) treatment, soil amended with 0.2 kg m⁻² ammonium bicarbonate and 0.3 kg m⁻² organic fertilizer (N: 2.0%, P: 0.9%, K: 0.9%) (FOF); 2) control 1, soil amended with 0.2 kg m⁻² ammonium bicarbonate and chemical fertilizer (FCF); 3) control 2, soil without fumigation and with

0.3 kg m⁻² organic fertilizer added (CKOF). Ammonium bicarbonate was sprinkled on soil evenly, and then the soil and fumigant were mixed followed by watering. After application of fumigant, all the treatments were covered with plastic film with 15-days-fumigation before fertilization. The amount of chemical fertilizer (urea: 12.9 g m⁻², superphosphate: 40.9 g m⁻², potassium sulfate: 5.4 g m⁻²) in control 1 was calculated based on the nutrient amount of organic fertilizer. Each replicate block of each treatment had 4 m² and included 20 tomato plants. All plants were watered once a week by a drip irrigation system. The tomato variety was general variety “Shi Ji Fen Guan” which was normally used in this area. Three months after the tomato seedlings were transplanted into the field, a bioassay for disease incidence was performed until the end of the experiment and was based on observations of typical wilt symptoms, including necrosis and the drooping of leaves. The disease incidence in the field was calculated by counting the number of tomato plants with bacterial wilt among the total number of tomato plants in each plot.

2.2. Soil sampling and soil property analysis

Soil sampling was performed in March and June of 2014. Samples collected after fumigation and before fertilization in March were defined as before planting, and Samples collected during tomato harvesting in June were defined as harvest. A nine-point sampling method was utilized to collect soil cores at 0–15 cm depth from the surface after fumigation in each split plot to form a composite sample. During harvesting, bulk soil samples were collected in the same way as before planting. For the rhizosphere soil, entire healthy plants were collected from the plots and shaken vigorously to remove excess soil, and the soil still adhering to the roots was considered to be rhizosphere soil (Fu et al., 2017). All the soil samples were kept in plastic bags on ice and transported to the laboratory and stored at 4 °C. After removing the visible organic matter and sifting the samples through a 2-mm sieve, one portion of each sample was frozen and stored at -80 °C until the nucleic acid was extracted, and the other portion was air dried for chemical analyses. All bulk soil chemical properties were measured according to Liu et al. (2018).

2.3. DNA extraction and total soil microbial abundance determination

Soil DNA was extracted from 0.25 g soil using the PowerSoil DNA Isolation Kit (MoBio Laboratories Inc., USA) following the manufacturer's protocol. The concentration and quality of the DNA were determined using Nanodrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The total number of bacteria and fungi were quantified using quantitative PCR (qPCR) according to Fierer et al. (2005). Three pairs of primer (shown in Table S1) sets that were specifically developed to target bacteria V3 16S gene (338 F and 518R), fungi ITS1 gene (ITS1 and 5.8S) and pathogen flic gene (FlicF and FlicR) were employed to assess their abundances. The qPCR analyses were carried out using an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, CA) with SYBR green I fluorescent dye detection in 20-μl volumes containing 10 μl of SYBR Premix Ex Taq (TaKaRa Bio Inc., Japan), 2 μl of template (10–20 ng μl⁻¹), and 0.4 μl of both the forward and reverse primers (10 mmol L⁻¹ each). All quantitative PCR reactions were performed using the standard temperature profile (Hu et al., 2016). For each sample, we performed 3 technical replicates.

2.4. Sequencing of bacterial and fungal ribosomal markers

Amplification of the bacterial 16S rRNA gene and fungal ITS gene was performed from the genomic DNA by PCR. The 520 F and 802R primer sets were used for the bacterial 16S rRNA gene V4 region amplification, while ITS5 and ITS1 were used for the fungal ITS sequence amplification. Detailed information can be found in Liu et al. (2018),

and the primers are provided in Table S1. Amplicons were sequenced on the Illumina MiSeq platform at the Personal Biotechnology Co., Ltd., Shanghai, China. All sequences were deposited in the NCBI Sequence Read Archive database with the accession number [SRP151886](#).

Samples were analysed using the QIIME version 1.8.0 (Caporaso et al., 2010) pipeline. Raw sequences were quality filtered by QIIME to remove the reads with a length of less than 250 bp and to discard the ambiguous bases and chimeras (Edgar et al., 2011). Then, the sequences were clustered into operational taxonomic units (OTUs, 97% similarity) using UCLUST (Edgar, 2010). Finally, the classification of the representative sequences for each OTU was performed using the RDP classifier (Wang et al., 2007). To obtain equivalent sequencing depths for further bacterial and fungal community analyses, each sample was rarefied to 20,883 sequences for the 16S rRNA genes and 13,487 sequences for the ITS sequences.

2.5. Statistical analysis

All statistical tests performed in this study were considered significant at $p < 0.05$. Alpha diversity was analysed in MOTHUR (Schloss et al., 2009). The multivariate analysis of the microbial community involved principal coordinate analysis (PCoA), multiple regression trees (MRTs) and structural equation modelling (SEM). Together with analysis of molecular variance (AMOVA) and permutational analysis of variance (PERMANOVA) to evaluate the significant differences in the bacterial and fungal community structures among the three treatments, PCoA was conducted to compare the major similarity and variance components of the bacterial and fungal community compositions among all the soil samples. Then, we also constructed MRTs to identify the explanatory variables that contributed to the community differences. Finally, we used SEM to test the potential suppressive hypothesis. For SEM, PCoA axis of bacteria, fungi and soil properties represented bacteria, fungi and soil properties, and qPCR result of *Ralstonia solanacearum* represented pathogen number. Subsequently, the model was calculated using “lavaan” package (Function: sem) in R. Furthermore, a linear regression model was conducted to identify potential sensitive biocontrol agents. All the multivariate analyses and linear regression models were carried out based on OTUs using R version 3.3.1 for Windows. Some other statistical analyses, such as one-way analysis of variance (ANOVA), Duncan’s test, two-sample *t*-test analyses and the calculation of Spearman correlation coefficients, were conducted using the IBM SPSS 20.0 software program (SPSS Inc., USA).

3. Results

3.1. Bacterial wilt disease incidences

Three months after transplanting, we observed significant differences in the wilt disease incidences among treatments (Fig. 1a).

Fig. 1. Results regarding bacterial wilt in tomato plants 3 months after transplantation. CKOF: organic fertilizer was amended in non-fumigated soil; FCF: chemical fertilizer was amended in fumigated soil; FOF: organic fertilizer was amended in fumigated soil. a. Photo of the experiment. b. Incidence of bacterial wilt disease. c. Quantitative PCR results for *Ralstonia* in the bulk soil and rhizosphere. All values are the mean of four replicates. Bars with different letters indicate significant differences among the four treatments as defined by Duncan’s test ($p < 0.05$).

Specifically, the lowest disease incidence was observed in the fumigation and organic fertilizer application treatment (FOF, 14%), whereas in the control treatments, only fumigation (with chemical fertilizer, FCF) or organic fertilizer (CKOF) led to 67% and 81% disease incidence, respectively (Fig. 1a). Interestingly, despite the almost 6-fold reduction in disease incidence in the combined treatment, the abundance of *R. solanacearum* remained high in both the bulk and rhizosphere samples irrespective of the treatment (Fig. 1b). Nevertheless, the combined treatment still showed significantly lower abundance when compared to FCF and CKOF in the rhizosphere and CKOF in the bulk soil. The disparity between the disease incidence and *R. solanacearum* abundance indicates that indirect mechanisms might play a role in our system.

3.2. Microbial diversity

After basal quality control, the number of high-quality sequences per sample varied from 20,884 to 110,483 for bacteria and 13,487 to 333,112 for fungi, and a total of 1,798,891 bacterial 16S rRNA and 2,812,981 ITS high-quality sequences were obtained from 36 soil samples. Moreover, at the 97% similarity cut-off level, 6,509 bacterial and 3,272 fungal OTUs were obtained, representing an average Good’s coverage of $97.3 \pm 0.7\%$ and $99.3 \pm 0.2\%$, respectively.

The bacterial and fungal richness (Chao1) and diversity (Shannon) in the rhizosphere were not influenced by the treatments before planting or during harvesting (Table 1). During harvest, the highest fungal richness and diversity were observed in the bulk soil of the individual treatments (organic fertilizer only, CKOF, or fumigation with chemical fertilizer, FCF), whereas the bacterial diversity and richness were highest with organic fertilizer alone (CKOF) and lowest in the combined treatment (FOF) (Table 1).

3.3. Microbial community structure

The principal coordinate analysis (PCoA) based on the Bray-Curtis distance clearly showed that the treatments had no significant effect on the bulk soil bacterial and fungal communities before planting (AMOVA, $p_{\text{bacteria}} = 0.749$, $p_{\text{fungi}} = 0.138$). However, the two communities responded differently to the treatments during the growing season, leading to significant differences in the bacterial and fungal community composition among CKOF, FCF and FOF in both the bulk and rhizosphere soils during harvest (AMOVA, $p < 0.001$) (Fig. 2). Overall, during harvest time, the soil samples from the FOF treatment grouped together and were distinctly separated from those from the CKOF and FCF treatments along the second component (PCoA2).

The MRT analysis explained 92.4% and 88.7% of the detected variation in the composition of the soil bacterial and fungal communities, respectively (Fig. 3). However, the treatments influenced the structure of the bacterial and fungal communities to different extents. The main driver of the bacterial communities was the soil compartment, followed by fumigation and then the type of fertilizer, whereas

Table 1
Bacterial and fungal richness (Chao) and diversity (Shannon) indices of the treatments.

Compartment	Treatment	Richness (Chao)		Diversity (Shannon)	
		Bacteria	Fungi	Bacteria	Fungi
Before planting	CKOF	2979.21 a [†]	746.19 a	6.26 a	4.03 a
	FCF	2912.27 a	746.66 a	6.15 a	4.14 a
	FOF	2914.45 a	720.68 a	6.11 a	4.19 a
Harvest Bulk soil	CKOF	2562.12 a	1160.75 a	6.86 a	5.06 a
	FCF	2081.51 b	1089.12 a	5.80 b	4.84 a
	FOF	1684.73 c	885.82 b	5.41 b	4.16 b
Harvest Rhizosphere	CKOF	2050.47 a	847.96 a	4.89 a	4.12 a
	FCF	2093.65 a	671.66 a	5.12 a	3.42 a
	FOF	2049.25 a	767.44 a	5.21 a	3.79 a

CKOF: organic fertilizer was amended in non-fumigated soil; FCF: chemical fertilizer was amended in fumigated soil; FOF: organic fertilizer was amended in fumigated soil. [†]: Values within the same column of each compartment followed by different letters are significantly different ($p < 0.05$).

fumigation was the main driver of the fungal communities, followed by the type of fertilizer and soil compartment. After comparing the contributions of the two strategies at harvest, we observed that fumigation and fertilization had similar effects on the bacterial and fungal composition (data not shown).

3.4. Relationships among the microbial communities, soil properties and soil disease

The structure equation model linking shifts in the bulk soil and rhizosphere microbial communities, soil properties, pathogen and disease incidence was supported by the data ($\chi^2 = 6.731$, d.f. = 5, $p = 0.241$; Fig. 4). According to the model, the tomato bacterial wilt disease incidence was mainly directly induced by the pathogen in the rhizosphere, the pathogen was negatively affected by the bacterial community in the rhizosphere, and the rhizosphere bacterial community was determined by the bulk soil bacterial community. Hence, the structural equation model suggested that the disease was controlled by a decrease in the pathogen via the rhizosphere bacterial community assembled by the observed shifts in the bulk soil bacterial community.

3.5. Sensitive bacterial community taxa and their relationships with disease incidence

Given the importance of suppressing *Ralstonia*, the rhizosphere bacterial community composition was further investigated. For identifying the sensitive bacterial taxa, we first pick up all the OTUs with relative abundance more than 1%. After that, we used LEfSe (Linear discriminant analysis Effect Size) to pick up the OTUs showed significant difference among three treatments. Finally, MaAsLin (Multivariate Association with Linear Models) was used to pick up the OTUs that have significant correlation with *Ralstonia solanacearum* abundance, and from those, 6 sensitive bacterial taxa were identified as OTU_11 (*Ralstonia*), OTU_21 (*Ralstonia*), OTU_23 (*Rhodanobacter*), OTU_67 (*Terrimonas*), OTU_19 (*Chitinophaga*), and OTU_2 (*Rhodanobacter*). Among these taxa, OTU_11 and OTU_21 showed higher relative abundance in CKOF and FCF than in FOF, while OTU_23, OTU_67, OTU_19 and OTU_2 had a lower relative abundance in CKOF and FCF than in FOF. Moreover, slightly fewer OTU_11 and OTU_21 and slightly more OTU_23, OTU_67, OTU_19 and OTU_2 were found in FCF than in CKOF, but only OTU_23 showed a significant difference (Fig. S2). Furthermore, Spearman correlation coefficients were calculated between the quantity of rhizosphere *Ralstonia* and the

Fig. 2. Bacterial and fungal community composition.

Principal coordinate analysis (PCoA) ordinations of the (a) bacterial and (b) fungal community composition based on the Bray-Curtis distance metric considering all soil samples. CKOF: organic fertilizer was amended in non-fumigated soil; FCF: chemical fertilizer was amended in fumigated soil; FOF: organic fertilizer was amended in fumigated soil. Circles refer to the samples before planting, triangles refer to the samples of bulk soil at harvest, and squares refer to the rhizosphere samples at harvest. Differences in the bacterial and fungal beta diversity of the CKOF, FCF and FOF treatment soils were determined by analysis of molecular variance (AMOVA). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Fig. 3. Variation in the composition of the soil bacterial and fungal communities.

Multiple regression tree (MRT) analysis of the treatment effects on the (a) bacterial and (b) fungal community composition. The identity and number of rhizosphere soil samples included in the analysis are shown under the tree. Numbers under the crosses of each split indicate the percentages of variance explained by the split. The R^2 , error, cross-validation error (CV Error), and standard error (SE) of the MRT analysis are listed under the tree. CKOF: organic fertilizer was amended in non-fumigated soil; FCF: chemical fertilizer was amended in fumigated soil; FOF: organic fertilizer was amended in fumigated soil. The letters B and R represent the bulk soil and rhizosphere, respectively.

relative abundances of the sensitive bacterial taxa that significantly responded to fumigation and fertilization. The quantity of rhizosphere *Ralstonia* was negatively correlated with OTU_23 (*Rhodanobacter*; $r = -0.80$, $p = 0.002$), OTU_67 (*Terrimonas*; $r = -0.77$, $p = 0.003$), OTU_19 (*Chitinophaga*; $r = -0.83$, $p < 0.001$) and OTU_2 (*Rhodanobacter*; $r = -0.70$, $p = 0.011$) and positively correlated with OTU_11 (*Ralstonia*; $r = 0.81$, $p = 0.001$) and OTU_21 (*Ralstonia*; $r = 0.84$, $p < 0.001$) (Fig. 5).

4. Discussion

Fumigation integrated with organic amendment was selected as a combined strategy in this study to suppress tomato bacterial wilt disease, as these two strategies can provide complementary biocontrol effects. Fortunately, the results in 2015 and 2016 showed the same trends as that in 2014 (Fig. S1). Yield from combined strategy also showed higher value than other treatments; thus, we assumed that fumigation by ammonium bicarbonate didn't have negative influence on tomato plants, and we also speculate that this combined strategy is a

safe and effective management to control tomato bacterial wilt disease. Fumigation can reduce pathogen abundance, and it can also influence the remaining native soil microbial communities. The use of organic fertilizer can, however, counteract the potential negative effects by improving microbial activity and influencing the diversity and structure of the microbial communities. Our results reveal that the combined treatment showed fantastic biocontrol capability, and we discuss the mechanisms driving the control of tomato bacterial wilt disease below.

4.1. Direct effects of fumigation and fertilization

The fumigant selected in this study is ammonium bicarbonate, a widely used nitrogenous fertilizer that can decrease soil microbial biomass through the release of ammonia (Li et al., 2016). Our results show that the combined treatment indeed led to a reduction in the abundance of *Ralstonia* (Fig. 1b). Although pathogenic and non-pathogenic *Ralstonia* could not be separately quantified via qPCR, the significant positive correlations between disease incidence and *Ralstonia* populations ($p = 0.003$; Table S2) indicated that the abundance

Fig. 4. Generalized multilevel path model of the direct and indirect pathways influencing rhizosphere pathogen and disease incidence. The model was supported by the data ($\chi^2 = 6.731$, d.f. = 5, $P = 0.241$). Arrows represent the flow of causality. Thick and thin arrows represent statistically significant ($P \leq 0.05$) and non-significant relationships, respectively. The path coefficients associated with each arrow are shown. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Fig. 5. Potential sensitive taxa.

Spearman correlations (r) between the relative abundances of bacterial taxa (relative abundance > 1%) and *Ralstonia* abundance. CKOF: organic fertilizer was amended in non-fumigated soil; FCF: chemical fertilizer was amended in fumigated soil; FOF: organic fertilizer was amended in fumigated soil. After FDR control, taxa with $q > 0.05$ are not shown.

of *Ralstonia* could serve as an indicator and that the combined strategy suppressed the *Ralstonia* abundance in the rhizosphere. Significantly lower relative abundances of OTU_11, and OTU_21, which belong to the genus *Ralstonia*, were found in the rhizosphere in the fumigation treatment amended with organic fertilizer and were both positively correlated with pathogenic *Ralstonia solanacearum*. Therefore, we considered that a population decline in pathogenic *Ralstonia* is one of the mechanisms underlying the control of plant disease, which is supported by many previous studies (Bailey and Lazarovits, 2003; Mao et al., 2017). As expected, the abundance of a pathogen in the rhizosphere directly influences plant health (Berendsen et al., 2012). However, other strategies seem to play a role in disease suppression, as the bio-control ability of the combined treatment was superior to the relative abundance of the pathogen, indicating that the shifts in the microbiota in the bulk soil caused the suppressiveness of the rhizosphere microbiota.

4.2. Indirect effects of fumigation and fertilization

The soil microbial community composition was largely affected by fumigation, which is in accordance with previous findings regarding fumigation with chloropicrin on ginger (Zhang et al., 2017) and with ammonium bicarbonate on cucumber (Li et al., 2016), in which fumigation led to shifts in the microbial community composition. Our results also indicate that fertilization had an impact on the microbial community structure, which is supported by previous observations that various organic amendments have important effects on microflora (Fu et al., 2017; Hartmann et al., 2015). Although we observed that the impact of fumigation was stronger, the application of organic matter following fumigation served as a co-deterministic driver of the

microbial community composition that induced general pathogen suppression. Interestingly, the effect of the treatment was only observed during harvest and not before planting. We hypothesize that the lack of response was caused by the detection of dead microbial DNA (Carini et al., 2017) shortly after the treatments were applied and that the influence was minimized due to higher microbial activity during the growing season.

4.3. The soil bacterial diversity but not the fungal diversity influences disease suppression

At harvest time, given that the rhizosphere is the main location where the microbial community interacts with the pathogen (Raaijmakers et al., 2009; Whipps, 2001), only a negative correlation between disease incidence and rhizospheric bacterial diversity was observed (Table S2). Similar correlations have been previously identified, in which higher bacterial diversity was associated with the increased suppression of *Ralstonia* (Hu et al., 2016) and the survival and growth of other pathogens (Dey et al., 2012). In soil, *Ralstonia solanacearum* can induce and invade many kinds of fungal cells, including chlamydospores (Spraker et al., 2016), suggesting that these fungi cannot suppress but may even instigate bacterial wilt. Together, these results indicate the importance of bacterial communities in contributing to the suppression of *Ralstonia*.

4.4. Sensitive bacterial taxa involved in disease suppression

The identified sensitive taxa included the genera *Rhodanobacter* (OTU_23, OTU_2), *Terrimonas* (OTU_67) and *Chitinophaga* (OTU_19). Although there has been no report of the *Rhodanobacter* genus

possessing specific biocontrol activity against *Ralstonia solanacearum*, three *Rhodanobacter* strains were reported as acting as biocontrol agents against the pathogen *Cylindrocladium spathiphylli* in the rhizosphere in compost-amended soil (De Clercq et al., 2006). *Terrimonas*, which was found in soil fumigated with basamid and biofumigated with compounds isolated from *Brassica juncea*, *Raphanus sativus* and *Tagetes patula*, exhibited a negative relationship with apple replant disease (Yim et al., 2017) and showed a greater abundance in the rhizosphere of healthy *Panax notoginseng* (Wu et al., 2015). *Chitinophaga* has been reported to exhibit fungicidal activities and induce antagonistic traits in other bacterial taxa (Chapelle et al., 2016). Furthermore, *Chitinophaga* can produce several kinds of potentially resistant substances, such as elansolid (Steinmetz et al., 2011) and lantibiotics (Mohr et al., 2015), to suppress pathogenic bacteria and fungi. Overall, the negative relationships between the pathogen and *Rhodanobacter*, *Terrimonas* and *Chitinophaga* suggest that these microbes are potential key taxa involved in disease suppression.

4.5. Overall response

The structural equation model demonstrated that the rhizosphere bacterial community, which was assembled by shifts in the bulk bacterial community, supported the suppressiveness of *Ralstonia*, which dominated the bacterial wilt disease incidence, and this result agreed with the bacterial diversity results discussed above. The assembly of the rhizosphere microbiome can be supported by previous studies that showed that different bulk soil microbiota can cause differences in the rhizosphere microbial community composition (de Ridder-Duine et al., 2005d; Liu et al., 2018). Moreover, in accordance with our results, suppressive effects of rhizosphere bacteria on *Rhizoctonia solani* (Mendes et al., 2011), *Fusarium oxysporum* (Fu et al., 2017) and *Ralstonia solanacearum* (Hu et al., 2016) have been previously demonstrated; thus, shifts in the rhizosphere bacterial community should be a key factor in the suppression of *Ralstonia*.

Based on the results presented above, we propose that suppression takes place via direct and indirect mechanisms and in the following order, as summarized in Fig. 6. When pathogens invade the rhizosphere microbiome, the first protective shield directly suppresses pathogen invasion through resource competition based on niche overlap (Wei et al., 2015) as well as the production of antibiotics by beneficial microbes (Hu et al., 2016) that already occur in the rhizosphere or are recruited by the plant in response to invasion (Pérez-Jaramillo et al., 2016). Once the first shield is broken, plant barriers subsequently act as the front line to block pathogen infection through physical defence (Zhan et al., 2014) and the secretion of low-molecular-weight antimicrobial chemicals (Baetz and Martinoia, 2014). Meanwhile, induced

systemic resistance (ISR) can also be activated by some beneficial microbiome components on the plant roots, triggering plant defence against pathogens (Pieterse et al., 2014). Unfortunately, as some pathogen cells can still invade the plant root, plant systemic acquired resistance (SAR) is finally primed to resist pathogen attack (Bernsdorff et al., 2016; Mittler and Blumwald, 2015), which can potentially induce the plant root to recruit a beneficial microbiome for pathogen suppression (Lebeis et al., 2015). In our results, although the density of *Ralstonia* in the rhizosphere in FCF was significantly lower than that in CKOF, the disease incidence was not significantly different (Fig. 1); thus, the shift in the rhizosphere microbiome stimulated plant defence (ISR). Hence, rhizosphere bacterial communities assembled through combined strategies effectively control pathogens not only through direct suppression (Fig. 6a) but also via indirect defence (Fig. 6b, d, e).

5. Conclusion

In summary, the results of the field experiment confirm that fumigation combined with organic amendment is an effective strategy for controlling bacterial wilt disease. The structural equation model suggests that it is the bacterial rather than the fungal community that dominates the suppressiveness to bacterial wilt disease, and this novel strategy effectively constructed a new soil bacterial community that formed a suppressive rhizosphere to suppress pathogenic *Ralstonia* and induce plant resistance. The beneficial microbial groups promoted (e.g., *Rhodanobacter*, *Terrimonas* and *Chitinophaga*) by the novel strategy contributed to the specific suppressiveness.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Fig. 6. Main mechanism of soil-borne pathogen suppression. a. The soil microbiome directly suppresses pathogens through nutrient and antibiotic competition. b. The plant directly suppresses pathogens through physical defence and chemical secretion. c. Plants recruit a microbiome to suppress pathogens. d. The microbiome induces the plant to increase its physical defence and chemical secretion to suppress pathogens. e. The damage caused by pathogens induces the plant to increase resistance and recruit a beneficial microbiome. Arrows and bars represent positive and negative relationships, respectively. Abbreviations: ISR, induced systemic resistance; SAR, systematic acquired resistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.apsoil.2019.103364>.

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