**Editors summary**

**Phage combination therapies reduce bacterial wilt disease incidence in tomato by up to 80%**

Phage combination therapies for bacterial wilt disease in tomato

Xiaofang Wang1,#, Zhong Wei1,#,\*, Keming Yang1, Jiangning Wang1, Alexandre Jousset1,2, Yangchun Xu1, Qirong Shen1, \*, Ville-Petri Friman1,3, \*

1Jiangsu Provincial Key Lab for Organic Solid Waste Utilization, Jiangsu Collaborative Innovation Center for Solid Organic Waste Resource Utilization, National Engineering Research Center for Organic-based Fertilizers, Nanjing Agricultural University, Weigang 1, Nanjing, 210095, PR China;

2Institute for Environmental Biology, Ecology & Biodiversity, Utrecht University, Padualaan 8, 3584CH Utrecht, The Netherlands;

3University of York, Department of Biology, Wentworth Way, York, YO10 5DD, UK.

\* Corresponding authors: Zhong Wei ([weizhong@njau.edu.cn)](mailto:weizhong@njau.edu.cn)), Qirong Shen (qirongshen@njau.edu.cn) and Ville-Petri Friman ([ville.friman@york.ac.uk](mailto:ville.friman@york.ac.uk))

# These authors contributed equally to this work

ABSTRACT

**Bacteriophage have been proposed as an alternative to pesticides to kill bacterial pathogens of crops. However, the efficacy of phage-biocontrol is variable and poorly understood in natural rhizosphere microbiomes at timescales considering both ecological and evolutionary processes.**

**We studied the biocontrol efficacy of different phage combinations on *Ralstonia solanacearum* infection in tomato. Increasing the number of phages in combinations decreased disease incidence by up to 80% in greenhouse and field experiments during single crop season. Decrease in disease incidence was explained by pathogen density reduction and selection for phage resistant but slow-growing pathogen strains, together with enrichment for bacterial species that were antagonistic towards *Ralstonia solanacearum*. Phage treatment did not affect existing rhizosphere microbiota. Specific phage combinations show promise as precision tools to engineer microbiomes and to control plant pathogenic bacteria.**

**INTRODUCTION**

It is estimated that around 10-20% of global food production is lost to plant diseases every year[1](#_ENREF_1), [2](#_ENREF_2). Better plant pathogen control methods are therefore urgently needed to maintain and improve crop yields in order to ensure future food security. Biocontrol, using bacterial inoculants to modify the composition of plant rhizosphere microbiota, has been proposed as an alternative to pesticides for pathogen elimination[3-5](#_ENREF_3). However, bacterial inoculants are often ineffective, owing to poor establishment in the rhizosphere, competition with native microbiota for resources, and interference with native microbiota[6](#_ENREF_6), [7](#_ENREF_7). Phage therapy, using viruses specific for plant pathogenic bacteria, could offer a more effective method for manipulating rhizosphere microbiota to protect plant from disease[8](#_ENREF_8), [9](#_ENREF_9). Compared with bacterial inoculants, the main benefits of phage are their host-specificity and ability to rapidly propagate in the presence of other microbiota as long as a host bacterium is present. Phage could be used as precision tools to target a pathogen, leaving the surrounding microbiota unaffected[9](#_ENREF_9). However, experimental evidence for phage efficacy in combating plant diseases in agriculturally relevant native rhizosphere microbiota is lacking.

Phage can control pathogens by ecological and evolutionary mechanisms. The main ecological mechanism is regulation of pathogen density. A reduction in pathogen abundance by phages can constrain the likelihood of infection, the expression of bacterial virulence genes[10](#_ENREF_10), [11](#_ENREF_11) or plastic phage defences[12](#_ENREF_12), [13](#_ENREF_13) that are switched on once certain pathogen population density threshold has been achieved. The main evolutionary mechanisms are fitness trade-offs between phage resistance and other important pathogen life-history traits. While the evolution of phage resistance has been considered in the context of failed biocontrol experiments[8](#_ENREF_8), it has seldom been considered as a tool to weaken the pathogen through trade-offs. For example, phage resistance is often traded against virulence because genes encoding phage receptors are also important for other functions such as nutrient acquisition, motility, antimicrobial resistance and plant colonisation[14-17](#_ENREF_14). Such trade-offs have been especially well studied for plant pathogenic *Ralstonia solanacearum* bacterium[18-20](#_ENREF_18), which is a causative agent of bacterial wilt and capable of infecting multiple important crop plant species globally[21](#_ENREF_21), [22](#_ENREF_22). With *R. solanacearum*, these trade-offs can be driven by high costs of expressing different virulence factors[20](#_ENREF_20) and global regulatory genes that control both metabolic and virulence pathways and networks[19](#_ENREF_19). While previous work has demonstrated that evolution of phage resistance is linked with *R. solanacearum* growth and virulence[16](#_ENREF_16), [23](#_ENREF_23), explicit demonstrations that phage can be used as evolutionary tools to weaken a pathogen via fitness trade-offs are rare.

Phage-mediated pathogen density reduction, or evolutionary trade-offs, could indirectly affect the diversity and functioning of other rhizosphere microbiota. For example, reduction in relative pathogen density by phage could increase niche space and nutrients for native microbiota, which might result in changes in the rhizosphere microbiome composition and diversity. This could have beneficial secondary effects for plant health because increased microbiome diversity is often correlated with reduced pathogen loads and disease incidence[24](#_ENREF_24), [25](#_ENREF_25). Furthermore, evolutionary trade-offs that weaken pathogen nutrient uptake or catabolism are expected to intensify resource competition of any pathogen with the native microbiota, which could result in a further reduction in pathogen abundance[16](#_ENREF_16), [26-28](#_ENREF_26).

Here we report experiments to ascertain the effects of phage therapy in the tomato plant rhizosphere on the pathogenic bacterium *Ralstonia solanacearum*. We assessed the use of phage to target the pathogen in a complex rhizosphere microbiome using greenhouse and field experiments, investigated whether evolution of phage resistance affects the pathogen competitiveness and growth via trade-offs and examined whether phage indirectly change the composition, diversity and functioning of the wider rhizosphere microbiome.

**RESULTS**

**Phage effects on disease incidence in the greenhouse and field**

We used phage combinations consisting of one to four phage types isolated from tomato fields in China that had distinct infectivity ranges despite high genetic similarity (>99% similarity; Fig. S1-4, Table S1-2 and online methods). Increasing the number of phages in a combination is expected to strengthen control of pathogen density by increasing the efficiency of infection and lowering rates of phage resistance evolution due to higher number of mutations required for the evolution of broad phage resistance range[26](#_ENREF_26), [27](#_ENREF_27). Although single phages reduced the incidence of bacterial wilt disease compared to control in the greenhouse experiment (F1,14 = 6.842, P= 0.021, Fig. 1A), disease incidence was reduced more by phage combinations that contained a higher number of phages (R2= 0.315, P= 6.518e-05, Fig. 1A). The reduction in disease incidence could be explained by a reduction in pathogen densities and this effect became stronger with an increasing number of phages present in the combination (R2= 0.186, P=0.003, Fig. 1B). Increasing the number of phages had also a negative effect on the total phage density (R2= 0.178, P=0.003, Fig. 1B), which confirms that phage densities depended on the host abundance as indicated by a positive relationship between phage and pathogen densities (R2= 0.506, P=7.708e-08, Fig. S5). All phage types had clear negative effects on the disease incidence and pathogen densities except for the phage NB-P21, which did not significantly affect pathogen densities (Table S3).

In the field experiment we observed similar results. All four phage types had substantial biocontrol efficacy in the field experiment (F3,191 = 0.385, P= 0.764, Fig. 1C) leading to an average 80% reduction in bacterial wilt disease severity (disease index) compared to the control treatment (F1,287 = 89.315, P < 0.001, Fig. 1C). However, our four-phage combination had the highest biocontrol efficacy leading to the lowest mean disease index among all the treatments (F2,287 = 47.057, P< 0.001, Fig. 1C) even though considerable variation was observed within treatments. Together these results suggest that while all phage types were effective at reducing bacterial wilt disease incidence on their own, they were more effective when applied as combinations, both in the greenhouse and field conditions.

**Evolution of phage resistance in the greenhouse experiment**

We next explored the evolution of phage resistance and its associated costs at the end of the greenhouse experiment. All phage types selected for pathogens that had increased resistance to ancestral phages (F1,44= 14.198, P< 0.001, Fig. 2A), and generally, pathogens evolved a broader resistance range when they had been exposed to phage combinations during the greenhouse experiment (R2=0.287, P<0.001, Fig. 2A). We also found that pathogens retained generally higher levels of resistance to both ancestral and coevolved phages when they had evolved in the presence of phage combinations (F2,41= 0.054, P=0.947, Fig. 2B). Together these results indicate that pathogens evolved the broadest phage resistance range when exposed to three-phage combinations.

Evolving resistance to phages was costly in terms of reduced pathogen growth in the absence of phages (carrying capacity: F1,44= 14.684, P< 0.001, Fig. 2C; maximum growth rate: F1,44= 6.293, P= 0.016, Fig. S6). Moreover, the magnitude of this cost increased when pathogens were exposed to multiple phages leading to a steeper negative correlation between phage resistance and growth (carrying capacity: R2=0.308, P=2.2e-16, Fig. 2D small inset in the top right corner). This growth cost was also linked with reduced competitive ability of resistant pathogens when measured in competition experiments with phage-susceptible ancestral pathogen genotype (F4,47=7.303, P<0.001; Fig. S7). Together these results suggest that phage combinations can select for resistant but slow-growing pathogens that are associated with reduced disease incidence.

**Effect of phage on the rhizosphere microbiota composition and function**

The presence of phage had a large effect on the composition of the rhizosphere microbiome measured at the end of the greenhouse experiment (F1,43= 2.101, P= 0.001, Fig. 3A). Specifically, increasing the number of phages present in phage combinations correlated with high bacterial community diversity (Shannon diversity index: R2= 0.449, P=7.428e-07, Fig. 3B; OTU richness: R2= 0.308, P=8.138e-05, Fig. S8) and high relative abundance of Chloroflexi (R2= 0.509, P=6.814e-08), Acidobacteria (R2= 0.214, P=0.001), Planctomycetes (R2= 0.157, P=0.005) and Firmicutes (R2= 0.233, P=0.001) phyla, and low relative abundance of Proteobacteria (R2= 0.140, P=0.008) and Bacteroidetes (R2= 0.184, P=0.003) phyla (Fig. 3C, S9). Phage also significantly increased the abundance of the Actinobacteria phylum compared to the control treatment regardless of the number of phages present in in phage combinations (F1,44= 12.761, P< 0.001, Fig. 3C, S9). To analyse these patterns in more detail, we constructed co-occurrence networks based on OTUs for single-phage and three-phage communities and used Netshift analysis to identify potentially important driver taxa behind community change[28](#_ENREF_28). After initial screening, 500 taxa (nodes) were retained in both networks (Fig S10). On average, single-phage networks were more connected and had shorter path lengths indicative of potentially more frequent interactions (Fig. S10 and Table S4). Only 9 out of 311 taxa associations were found in both communities. Instead, most of the taxa associations were completely different between single-phage and three-phage communities and the number of significant associations increased with the number of phages (Fig. 3D; 72 vs. 230 associations, respectively). Furthermore, Netshift analysis revealed 40 potential driver taxa linked with the change in microbiome composition (Fig. 3D). Of these taxa, 16 increased and 7 decreased in abundance in three-phage compared to single-phage communities and especially *Pseudobacteroides*, *Gaiellales*, *Luteimonas* and two other Actinobacteria (288-2\_norank and Elev-16S-1332\_norank) were found to play important roles in changing the network structure (Fig. 3D and Table S5).

To verify that these changes were not driven by phage directly, we used a short-term lab experiment to test how pathogen and three-phage combinations affected the composition and diversity of rhizosphere community using a soil wash prepared from the same soil used in the greenhouse experiment. We found that the presence of phages did not affect the composition or diversity of the microbiome in the absence of the pathogen (composition: F1,14= 1.165, P= 0.300, Fig. 4A; Shannon diversity index: F1,14= 1.502, P= 0.242, Fig. 4B), while the presence of pathogen alone had a large effect (composition: F1,5= 239.545, P< 0.001, Fig. 4A; Shannon diversity index: F1,5= 243.884, P< 0.001, Fig. 4B). The presence of phages constrained and even prevented the pathogen-mediated changes in the community composition and diversity (Fig. 4A, B). To explore this in more detail, we randomly isolated 400 cultivable bacterial strains from the same rhizosphere soil and tested their susceptibility to *R. solanacearum*-infecting phages individually (Fig. 4C, Table S6). We found that none of the four phages could infect any of the 400 isolates (Fig. S11). Together these results suggest that phages were only capable of infecting the pathogen and that the changes in the rhizosphere microbiome composition, diversity and species co-occurrence networks were driven by indirect feedbacks.

We next explored the functional properties of the bacterial taxa that were observed to increase in the presence of phages at the genera level. The bacterial genera abundances correlated mostly positively with the number of phages present in the community (22.30% positive vs. 6.68% negative significant correlations, Fig. S12A), and most of the significantly affected genera belonged to Proteobacteria, Actinobacteria, Firmicutes and Chloroflexi phyla (Fig. S12B). To determine how the enriched bacterial taxa interacted with the pathogen, we conducted pairwise competition co-culture experiments in the lab using ancestral pathogen and all 400 isolated non-pathogenic rhizosphere bacteria (Fig. 4C). We found that enriched bacteria belonging to *Acinetobacter* (n=12), *Bacillus* (n=67), *Comamonas* (n=7), *Ensifer* (n=10) and *Rhodococcus* (n=4) genera were mostly antagonistic to the pathogen leading to an average of 64.89 % reduction in pathogen growth (Fig. 4D). These bacteria could have thus potentially contributed to the suppression of *R. solanacearum* either via resource or interference competition during the greenhouse experiment.

**Ecological and evolutionary mechanisms underlying successful phage biocontrol**

Structural equation model analysis (PLS-SEM) and path-modelling estimation were used to disentangle the relative importance of ecological and evolutionary mechanisms behind successful phage biocontrol outcomes in the greenhouse. Specifically, we explored how the beneficial effects of phage combinations were mediated via pathogen density reduction and evolution of phage resistance-growth trade-offs. The final full model had a reasonable fit explaining 36% of the variation in disease incidence (χ2 =31.7 and NFI = 0.81) and increasing the number of phages present in the combinations increased the level of phage resistance and decreased the pathogen growth (carrying capacity, Fig. 5). Reduction in the pathogen carrying capacity was positively correlated with the reduction in pathogen abundance, which was in turn positively linked with the disease incidence (Fig. 5). This suggests that phage-mediated selection for highly resistant but slow-growing pathogens strongly contributed to the reduction in pathogen abundances and associated decrease in disease incidence. Furthermore, pathogen abundances correlated negatively with the microbiome diversity, while phage resistance evolution was positively associated with high microbiome diversity (Fig. 5). These results were further validated by constructing less complex SEMs (Fig. S13) that showed similar results: increasing the number of phages had a negative effect on the pathogen density via carrying capacity (λ= 0.514, P= 0.001, Fig. S13A), the microbiome diversity (Shannon index) was negatively correlated with the pathogen density (λ= -0.467, P= 0.015, Fig. S13B, Table S7) and that the changes in pathogen densities were more important for the disease incidence compared to microbiome diversity (λ=0.460, P= 0.010, Fig. S13C, Table S8). Together these results suggest that both ecological and evolutionary mechanisms were important in explaining the reduction in the disease incidence.

**DISCUSSION**

We show that phage can be used as a precision tool to improve plant health in the greenhouse and the field. Notably, effects of phage biocontrol were mediated by ecological mechanisms (phage killing the pathogen) and by evolutionary mechanisms, because reduction in disease was associated with selection for highly resistant but slow-growing pathogens. Moreover, effects of phage on the pathogen indirectly changed the composition and the diversity of the resident bacterial microbiome and enriched bacterial taxa that were highly antagonistic towards the pathogen. Our results highlight the importance of considering both ecological and evolutionary mechanisms in the context of microbiota when designing phage therapies for agricultural and biotechnological use.

Increasing the number of phages in a combination improved the biocontrol efficacy of phage combinations, by a more substantial reduction of pathogen density, disease incidence and disease index in both greenhouse and field experiments. Second, although phage combinations selected for resistance to both ancestral and coevolved phages, it was costly, leading to a reduction in pathogen growth and competitive ability. These results are consistent with previous studies demonstrating increased efficacy of phage combinations in controlling the densities of pathogenic bacteria[27](#_ENREF_27), [29-31](#_ENREF_29) and increased resistance range evolution due to cross-resistance mutations[26](#_ENREF_26), [32-34](#_ENREF_32). While our phage combinations consisted of genetically highly similar phages (>99% similarity), they had distinct infectivity profiles and effects on phage resistance evolution. Although we were not able to uncover exact phage infectivity mechanisms, we speculate that phages used different receptors or mechanisms to kill pathogens, as prior exposure to diverse phage combination was required to evolve high levels of resistance to all ancestral phages. We also found that pathogens were able to evolve resistance to both ancestral and coevolved phages when they were concurrently exposed to multiple phages. This result suggest that phage diversity could affect the type of coevolutionary dynamics and selection for generalist (arms race dynamics) and specialist (fluctuating selection dynamics) phage resistance strategies[32](#_ENREF_32), [35](#_ENREF_35). In the future, it will be important to study these effects over successive plant generations to observe if phage selection can constrain *R. solanacearum* outbreaks at longer timescales that might allow pathogens to re-evolve and restore their competitive ability.

Evolution of phage resistance was costly in terms of reduced pathogen maximum growth rate, carrying capacity and competitive ability against non-resistant ancestral strain. Such costs have often been observed across different phage-bacteria systems including *R. solanacearum* and its phages[16](#_ENREF_16), [36](#_ENREF_36). We found that the magnitude of the cost increased with increase in the phage resistance range. One potential mechanism for this could be that selection by different phages potentially impaired several phage receptors leading to an escalation of costs of resistance. For example, phages often bind to transmembrane proteins, which are also used to take up nutrients, and while mutations in these receptors can make bacteria resistant to phages, they often reduce the competitive fitness of the bacteria[14](#_ENREF_14), [37](#_ENREF_37). Such costs could be especially severe in complex soil microbiomes where resources are often limited and pathogens need to compete with a myriad of other bacteria. In addition to the reduction in the pathogen competitive ability, reduced growth likely weakened the pathogen ability to infect plants as *R. solanacearum* virulence gene expression is triggered by quorum sensing, which requires high pathogen population densities[38](#_ENREF_38). Changes in *R. solanacearum* metabolism could also be linked with its virulence due to overlapping regulatory networks and key regulatory genes[18](#_ENREF_18), [20](#_ENREF_20). Our structural equation modelling results indirectly support this by demonstrating that phage effects on pathogen densities and disease incidence were strongly channelled via resistance-mediated reduction in the carrying capacity of the pathogen. Identifying phages that drive evolutionary changes in key metabolic and virulence regulatory ‘hubs’ could be especially beneficial for therapeutic purposes. Moreover, our results show that the phage-bacteria interaction changed the ecology of pathogen-plant interaction leading to reduced disease incidence. Such eco-evolutionary feedbacks have previously been found in various systems[37-41](#_ENREF_37) and our results suggest they might also be important for plant disease dynamics in the agricultural context.

Increasing the number of phages present in combinations correlated strongly with changes in the composition and diversity of native bacterial rhizosphere microbiome. This effect was indirect as none of the phages were able to infect any of the natural isolates and three-phage combinations alone did not have a significant effect on the rhizosphere microbiome composition or diversity. While it is possible that our isolate collection did not fully represent the whole diversity or the conditions present in the rhizosphere soil, it is more probable that phage-mediated effects were driven indirectly via pathogen density reduction and selection for reduced pathogen competitiveness. This suggests that phages could be safe and environmentally friendly bioresource for controlling plant pathogenic bacteria. A similar positive relationship between rhizosphere microbiome diversity and plant health has previously been observed in the fields contaminated with *R. solanacearum*[25](#_ENREF_25) and such differences have been suggested to result from distinct root exudation patterns exerted by healthy and diseased plants[42](#_ENREF_42" \o "Gu, 2016 #3597). Notably, we found that several bacterial taxa, whose abundances correlated positively with the number of phages present in phage combinations, showed high antagonism towards the pathogen either via resource or interference competition[4](#_ENREF_4), [43](#_ENREF_43). Moreover, we were able to identify several candidate driver taxa that played a key role in bacterial co-occurrence networks and were enriched in three-phage compared to single-phage bacterial communities. For example, Bacillus genera, which has previously been shown to have high antibiotic activity specifically against phage resistant *R. solanacearum* strains[16](#_ENREF_16), increased in abundance along with the number of phages present in phage combinations and most of them were antagonistic to the pathogen. It is thus possible that phage effects were further enforced by enrichment of antagonistic bacteria present in the resident microbiome. In the future, it would be interesting to use metagenomic sequencing to study these patterns throughout the plant development focusing on wider microbiome diversity including fungi and protists.

Using phage for biocontrol in agriculture shows promise. Although the concept of using phage to control plant pathogenic bacteria is not new, commercial use of phage in agriculture is still limited[8](#_ENREF_8), [9](#_ENREF_9), [44](#_ENREF_44). We report that phage efficiency can be improved by using combinations and that phage can retain biocontrol efficiency regardless of the rapid evolution of phage resistance.

**Figure legends**

Figure 1. **Phage combinations and disease incidence.** The effect of phage combinations on disease incidence(A) and pathogen and phage densities (B) at the end of the greenhouse experiment (n=12 for single- and three-phage treatments and n=18 for two-phage treatment). Panel C shows the effect of single- and four-phage treatments on plant disease severity (disease index) at the end of the field experiment as violin plots (n=48; abbreviations on the X-axis refer to four different phage types). All relationships were analysed using linear regression analysis and ANOVA and R2 and P values presented refer to the most parsimonious models. In panels A and B, red dashed lines represent the values observed in the control treatments in the absence of phages (disease incidence in panel A and pathogen density in panel B, n=3 for the control line). In panel C, each side of the violin plot shows a kernel density estimation for the distribution of the data. Wider sections of the violin plot represent a higher probability and the skinnier sections represent a lower probability. Dots in plot showed the real data distribution. In all panels, n are based on biologically independent samples.

Figure 2. **Resistance evolution to ancestral and coevolved phages**. The mean phage resistance to ancestral (A and B) and coevolved phages (B) and the cost of resistance in terms of pathogen carrying capacity in the absence of phages after 24h of growth (C). Panel D shows a negative correlation between pathogen carrying capacity and phage resistance indicative of resistance-growth trade-off. Red dashed line shows the resistance of control pathogen selection lines where pathogen evolved in the absence of phages (A; n= 3) and the growth of ancestral pathogen strain in the absence of phage (C; n= 3). Lowercase letters above boxplots in panel A and C denote for significant differences between phage combination treatments (multiple comparisons were conducted using Tukey test, FDR adjusted P<0.05). All box-plots show interquartile range (25 to 75% of the data), the median as lines and outliers as dots (n=3 for the control, n=12 for single- and three-phage and n=18 for two-phage treatment; 8 clones isolated from each sample). Error bars in panel B represent SD of mean (n=12 for single- and three-phage and n=18 for two-phage treatment). In panel D, R2 and P values refer to the most parsimonious model, black line shows mean regression based on all data points and the small inset shows the increase in regression coefficient between pathogen carrying capacity and phage resistance in different phage combination treatments (\*\*\* FDR adjusted P<0.001, n.s. FDR adjusted P=0.191; n=3 for control , n=12 for single- and three-phage and n=18 for two-phage treatment; 8 clones isolated from each sample). In all panels, n are based on biologically independent samples.

Figure 3. **Effects of phage on rhizosphere communities**. Changes in the composition (A) and diversity (B) of rhizosphere bacterial community in different phage combination treatments (composition and diversity analyses were based on multidimensional scaling analysis and Shannon diversity index based on OTUs, respectively, and in panel A, N0-N3 denote for number of phages present in phage combinations (n=3 for no-phage treatment, n=12 for single- and three-phage treatments and n=18 for two-phage treatment). Red dashed line in B shows the no-phage control treatment and R2 and P values refer to the most parsimonious fitted model (n= 3). Panel C shows changes in the relative densities of the pathogen (black) and other bacterial phyla (other colours) in different phage combination treatments and the key on the left show significant changes and their direction (upward and downward arrows for increased and reduced abundances, respectively) for each phylum and the pathogen. Panel D shows the potential ‘driver taxa’ important for changes observed in bacterial co-occurrence networks between single- and three-phage combinations. Node sizes are proportional to their scaled NESH score (a score identifying the importance of given microbial taxa in the association network) and a node is coloured red if its importance (betweenness) increases when comparing microbiomes associated with single- and three-phage treatments. Total of 72 and 230 unique associations was found in single- and three-phage treatments, respectively, and only 9 associations were the same in both single- and three-phage treatments. All n are based on biologically independent samples.

Figure 4. **Phage specificity and effects on the suppressiveness of rhizosphere microbiota.** Changes in the composition (A ) and diversity (B) of the natural bacterial community in the absence and presence of the pathogen and three-phage combinations (In A and B: n=3 for Community and Community+Pathogen treatments and n=12 for Community+Phage and Community+Pathogen+Phage treatments, and in panel B, box-plots shows interquartile range between 25 to 75% of the data, median as a line and outliers as dots). Lowercase letters above boxplots in panel B denote for significant differences between treatments (multiple comparisons were conducted using Tukey test, FDR adjusted P<0.05). Panel C shows a phylogenetic tree based on 400 culturable isolates and the different node colours in the cladogram denote for four different phyla: Proteobacteria (44.00%; red), Firmicutes (23.25%; blue), Bacteroidetes (20.25%; purple) and Actinobacteria (12.50%; green) and different ring colours bacterial OTUs observed at different taxonomic levels. Panel D shows the inhibition of ancestral *R. solanacearum* pathogen by strains (OTUs) belonging to five genera that were enriched with increasing number of phages: box-plot shows interquartile range (25 to 75% of the data), the median as lines and individual strains as dots (n=12 for *Acinetobacter*, n=67 for *Bacillus*, n=7 for *Comamonas*, n=10 for *Ensifer*, and n=4 for *Rhodococcus*). In panel D, blue, grey and orange dashed lines show the mean inhibition of ancestral pathogen by bacterial isolates that were reduced, not significantly associated or increased in abundance with the increasing number of phages present in the phage combinations, respectively. All n are based on biologically independent samples.

Figure 5. **Mechanisms underlying phage-mediated effects on bacterial wilt disease**. Shown is a structural equation model path diagram disentangling the ecological and evolutionary mechanisms behind phage-mediated effects on the disease incidence. The red, blue and grey arrows denote for positive, negative and non-significant pathways, respectively, and the numbers beside arrows denote for the magnitude of these effects. Numbers within the circles show the percentage of variance explained by other variables and χ2 and NFI values denote for the fit of the model.

**Data availability statement**

All sequence data that of this study have been deposited in NCBI Sequence Read Archive (SRA) database and all the accession numbers are provided in online methods. Phage sequence accession numbers are: SRR8402465 (NJ-P3), SRR8403229 (NB-P21), SRR8403928 (NC-P34) and SRR8410130 (NN-P42). The rhizosphere microbiome data determined at the end of the greenhouse experiment have accession numbers running from SRR8417955 to SRR8417999 (45 samples, paired end sequencing). The rhizosphere microbiome data obtained in a separate lab experiment have accession numbers running from SRR8470488 to SRR8501098 (30 samples, paired end reads). All other data has been deposited to Dryad Digital Repository with following digital identifier: 10.5061/dryad.02v6wwpzq.

**Code availability statement**

No custom code or mathematical algorithm deemed central to our conclusions was used in the analysis.

**References**

1. Raaijmakers, J.M., Paulitz, T.C., Steinberg, C., Alabouvette, C. & Moenne-Loccoz, Y. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil* **321**, 341-361 (2009).

2. Strange, R.N. & Scott, P.R. Plant disease: A threat to global food security. *Annu. Rev. Phytopathol.* **43**, 83-116 (2005).

3. Berendsen, R.L., Pieterse, C.M.J. & Bakker, P.A.H.M. The rhizosphere microbiome and plant health. *Trends Plant Sci.* **17**, 478-486 (2012).

4. Hu, J. et al. Probiotic diversity enhances rhizosphere microbiome function and plant disease suppression. *Mbio* **7** (2016).

5. Wei, Z. et al. Trophic network architecture of root-associated bacterial communities determines pathogen invasion and plant health. *Nature communications* **6** (2015).

6. Brodeur, J. Host specificity in biological control: insights from opportunistic pathogens. *Evol. Appl.* **5**, 470-480 (2012).

7. Kyselková, M. & Moënne-Loccoz, Y. in Organic Fertilisation, Soil Quality and Human Health 93-140 (Springer, 2012).

8. Alvarez, B. & Biosca, E.G. Bacteriophage-based bacterial wilt biocontrol for an environmentally sustainable agriculture. *Front. Plant Sci.* **8**, 1218 (2017).

9. Buttimer, C. et al. Bacteriophages and bacterial plant diseases. *Frontiers in microbiology* **8**, 34 (2017).

10. Rutherford, S.T. & Bassler, B.L. Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harbor perspectives in medicine* **2** (2012).

11. Levin, B.R. & Bull, J.J. Population and evolutionary dynamics of phage therapy. *Nature Reviews Microbiology* **2**, 166-173 (2004).

12. Hoyland-Kroghsbo, N.M., Maerkedahl, R.B. & Svenningsen, S.L. A quorum-sensing-induced bacteriophage defense mechanism. *Mbio* **4**, e00362-00312 (2013).

13. Tan, D., Svenningsen, S.L. & Middelboe, M. Quorum sensing determines the choice of antiphage defense strategy in *Vibrio anguillarum*. *Mbio* **6**, e00627 (2015).

14. Lenski, R.E. Coevolution of bacteria and phage: are there endless cycles of bacterial defenses and phage counterdefenses? *J. Theor. Biol.* **108**, 319-325 (1984).

15. Bohannan, B.J.M. & Lenski, R.E. Linking genetic change to community evolution: insights from studies of bacteria and bacteriophage. *Ecology Letters* **3**, 362-377 (2000).

16. Wang, X.F. et al. Parasites and competitors suppress bacterial pathogen synergistically due to evolutionary trade-offs. *Evolution* **71**, 733-746 (2017).

17. Bohannan, B.J., Kerr, B., Jessup, C.M., Hughes, J.B. & Sandvik, G. Trade-offs and coexistence in microbial microcosms. *Antonie van Leeuwenhoek* **81**, 107-115 (2002).

18. Peyraud, R., Cottret, L., Marmiesse, L. & Genin, S. Control of primary metabolism by a virulence regulatory network promotes robustness in a plant pathogen. *Nature communications* **9** (2018).

19. Perrier, A. et al. Enhanced in planta fitness through adaptive mutations in EfpR, a dual regulator of virulence and metabolic functions in the plant pathogen *Ralstonia solanacearum*. *PLoS Pathog.* **12** (2016).

20. Peyraud, R., Cottret, L., Marmiesse, L., Gouzy, J. & Genin, S. A resource allocation trade-Off between virulence and proliferation drives metabolic versatility in the plant pathogen *Ralstonia solanacearum*. *Plos Pathogens* **12** (2016).

21. Hayward, A.C. Biology and epidemiology of bacterial wilt caused by *Pseudomonas Solanacearum*. *Annu. Rev. Phytopathol.* **29**, 65-87 (1991).

22. Jiang, G.F. et al. Bacterial Wilt in China: History, current status, and future perspectives. *Frontiers in Plant Science* **8** (2017).

23. Addy, H.S., Askora, A., Kawasaki, T., Fujie, M. & Yamada, T. Loss of Virulence of the phytopathogen *Ralstonia solanacearum* through infection by phi RSM filamentous phages. *Phytopathology* **102**, 469-477 (2012).

24. Chapelle, E., Mendes, R., Bakker, P.A.H.M. & Raaijmakers, J.M. Fungal invasion of the rhizosphere microbiome. *Isme Journal* **10**, 265-268 (2016).

25. Wei, Z. et al. Ralstonia solanacearum pathogen disrupts bacterial rhizosphere microbiome during an invasion. *Soil Biology and Biochemistry* **118**, 8-17 (2018).

26. Wright, R.C.T., Friman, V.P., Smith, M.C.M. & Brockhurst, M.A. Cross-resistance is modular in bacteria-phage interactions. *PLoS Biol.* **16** (2018).

27. Hall, A.R., De Vos, D., Friman, V.P., Pirnay, J.P. & Buckling, A. Effects of sequential and simultaneous applications of bacteriophages on populations of Pseudomonas aeruginosa in vitro and in wax moth larvae. *Appl Environ Microbiol* **78**, 5646-5652 (2012).

28. Kuntal, B.K., Chandrakar, P., Sadhu, S. & Mande, S.S. 'NetShift': a methodology for understanding 'driver microbes' from healthy and disease microbiome datasets. *ISME J.* **13**, 442-454 (2019).

29. Yu, L. et al. A guard-killer phage cocktail effectively lyses the host and inhibits the development of phage-resistant strains of Escherichia coli. *Appl. Microbiol. Biotechnol.* (2017).

30. Wei, C. et al. Developing a bacteriophage cocktail for biocontrol of potato bacterial wilt. *Virologica Sinica* **32**, 476-484 (2017).

31. Yen, M.M., Cairns, L.S. & Camilli, A. A cocktail of three virulent bacteriophages prevents Vibrio cholerae infection in animal models. *Nat. Commun.* **8** (2017).

32. Betts, A., Gray, C., Zelek, M., MacLean, R.C. & King, K.C. High parasite diversity accelerates host adaptation and diversification. *Science* **360**, 907-911 (2018).

33. Betts, A., Gifford, D.R., MacLean, R.C. & King, K.C. Parasite diversity drives rapid host dynamics and evolution of resistance in a bacteria-phage system. *Evolution* **70**, 969-978 (2016).

34. Hall, A.R., Scanlan, P.D., Morgan, A.D. & Buckling, A. Host-parasite coevolutionary arms races give way to fluctuating selection. *Ecology Letters* **14**, 635-642 (2011).

35. Gomez, P. & Buckling, A. Bacteria-phage antagonistic coevolution in soil. *Science* **332**, 106-109 (2011).

36. Yamada, T. et al. New bacteriophages that infect the phytopathogen *Ralstonia solanacearum*. *Microbiol-Sgm* **153**, 2630-2639 (2007).

37. Taylor, V.L., Fitzpatrick, A.D., Islam, Z. & Maxwell, K.L. The diverse impacts of phage morons on bacterial fitness and virulence. *Advances in virus research* **103**, 1-31 (2019).

38. Khokhani, D., Lowe-Power, T.M., Tran, T.M. & Allen, C. A single regulator mediates strategic switching between attachment/spread and growth/virulence in the plant pathogen *Ralstonia solanacearum*. *Mbio* **8**, e00895-00817 (2017).

39. Friman, V.P., Dupont, A., Bass, D., Murrell, D.J. & Bell, T. Relative importance of evolutionary dynamics depends on the composition of microbial predator-prey community. *ISME J.* **10**, 1352-1362 (2016).

40. Hiltunen, T. & Becks, L. Consumer co-evolution as an important component of the eco-evolutionary feedback. *Nat. Commun.* **5** (2014).

41. Brunner, F.S., Anaya-Rojas, J.M., Matthews, B. & Eizaguirre, C. Experimental evidence that parasites drive eco-evolutionary feedbacks. *PNAS* **114**, 3678-3683 (2017).

42. Gu, Y. et al. Pathogen invasion indirectly changes the composition of soil microbiome via shifts in root exudation profile. *Biology and Fertility of Soils* **52**, 997-1005 (2016).

43. Li, M. et al. Facilitation promotes invasions in plant-associated microbial communities. *Ecol Lett* (2018).

44. Balogh, B., Jones, J.B., Iriarte, F.B. & Momol, M.T. Phage therapy for plant disease control. *Current Pharm. Biotechnol.* **11**, 48-57 (2010).

ONLINE MATERIALS AND METHODS

**(a) Microbial strains and experimental conditions**

We used *Ralstonia solanacearum* strain QL-Rs1115 (GenBank accession GU390462) as a model plant pathogenic bacterium in our experiments [45](#_ENREF_45). *R. solanacearum* QL-Rs1115 was routinely grown at 30 °C in NB medium (glucose 10.0 g L-1, peptone 5.0 g L-1, yeast extract 0.5 g L-1, beef extract 3.0 g L-1) for 24 hours with shaking (170 rpm) before all the experiments. We chose four lytic phages (NJ-P3, NB-P21, NC-P34, NN-P42) that were isolated from four geographically distant tomato fields in China as our model phages (Table S1). The initial phage stocks were prepared by growing each phage individually with the stock *R. solanacearum* strain in NB medium for 24 hours as described above with the addition of centrifugation (5 mins at 10000×g force) and filtration (0.22 μm) steps to isolate and purify phages from bacteria. The phage titers were adjusted to 107 phage particles per mL and phage stocks were stored at 4 °C. Phages were also assembled into communities with 1, 2 and 3 phages types in all possible combinations using 100%, 50% and 33% of each phage single-culture, respectively (Table S2) and stored at 4 °C. All phages were sequenced for detailed molecular characterization as follows. Phage chromosomal DNA was isolated using the λ phage genomic DNA purification kit (ABigen, China) following manufacturer’s instructions. Whole-genome sequencing was performed with Illumina Hiseq 4000 platform. A de novo genome assembly was conducted using SOAPdenovo and GapCloser. The phage genome sizes varied between 42,528 bp (NJ-P3), 41,194 bp (NB-P21), 41,943 bp (NC-P34), and 42,278 bp (NN-P42) with average GC contents of 62.26% (NJ-P3), 62.22% (NB-P21), 61.99% (NC-P34), 62.10% (NN-P42). Gene predictions and annotations were carried out using GeneMarkS. All four isolated phages were closely related with each other (>99.93% similarity) and belonged to Caudovirales order and Podoviridae family based on morphology and sequence similarity with the other phages (Fig. S1-3). All phage types were distantly related with known lysogenic phages publicly available in NCBI and showed clear lysis of stock *R. solanacearum* strain on double-layer agar plates indicative of lytic nature. We also quantified phage infectivity ranges using 96 *R. solanacearum* strains isolated from the same four fields where the phages were originally isolated from (24 independent pathogen isolates per field; See supplementary Fig. S14 for phylogenetic relationship between the isolates). All phages had relatively broad but clearly different infectivity ranges and especially phage types NJ-P3 and NN-P42 showed high and broad infectivity profiles (Fig. S4). Phage sequences were deposited in the NCBI Sequence Read Archive (SRA) database and accession numbers are SRR8402465 (NJ-P3), SRR8403229 (NB-P21), SRR8403928 (NC-P34), SRR8410130 (NN-P42).

**(b) Assessing the efficacy of phage combinations during greenhouse and field experiments and collection of soil samples at the end of the greenhouse experiment**

We used a 45-days long greenhouse experiment with a tomato to test the biocontrol efficacy of phage combinations (1 to 3 phage types – community assembly described below) in the presence of natural rhizosphere microbiome. The greenhouse experiment was conducted between August and October 2017 with average minimum and maximum temperatures of 22°C and 28.0 ℃, respectively. Tomato seeds (Solanum lycopersicum cv. ‘Hezuo 903’) were first surface-sterilized (in 3% NaClO for 5 mins and in 70% ethyl alcohol for 1 min) and germinated on water-agar plates for two days. Seeds were then sown into seedling plates containing 200 g of seedling substrate (Huainong, Huaian Soil and Fertilizer Institute, Huaian, China). At the three-leaf stage, tomato plants were transplanted to eight-cell seedling trays with each cell containing 600 g of homogenized, non-sterile paddy soil collected from Yixing City, Jiangsu Province, China (119°44′E, 31°22′N). This natural soil contained a high diversity of bacteria but no *R. solanacearum* pathogen or its phages: no pathogen colonies were observed on selective M-SMSA plates and no phage plaques were found on double-layer agar plates when using the same 96 *R. solanacearum* isolates as we did for estimating phage infectivity ranges as hosts. The basic soil nutrient concentration was: 0.96 g/kg of total nitrogen, 0.53 g/kg of total phosphorus, 5.29 g/kg of total potassium, 8.74 mg/kg of available phosphorus and 80 mg/kg of available potassium. After 7 days of transplantation, suspension of *R. solanacearum* QL-Rs1115 pathogen was inoculated to the roots of the plants using pipette resulting in ~107 cells g-1 soil. Two days after the pathogen inoculation, plants were inoculated with different phage combinations (Table S2) at a final concentration of ~106 total phage particles g-1 soil for each phage treatment. The control treatment included only the pathogen without the addition of phages. Each phage combination treatment was replicated three times and each replicate seedling tray contained 8 tomato plants (total of 24 plants per phage combination resulting in 360 plants including control treatment). Tomato plants were maintained under standard greenhouse conditions with natural temperature variation ranging from 25 °C to 35 °C and watered regularly with sterile water. Seedling trays were arranged in randomized order and rearranged randomly at every 2 days. At the end of the experiment (38 days after pathogen inoculation), we recorded the disease incidence and collected rhizosphere soil samples from two randomly chosen healthy plants from each replicate seedling tray (for treatments less than 2 healthy plants (8.89%), slightly diseased plant was used to represent this treatment) resulting in total of 45 rhizosphere soil samples. The rhizosphere soil was collected using previously described methods[4](#_ENREF_4) as follows. One gram of each rhizophere soil sample was directly used to isolate phage populations by mixing with 9 mL of water, chloroforming, filtering and storing at 4 °C as described earlier. The other two grams of samples were stored in 15 % glycerol at -80 °C for determining *R. solanacearum* population densities, for DNA extraction to determine bacterial community composition (described in detail later) and to isolate pathogen colonies for subsequent fitness assays (phage resistance and cost of resistance) as described earlier [16](#_ENREF_16).

The results obtained in the greenhouse experiment were validated in a field experiment in Qilin, Nanjing, China (118° 57' E, 32° 03' N) [45](#_ENREF_45) where we tested the biocontrol efficiency of single-phage and 4-phage combinations relative to the control treatment (no phage). The experiment was conducted from August to September 2017 (average maximum and minimum temperatures are 29.2 ℃ and 23.0 ℃, respectively) in a field that has been used for culturing tomato for 15 years. The type of soil was yellow-brown soil, which is typically used for growing vegetables. It contained 24.0 g/kg of organic matter, 1.7 g/kg of total nitrogen, 173.1 mg/kg of available phosphorus and 178 mg/kg of available potassium and had pH of 5.8. No pesticides were used during the experiment and a standard chemical fertilization was applied. The field has been naturally infected by *Ralstonia solanacearum* for more than 10 years and had suffered high wilt disease in 2016 (∼80% for autumn season and ∼40% for spring season), which makes the cultivation of any vulnerable crops, such as tomato or potato, unfeasible. The tomato seedlings (cultivar Hezuo 903) were first grown in nursery trays for 30 days (using substrate from Huaian Agricultural Technological Development Ltd., Huanyin, Jiangsu, China) before transplantation to the field (270–300 m2). After 7 days after transplantation, phages (~109 PFU per plant) were applied to the roots of tomato plants (plant base) using pipette. In addition to four independently single-phage and four phage combination treatments (Table S2), a control treatment was established without addition of phages (plain M9 buffer). Each treatment contained 48 tomato plants that were randomly selected among all the transplanted plants within 20 m2 experimental area (approximately 1000 plants transplanted in total). Severity of bacterial wilt disease incidence was recorded 45 days after inoculation as a mean of disease index of each plant using a scale ranging from 0 to 4 (0 = no signs of wilting, 1 = 1–25% leaf area wilted, 2 = 26–50% leaf area wilted, 3 = 51–75% leaf area wilted and 4 = 76–100% leaf area wilted).

**(c) Quantification of *R. solanacearum* and phage densities at the end of the greenhouse experiment**

We determined *R. solanacearum* densities using quantitative polymerase chain reaction (qPCR). The *R. solanacearum* DNA was extracted using a Power Soil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA) following manufacturer’s protocol. The DNA concentrations were determined by using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA) and extracted DNA was used for *R. solanacearum* density measurements using specific primers (forward, 5’-GAA CGC CAA CGG TGC GAA CT-3’; reverse, 5’-GGC GGC CTT CAG GGA GGT C-3’) targeting the fliC gene, which encodes the *R. solanacearum* flagellum subunit[46](#_ENREF_46). The qPCR analyses were carried out with a StepOnePlus™ Real-Time RCR Instrument using SYBR green fluorescent dye detection and three technical replicates as described previously[4](#_ENREF_4). Phage densities were quantified using spotting assay on soft double-agar overlays[16](#_ENREF_16). Briefly, purified phage populations were diluted and spotted on agar plates with an even overlay of stock *R. solanacearum* strain. After 24 h of growth at 30 °C, phage densities were calculated by counting the plaque forming units (PFUs).

**(d) Quantification of phage resistance and the cost of resistance with evolved *R. solanacearum* isolates**

To determine the evolution of phage resistance during the greenhouse experiment, *R. solanacearum* bacteria were isolated from the cryopreserved rhizosphere sub-samples plating serial dilutions on semi-selective agar medium (M-SMSA)[47](#_ENREF_47). After 48 h of growth at 30 °C, eight colonies were randomly isolated from each phage combination treatment replicate and grown individually for 24h at 30 °C in NB medium on 96-well microplates before cryopreserving at –80°C in 15% [16](#_ENREF_16). Pathogen resistance was determined individually against each ancestral phage the pathogen had been exposed to during the greenhouse experiment. Phage resistance was then calculated as an average resistance to all these phages. Pathogen resistance to contemporary phages was measured against the whole coevolved phage populations originating from the same replicate population as the pathogen at the end of the greenhouse experiment. These resistance assays thus included multiple phages in two and three phage combination treatments without further knowledge about their respective frequencies. In both cases, phage resistance was determined as pathogen growth reduction by phages in liquid media as described[26](#_ENREF_26). Briefly, the growth of ancestral and evolved *R. solanacearum* bacterial colonies (inoculum of ~107 cells mL-1) were measured both in the absence and presence of each ancestral phage and contemporary phage population (inoculum of ~106 phage particles mL-1) on 96-well microplates at 30 °C using spectrophotometer at 24 h time point (OD600). The same bacterial colonies were also used to quantify the cost of resistance in terms of bacterial growth in the absence of phages using model root exudate medium (RE medium) composed of OS minimal medium supplemented with 10 mM concentration of 48 different carbon resources including sugars, amino acids and organic acids commonly observed in tomato root exudates[5](#_ENREF_5), [48](#_ENREF_48). The cost of resistance was determined as a reduction in bacterial maximum density for 24 h at 4 h intervals (OD600) compared to the ancestral strain[27](#_ENREF_27).

**(e) Comparing the competitive ability of evolved phage resistant *R. solanacearum* strains with susceptible ancestral pathogen strain**

The competitive ability of evolved phage resistant pathogen strains was determined in direct co-culture competition assays with red fluorescent labelled ancestral *R. solanacearum* (QL-RFP) strain. To this end, we measured the growth of the ancestral QL-RFP (inoculum of ~106 cells mL-1) strain in the absence and presence of each evolved pathogen isolate (n=361) on 96-well microplates at 30 °C for 24 h using red fluorescence signal (excitation: 587 nm, emission: 610 nm; inoculum of ~106 cells mL-1 for both ancestral and evolved strains). Evolved pathogen strain competitive ability was measured as the difference in the growth of ancestral QL-RFP strain in the absence (OD600a) and presence (OD600p) of evolved strains using the following formula: Competitive ability = (OD600a – OD600p)/OD600a\*100

**(f) Determining changes in rhizosphere microbiome composition using Illumina MiSeq sequencing**

The impact of phage combinations on rhizosphere bacterial microbiome composition was determined using multiplexed MiSeq sequencing[25](#_ENREF_25). The V4 hypervariable region of the 16S rRNA gene was amplified with the primer pair 563F (5’-AYT GGG YDT AAAGVG-3’) and 802R (5’-TAC NVG GGT ATC TAA TCC-3’)[49](#_ENREF_49). Sequencing reads were assigned to each sample using unique barcodes and reads were processed with QIIME open-source bioinformatics pipeline[50](#_ENREF_50). Filtering of noisy sequences, chimera checking and operational taxonomic unit (OTU) cutoff was assigned at 97% identity level using USARCH[51](#_ENREF_51) and RDP database with the online version of the RDP classifier[52](#_ENREF_52). Chimeric sequences that were identified using both de novo and reference-based chimera checking methods were removed from the data. Sequence data were deposited in the NCBI Sequence Read Archive (SRA) database and accession numbers are shown in the Supplementary material.

**(g)** **Testing direct effects of three-phage combination on bacterial community composition and diversity using culture-dependent and -independent methods**

We used a culture-independent method to directly test if the presence of three-phage combinations (Table S2) directly affected the composition and diversity natural rhizosphere microbiome in the absence of pathogen. Briefly, rhizosphere microbiome inoculum was prepared by creating a 1:9 paddy soil-sterile water mixture using the same soil that was used for the greenhouse experiment. We then inoculated 8 μL of the rhizosphere microbiome inoculum (~108 cells mL-1) to 48-well microplates containing 792 μL 25% NB medium per well after inoculating *R. solanacearum* and three-phage combinations alone or together at final concentrations of ~106 CFU pathogen cells and ~105 PFU of phages. Rhizosphere microbiome inoculum alone was used as a control. After 48 h of culturing at 30 °C with shaking (170 rpm), DNA was extracted and used for bacterial diversity and abundance measurements using specific primers (forward, 5’- ACT CCT ACG GGA GGC AGC AG-3’; reverse, 5’- ATT ACC GCG GCT GCT GG-3’) targeting the 16S rRNA gene[53](#_ENREF_53) followed by multiplexed MiSeq sequencing as described above. We then tested the susceptibility of non-pathogenic bacterial isolates to *R. solanacearum*-infecting phages by using culture-dependent method. To achieve this, we isolated a random selection of culturable bacteria from the same paddy soil, which was used for greenhouse experiment using nonselective agar media (TSA, [tryptone](https://en.wikipedia.org/wiki/Tryptone) 15 g L-1, [soytone](https://en.wikipedia.org/w/index.php?title=Soytone&action=edit&redlink=1) 5 g L-1, 5 g L-1 NaCl, [agar](https://en.wikipedia.org/wiki/Agar) 20 g  L-1, pH 7.0). Serial diluted soil suspensions were spread on agar media and incubated at 30 ℃ for 48 h. Four replicate soil samples from the same soil stock were used and 100 bacterial colonies were randomly picked from each replicate resulting in total of 400 isolates, which were identified by sequencing the whole 16S rRNA gene with the primer pair 27F (5’- AGA GTT TGA TCC TGG CTC AG-3’); 1492R (5’-GGT TAC CTT GTT ACG ACT T- 3’)[54](#_ENREF_54). The susceptibility of non-pathogenic strains to phages was tested using streak assay[55](#_ENREF_55) by testing if bacterial growth was inhibited when streaked across a ‘line’ of dried phage (40 μL) on NB agar plate. Strains were scored as resistant if there was no detectable inhibition of growth by the phage compared to control treatments (bacteria streaked on plates without phages).

**(h) Determining the *R. solanacearum* pathogen inhibition by the non-pathogenic bacterial isolates**

To study if culturable non-pathogenic bacterial isolates had negative effects on the pathogen (either via interference or resource competition), we compared the reduction in pathogen density when co-cultured with non-pathogenic bacteria in pairwise cultures versus when grown alone. We specifically concentrated on bacteria that increased in response to increasing phage number at genera level. All non-pathogenic strain densities were adjusted to ~107 cells per mL and the density of mCherry fluorescence-tagged *R. solanacearum* QL-Rs1115-RFP to ~106 cells per ml. Co-cultures with even starting volumes (50:50%) were set up in 96-well plates with 25% liquid NB medium and incubated at 30 ℃ with shaking (170rpm). The pathogen strain was also grown alone as a control and all treatments were measured in triplicate. After 48 h, total bacterial densities were measured as optical density (OD600 nm) and pathogen densities quantified as red fluorescence intensity (mCherry, excitation: 587 nm, emission: 610 nm) using SpectraMax M5 spectrophotometer. The relative density of the pathogen was calculated as the proportion by dividing the relative fluorescence unit (RFU) intensity with the total bacterial density (mCherry /OD600)[56](#_ENREF_56). Pathogen inhibition was defined as the percentage reduction in the pathogen growth by non-pathogenic bacteria relative to pathogen growth in the absence of non-pathogenic bacteria.

**(i) Statistical Analysis**

Data was analysed with a combination of linear mixed models and linear regression where dependent variables (pathogen or phage densities, disease incidence, phage resistance or carrying capacity and OTU richness or Shannon index) were explained by phage combination treatment (number of phage types) or evolutionary history of the pathogen (evolved in the absence or presence of phages). Before all analyses, pathogen and phage density data were log10-transformed to fulfil parametric model assumptions. When individual clones were used in the analyses, they were always nested under replicates. Patterns of similarity among samples were visualized with non-metric multidimensional scaling (NMDS) using the metaMDS function in the R vegan package[57](#_ENREF_57). The diversity of rhizosphere microbial communities was estimated as OTU richness and Shannon diversity index (at OTU level) and the phage type identity effects (presence/absence in communities) on pathogen density and disease incidence were examined using a function ‘sAICfun’ in the library ‘devtools’. Top 500 OTUs belonging to single-phage and three-phage treatments were retained for the analysis and similarity matrices were calculated based on Spearman rank correlation. The edges in the co-occurrence networks represent statistically significant (FDR<0.05) Spearman correlations with the absolute correlation coefficient values above 0.8. The nodes in the co-occurrence networks represent individual OTUs. Network analysis was only conducted between single- and three-phage treatments to specifically focus on phage effects on the microbiome. Networks were drawn using Gephi[58](#_ENREF_58) and “NetShift” method was used to identify potential keystone driver taxa underlying differences in microbiomes exposed to single-phage and three-phage communities[28](#_ENREF_28). This method allows quantifying directional changes in the individual node interactions by exploring significant overall changes in community patterns using online NetShift tool. The ‘driver taxa’ were identified based on the NESH score, Jaccard Index and Betweenness values[28](#_ENREF_28). We also used PLS-SEM analysis to compare the relative importance of ecological and evolutionary outcomes in determining plant disease incidence using path-modelling estimation with PLS and Bootstrapping Algorithm. Specifically, we focused on exploring how the number of phages present in phage combinations affected disease incidence via effects on pathogen density, microbiome diversity and the magnitude of phage resistance and cost of resistance (phage density was not included into to the model due to high correlation with the pathogen density to avoid multicollinearity; Fig S5). All data were analysed using SPSS Statistics version 20, R 3.5.1[59](#_ENREF_59) and SmartPLS 3 software[60](#_ENREF_60).

**(j) Accession numbers for sequence data**The rhizosphere microbiome data determined at the end of the greenhouse experiment have following accession numbers (45 samples, paired end sequencing):

|  |  |  |  |
| --- | --- | --- | --- |
| Treatment | Accession no | Paired end read 1 | Paired end read 2 |
| Control | SRR8417988 | 1-1.1.fq | 1-1.2.fq |
| Control | SRR8417981 | 1-2.1.fq | 1-2.2.fq |
| Control | SRR8417968 | 1-3.1.fq | 1-3.2.fq |
| 1-phage | SRR8417987 | 2-1.1.fq | 2-1.2.fq |
| 1-phage | SRR8417980 | 2-2.1.fq | 2-2.2.fq |
| 1-phage | SRR8417967 | 2-3.1.fq | 2-3.2.fq |
| 1-phage | SRR8417991 | 3-1.1.fq | 3-1.2.fq |
| 1-phage | SRR8417979 | 3-2.1.fq | 3-2.2.fq |
| 1-phage | SRR8417970 | 3-3.1.fq | 3-3.2.fq |
| 1-phage | SRR8417989 | 4-1.1.fq | 4-1.2.fq |
| 1-phage | SRR8417984 | 4-2.1.fq | 4-2.2.fq |
| 1-phage | SRR8417969 | 4-3.1.fq | 4-3.2.fq |
| 1-phage | SRR8417997 | 5-1.1.fq | 5-1.2.fq |
| 1-phage | SRR8417983 | 5-2.1.fq | 5-2.2.fq |
| 1-phage | SRR8417972 | 5-3.1.fq | 5-3.2.fq |
| 2-phage | SRR8417996 | 6-1.1.fq | 6-1.2.fq |
| 2-phage | SRR8417995 | 6-2.1.fq | 6-2.2.fq |
| 2-phage | SRR8417971 | 6-3.1.fq | 6-3.2.fq |
| 2-phage | SRR8417999 | 7-1.1.fq | 7-1.2.fq |
| 2-phage | SRR8417955 | 7-2.1.fq | 7-2.2.fq |
| 2-phage | SRR8417974 | 7-3.1.fq | 7-3.2.fq |
| 2-phage | SRR8417998 | 8-1.1.fq | 8-1.2.fq |
| 2-phage | SRR8417993 | 8-2.1.fq | 8-2.2.fq |
| 2-phage | SRR8417973 | 8-3.1.fq | 8-3.2.fq |
| 2-phage | SRR8417986 | 9-1.1.fq | 9-1.2.fq |
| 2-phage | SRR8417994 | 9-2.1.fq | 9-2.2.fq |
| 2-phage | SRR8417966 | 9-3.1.fq | 9-3.2.fq |
| 2-phage | SRR8417985 | 10-1.1.fq | 10-1.2.fq |
| 2-phage | SRR8417958 | 10-2.1.fq | 10-2.2.fq |
| 2-phage | SRR8417965 | 10-3.1.fq | 10-3.2.fq |
| 2-phage | SRR8417978 | 11-1.1.fq | 11-1.2.fq |
| 2-phage | SRR8417959 | 11-2.1.fq | 11-2.2.fq |
| 2-phage | SRR8417960 | 11-3.1.fq | 11-3.2.fq |
| 3-phage | SRR8417977 | 12-1.1.fq | 12-1.2.fq |
| 3-phage | SRR8417956 | 12-2.1.fq | 12-2.2.fq |
| 3-phage | SRR8417961 | 12-3.1.fq | 12-3.2.fq |
| 3-phage | SRR8417976 | 13-1.1.fq | 13-1.2.fq |
| 3-phage | SRR8417957 | 13-2.1.fq | 13-2.2.fq |
| 3-phage | SRR8417962 | 13-3.1.fq | 13-3.2.fq |
| 3-phage | SRR8417975 | 14-1.1.fq | 14-1.2.fq |
| 3-phage | SRR8417990 | 14-2.1.fq | 14-2.2.fq |
| 3-phage | SRR8417963 | 14-3.1.fq | 14-3.2.fq |
| 3-phage | SRR8417982 | 15-1.1.fq | 15-1.2.fq |
| 3-phage | SRR8417992 | 15-2.1.fq | 15-2.2.fq |
| 3-phage | SRR8417964 | 15-3.1.fq | 15-3.2.fq |

The rhizosphere microbiome data obtained in a separate lab experiment have following accession numbers (30 samples, paired end reads):

|  |  |  |  |
| --- | --- | --- | --- |
| Treatment | Accession no | Paired end read 1 | Paired end read 2 |
| RS | SRR8501090 | A1.1.fq | A1.2.fq |
| RS | SRR8501089 | A2.1.fq | A2.2.fq |
| RS | SRR8501092 | A3.1.fq | A3.2.fq |
| RS+3-phage | SRR8501091 | A34.1.fq | A34.2.fq |
| RS+3-phage | SRR8501094 | A35.1.fq | A35.2.fq |
| RS+3-phage | SRR8501093 | A36.1.fq | A36.2.fq |
| RS+3-phage | SRR8501096 | A37.1.fq | A37.2.fq |
| RS+3-phage | SRR8501095 | A38.1.fq | A38.2.fq |
| RS+3-phage | SRR8501098 | A39.1.fq | A39.2.fq |
| RS+3-phage | SRR8501097 | A40.1.fq | A40.2.fq |
| RS+3-phage | SRR8501087 | A41.1.fq | A41.2.fq |
| RS+3-phage | SRR8501086 | A42.1.fq | A42.2.fq |
| RS+3-phage | SRR8501085 | A43.1.fq | A43.2.fq |
| RS+3-phage | SRR8501084 | A44.1.fq | A44.2.fq |
| RS+3-phage | SRR8501088 | A45.1.fq | A45.2.fq |
| 3-phage | SRR8470500 | A49-1.fq | A49-2.fq |
| 3-phage | SRR8470499 | A50-1.fq | A50-2.fq |
| 3-phage | SRR8470502 | A51-1.fq | A51-2.fq |
| 3-phage | SRR8470501 | A52-1.fq | A52-2.fq |
| 3-phage | SRR8470496 | A53-1.fq | A53-2.fq |
| 3-phage | SRR8470495 | A54-1.fq | A54-2.fq |
| 3-phage | SRR8470498 | A55-1.fq | A55-2.fq |
| 3-phage | SRR8470497 | A56-1.fq | A56-2.fq |
| 3-phage | SRR8470494 | A57-1.fq | A57-2.fq |
| 3-phage | SRR8470493 | A58-1.fq | A58-2.fq |
| 3-phage | SRR8470492 | A59-1.fq | A59-2.fq |
| 3-phage | SRR8470491 | A60-1.fq | A60-2.fq |
| Control | SRR8470490 | A61-1.fq | A61-2.fq |
| Control | SRR8470489 | A62-1.fq | A62-2.fq |
| Control | SRR8470488 | A63-1.fq | A63-2.fq |

ACKNOWLEDGEMENTS

This research was financially supported by the National Natural Science Foundation of China (41671248; ZW), the National Key Research and Development Program of China (2018YFD1000800; ZW), the National Key Basic Research Program of China (2015CB150503; QS) and the 111 project (B12009; QS). AJ is supported by the Netherlands Organisation for Scientific Research (NWO) project ALW.870.15.050. VPF is supported by the Wellcome Trust [ref: 105624] through the Centre for Chronic Diseases and Disorders (C2D2) and Royal Society Research Grants (RSG\R1\180213 and CHL\R1\180031) at the University of York.

AUTHOR CONTRIBUTIONS

VPF, XFW and ZW developed the ideas and designed the experimental plans. XFW, JNW, ZW performed the experiments. XFW, KEY, AJ, ZW, YX, QS and VPF analyzed the data. All the authors wrote the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

**References:**

45. Wei, Z. et al. Efficacy of Bacillus-fortified organic fertiliser in controlling bacterial wilt of tomato in the field. *Appl Soil Ecol* **48**, 152-159 (2011).

46. Schonfeld, J., Heuer, H., van Elsas, J.D. & Smalla, K. Specific and sensitive detection of *Ralstonia solanacearum* in soil on the basis of PCR amplification of fliC fragments. *Appl. Environ. Microbiol.* **69**, 7248-7256 (2003).

47. Elphinstone, J., Hennessy, J., Wilson, J. & Stead, D. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. *EPPO Bulletin* **26**, 663-678 (1996).

48. Ji, P. & Wilson, M. Assessment of the importance of similarity in carbon source utilization profiles between the biological control agent and the pathogen in biological control of bacterial speck of tomato. *Appl. Environ. Microbiol.* **68**, 4383-4389 (2002).

49. Cardenas, E. et al. Significant association between sulfate-reducing bacteria and uranium-reducing microbial communities as revealed by a combined massively parallel sequencing-indicator species approach. *Appl. Environ. Microbiol.* **76**, 6778-6786 (2010).

50. Caporaso, J.G. et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* **7**, 335 (2010).

51. Macdonald, C.A. et al. Long-term impacts of zinc and copper enriched sewage sludge additions on bacterial, archaeal and fungal communities in arable and grassland soils. *Soil Biol. Biochem.* **43**, 932-941 (2011).

52. Margesin, R., Płaza, G.A. & Kasenbacher, S. Characterization of bacterial communities at heavy-metal-contaminated sites. *Chemosphere* **82**, 1583-1588 (2011).

53. Fierer, N., Jackson, J.A., Vilgalys, R. & Jackson, R.B. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* **71**, 4117-4120 (2005).

54. Eden, P.A., Schmidt, T.M., Blakemore, R.P. & Pace, N.R. Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *Int. J. Syst. Evol. Microbiol.* **41**, 324-325 (1991).

55. Friman, V.P. & Buckling, A. Phages can constrain protist predation-driven attenuation of *Pseudomonas aeruginosa* virulence in multienemy communities. *Isme Journal* **8**, 1820-1830 (2014).

56. Yang, T. et al. Resource availability modulates biodiversity‐invasion relationships by altering competitive interactions. *Environ. Microbiol.* (2017).

57. Dixon, P. VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* **14**, 927-930 (2003).

58. Bastian, M., Heymann, S. & Jacomy, M. in Third international AAAI conference on weblogs and social media (2009).

59. Team, R.C. R: A language and environment for statistical computing. (2015).

60. Hair, J.F., Ringle, C.M. & Sarstedt, M. Partial least squares structural equation modeling: rigorous applications, better results and higher acceptance. *Long Range Plann* **46**, 1-12 (2013).