Developing bacteriophage therapy for the plant pathogenic bacterium *Ralstonia solanacearum*

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Abstract

Ralstonia solanacearum (*R. solanacearum*) is a soil dwelling species of gram-negative β-proteobacteria. It is an extremely destructive plant pathogen, with a vast host range of over 200 different plant species, including vital agricultural crops. Current control methods are often ineffective at constraining the pathogen and heavily rely on the application of agrochemicals. The adverse environmental impacts of such agrochemicals in conjunction with their high economic cost and poor efficacy has incentivised new approaches for controlling *R. solanacearum*. Numerous studies have highlighted the potential of viral parasites of *R. solanacearum*, known as bacteriophages, to control the pathogen and reduce disease incidence in afflicted plants. To effectively implement bacteriophages as a viable control method, potential phage must be characterised in relation to how they interact with *R. solanacearum* and the plant. Also, it is important to quantify how rapidly *R. solanacearum* might evolve resistance to phages and how this could affect disease control outcomes. To achieve this, I conducted infection assays to determine infectivity ranges of ten phage isolates against ten *R. solanacearum* strains and performed evolution experiments across three different systems: a liquid media model and two tomato plant models grown in hydroponic and soil conditions. The infection assays identified one phage (W4) as a potential control agent against *R. solanacearum* (strain 146). The interactions of this phage and *R. solanacearum* strain were examined in detail across the three systems. Following treatment with W4 phage, the density of *R. solanacearum* was significantly reduced in all three models. This subsequently led to reduced disease symptoms in the hydroponic but not in the soil system. Phage resistance assays after completion of the experiments showed the emergence of phage resistance in all three systems. Furthermore, in both the hydroponic and liquid media models, significant trade-offs were observed between phage resistance and bacterial growth. In contrast to these results, we observed evolution of high levels of phage resistance with no fitness trade-offs in the soil plant model. This work presents W4 as an interesting phage strain with the ability to suppress *R. solanacearum* in liquid media and hydroponic environments. It also highlights the need to use more realistic systems to better predict how phage and *R. solanacearum* might interact in the field and natural environments.

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Introduction

1.1 *R. solanacearum* and it's agricultural impact

As the human population continues to grow at an unprecedented rate, there is rising concern regarding global food security. Currently, 700 million people worldwide are clinically malnourished. With the human population due to hit 9 billion by 2050, alongside a changing climate reducing viable farmland, it is estimated that crop yields need to increase by 60% to prevent large scale starvation.[1,2] One significant cause of crop loss worldwide are plant pathogens. Collectively, these pathogens are responsible for approximately 15% of annual crop losses, therefore, improved pathogen treatment strategies are an attractive avenue for increasing crop yield.[3]

One pathogen in drastic need of improved control methods is *Ralstonia solanacearum* (*R. solanacearum*). *R. solanacearum* is a gram-negative β-proteobacteria and is part of the *R. solanacearum* species complex (RSSC). The RSSC is comprised of 4 phylotypes and 2 sub phylotypes. Currently these phylotypes are divided into three species: *Ralstonia pseudosolanacearum* (phylotype I and III), *R. solanacearum* (phylotype IIA and IIB) and *Ralstonia syzygii* (phylotype IV)[4]. In Europe phylotype IIB is the most prolific causing severe wilt in afflicted plants, resulting in dramatic yield reduction and plant death[4]. In addition, in the UK phylotype IIB is the causative agent of brown potato rot[4]. Furthermore, *R. solanacearum* has a vast host range of over 200 plant species, including several agricultural crops such as tomato, banana, soybean, tobacco, potato and aubergine[5]. This extensive host range combined with a lack of effective control methods have made *R. solanacearum* the 2nd most economically damaging plant pathogen worldwide, causing an estimated $1 billion in damage each year[6].

1.2 The pathogenic life cycle of *R. solanacearum*

Ralstonia solanacearum is typically found as a saprophyte within the soil[7,8](**Figure 1**). The bacterium gains entrance into the plant through natural wounds or cracks in the roots (**Figure 1**). Once inside the root cortex the bacterium employs a type III secretion system (T3SS) which is mediated by the *hrp* gene locus[5]. The T3SS then delivers over 70 different effector proteins into the plant cell cytoplasm, these effector proteins have a wide range of different effects on the plant cells, including, suppressing plant defences, modulating host metabolism, and avoiding plant recognition of pathogen associated molecular patterns (PAMPS)[8]. It has been shown that the T3SS and its associated effector proteins are essential for *Ralstonia* virulence[9]. The bacterium then translocates to the xylem of the plant where it begins to replicate and undergoes a phenotypic switch mediated by quorum sensing at the *phc* locus[10]. This results in the production of huge amounts of extracellular polysaccharide (EPS) and a mucoidal phenotype. EPS production leads to blockage of the xylem leading to a massive reduction in waterflow within the plant and the classical wilting symptoms associated with *R. solanacearum* infection. Obstruction of the vasculature of the plant eventually results in plant death and subsequent sheading of the bacterium to the soil[5] (**Figure 1**).

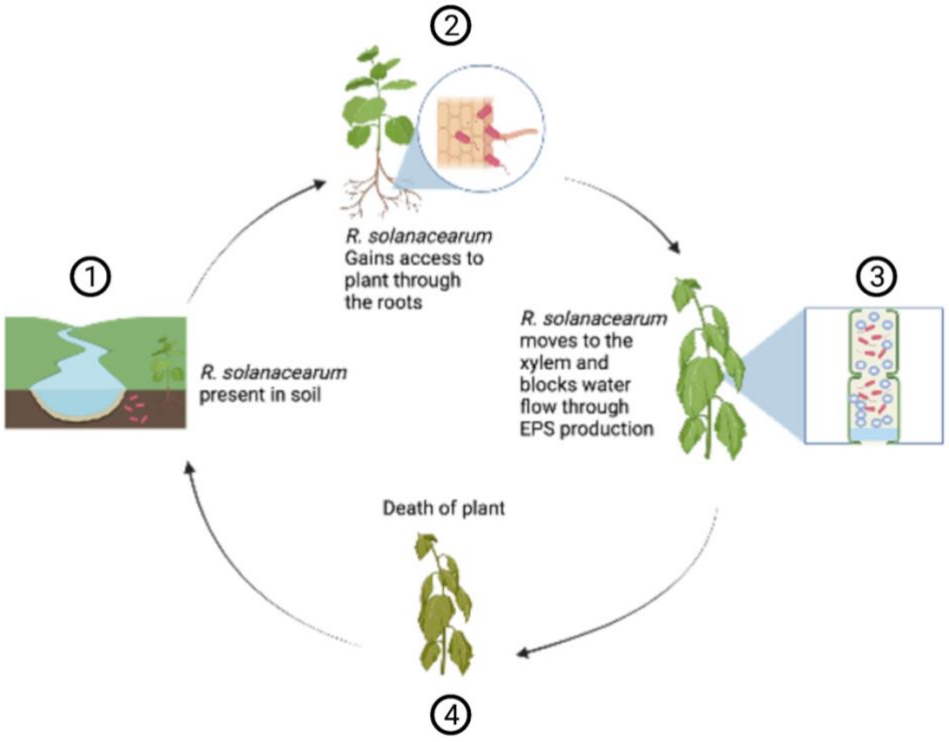

Figure 1. *R. solanacearum* cycle of pathogenesis. *R. solanacearum* is present in the soil where it gains access to plants through natural cracks or openings within the roots. Once inside the plant, the bacterium employs a type III secretion system and associated effector proteins to suppress the plant immune response. *R. solanacearum* then translocates to the xylem of the plant where it begins to replicate and undergo a phenotypic switch mediated by quorum sensing, leading to massive production of extracellular polysaccharide (EPS). This EPS production coupled with bacterial growth causes blocking of the vasculature and subsequent death of the plant. Pathogen is then released back to the environment and can initiate another cycle of infection when suitable hosts become available (Figure made using biorender).

1.3 Current *R. solanacearum* control methods

Due to the economic devastation *R. solanacearum* causes, a myriad of therapeutic approaches have been developed to bring the pathogen under control[11].The most widely used of these methods are crop rotation and application of pathogen-suppressing chemicals[7]. However, these are often ineffective as *R. solanacearum* can persist in pesticide runoff or alternative asymptomatic hosts[8]. The poor efficacy of current treatments in conjunction with their high economic cost and environmental impact has incentivised more efficient and less environmentally damaging treatments for *R. solanacearum*[11]. This new mentality towards bacterial wilt control has highlighted alternative methods, most promisingly *Bacillus* biocontrol bacterium and bio fumigation[11-14]. T-5 *Bacillus* has been shown to suppress the growth of *R. solanacearum* under lab conditions. Most promisingly when examined *In planta*, it was shown to prevent colonisation of tomato roots by *R. solanacearum* in turn preventing the development of disease[14]. Biofumigation is an agronomic practice involving the application of volatile organic compounds released from plant residues to suppress plant pathogens[11]. Despite the many benefits of these therapies, they are still hindered by poor efficacy within field

trials[13]. One further alternative control method could be the application of viral parasites of *R. solanacearum* known as bacteriophages (phages), to specifically target and kill the pathogen[16-18].

1.4 Bacteriophages and their potential as a biocontrol agent for *R. solanacearum*

Phages are viruses whose infection and replication cycle takes place within bacteria. They are found ubiquitously throughout the environment, including the plant rhizosphere[19,20]. Despite their extensive diversity, phage can be grouped into two broad catergories based on their life cycles, lysogenic and lytic[21]. Lytic phages bind to elements of the bacterial cell surface membrane such as lipopolysaccharide (LPS), pilus, flagella or extracellular proteins using specialised tail fibre proteins. Upon successfully binding, phage inject their DNA into the bacterial cell[21]. The phage genome is transcribed and translated causing the production of more virions within the bacterial cell. The virions then induce bacterial cell lysis, death of the bacterial cell and release of new phage virions[21]. In contrast to this life cycle, the genome of lysogenic phage integrates into the bacterial chromosome (**Figure 2**). This integrated state is known as a prophage and is stably maintained within the bacterial chromosome by prophage encoded repressor proteins. The prophage DNA is replicated and inherited during bacterial cell division, and upon encountering cellular stress activation of the SOS response can result in de-repression of the prophage operon and subsequent excision of the prophage genome and entrance into the lytic cycle (**Figure 2**). Furthermore, the prophage DNA can confer beneficial traits to the bacterial cell such as antibiotic resistance or higher levels of virulence, enhancing bacterial fitness[21].

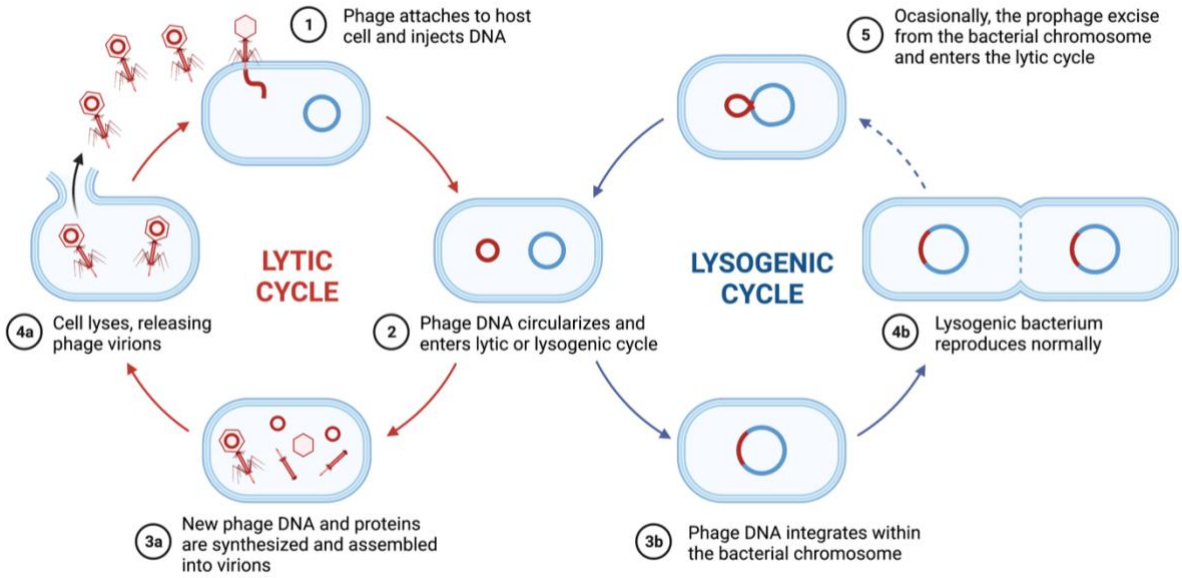

Figure 2. The two major life cycles of bacteriophage: Lytic (left) and lysogenic (right) cycles. The lytic life cycle involves the entrance of the viral chromosome into the bacterial cell. The viral chromosome causes the production of more virions within the bacterial cell, the viral particles are assembled into phages, which then lyse the bacterium and are released back to the environment. In contrast to lytic lifecycle, temperate phages integrate their genetic material into the host genome during lysogenic cycle. Phages then replicate during normal bacterial cell division. Often triggered by stress, the temperate phage chromosome can excise from the bacterial genome and enter the lytic cycle (Figure made using biorender).

As lytic bacteriophages have the ability to efficiently kill bacterial cells, naturally occur in the plant rhizosphere and are very specific to individual bacterial strains, they have immense potential as a treatment for bacterial disease causing agents such as, *R. solanacearum*[22]. The lytic nature of the

bacteriophage lifecycle immediately highlights their inherent use as a control method by direct killing of the pathogen. In addition, there are several other benefits regarding the use of bacteriophages[22,23]. These include a reduction in the use of pathogen suppressing chemicals which in turn reduces environmental and ecosystem disruption caused by such chemicals[22-25]. Furthermore, phages are very specific to individual bacterial strains which allows targeted killing of pathogenic bacteria whilst reducing off target effects on the beneficial plant microbiota. Pahges also have the unique ability of being able to coevolve with the pathogen retaining their infectivity in case of resistance evolution by the pathogen[23,24].

1.5 Bacteriophage resistance and Coevolution

Due to the immense selection pressure that phages inflict on *R. solanacearum*, resistance to phage can evolve rapidly[26-30]. There are a myriad of avenues through which *R. solanacearum* can achieve phage resistance, which include loss or mutation in phage binding receptors, upregulation of restriction modification defence systems, abortive infection, or activation of other phage defence systems[27]. The emergence of bacteriophage resistance in *R. solanacearum* has frequently been viewed as a negative outcome to phage therapy. However, recent research has shown phage resistance can result in significant fitness trade-offs in bacterial populations, where resistant bacteria persist due to escaping phage predation even though is detrimental in the absence of phages. Furthermore, it has been reported that trade-offs associated with phage resistance can result in avirulent strains[24]. This is thought to be due to the high metabolic cost of virulence genes and has been shown to occur in the rhizosphere tomato plants where treatment with combinatorial phage treatments resulted in avirulence of *R. solancearum*[24,29]. These avirulent *R. solanacearum* had mutations in the *phcA* loci, which is the quorum sensing pathway responsible for *R. solanacearum* EPS production and is a crucial virulence factor[31]. In turn this has led to a new perspective on phage therapy where using phage which select for avirulent varieties of *R. solanacearum* could be the most beneficial treatment[11]. Alternatively, the emergence of phage resistance can in turn trigger selection for increased phage infectivity, leading to phage-bacteria coevolution[26-30].

Coevolution is defined as the “process of reciprocal adaptation and counter-adaptation between ecologically interacting species”[26]. Between *R. solanacearum* and phage these coevolutionary patterns of adaptation and counter-adaptation typically manifests as arms race dynamics, where bacterial resistance and phage infectivity escalate over time (generalist phage) or fluctuating selection dynamics which is observed as no clear direction of infectivity or resistance and phage bacterial genotypes match in time (specialist phage)[24,27,30]. Coevolving with their host bacteria can allow phages to retain their ability to control pathogen infections through coevolving to become more infective[30].

1.6 Overall aims of this study

Despite several benefits, there remain large gaps in our knowledge, particularly in how to maximise the effectiveness of phage as a potential biocontrol agent. Specifically, one of the main challenges in utilising phage therapy as an effective agricultural tool is better understanding phage-pathogen interactions, such as, the emergence of phage resistance and the potential coevolutionary dynamics between phage and bacteria, which could limit phage efficacy. Moreover, most of our knowledge on phage-bacteria interactions comes from simple lab experiments that fail to account for the complexity

of natural environments where phage would be applied. As a result, to gain insight into phage-pathogen interactions in *R. solanacearum* I set to explore following questions: (**i**) to determine the infectivity range of phage isolates within our collection against *R. solanacearum* isolates, (**ii**) assess the emergence and associated cost of resistance between *R. solanacearum* and its phage in *liquid media* and in the plant rhizosphere, (**iii**) Examine the ability of phage to alleviate *R. solanacearum* symptoms, and (**iv**) initiated preliminary work to determine what genetic changes are involved in *R. solanacearum* phage resistance and what impacts these might have on disease outcomes.

Methods

2. 1 *R. solanacearum* strains and bacteriophage isolates

2.1.1 R. solanacearum strains used throughout the study

We selected 10 *R. solanacearum* strains to test our phage against a diverse *R. solanacearum* background (**Table 1.**). The majority of strains selected for experiments were of the IIB phylotype due to its prevalence within Europe and particularly its agricultural impact within the UK (causative agent of brown potato rot). Alongside the IIB strains two *R. solanacearum* strains were included from phylotype III and I (001, 081) to examine if our phage isolates were specific to the IIB phylotype. Furthermore, two phylotype IIB strains (146, 380) were included from Kenya and Nigeria to examine if geographic variation had an impact on the phage infectivity. Using this range of *R. solanacearum* strains would thus inform if our phage isolates were specialist (only able to infect a few specific strains) or generalist (able to infect many distinct strains).

Table 1. *R. solanacearum* strains used in this study with information of their isolation location.

***Ralstonia* strain**	**Location of isolation (Country)**	**Phylotype**	**Isolated from**	**Species**
001	Angola	III	*Solanum tuberosum*	*Ralstonia pseudosolanacearum*
081	French Guyana	I	*Solanum lycopersicum*	*Ralstonia solanacearum*
146	Nigeria	IIB	*Solanum tuberosum*	*Ralstonia solanacearum*
199	UK	IIB	*Solanum tuberosum*	*Ralstonia solanacearum*
206	UK	IIB	*Solanum dulcamara*	*Ralstonia solanacearum*

281	UK	IIB	Water	*Ralstonia solanacearum*
337	UK	IIB	Water	*Ralstonia solanacearum*
347	UK	IIB	*Solanum dulcamara*	*Ralstonia solanacearum*
380	Kenya	IIB	*Pelargonium hortorum*	*Ralstonia solanacearum*
385	UK	IIB	*Solanum dulcamara*	*Ralstonia solanacearum*

2.1.2 Bacteriophage isolates used throughout the study

Bacteriophage isolates used in this study originated from two geographic locations. Five phage isolates from different locations along the Thames (UK phage isolates, **Table 2**) and five Chinese phage isolates from Nanjing were used throughout this study (Chinese phage isolates, **Table 2**). This provided a range of unique phages to examine infectivity range against the 10 *R. solanacearum* strains.

Table 2. The bacteriophages used in this study with information of location of isolation.

Bacteriophage type	Location of isolation (Country)	Isolated from
W4	UK	Thames river
W5	UK	Thames river
W9	UK	Thames river
W10	UK	Thames river
SD10	UK	Thames river
42	China	Nanjing, Qilin fields from *Solanum lycopersicum* rhizosphere
44	China	Nanjing, Qilin fields from *Solanum lycopersicum* rhizosphere

45	China	Nanjing, Qilin fields from *Solanum lycopersicum* rhizosphere
47	China	Nanjing, Qilin fields from *Solanum lycopersicum* rhizosphere
CP20	China	Nanjing, Qilin fields from *Solanum lycopersicum* rhizosphere

2. 2 Culture media

All media recipes are described in **Table 3**. CPG media was used as the standard growth media for *R. solanacearum* cultures, on both plates and liquid cultures (**Table 3**). Moreover, SMSA semi-selective media containing antibiotics was utilised for selective isolation of *R. solanacearum* colonies from soil and hydroponic experiments (**Table 3**). Murashige and Skoog basal media (MS-S) was used for the growth of tomato seeds (Solid) and liquid adjusted to Ph 6.0 for hydroponic tomato plant growth (**Table 3**).

Table 3. Media recipes for used culture media.

Ingredient	**CPG**	**SMSA**	**MS-S**
Casamino acids	1g	1g	-
Peptone	10g	10g	-
Glucose	5g	-	-
Glycerol	-	5ml	-
Crystal violet	-	5mg	-
Polymyxin B sulphate	-	100mg	-
Bacitracin	-	25mg	-
Chloramphenicol	-	5mg	-
Penicillin	-	0.5mg	-
Tetrazolium salts	-	5mg	-

Deionized water	1000ml	1000ml	1000ml
Agar (for solid media)	15g	15g	15g
Muragushi and skoog	-	-	4.41g

2. 3 Cryopreservation and strain culturing

R. solanacearum isolates were stored in suspension with CPG (25% glycerol at final concentration) at -80°C. These frozen stocks were recovered through streaking onto CPG agar plates and incubated at 28°C overnight. For liquid cultures, a single colony was picked and inoculated in 10ml liquid CPG media overnight (28°C with 50RPM shaking).

2.4 Spot plating plaque assays for determining phage infectivity

The infectivity range of phage isolates was inferred by their ability to lyse *R. solanacearum* strains on an CPG agar plates. A 350µl volume of overnight *R. solanacearum* culture was added to 10ml of 50 °C soft CPG media. This was overlayed onto a solid CPG plate and left to dry. Once dried, 5µl of phage sample was spotted onto the plate and left to dry. The plates were incubated at 28°C statically. Successful infection of *R. solanacearum* by phages was determined by an observable clear plaque 48 hours after incubation.

2. 5 Bacteriophage isolation using chloroform extraction

Bacteriophages were isolated from samples via extraction with chloroform. A 100µl aliquot of chloroform was added to 900µl of sample. This was then vortexed for 1 minute, followed by centrifugation for 3min at 13,600 RPM. After centrifugation, the supernatant layer containing phage was pipetted into a fresh 2ml Eppendorf and isolated phages were stored at 4 °C.

2. 6 Bacteriophage propagation

Bacteriophage were grown to a high density for experimental use. The desired host (from overnight culture) and phage lysate were co-cultured in 35ml of CPG media for 48hr, 28°C, 50RPM. The culture was then spun and filtered through 0.2µm filter to separate phage from bacteria. Phage densities were quantified as plaque forming units per ml (PFU/ml) as described in methods later at **2.7.2**. The phage culture was then diluted to 3 x 10^6 PFU/ml which was the working phage concentration for all our experiments unless stated otherwise.

2. 7 Determining bacterial and phage densities

2.7.1 Bacterial densities

Two approaches were used to estimate the density of *R. solanacearum* populations. The first used a spectrophotometer and optical density (OD at 600 nm wavelength). OD was measured through inoculating CPG containing wells in a 96-well plate with an overnight culture of *R. solanacearum*. Plates were then incubated statically at 28°C and the OD was measured for each well at 24-hour intervals using a Tecan infinite 200 pro microplate reader (spectrophotometer).

The other approach used to determine the density of *R. solanacearum* populations was through calculating the colony forming units per ml (CFU/ml). Firstly, a serial dilution using dH_2O was performed on desired samples. Dilutions 10^{-3} to 10^{-7} had 100µl plated onto CPG solid agar plates. The number of colonies were counted, and the CFU/ml calculated.

2.7.2 Phage densities

Plaque forming units per ml (PFU/ml) were used to assess the density of phage. To calculate PFU/ml, a sample containing phage was serial diluted using sterile dH_2O. Given *R. solanacearum* hosts were grown for 24 hours and 300 µl of culture was then added to 15ml of CPG soft agar. A 100µl aliquot of diluted phage was then added to the soft agar mix. This was then poured onto solid CPG agar plates. The plates were then incubated for 24 hours at 28°C, removed from the incubator and the number of plaques counted as PFU/ml.

Model systems and selection experiments

2.8 Liquid media experimental set up

A 16-day serial passaging experimental evolution model was developed to examine how phage and their host *R. solanacearum* coevolve in liquid media under lab conditions on 96-well microplates (**Figure 3**). To assess evolutionary dynamics in CPG culture media, 10µl of overnight *R. solanacearum* 146 culture (5 x 10^6 CFU/ml) was inoculated into a 96-well plate containing 188µl CPG. Eight replicate wells were inoculated with 2µl of W4 phage (3 x 10^6 PFU/ml), another eight replicates were retained as no-phage controls and had 2µl of CPG added. Microplates were incubated statically at 28°C throughout the experiment and the bacterial density was measured (OD600nm) at 24-hour intervals. *R. solanacearum* and phages were serially passaged every 4 days by adding a 10µl inoculum to 190µl of fresh CPG media on 96-well plates, which were then again placed at 28°C. Evolved populations were cryopreserved by adding 100µl of 50% glycerol to each well and placing plates at -80°C. Four serial passages were performed during the selection experiment (16 days). After 16 days, each replicate population was then serially diluted (10^{-1} to 10^{-8}) and dilutions were plated onto CPG agar plates. Individual colonies (N=12 for each replicate) were selected randomly and transferred into a 96-well plate containing 100µl of CPG. Clones were then incubated for 48 hours at 28°C, after 70µl of sub-samples were mixed with 50% glycerol and cryopreserved at -80°C for quantifying phage resistance and growth later.

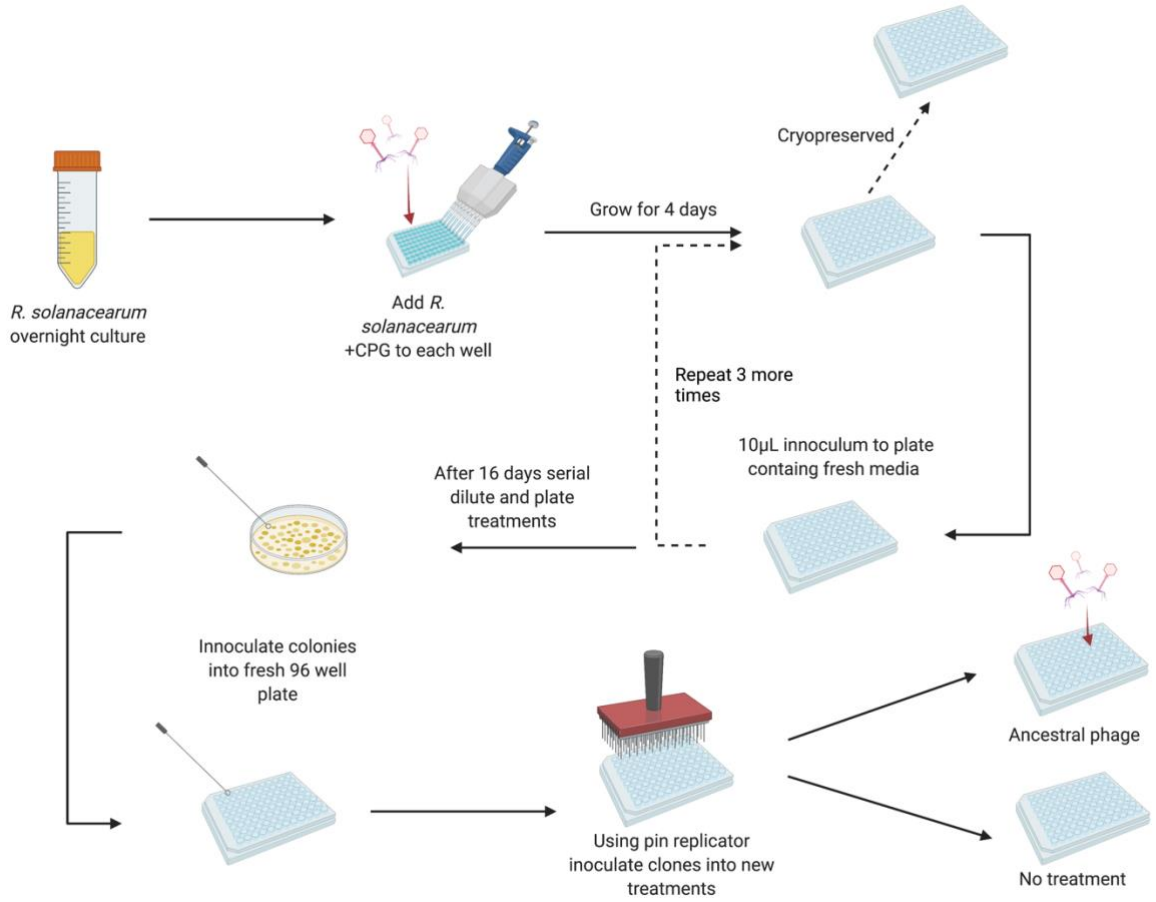

Figure 3. Experimental set up for Liquid media evolution experiment. *R. solanacearum* strain 146 was inoculated into a 96-well plate containing CPG in the absence or presence of phage W4. The bacterial density (OD600nm) was measured every 24 hours, and after 4 days, a 10µl inoculum was passaged into a fresh 96-well plate and the previous plate cryopreserved. After 4 serial passages (~16 days), each replicate population was plated onto CPG agar plates and 12 individual colonies were transferred to a new 96-well plate and grown for 48 hours. These clones were then exposed to ancestral phage as well as no treatment to quantify evolutionary changes in phage resistance and potential cost of resistance.

2.8.1 Phage resistance assays in the liquid media system

Following completion of the selection experiment, phage resistance assays were performed to quantify phage resistance evolution and the potential cost of resistance. The final cryopreserved plates containing clones of each replicate were defrosted. These bacterial samples were inoculated into two 96-well plates containing 198µl CPG using a pin replicator. A 2µl volume of ancestral phage W4 at density of 3 x 10^6 PFU/ml was added to one plate to assess resistance (N=96) and 2µl of CPG media added to the other 96-well plate to assess the cost of resistance (N=96). The OD (600nm) was measured at 24-hour intervals as a measure of bacterial density for 72 hours. Phage resistance and cost of resistance was determined through comparing the total bacterial growth (area under the curve calculated as integral) of ancestral bacteria treated with phage and *R. solanacearum* clones isolated from the end of the experiment.

2.8.2 Time-shift assays to estimate phage-bacteria coevolution

Time-shift assays were conducted to examine the coevolutionary relationships between phage W4 and *R. solanacearum*. Cryopreserved plates at the timepoints 4 days, 8 days, 12 days, and 16 days were defrosted and phages were isolated from all populations using an aliquot of 100µl, which was diluted to 900µl in dH_2O and phage were extracted by chloroform extraction as described earlier (**2.5**). *R. solanacearum* were isolated from the 8- and 16-day timepoints through serial diluting (10^{-4} to 10^{-8}) and plating onto CPG media. Evolved bacterial colonies (N=12 for each replicate) were picked from these plates and inoculated into a 96-well plate containing 140µl of CPG media and incubated for 48 hours at 28°C. Pin replicators were used to inoculate evolved *R. solanacearum* clones into new 96-well plates, which were then exposed to ancestral W4 phage and W4 phage from each timepoint within each replicate selection line (sympatric populations) by pipetting 2µl of phage at 3 x 10^6 PFU/ml density. The plates were incubated statically at 28°C and the bacterial density was measured as OD (600nm) at 24-hour intervals for 72 hours. The total bacterial growth (calculated as area under the curve; as integral) was calculated for each treatment. Phage coevolutionary changes were quantified by comparing the total bacterial growth between phages at isolated from different timepoints.

2.9 hydroponic system experimental set up

To examine how our findings translate into an *In planta* environment, we developed a hydroponic system, where plants were grown in falcon tubes containing liquid culture media which was sampled periodically. To develop this system, preliminary work to assess the optimal conditions for this system was conducted.

2.9.1 Hydroponic experimental set up

The hydroponic system was used to investigate if phage W4 can successfully alleviate *R. solanacearum* symptoms, alongside examining phage resistance evolution in the presence of a plant. To answer these questions, firstly, micro tom tomato seeds were sterilised by performing a series of washes with 70% ethanol, a bleach solution (50% bleach (Fisher scientific), 50% dH_2O and 0.05% Triton X (Thermo scientific)) and finally dH_2O. The sterile tomato seeds were sown onto MS-S agar plates and incubated at 28°C for 12hr day/night cycles. After 20 days, *R. solanacearum* was inoculated in 10ml CPG from glycerol stock and incubated at 28°C 50RPM shaking overnight. After 21 days following sowing the MS-S plates were removed from the incubator and each germinated seedling was placed into a 50ml falcon tube containing 45ml of pH 6.0 MS-S media. Parafilm was wrapped around the top of the falcon tube to suspend the seedling in the media. The falcon tubes were placed into tube racks and placed in autoclaved sunbags (Sigma) to ensure sterile conditions, sealed with a foldback clip, and placed in a plant growth chamber at 28°C for 12hr day/night cycles to acclimatise. After 1-week of acclimatisation all the plants (N=10) were inoculated with 1ml of *R. solanacearum* culture at an 0.3 OD (600nm) (~ 3 x 10^6 CFU/ml) and half of the plants were also treated with 10µl of phage W4 at a density of 3 x 10^6 PFU/ml added (N=5). The plants were then returned to the plant growth chamber at 28°C for 12hr day/night cycles for 24 days. To assess plant disease symptoms, disease severity was calculated, and the dry plant biomass was measured (Detailed bellow **2.8.7** & **2.8.8**). The effect of phage on *R. solanacearum* density was measured by quantifying the phage and bacterial densities (CFU/ml and PFU/ml as discussed previously **2.7.1** and **2.7.2**) over the course of the experiment. At

the end of the experiment (24 days) phage resistance assays were conducted as detailed in **2.9.5** to determine phage resistance and the associated potential cost of resistance. fitness assays were conducted.

2.9.2 Measuring disease severity within the hydroponic system

Throughout the experiment disease severity was quantified by measuring the % amount of wilting foliage every 8 days. These were grouped into the following indices, disease index 0 having no wilting symptoms, index 1, 25% foliage wilting, index 2, 50%, index 3, 75%, index 4, 100% and index 5 is plant death. As a further measure of plant health, dry plant biomass was calculated at the end of the experiment (24 days) as follow. Plants were removed from their tubes and sealed in brown envelopes. These were placed at 50 °C for 3 days, the plants were then removed and weighed.

2.9.3 Calculating bacterial and phage densities in the experiment using hydroponic system

Phage and *R. solanacearum* densities were calculated every eight days, resulting in three sampling time points during the experiment (24 days). To calculate bacterial and phage densities, two ml of media was removed from each plant replicate in sterile conditions within laminar flow. One ml of sample was used to isolate the phage using chloroform extraction. The PFU/ml was then calculated for each sample and averaged across each treatment (**2.7.2**). The other ml of sample was serially diluted and 100µl of diluted samples plated onto SMSA semi-selective agar. Colonies were counted for each sample and averaged across each treatment (**2.7.1**).

2.9.4 Phage resistance assays for the hydroponic system

Following completion of the selection experiment in hydroponic system, phage resistance assays were performed to examine if resistance to phage had evolved. One ml of culture media was removed from each plant and serial diluted at the final sampling time point. The dilutions 10^{-4} to 10^{-8} were plated onto SMSA semi-selective agar and after 24hr of incubation, 12 colonies per replicate were randomly picked and inoculated into 96-well plate wells containing 140µl of liquid CPG. The 96-well plates were incubated for 24hr, 100µl of 50% glycerol added to each well and plates stored at -80°C until required. To assess phage resistance, frozen bacterial colonies were inoculated into two 96-well plates containing 198µl CPG using a pin replicator. A 2µl volume of phage W4 at density of 3 x 10^6 PFU/ml was added to one plate (N=96). The remaining plate had 2µl of CPG added to another plate to assess the potential cost of resistance in the absence of phage selection. Two 96-well plates of ancestral bacteria one grown in the presence of W4 (N=96) at a density of 3 x 10^6PFU/ml and the other with no selection were also started. The plates were incubated statically at 28^{o}C and bacterial density measured as OD (600nm) at 24-hour intervals. To assess emergence of resistance alongside potential cost of resistance the evolved clone treatments were compared to there ancestral counterpart treatments through T-test comparisons.

2.10 Setting up tomato soil experimental system

Bacteria-phage interactions were also studied using a tomato soil system, which is reflective of agricultural conditions. Briefly, tomato seeds were sterilised as described in **2.10**, and 10 seeds were

then sown into pots containing sterile soil (John Innes no 2) and watered with sterile dH_2O every 4 days. The pots were place in a plant growth chamber at 28°C for 12hr day/night cycles for 21 days. After 21 days, *R. solanacearum* strain 146 was inoculated into 10ml CPG and incubated at 28°C 50RPM shaking for 24 hours. The tomato plants were inoculated by cutting each plant at the base of the stem and pipetting 1ml of *R. solanacearum* culture at OD (600nm) 0.3 (~ $3x\ 10^6$CFU/ml) onto the wound site (N=10). In addition, 10µl of W4 Phage (3×10^6 PFU) was added to half the plants (N=5). The plants were placed in subags (sigma), to ensure sterile conditions, sealed with a foldback clip, and place in the plant growth chamber at 28°C for 12hr day/night cycles for 24 days. Every four days throughout the experiment the disease severity was measured as described below in **2.10.1.** After completion of the experiment the plants were weighed (**2.10.1**), bacterial and phage densities quantified (**2.10.2**) and phage resistance assays conducted on *R. solanacearum* from the system (**2.10.3**).

2.10.1 Measuring disease severity in the soil system

To quantify disease severity the same disease index was used as in the hydroponic system to quantify plant wilting (**2.9.3**). In addition, as with the hydroponic system plants were also weighed at the end of the experiment (24 days) **(2.9.3**). Plants were removed from there pots. The soil was washed from the roots by soaking in dH_2O. The plants were then sealed in brown envelopes and placed at placed at 50 °C for 3 days, the plants were then removed and weighed.

2.10.2 Quantifying bacterial and phage densities in the soil system

At the final timepoint of the soil system (24 days), the density of *R. solanacearum* and phage in the soil and stem of each plant was calculated. Bacterial density in the soil was calculated as follows, 1g of soil was weighed into a falcon tube and 20ml of sterile water added. The falcons were vortexed for 3min and left to settle for 1hr. A 1ml volume of this solution was then diluted (10^{-1} to 10^{-8}) and 100µl of each dilution plated onto SMSA semi-selective plates. The colonies were counted on each plate and CFU/ml calculated as described previously (**2.7.1**). The phage density was calculated from this same soil sample by removing 900µl of sample and performing phage chloroform extraction and calculation of PFU/ml as described in (**2.7.2**). The bacterial density in the stem of the plant was calculated by cutting a 0.5g section of the stem near the base of the plant. The cut stem was placed into a 2ml Eppendorf containing 1ml of sterile dH_2O and two tissuelyser (Quiagen) metal beads. The samples were left to stand in the water for 1 hour to soften the tissue. The tubes were placed into a Qiagen tissuelyser II and tissue lysed at max speed for 4 minutes. Following lysis, 100µl of these samples was serial diluted (10^{-1} to 10^{-8}) and 100µl plated onto SMSA semi-selective media. The colonies were counted, and CFU/ml calculated. To quantify the phage from these samples the remaining 900µl was chloroform extracted and the PFU/ml calculated from this extraction as in **2.7.2**.

2.10.3 Phage resistance assays for bacterial colonies isolated from the soil system

Following completion of the experiment, phage resistance assays were conducted on *R. solanacearum* isolated from the soil and stem of the plant. Twelve colonies were picked for each replicate plant, both treatment (Phage and no phage) and location (Stem and soil) were inoculated into a 96-well plate containing 140µl of CPG media. Phage resistance was then determined as previously (**2.8.1**)

2. 11 Statistical analyses

All growth curves obtained were analysed through calculating the total bacterial growth as the area under the curve (integral). This calculation was performed using custom R scripts detailed bellow.

```
# Area dataset script

        area_dataset <- old_dataset %>%

# Makes the specified columns (time and OD) numeric (needs to be numeric for auc function)

         modify_at(c('OD', 'Time'), as.numeric) %>%

# Specify the factors that you want to group by (i.e. condition, isolate identity, replicate)

         group_by(ID, Condition, Replicate) %>%

# Create a nested data frame where each cell in one column is a data frame

        nest %>%

# Create new columns with the area under the curve using the auc function

        mutate(area = map(data, ~auc(.$Time, .$OD))) %>%
        select(-data) %>%

# Remove the data column

         unnest
```

The total bacterial growth calculated using the above script was compared between treatments using One-Way ANOVA or pairwise T-tests to determine if there was a significant difference between treatments. Plant disease index measurements were taken over the course of experiments as described in (**2.9.3**). The average disease index across each treatment at the end of the experiment was calculated and compared across treatments through pairwise T-tests. A further quantifier of plant health used throughout this study was the dry plant biomass. Following measurement of the dry plant biomass as described in (**2.9.3**). The average dry weight biomass was compared between treatments using pairwise T-tests to determine statistical significance. All graphs and statistical tests were conducted in R version 3.6.2[31].

Results

3.1) Multiple phage exhibit generalist infectivity against R. solanacearum

The first step in characterising potential biocontrol phages is to determine their host range. As a result, we first tested which *R. solanacearum* strains each phage could clearly infect. To ascertain the infectivity range of our phage isolates (**Table 2**) against our *R. solanacearum* collection (**Table 1**), the lytic activity of ten phage isolates (from the UK and China) were assayed against ten *R. solanacearum* strains (isolated from the UK, Angola, French Guyana, Nigeria and USA) using soft agar spotting method. These assays revealed that five phage exhibited generalist infectivity, being able to infect most of the bacterial isolates within our collection (generalist phage: W4, W9, W10, SD10 and CP20; Table 2). The remaining five phage showed more specialised infectivity patterns, capable of infecting only two out of ten bacterial strains (**Table 4**). Based on these results, we selected W4, W5, 42 and 47 phages for further studies in order to have a mixture of specialist and generalist and phages from different isolation locations (W4, W5: River water, 42, 47: Tomato rhizosphere).

Table 4. The lytic activity of phages isolated from the UK and China against our *R. solanacearum* collection.

		UK phage isolates					Chinese phage isolates				
Ralstonia strain	*Ralstonia* isolation location	W4	W5	W9	W10	SD10	42	44	45	47	CP20
001	French Guyana	-	-	-	-	-	-	-	-	-	-
081	Nigeria	+	-	+	+	+	+	+	+	+	+
146	UK	+	+	+	+	+	+	+	+	+	+
199	UK	+	-	+	+	+	-	-	-	-	+
206	UK	+	-	+	+	+	-	-	-	-	+
281	UK	+	-	+	+	+	-	-	-	-	+
337	UK	+	-	+	+	+	-	-	-	-	+
380	USA	+	-	+	+	+	-	-	-	-	+
385	UK	+	-	+	+	+	-	-	-	-	+

Key: + Indicates the observation of a clear plaque following spot plating.
- Indicates no observation of a clear plaque following spot plating.

3.2) Phage W4 effectively suppresses R. solanacearum strains 380, 385 and 146

While the soft agar assays could reveal which phage were capable of infecting different *R. solanacearum* isolates, this method carries limitations, as little information is given related to the

dynamics of inhibition. Therefore, to look at how phage are affecting bacterial growth in more detail growth curves of strains were conducted in liquid CPG media in the presence and absence of phage. A few important pathogen strains from varying geographic areas were selected (081, 146, 380, 385). We decided to assay these strains against the phage W4 due to its broad infectivity range and successful inhibition of all these strains on CPG agar plates. The strains were grown in CPG liquid media and the bacterial density (OD600nm) was measured at 10-minute intervals for 70 hours. Strains 146, 380 and 385 were all clearly inhibited by phage W4 (**Figure 5A/B**) and were all rapidly driven to very low densities in comparison to their counterparts grown in the absence of W4 phage (ANOVA: $F_{3,13148} = 38277$, $p < 0.001$). However, strain 081 was unaffected by the addition of W4 phage (ANOVA: $F_{3,13148} = 38277$, $p > 0.05$) compared to when it is grown in the absence of phage (**Figure 5A**). Based on the susceptibility of strain 146 against all our phage isolates (section 3.1) in addition to these analyses, we decided to use *R. solanacearum* strain 146 in the further experiments.

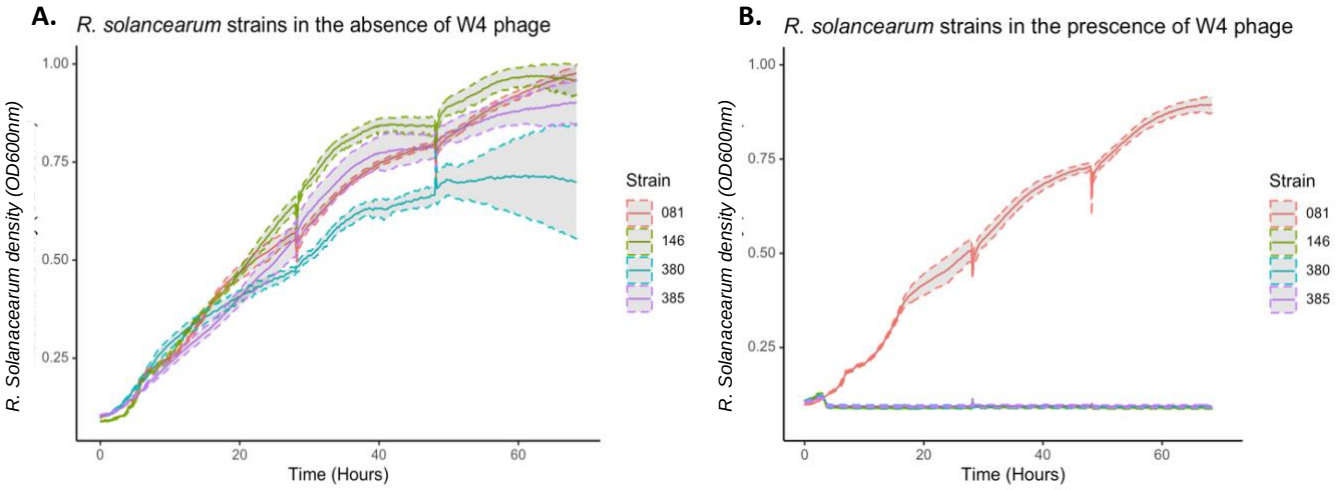

Figure 5. The growth of four *R. solanacearum* strains in the absence (A) and presence (B) of W4 phage. The *R. solanacearum* strains 380, 385, 081 and 146 were exposed to W4 phage (A) and no phage (B) in 96-well plates with 8 replicates for each treatment. The bacterial density as OD (600nm) was measured at 10-minute intervals for 72 hours.

3.3) W4 phage shows the most efficient suppression of R. solanacearum strain 146

To further quantify the effect of different phage isolates on bacterial growth dynamics, *R. solanacearum* strain 146 was assayed against four different phages. The phage W4 was chosen due to its generalist activity and effective suppression of multiple *R. solanacearum* strains in liquid CPG media, while W5 was chosen based on its alternative specialist infectivity range. The phage isolates 42 and 47 were also used, based on their alternative isolation location (Tomato rhizosphere). The *R. solanacearum* strain 146 was grown in liquid media and the bacterial density (OD600nm) was measured at 24-hour intervals. To compare overall growth between treatments the area under the curve for each treatment was calculated for each condition (as integral, i.e., 'area under the curve') **(Figure 6B)**. Overall, phages; W4, 42 and 47 reduced bacterial growth compared to the no-phage control treatment (ANOVA: $F_{5,42} = 75.89$, $p < 0.001$) except for W5 ($p = 0.33$). The generalist phage W4 was the most effective at reducing bacterial growth, driving *R. solanacearum* to very low densities in all replicates, while the other phages W5, 42 and 47 showed varying levels of bacterial growth suppression. For the remainder of our experiments going forward we decided to use phage W4 due to its broad infectivity range and ability to effectively suppress *R. solanacearum* strain 146 growth.

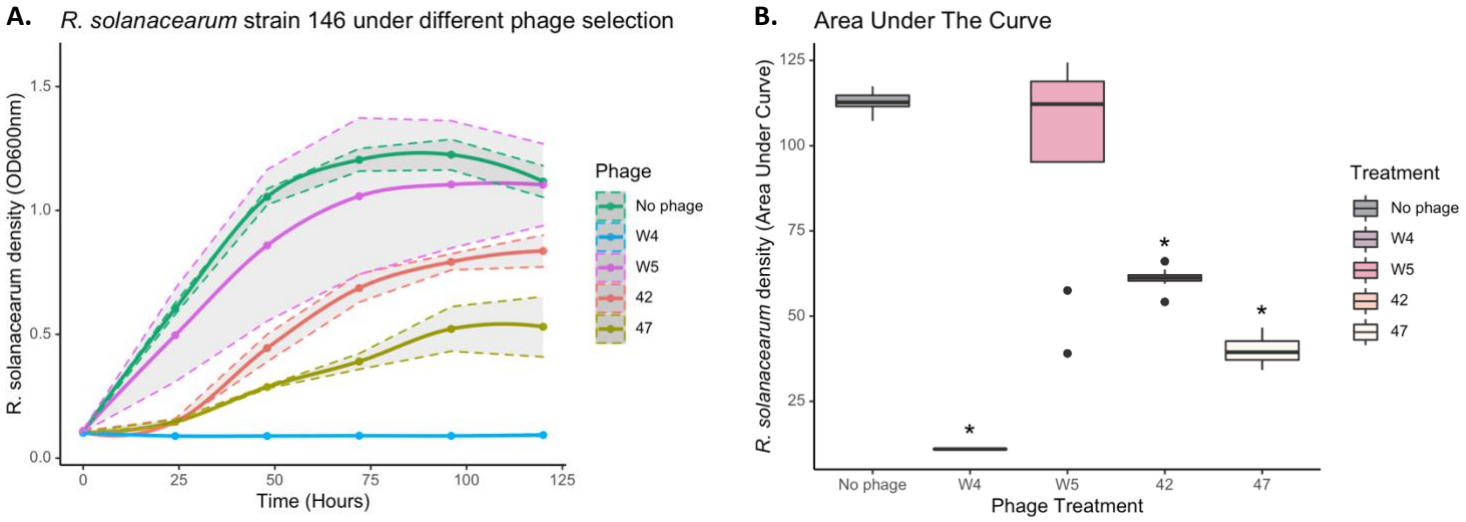

Figure 6. *R. solanacearum* strain 146 growth in the absence and presence of different phage. (A) *R. solanacearum* strain 146 growth dynamics in liquid CPG media in the absence and presence of different phage over 120 hours. The OD (600nm) was measured every 24 hours. The solid line indicates an average of 8 replicates and shading indicates ± confidence interval. **(B)** The area under the curve as an average of 8 replicates. The * denotes significance relative to 'No phage' treatment at level P < 0.001.

3.4) Liquid media evolution experiment indicates trade-off between phage resistance and bacterial growth

A critical knowledge gap crucial for the development of an efficacious phage therapy is the emergence of phage resistance within treated *R. solanacearum* populations. In order to examine if phage resistance emerges, and if there are fitness trade-offs attributed to W4 phage resistance, we experimentally cocultured phage W4 and *R. solanacearum* strain 146 in a 16-day serial passaging experimental evolution model in liquid media. In addition to phage resistance and trade-offs we also examined if coevolution between *R. solanacearum* 146 and phage W4 was occurring (**Figure 10)**.

Firstly, we examined the density of *R. solanacearum* 146 over the duration of the 16-day experiment to quantify the ability of phage W4 to suppress *R. solanacearum* growth (**Figure 7A**). As shown clearly in **Figure 7A** the density of *R. solanacearum* for the nontreatment progressively increased over the duration of the experiment, whereas the density of the W4 treated *R. solanacearum* remained lower and relatively constant (**Figure 7A**). To accurately compare these differences in growth between treatments the overall total growth rate of *R. solanacearum* was calculated (area under the curve as integral) **Figure 7B**. Overall growth for the no phage treatment in relation to W4 was observed to be significantly higher (T-test reporting: T=55.181, df= 303.1, p < 0.001). These results suggest that W4 phage is effective at suppressing bacterial growth over 18 days in CPG liquid media.

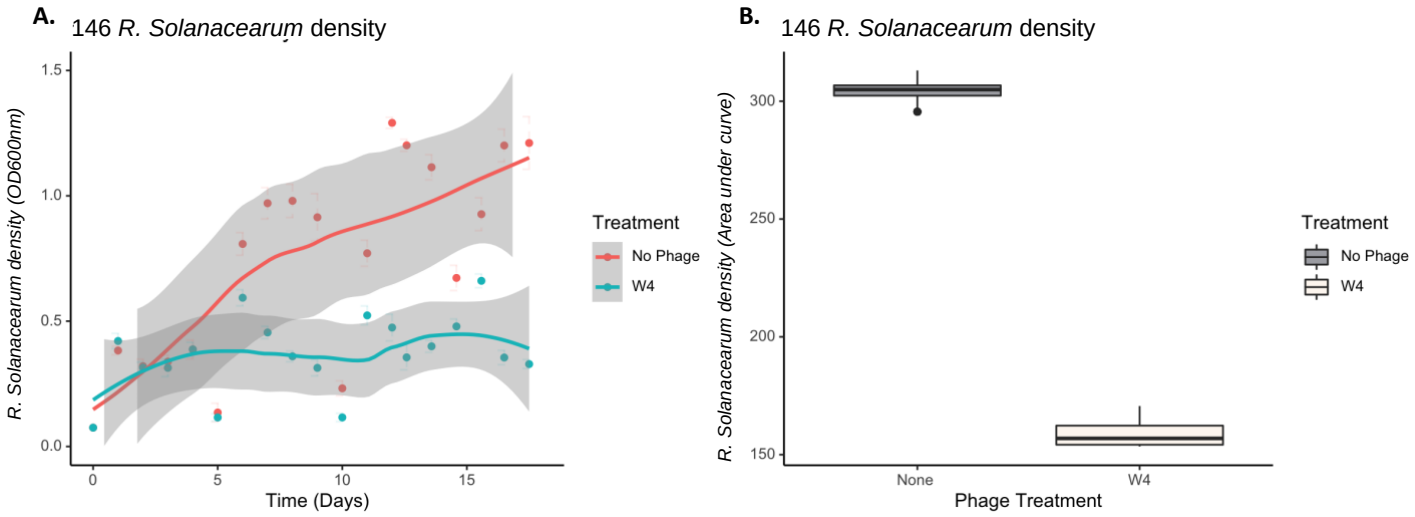

Figure 7. (A.) Growth of *R. solanacearum* quantified as OD (600nm) measured daily for 8 days ± phage W4, measurements were taken in liquid media evolution experiment. The bacterial density (OD600nm) was measured at 24-hour intervals throughout the liquid media system evolution experiment for each treatment. The OD (600nm) reads across 8 replicates for each treatment were averaged and shown above. The shaded area represents the mean ± confidence interval. **(B.) Total growth of treatments was calculated as the area under the curve (as integral).** Phage effect on *R. solanacearum* density during the experiment was calculated as the area under the curve.

To determine if W4 phage resistant *R. solanacearum* had emerged, bacterial clones from the end timepoint (16-days) of the experiment were isolated from the W4 treatment and no-phage treatment. These were defined as different evolutionary histories "W4" and "None" respectively. Following isolation, the clones were treated with W4 ancestral phage and no treatment control **Figure 8A**. The total growth for each treatment was calculated as area under the curve (as integral and compared via ANOVA). *R. solanacearum* which had W4 selection as an evolutionary history showed a dramatic improvement in growth relative to the none evolutionary history when exposed to ancestral W4 phage (**Figure 8A**) (ANOVA: $F_{5,24}$ = 29.1, p <0.001). This suggests that significant levels of resistance to W4 phage have arisen within the *R. solanacearum* population. However, when phage selection was removed from both treatments the *R. solanacearum* with a W4 evolutionary history had a much lower total growth in comparison to the none evolutionary history treatment (ANOVA: $F_{5,24}$ = 29.1, p <0.001). This indicates that there is a trade-off between W4 resistance and *R. solanacearum* growth.

To further quantify the difference in bacterial growth the difference in bacterial density as OD (600nm) from the final timepoint was calculated (**Figure 8B**). This showed a significant reduction in bacterial growth for the W4 evolutionary history no phage treatment compared to the control (none evolutionary history no-phage treatment). Alongside this there was also a significant reduction in bacterial growth for the no evolutionary history W4 treatment compared to the W4 evolutionary history W4 treatment (ANOVA: $F_{5,24}$ = 24.1, p <0.001; p < 0.001 for pairwise comparison). This further suggests that a fitness trade-off is present between W4 phage resistance and bacterial fitness.

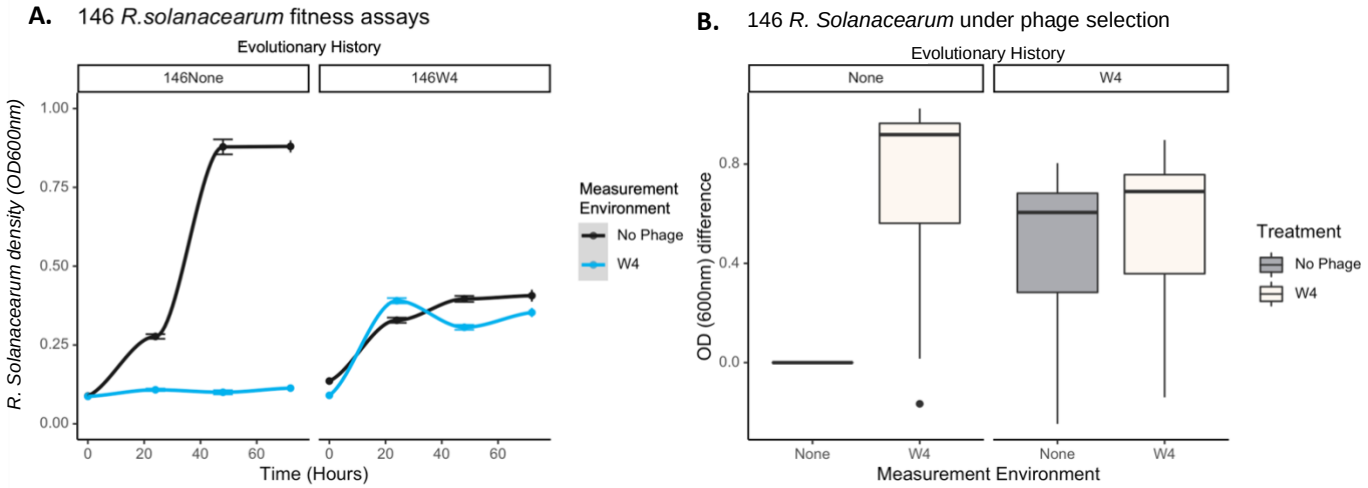

Figure 8. (A.) Growth of *R. solanacearum* evolved clones in the absence and presence of ancestral W4 phage. *R. solanacearum* clones were evolved with W4 phage for 18 days. The alone-evolved and W4-evolved *R. solanacearum* clones were then exposed to ancestral W4 phage or grown in the absence of phages in CPG media and bacterial density measured (OD 600nm) at every 24 hours for 3 days. **(B.) Boxplot of evolved *R. solanacearum,* OD (600nm) difference relative to no selection treatment.** * Denotes significance $p < 0.001$ relative to none treatment.

It is important to examine the coevolutionary dynamics between *R. solanacearum* and phage to build an understanding of how the pathogen and phage interact. Therefore time-shift assays were conducted from the liquid media evolution experiment to shed light on these interactions. *R. solanacearum* (strain 146) clones from four different evolutionary histories were isolated; ancestral bacteria, bacterial clones isolated after 16 days of growth in CPG media, 8 days in CPG with phage and 16 days of growth in CPG with phage W4. The bacterial clones were assayed against phage W4 isolated from different evolutionary timepoints (Ancestral, 4 days, 8 days, 12 days and 16 days) **Figure 9**. If the phages have coevolved to become more infective towards their host, then phages isolated from the 16-day timepoint should prove to be more infective against the ancestral bacteria. Despite this following calculation of the total bacterial growth (as integral, i.e., 'area under the curve') phage from the 16-day (final) timepoint showed the lowest suppression of bacterial growth against the ancestral bacteria, (**Figure 9A**) relative to the ancestral W4 phage (ANOVA reporting: $F_{5,2302} = 547.5$, $p < 0.001$). This suggests that there is no escalation of phage infectivity against the *R. solanacearum* host.

Unusually when assaying the phages against the day-16 control bacterium (**Figure 9B**) the bacteria appeared to be resistant to the ancestral W4 phage. Interestingly, it appeared that all the phage treatments in the day 16-control experiment grew to much higher OD (600nm) than the ancestral *R. solanacearum* (**Figure 9B**). To confirm this total bacterial growth was calculated for each treatment and compared to the growth of their ancestral counterparts. This revealed a significant difference in growth between all treatments (ANOVA reporting: $F_{5,2298} = 509$, $p > 0.001$) except for the no phage treatment ($p = 0.21$). This is contrary to what we would expect and is likely due to spontaneous resistance arising or contamination of the samples.

Based on these previous observations we wanted to examine if phage were more infective against bacterial populations contemporary to them. These assays showed that no phages were able to

suppress the growth of day-8 and day-16 evolved *R. solanacearum* clones compared to their respective no phage treatments ANOVA reporting: $F_{5,2298} = 6 \times 10^{27}$, $p > 0.05$) **(Figure 9C/D)**. This is indicative of high levels of phage resistance throughout these populations. However there appeared to be a trade-off associated with this resistance, comparing the no-phage treatments of the day-8 and day-16 timepoints to ancestral and day-16 control *R. solanacearum* the no-phage treatments grew significantly worse, despite no phage selection being present; confirmed by area under the curve analysis (as integral) (ANOVA reporting: $F_{10, 2298} = 543.4$, $p < 0.001$). Interestingly it appeared that these trade-offs became more severe over the duration of the experiment, as day-16 *R. solanacearum* grew significantly worse relative to day-8 *R. solanacearum* across all treatments (ANOVA reporting: $F_{10, 2298} = 483.4$, $p < 0.001$). **(Figure 9C/D)**.

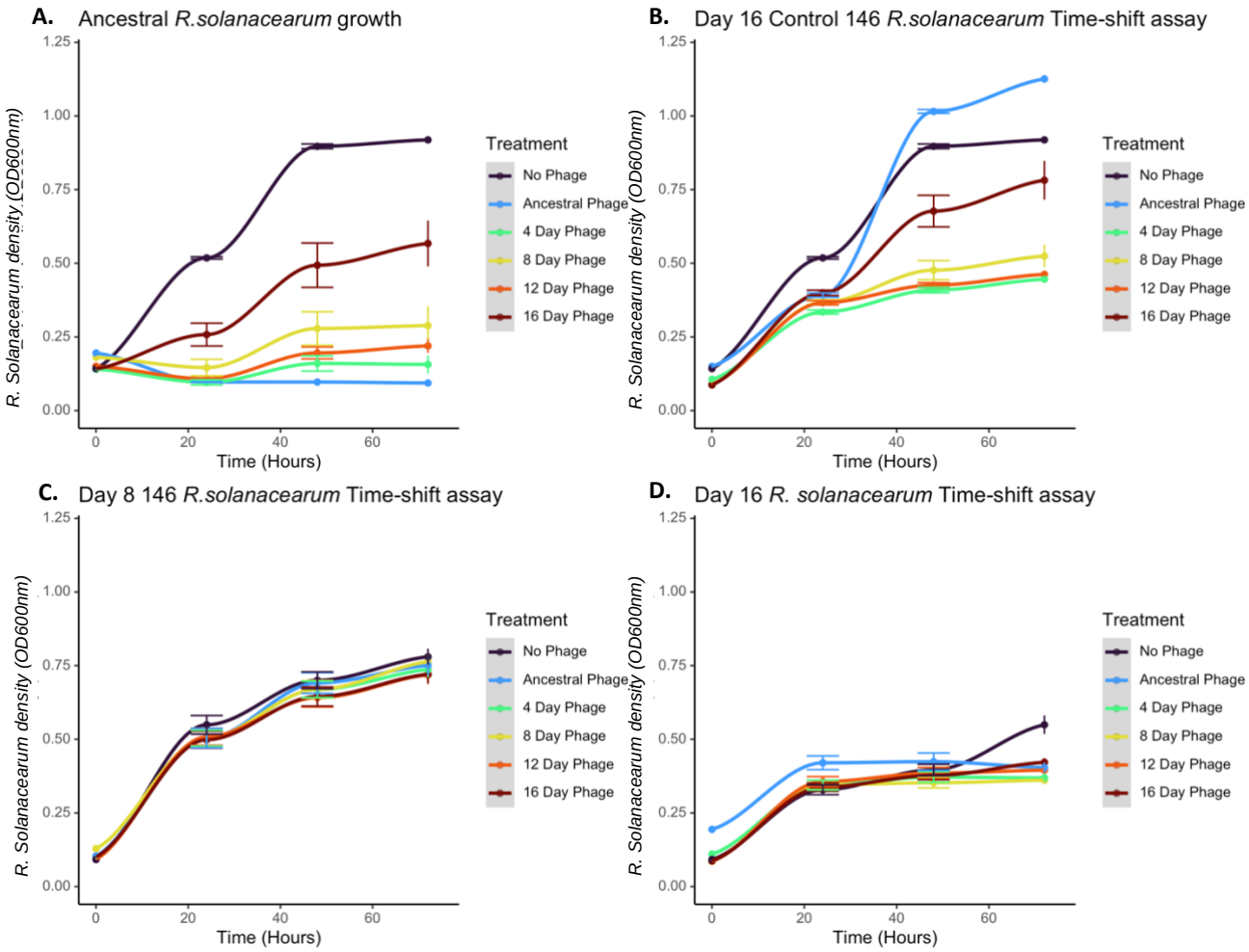

Figure 9. *R. solanacearum* strain 146 clones isolated at different time points exposed to phages isolated at different evolutionary intervals (past, contemporary, and future). Bacterial density was measured as OD (600nm) at 24-hour intervals. All assays were conducted in 96-well plates in CPG media. **(A.)** Ancestral *R. solanacearum* **(B.)** *R. solanacearum* clones isolated from a media control at the final time point (16 days). **(C.)** *R. solanacearum* clones isolated from the W4 treatment at the 8-day time point. **(D.)** *R. solanacearum* grown in the presence of W4, isolated from the final timepoint in (16 days).

3.5) Preliminary work and development of hydroponic system

The ability of *R. solanacearum* to grow in the plant growth media Murashige and Skoog (MS) was first assessed. MS liquid media was supplemented with sucrose (5g, 10g and 20g per L) to ensure an adequate carbon source for bacterial growth. *R. solanacearum* was inoculated into a 96-well plate containing the supplemented MS liquid media and dH_2O, with eight replicates for each sucrose concentration as well as the control dH_2O treatment (N=8). The bacterial growth was quantified as optical density (OD600nm) at 24-hour intervals. It was found that despite sucrose supplementation the growth of *R. solanacearum* on this media was extremely poor (**Figure 4A**). The 10g/L of sucrose treatment had the highest level of growth reaching a peak OD (600nm) of 0.2. However relative to the dH_2O control treatment no significant difference in total growth (calculated as area under the curve by integral) was observed (T-test reporting: $T_{5,36} = 13.02$, $p < 0.05$). Following these results, we hypothesised that the combination of low pH (pH5.5 for normal MS media) and nutrients in MS media might be inhibiting *R. solanacearum* growth. Upon buffering MS media to more neutral pH of 6 and 7 with 10g/L of sucrose, we found that bacterial growth improved dramatically (**Figure 4B**). Area under the curve analysis (as integral) followed by T-tests showed there was a significant improvement in *R. solanacearum* growth in both pH 6 and 7 (T-test reporting: $T_{5,36} = 8.61$, $p < 0.001$) compared to the unbuffered media, with pH 7 media having the greatest improvement in bacterial growth. Whereas there was no significant difference in pH 5.5 media ($T_{5,36} = 27.5$, $p > 0.05$) relative to the unbuffered media. Furthermore, we wanted to ensure that W4 phage was still effective at inhibiting *R. solanacearum* across this range of pH values. Therefore, we treated the buffered samples containing *R. solanacearum* with bacteriophage W4. As clearly shown in **Figure 4C**, phage W4 was still effective at suppressing *R. solanacearum* across all pH treatments (confirmed by area under the curve analysis followed by ANOVA: $F_{5,192} = 59$, $p < 0.001$) Therefore, going forward, we decided to use MS-S media buffered to a pH of 6 for the hydroponic plant experiments to maintain a slightly acidic environment preferred by tomatoes whilst ensuring appropriate growth of *R. solanacearum*.

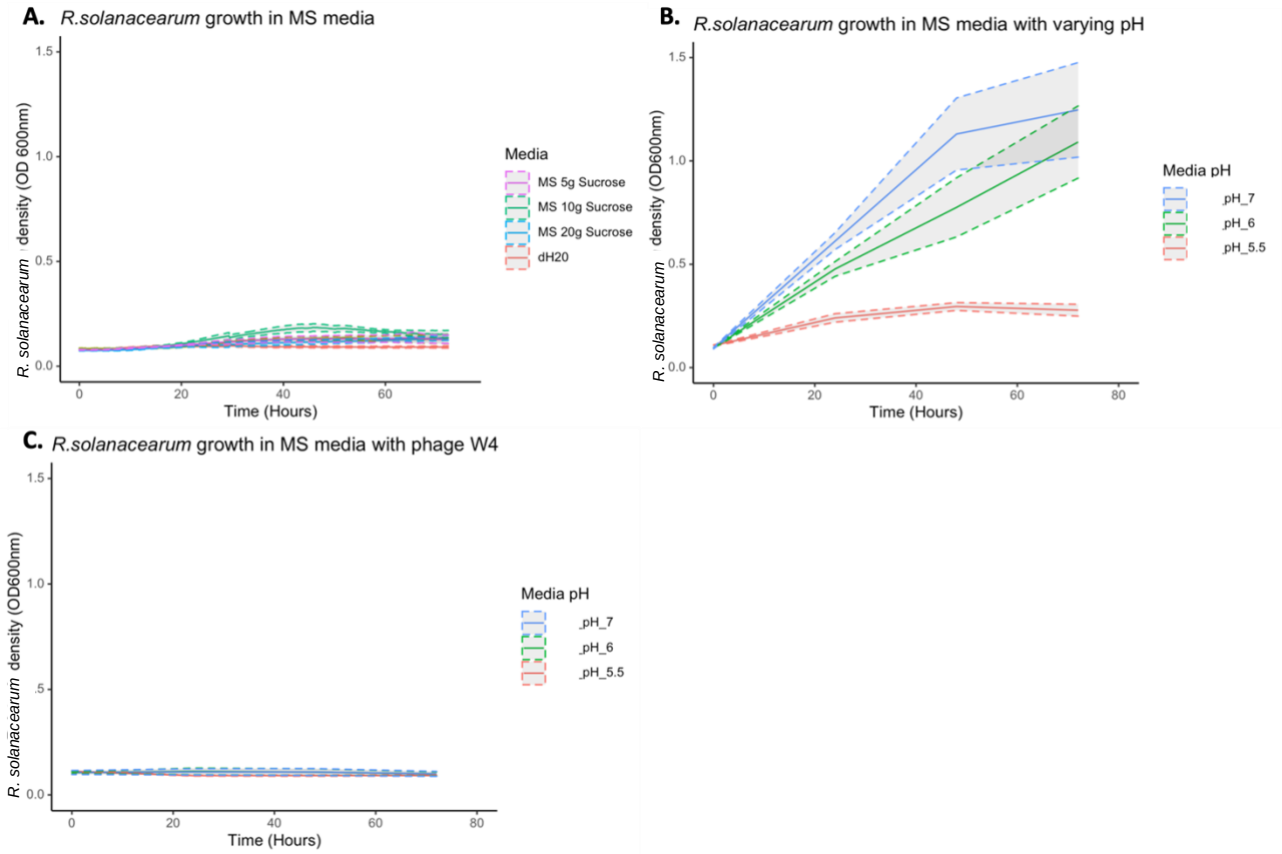

Figure 4. *R. solanacearum* growth in MS media. **(A)** MS media was supplemented with varying amounts of sucrose and inoculated with *R. solanacearum*. bacterial growth was quantified as optical density (OD600nm) at 24-hour intervals measured over 72 hours. **(B)** *R. solanacearum* growth in buffered MS media 10g/L sucrose. MS media was supplemented with 10g/L of sucrose and buffered to pH 6 and pH 7. The bacterial density was measured as optical density (OD600nm) was measured every 24 hours for 72 hours. **(C)** Suppression of *R. solanacearum* growth by phage W4. *R. solanacearum* was grown in MS media supplemented with sucrose and buffered. 10ul of W4 phage was added to each sample. OD(600nm) was measured every 24 hours for 72 hours. Dashed lines represents ± confidence interval.

3.6) The Hydroponic system shows improved plant survival and a reduction in R. solanacearum fitness

To test if the coevolutionary patterns and fitness trade-offs observed in the liquid media experiment are also observed in the presence of a plant, we validated phage W4 efficiency in the tomato rhizosphere using a hydroponic system. This allowed us to answer the following; firstly, can phage successfully alleviate the symptoms of *R. solanacearum* improving disease outcome? Does a fitness trade-off persist between W4 and *R. solanacearum* and is this trade-off related to a reduction in bacterial virulence?

The average disease index for plants in the hydroponic system was calculated every 8 days and is shown in **Figure 10A.** Both phage treated and untreated plants showed similar levels of disease progression with the average disease index and hence wilting foliage increasing steadily over the duration of the experiment **Figure 10A**. However, the level of disease severity after 24 days was significantly improved by application of phage W4 (t-test comparison of final timepoint reporting: $T_{5,23} = 3.7$, $p < 0.05$). Due to the subjective nature of quantifying wilting foliage the dry plant biomass of each plant was measured at the end of the experiment (24 days) **Figure 10B**. Overall, the phage treated plants had a significantly higher average dry plant biomass than the untreated plants (t-test comparison reporting: $T_{5,23} = 3.7$, $p < 0.05$). Therefore, the application of W4 phage clearly improved plant health relative to no phage treatment.

The relative abundance of *R. solanacearum* and phage can provide insight into how they are interacting in the culture media and if phage were reducing *R. solanacearum* density leading to an improvement in disease symptoms. Density of *R. solanacearum* in the culture media was quantified as CFU/ml, for the duration of the experiment **Figure 10C**. This showed an initial increase in *R. solanacearum* density at the start of the experiment for both treatments, followed by a later reduction in *R. solanacearum* density **Figure 10C**. Despite the W4 treatment appearing to have a much lower total growth rate, comparison of the total growth of *R. solanacearum* between the two treatments showed this was not significant (calculated as area under the curve, t-test reporting: $T_{5,23} = 3.9$, p-value = 0.8476).

The density of W4 (PFU/ml) was calculated over the duration of the hydroponic experiment **Figure 10D**. A similar trend as with *R. solanacearum* density was observed, with an initial slight rise in PFU/ml followed by a decline to 7×10^5 PFU/ml, where the phage density remained similar. Since *R. solanacearum* density was not affected significantly by phage, this could mean that the improvement seen in plant disease outcome could be attributed to trade-offs resulting from phage resistance.

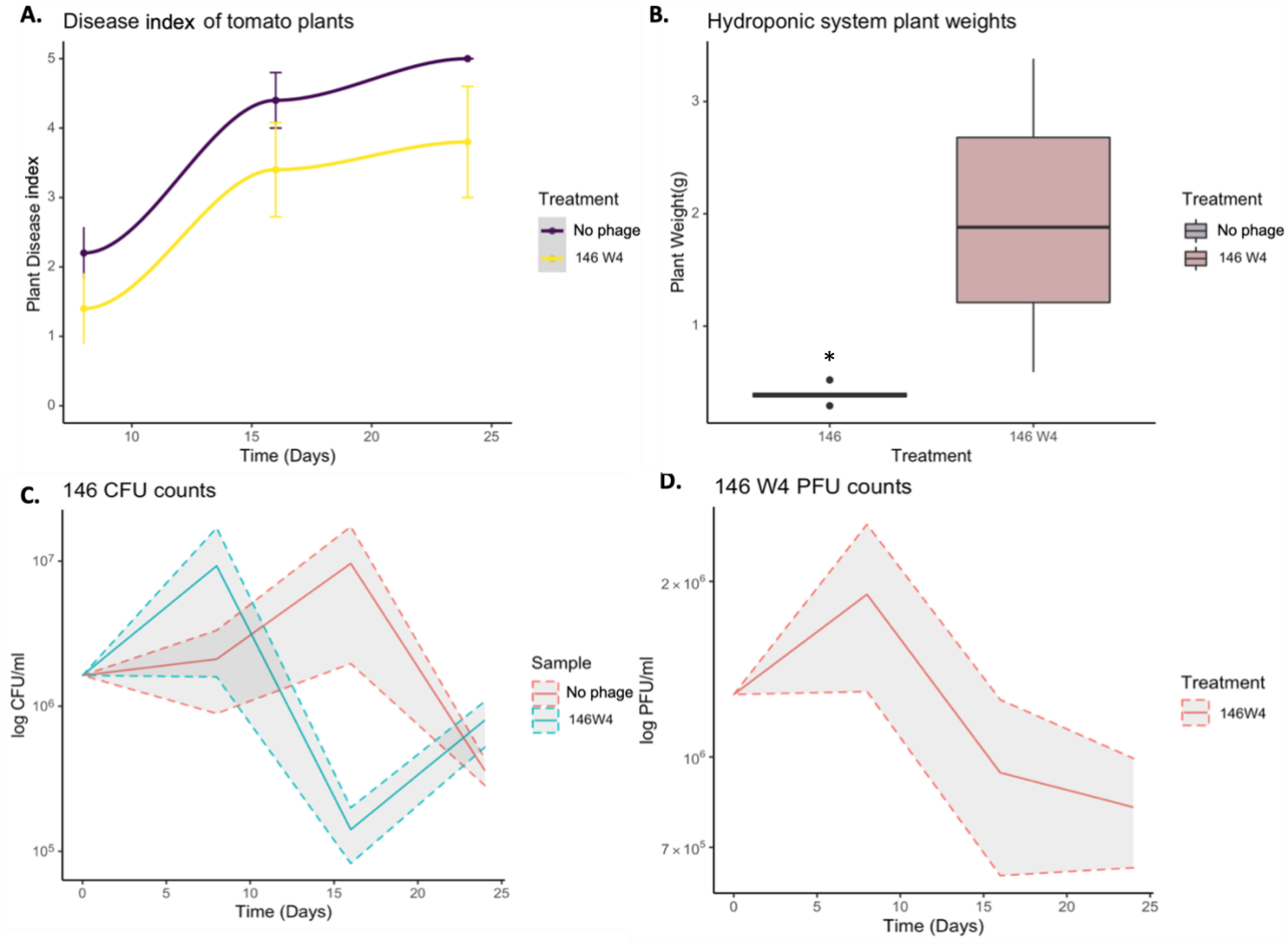

Figure 10. (A.) The average disease stage of hydroponic tomato plants. The disease stage of tomato plants was measured every 8 days following inoculation with *R. solanacearum* strain 146 or 385 and phage treatments W4 or CP20. **(B.)** Plant weights following completion of the hydroponic system. * Denotes significance of $p < 0.01$. **(C.) The *R. solanacearum* CFU counts**. *R. solanacearum* CFU was calculated from the hydroponic culture media every 8 days. **(D.) Phage PFU counts**. Bacteriophage PFU in the hydroponic culture media was calculated every 8 days.

As shown previously phage W4 was successful at alleviating *R. solanacearum* symptoms and a fitness trade-off was shown under *Liquid media* conditions. However, the question of whether this trade-off persists with the addition of a plant remains. We set out to examine if a fitness trade-off was present from the final timepoint of the hydroponic system. **Figure 11.** shows that *R. solanacearum* isolated from the hydroponic system appeared to be resistant to W4 phage. Area under the curve analysis confirmed that the total growth of *R. solanacearum* isolated from the W4 treatment was significantly higher when re-treated with phage W4 relative to ancestral bacteria (t-test reporting: $T_{5,23} = 42$, $p < 0.05$). However, as observed in the liquid media system, it also appears that there is a fitness cost associated with W4 phage resistance as clones grown in the presence of phage grew worse in comparison to the non-treatment even without W4 selection **Figure 11**. Area under the curve analysis confirmed that there was a significant difference in total bacterial growth between the two no-phage treatments (Reporting: $T_{5,23} = 39$, $p < 0.05$). This confirms that the fitness trade-off observed in the liquid media system persists in the presence of a plant. Furthermore, based on the reduced disease severity seen within the hydroponic system this trade-off could result in improve *in planta* disease symptoms of *R. solanacearum.*

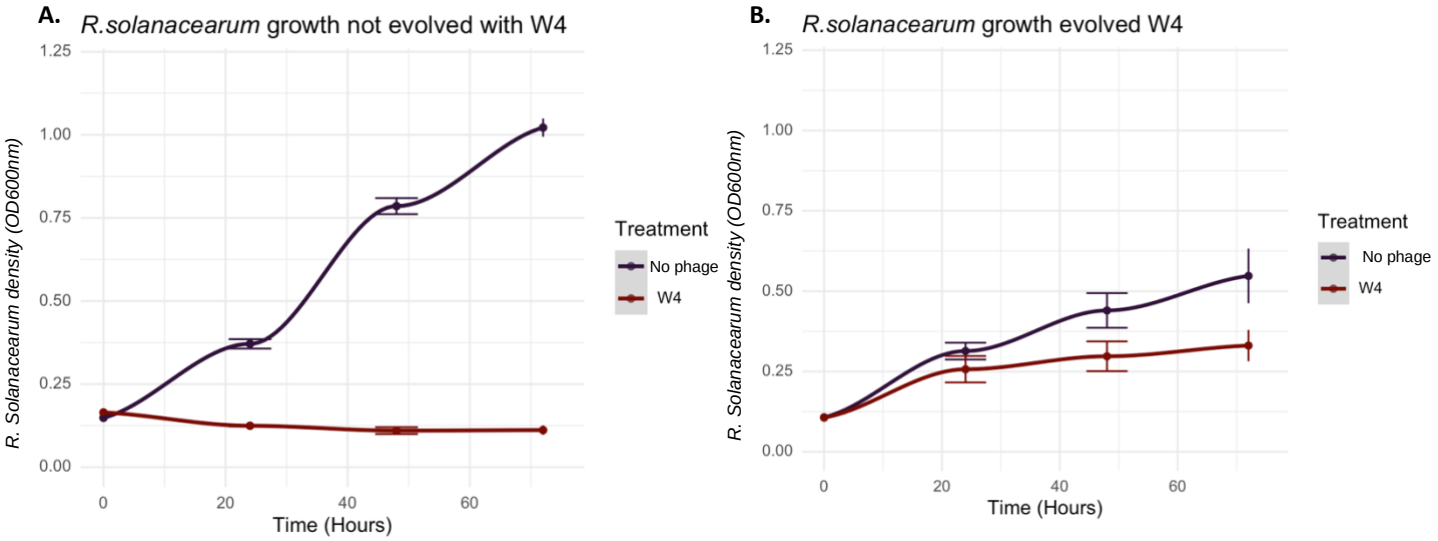

Figure 11. Fitness assays for evolved hydroponic system clones. (**A.) Untreated *R. solanacearum*. (B.) *R. solanacearum* treated with phage W4** 12 *R. solanacearum* clones were isolated from the final timepoint of the hydroponic system. These were assayed against ancestral phage. The OD (600nm) was measured at 24-hour intervals.

3.7) Soil system model indicates high phage abundance within the soil and an increase in bacterial fitness

Despite promising results from the hydroponic system, the culture media used within the hydroponic system is not necessarily a reflective indicator of real-world conditions. Therefore, to use a more natural system, and to examine how results translate to an environment closer related to how plants grow in the field, a soil system model was used. In addition to disease outcome, we also wanted to assess how phage W4 and *R. solanacearum* were interacting in this system. Based on this and results from the previous systems we set out to determine the following: does phage W4 continue to alleviate *R. solanacearum* symptoms within a soil system model? Can phage W4 successfully enter the plant or is it located mostly in the soil and how is bacterial fitness impacted in each location?

To examine if phage can effectively get inside the plant and reduce *R. solanacearum* density, tomato plants were inoculated with *R. solanacearum* alone and *R. solanacearum* with phage W4. The plants were grown for 24 days; *R. solanacearum* and phage were then isolated from the soil and stem of each plant. Phage densities (PFU/ml) were revealed to be much higher in the soil compared to the stem **(Figure 12A**) (T test reporting: $T_{1,8} = 5.039$, $p < 0.05$). This also appears to be reflected by the density of *R. solanacearum* (CFU/ml) in the soil, where there was a significantly lower CFU/ml present in the W4 soil treatment (T test reporting: $T_{1,8} = 4.12$, $p < 0.01$). Even though the *R. solanacearum* density in the untreated soil was higher, both stem treatments contained significantly more *R. solanacearum* than the phage treated soil (ANOVA: $F_{3,16} = 15.99$, $p < 0.05$). Furthermore, there was no observed difference in bacterial density between the stems of phage treated and untreated plants (ANOVA: $F_{3,16} = 15.99$, $p > 0.05$). This indicates that phage was unable to enter the stem in significant enough densities to suppress *R. solanacearum* growth relative to the no-phage treatment.

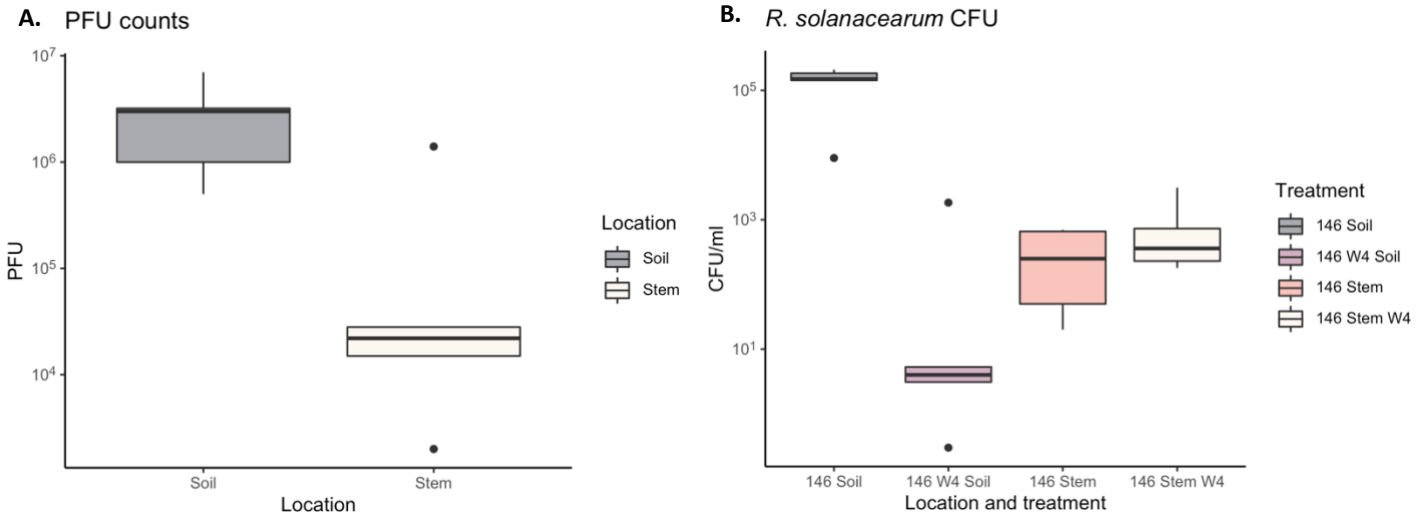

Figure 12. (A.) The PFU/ml by location in the soil system. The PFU/ml was calculated from the soil and the stem of the plant to assess if phage were still present in the plant after 24 days. **(B.) The CFU/ml by location in the soil system.** *R. solanacearum* strain 146 was isolated from the stem and soil of the plant and the CFU/ml was calculated and averaged across all treatments.

Due to the lack of a significant reduction in bacterial density in the stem of the plant we wanted to examine if phage resistance had evolved in these environments and if this was attributed to the reduction in PFU/ml. Therefore, *R. solanacearum* clones were isolated from the stem and soil of the plant. Phage resistance assays were conducted against these clones (**Figure 13**). *R. solanacearum* isolated from the soil which had been treated with phage W4 was the only treatment which showed resistance to W4 phage. Area under the curve analysis showed a dramatic increase in phage resistance for the soil treatment compared to the soil control (T-test reporting: $T_{9,54} = -4.9$, $p < 0.001$). Resistance to W4 phage in the soil was attributed to no change in bacterial fitness relative to the soil control (T-test reporting: $T_{9,54} = -4.9$, $p < 0.001$). There was no observable change in fitness or phage resistance for the *R. solanacearum* isolated from the plant stem.

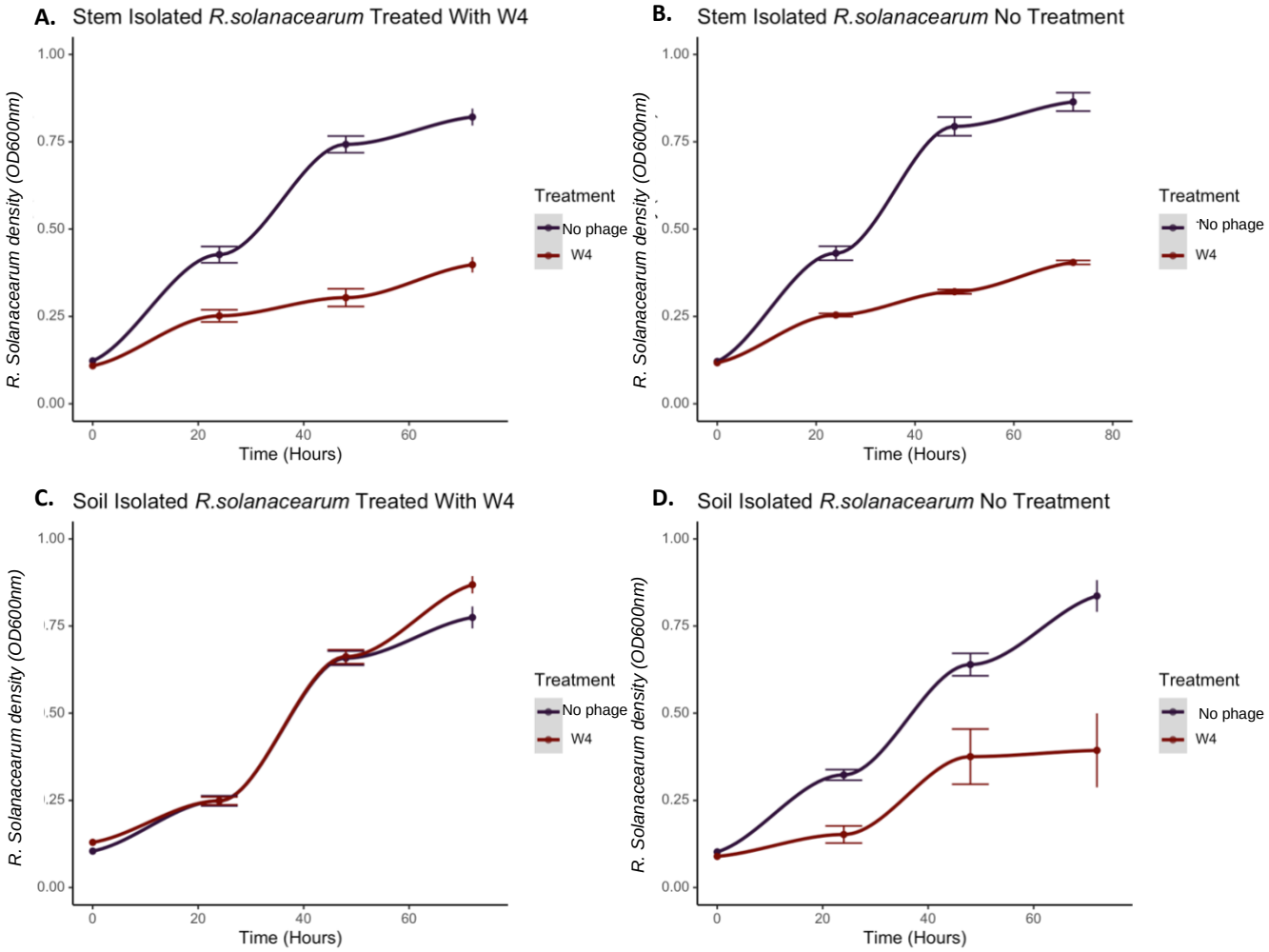

Figure 13. *R. solanacearum* isolated from different treatments and locations in the soil system experiment. (A) *R. solanacearum* isolated from the stem +W4 treatment**, (B.)** *R. solanacearum* isolated from the stem with no W4 treatment, **(C.)** *R. solanacearum* isolated from the soil with W4 treatment, **(D.)** *R. solanacearum* isolated in the absence of W4. All values are mean ± confidence interval.

One of the most critical determinants of an effective control treatment is the ability to improve disease outcome. To quantify disease progression and outcome in the soil system model we used the same system as with the hydroponic system. This showed no significant difference in plant disease progression between the two treatments, although, a greater variation was observed within the *R. solanacearum* alone treatment with one plant dying (**Fig 14A**). Despite this no significant difference was observed between the two (T-test reporting: $T_{7.8}$ = -0.2, p > 0.05). As an additional metric of disease outcome, the dry biomass of each plant was weighed (**Fig 14B)**. Similar to the disease index results, the plant weights revealed a larger variation in the 146 treatments alone comparatively to the phage treatment. However, despite a lower average plant biomass in the non-phage treatment a T-test confirmed that the results were non-significant (T-test reporting: $T_{7.8}$ = -7.2, p > 0.05).

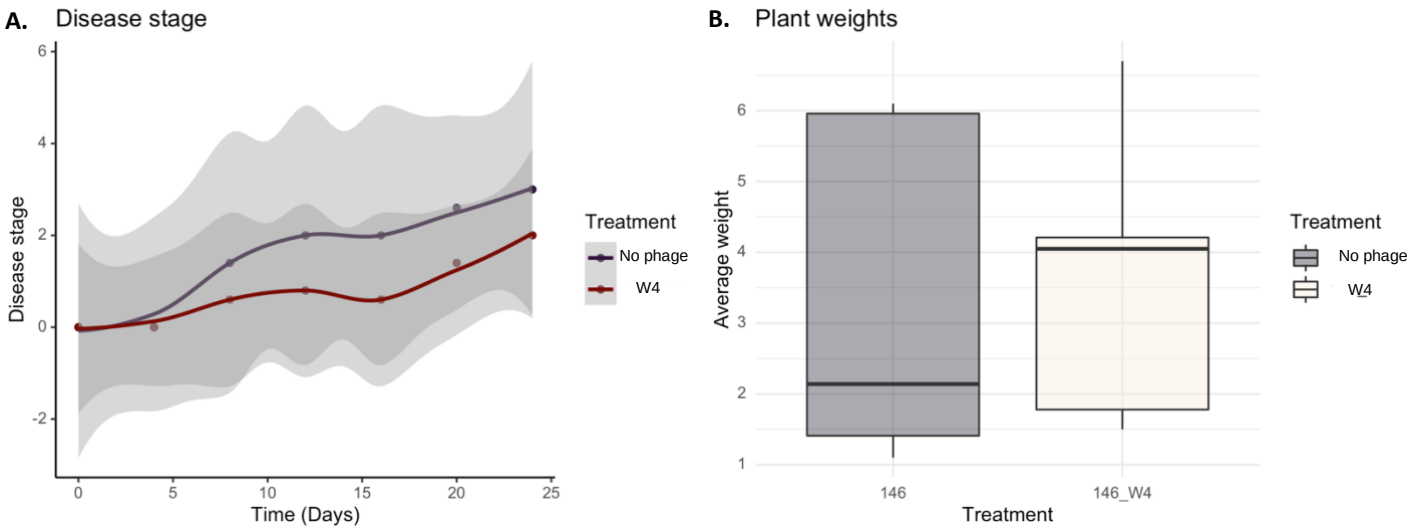

Figure 14. (A) The disease stage of tomato plants. Tomato plants were inoculated with *R. solanacearum* alone and *R. solanacearum* and phage. The disease stage of each plant was measured every 4 days for 24 days. The disease stage was averaged across 5 plants. **(B) The average dry weight biomass of tomato plants.** After 24 days tomato plants were removed from the soil, dried, and then weighed. The average dry plant biomass was plotted.

Discussion

There is a vast amount of literature detailing the potential of bacteriophage to treat devastating agricultural and clinical pathogens[7,12,13]. Hence, this has led to the creation of various initiatives such as Phage's for global health with the goal of bringing efficacious phage therapies to market. The development of an effective biocontrol phage therapy for *R. solanacearum* relies on understanding how phage and their host *R. solanacearum* interact[13,19]. Poor characterisation due to using systems poorly reflective of real-world conditions or a lack of understanding the evolutionary mechanisms at play results in mixed efficacy of treatments[11,15]. Understanding these interactions can facilitate discovery of more robust phage treatments for bacterial wilt effectuated by *R. solanacearum*. Our study aimed to effectively characterise phage strains isolated from the Thames River and tomato rhizosphere for their potential to treat *R. solanacearum*. To achieve this, we assessed phage bacterial evolutionary patterns across three different systems. This illustrated the importance of using conditions reflective of the field environment. In turn we also provided evidence of phage W4 as a potential treatment for *R. solanacearum* strain 146.

Firstly, the infectivity range of our phage isolates were tested against our *R. solanacearum* collection. Our results showed relatively large infectivity ranges for most of our isolates with four of our UK isolates being able to infect a wide range of UK *R. solanacearum* strains. In addition, 4 of the UK phage isolates were also able to infect strains which were geographically distinct to them. In contrast, 4 of the Chinese phage isolates were only able to infect a select few strains. The CP20 strain was the only Chinese isolate which exhibited a generalist infectivity range. There are a wide range of molecular determinants which effect the infectivity range of phage[34]. One of the most decisive of these is the specificity of the phage tail fibre protein as this protein dictates what the phage binds to during its infection cycle. The generalist ranges of our UK isolates and CP20 could be due to polyvalent activity of their tail fibre proteins[21,34]. Alternatively, these phages may be targeting a conserved bacterial surface molecules such as lipopolysaccharide as is reported in *Esericheia coli*[35]. In addition, other molecular determinants downstream such as endolysin resistance or restriction modification systems could be affecting host range[34,36]. After establishing our phage infectivity ranges the question of whether to favour phage with generalist or specialist infectivity ranges is an important question. It has been documented that specialist phage have a higher individual fitness against their hosts[37-39]. However there remains disadvantages with using specialist phage, such as, the rapid emergence of resistance against specialists, and you need to know the exact strain you are targeting.

The spot plating method used previously to determine phage infectivity range gives little insight into the dynamics of bacterial growth inhibition and emergence of phage resistance. To gain a more precise insight into if resistance is emerging and how *R. solanacearum* are growing in the presence of phage, we used a 96-well plate set up. This allowed us to periodically measure bacterial density (OD600nm). Four *R. solanacearum* strains; 081, 146, 380 and 385 were used on account of their high levels of virulence and distinct geographic areas of isolation. Phage W4 was assayed against these strains on account of its generalist infectivity range. The phage W4 was shown to be highly effective at inhibiting the growth of 146, 380 and 385 with very low densities recorded in any of these samples. This showed that W4 had a high fitness against these strains despite exhibiting a generalist infectivity range and was able to lyse these strains over the duration of the experiment. However, strain 081 appeared to

be immediately resistant to the phage despite forming clear plaques on agar plates. This potentially could be attributed to phase variation within the strain leading to a subset of phage resistant bacteria[40].

Following on from these results we wanted to examine our different phage isolates in more detail and see which of our phage were most effective at inhibiting *R. solanaceaum*'s growth. We selected a mixture of generalist and specialist phage and assayed them against the host *R. solanacearum* strain 146. Even through the same density of phage (PFU/ml) was used for each strain the generalist phage W4 exhibited much higher fitness compared to the other phage, W5, 42 and 47. It was shown to significantly reduce the growth of 146 relative to the no phage treatment in addition it significantly reduced *R. solanacearum* growth relative to the other phage treatments. This could be potentially due to W4 targeting a receptor a highly conserved receptor[35,38,41]. As such resistance was unable to emerge as rapidly as with the other phage strains. Since W4 was the most effective phage treatment and had a broad infectivity range we decided to characterise this phage further.

Unlike conventional agrochemical treatments phage have the advantage of being able to coevolve and adapt with their bacterial hosts[26-30]. We therefore wanted to characterise this unique advantage and assess the coevolutionary interactions between our phage W4 *R. solanacearum* 146 pairing to see if resistance emerges, and if so, can phage overcome this resistance. To do this we used a long-term evolutionary experiment in liquid media. Over the course of the experiment the bacterial density (OD600nm) of *R. solanacearum* was measured. This revealed that the W4 treatment was effective at suppressing the density of *R. solanacearum* for the duration of the experiment relative to the none treatment. Despite significant growth suppression *R. solanacearum* 146 was able to grow to much higher densities over the duration of this experiment than seen previously suggesting that some level of phage resistance has emerged. To quantify this resistance, phage resistance assays were conducted on *R. solanacearum* isolated from the end of the experiment. This revealed that *R. solanacearum* had developed some level of resistance to phage W4 compared to the ancestral bacteria. There are a myriad of ways through which *R. solanacearum* could have achieved resistance to phage W4. Throughout the lieterature previous studies exploring pahge *R. solanacearum* interactions have shown these can range from slight changes in bacterial metabolism to complete loss of metabolic pathways[17,26,28].

To examine the emergence of resistance and its associated cost we performed bacterial growth fitness assays on our evolved and non-evolved strains. This revealed that *R. solanacearum* after being grown in the presence of W4 phage had a significantly reduced fitness. Even when W4 phage was removed from the bacterium's environment its growth was significantly reduced in comparison to the ancestral bacterium. In contrast the control bacterium was still heavily inhibited by phage and grew significantly better when the phage selection was removed. These results suggest that *R. solanacearum* is evolving high levels of resistance, but this is at a strong fitness cost associated with this resistance leading to a reduction in bacterial density.

Despite showing promising inhibition of bacterial growth, the question of if W4 phage can coevolve to overcome the resistance of their bacterial hosts remains. To further shed light on the coevolutionary dynamics between phage and *R. solanacearum*, bacteria and phage were isolated from different time points and assayed against each other in liquid culture. This experiment revealed a

series of things, firstly ancestral *R. solanacearum* was inhibited to different extents by phages at different timepoints, with ancestral phage proving most effective. Suggesting W4 has evolved to strains at their specific timepoints. This is indicative of fluctuating selection dynamics (FSD), where bacterial and phage genotypes match through time[23,30]. Interestingly it has been reported within *pseudomonas aeruginosa* that FSD corresponds to the type of bacterial receptor which is being targeted[42]. It was shown that phage which target pili are more likely to exhibit FSD with their bacterial hosts[42]. Furthermore, the *R. solanacearum* from the final and second time points seemed to be resistant to all W4 phage, showing high levels of resistance against all phages. However, there seemed to be a significant trade-off with bacterial growth associated with this resistance, as 146 isolated from the day-8 and day-16 timepoints grew to much lower densities overall. If phage W4 is binding to bacterial pili, then loss of these pili can result in a significant reduction in bacterial fitness due to impeding swimming motility[42]. In addition, 146 isolated from the final time point grew to much lower density than any of the other isolated bacteria. This could be due to the cumulative effect of various trade-offs to generate sufficient resistance against phage from different time points, which in turn leads to a progressive decline in bacterial growth.

There are a host of reports suggesting that the cost of phage resistance can result in an avirulent *R. solanacearum* phenotype[32,42,43]. A potential mechanism for this is mutations within the *phc* gene. This is a critical virulence gene responsible for *R. solanacearum* EPS production and appears as a common mutation within stressful environments[32]. The mechanism underlying this is due to enhanced bacterial metabolism as a result of losing this pathway[32]. Therefore, after determining the strong effects phage W4 has on *R. solanacearum* 146 fitness, we set out to analyse if this resulted in improved plant disease outcome. The hydroponic system allowed us to track the density of *R. solanacearum* and phage whilst observing the effects on the plant. The density of *R. solanacearum* in the culture media in the non-phage treatment dropped dramatically 12 days into the experiment, this could be due to death of the plants and therefore a depletion of resources for the bacterium to grow. In the phage treatment there was a drop in bacterial density much earlier, likely due to the lytic activity of the phage. It was shown at the end of the experiment that the weights of the tomato plants treated with W4 were much higher and plant survival after 24 days significantly improved. As there was no observable difference in the density of *R. solanacearum* at the end of the experiment this increased *In planta* survival is most likely due to reduced bacterial fitness and virulence as a result of W4 phage resistance, as opposed to phage lytic activity[27.33]. To quantify this phage resistance assays were performed on *R. solanacearum* isolated from the final timepoint. This showed a significant reduction in fitness for *R. solanacearum* treated with W4 phage, therefore the observed improvement in tomato plant survival is likely attributed to reduced bacterial fitness.

After the promising results in the hydroponic and Liquid media systems we wanted to see how these results translated into an environment more reflective of real-world conditions. A soil system model using tomato plants inoculated with *R. solanacearum* 146 and W4 phage was utilised to achieve this. To examine the inhibitory effect W4 phage was having on *R. solanacearum* and if W4 phage could get inside the plant the PFU/ml and CFU/ml were calculated for all treatments and location (soil or stem) of the plant. This showed that phage was having significant lytic activity in the soil with a high PFU/ml and a very low density of *R. solanacearum*. The stem however had a significantly lower PFU/ml, furthermore there was no significant difference between the density of *R. solanacearum* in the stems of plants treated with or without W4. This would indicate that phage densities in the stem aren't high enough to effectively suppress *R.* solanacearum growth. Alternatively, significant levels of phage

resistance could have arisen. To quantify if phage resistance had emerged in the stem and the soil of each plant, we conducted fitness assays. Our fitness assays showed that *R. solanacearum* isolated from the stem had no significant difference in fitness between phage and non-phage treatments. Furthermore, in the soil extremely high levels of phage resistance were generated at no fitness cost. This suggests *R. solanacearum* can evade phage within the stem of the plant. The exact mechanism by which it can do this is unclear and could be due to phage not reaching high enough densities within the stem to effectively control the pathogen. Therefore *R. solanacearum* can escape phage predation through spatial escape. Unexpectedly, the *R. solanacearum* isolated from the soil was highly resistant to phage without any trade-off. The high densities of *R. solanacearum* in the stem therefore corresponded to dry plant biomass and disease symptoms having no significant difference compared to the non-phage treatments. It could be that *R. solanacearum* are able to effectively escape phage selection through alternative methods to the other two systems. This could be through spatial escape as phage aren't as able to get into the stem of the plant. On the other hand, the soil system is not as nutrient rich as the other two systems leading to a greater selection pressure on the bacteria and hence only de novo mutations resulting in highly fit bacterial populations are tolerated.

We have generated exciting data which demonstrates the need to use systems accurately reflective of field conditions when assessing phage treatments. In addition, we have demonstrated phage W4 as a potential treatment for *R. solanacearum* strain 146 in hydroponic and liquid media conditions. However, this research would strongly benefit from a range of future work. Some potential avenues for this would be sequencing of *R. solanacearum* isolates from each system. This would help develop an understanding of the mechanistic changes underlying the observed fitness trade-offs and associated phage resistance. Confirming this could yield insight into how phage can induce an avirulent genotype alongside why disease symptoms in the hydroponic system were alleviated and plant growth was improved yet the inverse was seen in a soil system model. It would also be beneficial to understand how the phage are adapting to their bacterial hosts over time through sequencing phage at different time points. If sequencing fails to uncover a mechanistic explanation to our results, it is possible that expression profiles between strains may differ in terms of phage defence systems or cellular receptors, as has been reported throughout the literature[36,40,43,44]. In this case RNAseq would provide an attractive option for future work.

The development of an effective bacteriophage biocontrol product heavily relies on characterisation of both the bacteriophage utilised and the pathogen target. This characterisation helps facilitate the discovery of which phage or combination of phage have the greatest pathogen-supressing abilities. In addition, resistance to phage often evolves rapidly. The current knowledge surrounding bacteriophage resistance and its effects on *R. solanacearum* remain poor, hence, the efficacy of phage treatments has remained unpredictable. To address this issue *R. solanacearum* strain 146 and bacteriophage W4 were examined in context of pathogen phage interactions. In turn we provide evidence of exposure to W4 effectively suppressing *R. solanacearums* growth, improving disease outcome and the cost of W4 resistance being attributed to fitness trade-offs. These findings indicate that W4 could be implemented as an effective control agent for *R. solanacearum* strain 146. Taken in conjunction with other studies the potential of phage therapy as a biocontrol is becoming to come to fruition.

Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this or any other university. All sources are acknowledged as references.

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