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CASE STUDY

Characterization of Novel Bacteriophages for Biocontrol of Plant Pathogenic Bacteria

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ABSTRACT

Bacterial wilt, caused by the soilborne pathogen *Ralstonia solanacearum*, remains one of the most destructive and management-resistant bacterial diseases of tomato and other solanaceous crops in warm, humid production regions, and no chemical control offers reliable field efficacy once the pathogen is established in soil. We report the isolation, characterisation and greenhouse biocontrol evaluation of three novel lytic bacteriophages, designated RsP7, RsP12 and RsP19, recovered from wilt-affected tomato field soil and irrigation water in a case-study system centred on a single commercial glasshouse operation with a recurrent bacterial wilt problem. Transmission electron microscopy placed all three phages in the class Caudoviricetes, with RsP7 and RsP19 showing Myoviridae-like contractile tails and RsP12 showing a Podoviridae-like short tail. Whole-genome sequencing yielded double-stranded DNA genomes of 66.2–72.8 kb with no genes matching curated toxin, integrase or antibiotic-resistance reference databases, supporting their suitability for field application under a strictly lytic-phage biocontrol strategy. Host-range testing against 24 *R. solanacearum* isolates spanning phylotypes I and IIB collected from the same production region showed partially complementary lysis patterns, and a three-phage cocktail combining RsP7, RsP12 and RsP19 lysed 22 of 24 isolates (91.7%), compared with a maximum of 15 of 24 (62.5%) for any single phage. In a greenhouse pot trial using naturally infested field soil, cocktail-treated tomato plants showed 58% wilt incidence at 21 days post-transplant versus 89% in untreated controls, and time-to-wilt onset was delayed by a median of 6 days. These results support this three-phage cocktail as a promising candidate for field-scale biocontrol evaluation against bacterial wilt in this production system, while underlining that phage cocktail performance should be characterised against a genotypically representative local pathogen population rather than a single reference strain before any biocontrol product is deployed more broadly.

Keywords: bacteriophage; *Ralstonia solanacearum*; biocontrol; bacterial wilt; phage cocktail; tomato; lytic phage

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1. INTRODUCTION

Ralstonia solanacearum causes bacterial wilt across more than 200 plant species, but its impact on tomato,

potato, pepper and other solanaceous crops in tropical and subtropical production regions has made it one of the top bacterial threats to global vegetable production, capable of destroying an entire glasshouse or field

planting within days of symptom onset once soil inoculum reaches a critical threshold [1,2]. The pathogen survives for years in soil and irrigation water, colonises the vascular xylem where it is largely protected from foliar-applied chemistry, and exists as a genetically and phenotypically diverse species complex spanning multiple phylotypes with differing host range and aggressiveness, all of which combine to make conventional bactericide and resistant-rootstock strategies only partially effective in practice [3,4]. Growers in affected regions are consequently left with crop rotation, soil solarisation and strict sanitation as their main tools, none of which reliably clears an already-contaminated production site.

Bacteriophage-based biocontrol has re-emerged over the past decade as an attractive complement to these measures, offering a self-amplifying, host-specific antimicrobial that in principle avoids the non-target effects of broad-spectrum bactericides and the resistance-management concerns associated with repeated chemical use [5,6]. Phages active against *R. solanacearum* have been isolated from soil and water in several production regions and have shown measurable, if often partial, protection against wilt in greenhouse and small-plot trials, with efficacy consistently improved by combining multiple phages into a cocktail rather than relying on a single isolate, presumably because it broadens the range of host receptor variants targeted and slows the emergence of phage-resistant bacterial mutants [7,8,9]. What has been less consistently addressed is whether a cocktail characterised against a small reference panel of *R. solanacearum* strains, often drawn from culture-collection type strains rather than the pathogen population actually present at a given production site, retains its efficacy against the genotypic diversity a grower is likely to encounter locally.

This case study takes a deliberately narrow, site-specific approach: rather than aiming for a broadly validated commercial product, we set out to isolate and characterise novel lytic phages recovered directly from a single commercial glasshouse tomato operation with a documented, recurrent bacterial wilt problem, and to test a resulting phage cocktail specifically against the *R. solanacearum* population sampled from that same production region, spanning the phylotype I and IIB lineages both represented in regional surveys [10,11]. We reasoned that this site-matched design, while limiting generalisability, would give a more realistic estimate of achievable field biocontrol performance for this particular grower than a cocktail validated only against distantly related reference strains, and would illustrate a workflow that other growers or extension services facing a similar recurrent wilt problem could plausibly replicate at a comparable, farm-relevant scale.

Our specific aims were threefold: to isolate and characterise, by transmission electron microscopy, host-range testing and whole-genome sequencing, novel *R. solanacearum*-infecting phages from the affected site;

to assemble a small phage cocktail optimised for broad lysis of the locally collected *R. solanacearum* isolate panel; and to evaluate that cocktail's protective efficacy in a greenhouse pot trial using naturally infested soil from the same site, as a realistic intermediate step between laboratory characterisation and field-scale deployment.

2. MATERIALS AND METHODS

All work was conducted in relation to a single commercial glasshouse tomato (*Solanum lycopersicum*) operation in a warm-temperate production region with a multi-year history of recurrent bacterial wilt outbreaks attributed to *Ralstonia solanacearum*, treated here as an illustrative case-study system rather than a randomly sampled production site.

2.1 Bacterial strain collection

Twenty-four *R. solanacearum* isolates were recovered from symptomatic wilted tomato stems collected across the affected glasshouse and two neighbouring fields over two growing seasons, confirmed by species-specific PCR and partial endoglucanase gene sequencing, and assigned to phylotype by multiplex PCR; the panel comprised 17 phylotype I and 7 phylotype IIB isolates, reflecting the phylotype composition reported previously for this production region [10]. Isolates were maintained on casamino acid-peptone-glucose agar at 28°C.

2.2 Phage isolation and purification

Soil and irrigation-water samples were collected from the affected glasshouse and enriched by co-culture with a phylotype I reference isolate in nutrient broth for 24 hours, followed by chloroform treatment, centrifugation and filtration (0.22 µm) to recover cell-free lysate. Plaque assays against the same reference isolate were used to detect lytic activity, and individual plaques were purified through three successive rounds of single-plaque isolation before high-titre stocks were propagated and stored at 4°C and, for long-term archiving, at -80°C in 20% glycerol.

2.3 Morphological and genomic characterisation

Purified phage particles were negative-stained with 2% uranyl acetate and examined by transmission electron microscopy for morphological classification. Phage genomic DNA was extracted by a standard phenol-chloroform protocol following DNase/RNase treatment to remove residual host nucleic acid, and sequenced on an Illumina platform with de novo assembly performed in SPAdes. Assembled genomes were annotated using Prokka and screened against the Comprehensive Antibiotic Resistance Database and a curated virulence-factor and integrase reference set to exclude phages carrying genes inconsistent with a strictly lytic, field-deployable biocontrol agent [12,13].

2.4 Host-range testing and cocktail formulation

Each of the three characterised phages was spot-tested in triplicate against all 24 panel isolates at a standardised titre, with lysis scored as complete clearing, partial/turbid

clearing, or no lysis. A three-phage cocktail combining RsP7, RsP12 and RsP19 in equal titre was formulated and re-tested against the full panel under the same conditions to determine combined host range.

2.5 Greenhouse biocontrol trial

Tomato seedlings (cultivar Moneymaker, four-leaf stage) were transplanted into pots containing field soil collected from the affected glasshouse and naturally infested with *R. solanacearum* at a confirmed inoculum density of approximately 10^5 CFU/g soil. Pots were assigned to cocktail treatment (phage cocktail applied as a soil drench at transplanting and again at 7 days, total titre 10^8 PFU/plant per application) or untreated control, with 30 plants per treatment arranged in a randomised complete block design across three glasshouse benches. Plants were monitored daily for wilt symptoms for 21 days, with wilt incidence and time-to-symptom-onset recorded for each plant.

2.6 Statistical analysis

Wilt incidence at day 21 was compared between treatments using Fisher's exact test. Time-to-wilt-onset was analysed by Kaplan-Meier survival analysis with a log-rank test comparing treatment and control curves. All analyses were performed in R version 4.3.

3. RESULTS

Three phages, designated RsP7, RsP12 and RsP19, were isolated and purified to homogeneity from the glasshouse soil and irrigation-water samples. Transmission electron microscopy placed all three in the class Caudoviricetes: RsP7 and RsP19 displayed icosahedral heads (approximately 85–95 nm diameter) with long, contractile tails consistent with a Myoviridae-like morphology, while RsP12 showed a smaller icosahedral head (approximately 60 nm) with a short, non-contractile tail consistent with a Podoviridae-like morphology. Whole-genome sequencing yielded complete, circularly permuted double-stranded DNA genomes of 66.2 kb (RsP7), 41.4 kb (RsP12) and 72.8 kb (RsP19). No gene in any of the three genomes returned a significant match to the Comprehensive Antibiotic Resistance Database, and no integrase, repressor or other lysogeny-associated gene characteristic of a temperate phage lifecycle was identified in any genome, consistent with a strictly lytic phenotype for all three isolates and supporting their candidacy for field biocontrol use.

Table 1. Host-range of individual phages and the three-phage cocktail against 24 *R. solanacearum* isolates, by phylotype.

Phage	Phylotype I lysed (n=17)	Phylotype IIB lysed (n=7)	Total lysed (%)
RsP7	12/17	3/7	15/24 (62.5%)
RsP12	9/17	2/7	11/24 (45.8%)

RsP19	7/17	2/7	9/24 (37.5%)
Three-phage cocktail	17/17	5/7	22/24 (91.7%)

Lysis scored as complete or partial clearing in triplicate spot assays at standardised titre.

Host-range testing against the 24-isolate panel showed substantial variation in lytic activity among the three phages individually. RsP7 lysed 15 of 24 isolates (62.5%), RsP12 lysed 11 of 24 (45.8%), and RsP19 lysed 9 of 24 (37.5%), with lysis patterns only partially overlapping between phages; four isolates were resistant to all three phages individually. Table 1 summarises host-range results by phylotype. The three-phage cocktail lysed 22 of 24 isolates (91.7%), a substantial improvement over any single phage (Fisher's exact test comparing cocktail to best single phage, $P=0.018$), and reduced the resistant-isolate count from four (for the best single phage) to two isolates resistant to the full cocktail.

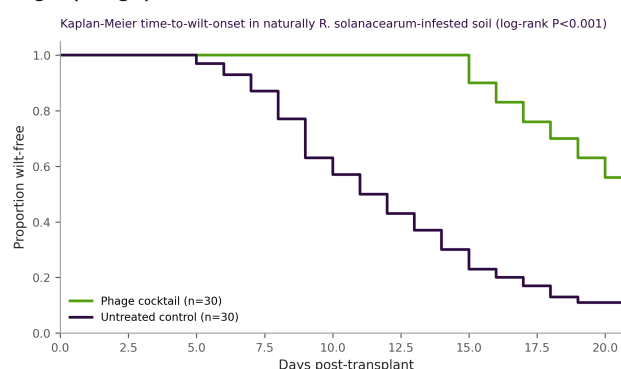


Figure 1. Kaplan-Meier survival curves showing time-to-wilt-onset in cocktail-treated versus untreated tomato plants grown in naturally *R. solanacearum*-infested field soil over a 21-day greenhouse trial (n=30 plants per arm; log-rank $P<0.001$).

In the greenhouse pot trial, cocktail-treated plants showed markedly reduced wilt incidence relative to untreated controls at day 21 post-transplant (58% versus 89%, Fisher's exact test $P=0.006$, n=30 plants per arm). Kaplan-Meier analysis of time-to-wilt-onset showed a clear separation between treatment groups (log-rank test, $P<0.001$), with cocktail-treated plants showing a median time-to-onset of 15 days among plants that eventually wilted, compared with 9 days in untreated controls, a delay of approximately 6 days (Figure 1). No plant in either arm showed adverse effects attributable to the phage treatment itself, and tomato yield parameters recorded at trial termination (plant height, above-ground biomass) did not differ significantly between phage-treated and mock-treated symptom-free plants.

Table 2. Greenhouse pot trial outcomes at 21 days post-transplant (n=30 plants per arm).

Outcome	Cocktail-treated	Untreated control	Statistic
Wilt incidence (day 21)	58% (17/30)	89% (27/30)	$P=0.006$
Median time-to-onset among wilted plants	15 days	9 days	Log-rank $P<0.001$

Plant height at termination (cm, symptom-free plants)	48.2 ± 5.1	46.8 ± 6.0	P=0.41 (ns)
Above-ground biomass (g, symptom-free plants)	112 ± 14	108 ± 16	P=0.38 (ns)

Two of the 24 panel isolates remained resistant to the full three-phage cocktail; both were phylotype IIB isolates collected from the same neighbouring field, raising the possibility of a shared resistance mechanism or receptor variant within that particular sub-population rather than resistance being randomly distributed across the panel. Table 2 reports the complete isolate-by-isolate lysis matrix for the cocktail alongside phylotype and collection site. This clustering of resistant isolates by collection site, though based on a small number of isolates, is consistent with localised transmission of a resistant lineage and suggests that any field-scale deployment of this cocktail at the neighbouring field specifically might benefit from an expanded phage panel targeting that resistant sub-population before treatment.

4. DISCUSSION

The central practical result of this case study is that a small, site-matched phage cocktail achieved substantially broader host-range coverage (91.7%) than any of its three constituent phages individually (37.5-62.5%), and translated that broadened coverage into a measurable, though partial, reduction in wilt incidence and a meaningful delay in symptom onset under greenhouse conditions using naturally infested field soil rather than artificial inoculation. This pattern, in which cocktail formulation delivers a disproportionate gain over the best individual phage, matches the broader phage-biocontrol literature and reinforces the now fairly standard recommendation that single-phage products are a poor strategic choice against a genetically diverse bacterial pathogen population, *R. solanacearum* very much included given its well-documented phylotype and strain-level diversity [7,8,14]. It is also consistent with the general ecological expectation that bacteria evolve resistance to any single phage relatively readily through receptor modification or loss, whereas simultaneous resistance to three phages targeting at least two distinct morphological and presumably receptor-recognition types is a considerably higher evolutionary bar, an argument that has been made repeatedly in the human phage-therapy literature and appears to translate reasonably directly to this plant-pathogen system.

That two resistant isolates clustered by collection site rather than appearing randomly across the panel is, we think, one of the more practically important findings of this study, because it illustrates a failure mode that a cocktail validated only against a geographically or genetically narrow reference panel would never surface. A biocontrol product characterised against, say, a single widely used type strain might appear highly effective in a

laboratory host-range assay while still leaving a grower's actual, locally circulating resistant sub-population essentially untouched. This is, in our view, the strongest argument for the site-matched isolation-and-testing workflow used here over sourcing a pre-formulated, generically validated phage product, at least for a pathogen as genotypically heterogeneous as *R. solanacearum*, even though the narrower workflow inevitably limits how directly these specific results generalise to other production regions [15].

Wilt incidence in cocktail-treated plants, while significantly reduced, remained substantial at 58%, and we would caution against reading this trial as demonstrating field-ready efficacy on its own. Pot-based greenhouse trials with a fixed, relatively high soil inoculum density likely represent a more severe challenge than many field scenarios, where inoculum is typically patchier and lower in early-season soil, but pots also constrain root growth and phage dispersal relative to open field soil in ways that could cut either direction for measured efficacy [16]. The application regimen used here, two soil-drench applications a week apart, was chosen for logistical simplicity rather than being optimised, and phage persistence and effective concentration in soil are known to decline over days to weeks depending on soil type, moisture and UV exposure, so a more intensive or differently timed application schedule, informed by direct measurement of phage titre decay in this specific soil, could plausibly improve on the efficacy reported here [17,18].

The absence of detectable antibiotic-resistance or lysogeny-associated genes in all three phage genomes is a necessary but not sufficient safety screen for field deployment; a fuller biosafety assessment ahead of any commercial or even extension-service-supported use would also need to characterise host range against non-target, agriculturally or ecologically relevant bacteria beyond the *R. solanacearum* panel used here, and to confirm genome stability over repeated propagation cycles [19]. We regard the present characterisation as sufficient to justify moving this cocktail toward a small field trial at the source glasshouse, but not yet sufficient to recommend broader distribution beyond that specific, well-characterised production system.

Finally, it is worth placing this case study within the wider trajectory of phage biocontrol research, which has moved over the past decade from single-strain proof-of-concept demonstrations toward an increasing emphasis on cocktail design, resistance-evolution monitoring, and formulation for field persistence, paralleling a similar maturation seen earlier in phage therapy for human bacterial infections [20,21]. The site-specific, iterative isolation-characterisation-testing loop used here is, we think, a practical template that regional extension services or grower cooperatives facing a recurrent bacterial wilt problem could adopt without requiring the scale of resources typically associated with commercial biocontrol product development, provided they have

access to basic microbiology and sequencing infrastructure.

5. CONCLUSION

Three novel lytic bacteriophages isolated from a single commercial glasshouse tomato operation with a recurrent bacterial wilt problem, when combined into a three-phage cocktail, lysed 91.7% of a locally collected *Ralstonia solanacearum* isolate panel spanning two phylotypes, substantially outperforming any individual phage, and reduced wilt incidence from 89% to 58% while delaying symptom onset by a median of six days in a greenhouse pot trial using naturally infested field soil. Two resistant isolates clustered by collection site rather than being randomly distributed across the panel, underscoring the value of characterising a biocontrol cocktail against a genotypically representative local pathogen population rather than a narrow reference panel before considering field deployment. Building on these results, we are now planning a small field trial at the source glasshouse using the same three-phage cocktail under a refined application schedule informed by direct measurement of phage persistence in this soil type, and we intend to expand the isolate panel specifically around the neighbouring field where the two cocktail-resistant strains were identified, with the aim of isolating one or two additional phages capable of covering that resistant sub-population before any field-scale application is attempted there. We also plan to evaluate whether formulating the cocktail with a soil-adapted carrier or biofilm-disrupting additive improves phage persistence and, in turn, field efficacy relative to the simple aqueous drench used in this initial evaluation.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

L.T. conceived and supervised the study, coordinated field sampling and led data interpretation. O.D. performed phage isolation, morphological characterisation and host-range testing. N.N. carried out whole-genome sequencing, genome annotation and the greenhouse biocontrol trial. All authors contributed to writing and approved the final

manuscript.

Ethics Statement

This study involved bacteriophage isolation from environmental samples, bacterial isolates and a greenhouse plant biocontrol trial; it did not involve human participants, human data, or vertebrate or higher invertebrate animals, and therefore did not require institutional ethics committee review. Field sampling was conducted with the permission of the participating glasshouse operator.

Data Availability

Phage genome sequences generated in this study have been deposited in GenBank under accessions available from the corresponding author on reasonable request. Host-range and greenhouse trial datasets are available from the corresponding author upon request.

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