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Bacteriophage-Mediated Reduction of Bacterial Speck on Tomato Seedlings

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Abstract

Background: One crucial first step in bacteriophage therapy is choosing a phage to apply, which involves screening for effectiveness in a meaningful way. Increasingly, research suggests that *in vitro* tests of phage-mediated bacterial lysis poorly translate to *in planta* effectiveness.
Materials and Methods: We tested a seedling-based method for rapidly screening phage effectiveness *in vivo*. In three trials, phages were prophylactically applied to tomato seedlings in sterile conical tubes before flooding with the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000. We recorded seedling disease progression and quantified endpoint bacteria and phage densities.
Results: Phages replicated in all trials, but reduction of disease symptoms and endpoint *P. syringae* density varied across trials with different application densities.
Conclusions: This resource-efficient method rapidly identified an effective phage and application density to mitigate disease on seedlings. We propose that this method could be used to screen candidate phages before testing in agricultural conditions.

Keywords: phage therapy, disease control, *Pseudomonas syringae*, tomato, seedling

Introduction

Agricultural yields are threatened by declining soil health, climate change, insect pests, and microbial pathogens.[1–5] Although global yield of major crops has increased for decades, the current rate of increase is predicted to be insufficient to meet nutritional needs by 2050.[6,7] Bacterial pathogens can impact yield through both destructive epidemics and more subtle losses such as fruit quality reduction.[8–12] Current control methods include breeding for genetic resistance, chemical application, antibiotics, and modifications of growing practices to reduce transmission.[13] However, the rise of antimicrobial resistance has increasingly challenged the effectiveness of many management strategies. Bacteria have evolved resistance to both chemical treatments and antibiotics, and have evolved counteradaptations that allow them to overcome host genetic resistance.[14–16]

The *Pseudomonas syringae* species complex has been a particularly difficult management challenge and was responsible for several recent disease outbreaks, notably including kiwifruit bacterial canker (reviewed in Lamichhane et al.).[17–19] The species complex as a whole infects a wide range of agriculturally important hosts, and the host range of individual strains varies from specialized to generalized.[20] The pathovar *P. syringae* pv. *tomato* infects tomato (*Solanum lycopersicum*) and causes bacterial speck disease, which produces lesions on leaf and fruit surfaces.[21] Infection by this pathogen can reduce yield by impacting host photosynthetic ability and fruit quality.[21,22] *P. syringae* can transmit vertically through seeds, horizontally through water droplets to nearby plants, and even long distance through aerosolization, making it difficult to completely prevent infection.[23,24] Disease control strategies have included breeding for genetic resistance and long-term application of copper compounds, both of which have suffered from reduced efficacy due to pathogen evolution.[17,25–31] More recently, growers and researchers have turned to exploring biocontrol methods such as bacteriophage (phage) therapy as an option for *P. syringae* management.[32,33]

Lytic phages have been underexplored for treating plant diseases, but interest has been reinvigorated with recent successes in clinical phage therapy and the spread of antibiotic resistance.[32,34] To infect a cell, phages first bind to a receptor on the cell surface, inject their genomic material, use

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host resources to synthesize proteins and new copies of their genome, and ultimately burst the cell to release viral particles. Phages are a promising treatment due to their ability to replicate, coevolve with bacteria, and their limited impact on the resident microbial community (due to their narrow host ranges).[35] However, their narrow host ranges also make it challenging to rapidly characterize phages that infect novel outbreak-causing bacterial strains.[36] In addition, *in vivo* effectiveness of phage therapy candidates has often been unpredictable from results *in vitro*,[37–39] but *in vivo* trials can be time consuming and resource intensive.[40] Success of agricultural phage therapy trials has been mixed, with some understanding of the environmental factors that can impact phage persistence (such as UV, temperature, and desiccation) and effective methods for reducing phage decay (using protective formulations and evening applications), but with little mechanistic understanding of the variation across studies and systems.[41–43]

We sought to develop a seedling-based method for measuring phage-mediated reduction of bacterial speck disease on tomato. We performed three trials wherein we assessed the effectiveness of phage pretreatment at reducing disease symptoms and endpoint bacterial densities. One of the two tested phages successfully replicated in all trials, and reduced disease symptoms and pathogen density in two of three trials. This method is rapid (can identify effective phages in <2 weeks) and space efficient (each seedling requires the space of a single conical tube), allowing researchers to identify suitable candidate phages *in vivo* using minimal time and resources.

Materials and Methods

Seed sterilization and germination

Tomato seeds (*S. lycopersicum* cultivar Moneymaker) were sterilized in 70% ethanol for 1 min, followed by a 20 min soak in sterilization solution (one part 8.25% bleach, three parts 0.2% Tween 20 in water). Seeds were then washed in an excess of autoclaved MilliQ water, and placed in loosely capped sterile 15 mL tubes with 7 mL water agar (one seed per tube). Tube racks were covered in aluminum foil and placed in a 21°C chamber and checked daily for signs of germination. Postshoot emergence, tubes were moved to a 28°C chamber with a 15 h day–9 h night cycle.

Bacteria and phage preparation

Bacterial cultures were grown from a freezer stock of *P. syringae* pv. *tomato* DC3000 that originated from a single colony. Cultures were shaken in King's B (KB) liquid medium at 28°C until stationary phase. Cells were then pelleted and washed in sterile 10 mM $MgCl_2$ to remove culture medium. Bacterial suspensions were then adjusted to the desired optical density (OD600 of 0.002 in the first trial, and 0.0002 in the others) with 0.015% filter sterile Silwet.

Two phages were used for this study, *Podoviridae* phage FRS (hereafter P1) and *Myoviridae* phage SHL (hereafter P2), both of which were isolated from water by OmniLytics, Inc. as candidate biocontrol agents and were characterized using microscopy and whole genome sequencing by the Koskella laboratory. In the first trial, phages were prepared using a ''webby plate'' of each phage stock on a lawn of DC3000. Phages were recovered by swirling 10 mM $MgCl_2$ on each plate. The recovered volume was filtered (0.45 μm) and quantified by dilution series on double agar overlay plates to determine plaque forming units (PFUs). This method yielded low concentration phage stocks that contained visible amounts of plating media (yellow). Therefore, in subsequent trials, phages were prepared by coculturing with DC3000 in liquid KB. Cocultures were shaken overnight at 28°C, filtered, and quantified by PFUs. Lysates were then diluted in a large volume of 10 mM $MgCl_2$ to the desired concentration for seedling inoculations. In the third trial, we included an ''inactivated'' phage treatment as an additional control. A lysate of P1 was autoclaved for 30 min at 121°C with 15 psi pressure, which completely eliminated infectious phage particles (as determined by PFUs), and this solution was used as the inactivated phage treatment.

Throughout the text and figures, we use the following abbreviations for treatment names: DC3000 only is ''B only,'' DC3000 with FRS phage is ''B+P1,'' DC3000 with SHL phage is ''B+P2,'' DC3000 with autoclaved FRS phage is ''B+inactivated P1,'' FRS phage only is ''P1 only,'' SHL phage only is ''P2 only,'' autoclaved FRS phage only is ''Inactivated P1 only,'' and the magnesium chloride control is ''$MgCl_2$ control.''

Inoculation and seedling incubation

In each trial there were two inoculation rounds, with all steps performed aseptically. First, either active phage, inactivated phage, or 10 mM $MgCl_2$ was added to each seedling (depending on the randomly assigned treatment). Seven to 12-day-old seedlings were flooded with 7 mL of the appropriate solution and placed on an orbital shaker at room temperature for 4 min. We then removed the solution and dried each tube in a biosafety cabinet. In the next round of inoculations, either 7 mL of the prepared bacterial suspension or 10 mM $MgCl_2$ was added to each tube as appropriate for the treatment. Tubes were shaken for 4 min before liquid removal and drying. This inoculation method minimizes the possibility of bacteria–phage interactions occurring in residual liquid on the tube walls or water agar surface. Seedlings were then placed in a 28°C chamber with a 15 h day–9 h night cycle. Disease symptoms were scored blindly and approximately daily, following the protocol of Morella et al.[44] Trials differed in their tested treatments and applied bacteria and phage densities (Table 1).

Endpoint bacteria and phage quantification

At the endpoint of each trial, each whole seedling was individually weighed and homogenized in 10 mM $MgCl_2$ with two sterile ceramic beads in a FastPrep-24™ 5G (MP Biomedicals, USA) set to 4 m/s for 60 s. We quantified bacterial densities by dilution plating and counted colony forming units (CFUs) after 48 h of incubation at 28°C. In addition, we quantified bacteria and P1 densities using droplet digital™ polymerase chain reaction (ddPCR; Bio-Rad, USA), a DNA-based quantification technique. For each reaction, we used 3 μL of 1:10 homogenate diluted in water as template, and included fluorescent probes specific to Pseudomonads and phage P1 (as in Morella et al., and using the same setup and reaction conditions).[45] After the third trial, we performed a small-scale phage resistance assay by isolating

Table 1. Treatment and Inoculation Details for Each Trial

Trial no.	*Seedling age, days*	*Treatments* (n)	*Bacterial density (OD600)*	*Applied MOI (phage:bacteria)*
1	12	$MgCl_2$ control (3), P1 only (3), P2 only (3), B only (8), B+P1 (8), B+P2 (8)	0.002	0.015:1
2	7	$MgCl_2$ control (3), P1 only (6), B only (6), B+P1 (6)	0.0002	8.15:1
3	11	$MgCl_2$ control (3), P1 only (7), Inactivated P1 only (7), B only (7), B+P1 (7), B+inactivated P1 (7)	0.0002	455:1

P1: phage 1 (FRS); P2: phage 2 (SHL); B: bacteria (*P. syringae* pv. *tomato* DC3000).
MOI, multiplicity of infection; OD600, optical density at 600 nm.

eight colonies from five of the replicate B+P1 samples, and four colonies from the sixth. We streaked overnight cultures of each colony across a high titer line of P1 on a hard agar plate (cross-streak method), and incubated for 48 h at 28°C. Colonies that could grow over the phage line were recorded as resistant, and those that could not were recorded as sensitive.

Statistics and figures

Figure 1 is an overview of our experimental methods and was made using BioRender. Generation of plots for all other figures and statistical analysis was done in R version 3.6.2 using packages lme4, emmeans, PMCMR, and dunn.test.[46–50] Figure panels were compiled in Adobe Illustrator. The effect of treatment on disease progression over time in each trial was analyzed as a mixed model testing for a treatment by day interaction, with a random effect for each seedling. We then tested for the effect of treatment on the area under the disease progression curve (AUDPC), endpoint bacterial densities (CFU and ddPCR), and endpoint phage densities in each trial separately using either linear models or Kruskal–Wallis tests (for models with poor fit, as determined by visual inspection of residuals). A linear model of the relationship between CFU and ddPCR copies per milligram seedling was generated for all bacteria-inoculated samples, while controlling for the effects of treatment and trial. Similarly, we quantified the relationship between endpoint bacteria and phage densities in a single treatment of interest (B+P1) using a linear model controlling for the effect of trial. Post hoc analysis for linear models was done comparing estimated marginal means, with a Tukey adjustment for multiple testing. For the nonparametric Kruskal–Wallis tests, post hoc analysis was done using Dunn's tests with a Bonferroni correction for multiple testing of planned comparisons. An alpha level of 0.05 was used in all statistical tests. All data files are available in the Dryad data repository (DOI: 10.6078/D15T4M).

Results

Phage-mediated reduction of disease symptoms

In all trials, the effect of inoculum treatment on disease score depended on day postinoculation (trial 1: $F(20, 102.36) = 6.47$, $p < 0.001$; trial 2: $F(12, 68) = 2.04$, $p = 0.0338$;

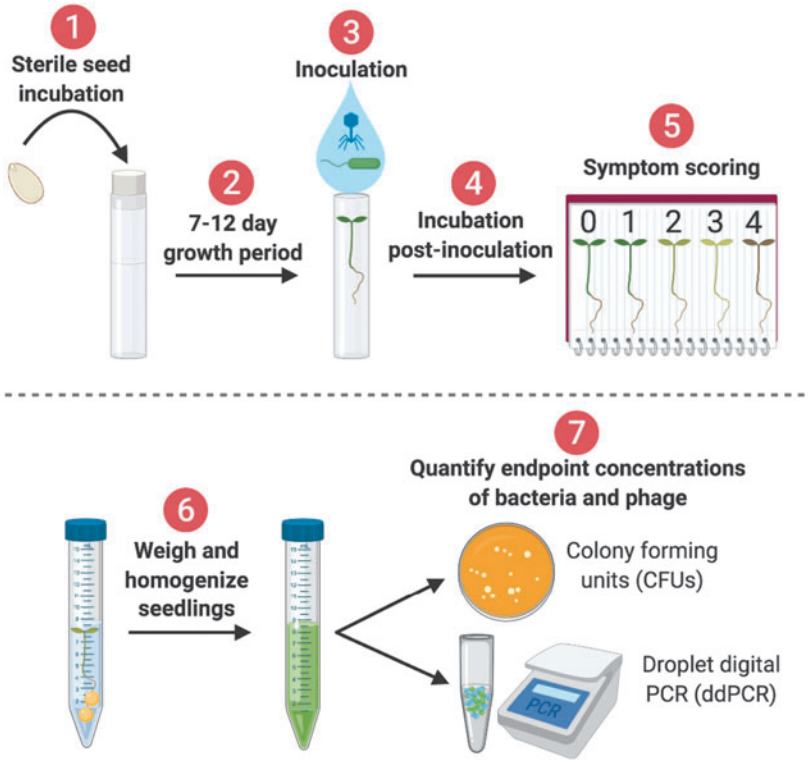

FIG. 1. Diagram of seedling inoculation and sampling methods, created with BioRender.com. Note that representative images of seedling symptoms associated with each disease score can be found in Morella et al.[44]

trial 3: $F(30, 192) = 8.07$, $p < 0.001$; Fig. 2A). In the first trial, no treatments differed in their starting disease scores (all $p > 0.98$), but all bacteria-inoculated treatments had higher final disease scores than those inoculated without bacteria (all $p < 0.005$). Phage treatment did not reduce disease symptoms on the final day (B+P1 and B+P2 each compared with B only, $p > 0.8133$), but on the second to last day, B+P1 disease scores were significantly lower than those of B only ($p = 0.0209$). With this preliminary result, we chose to move forward testing only P1 at a higher dose in subsequent experiments. In trials 2 and 3, phage P1 significantly reduced final disease symptoms compared with bacteria inoculated without phage (in both trials $p < 0.001$). In addition, B+P1 symptoms were not significantly different from controls (magnesium chloride and phage only) in both trials ($p > 0.74$). Autoclaved phage did not significantly reduce final disease symptoms compared with bacteria alone ($p = 0.99$).

Analysis of the AUDPC results suggested the same qualitative findings as the mixed model analysis of disease scores over time (Fig. 2B). In all trials and comparing across all treatments (including controls), treatment significantly impacted AUDPC (trial 1: $F(5, 25) = 9.91$, $p < 0.001$; trial 2: $H(3) = 12.94$, $p = 0.0048$; trial 3: $F(5, 32) = 7.84$, $p < 0.001$). In trial 1, neither phage P1 nor phage P2 had an impact on disease symptoms compared with bacteria only (P1:

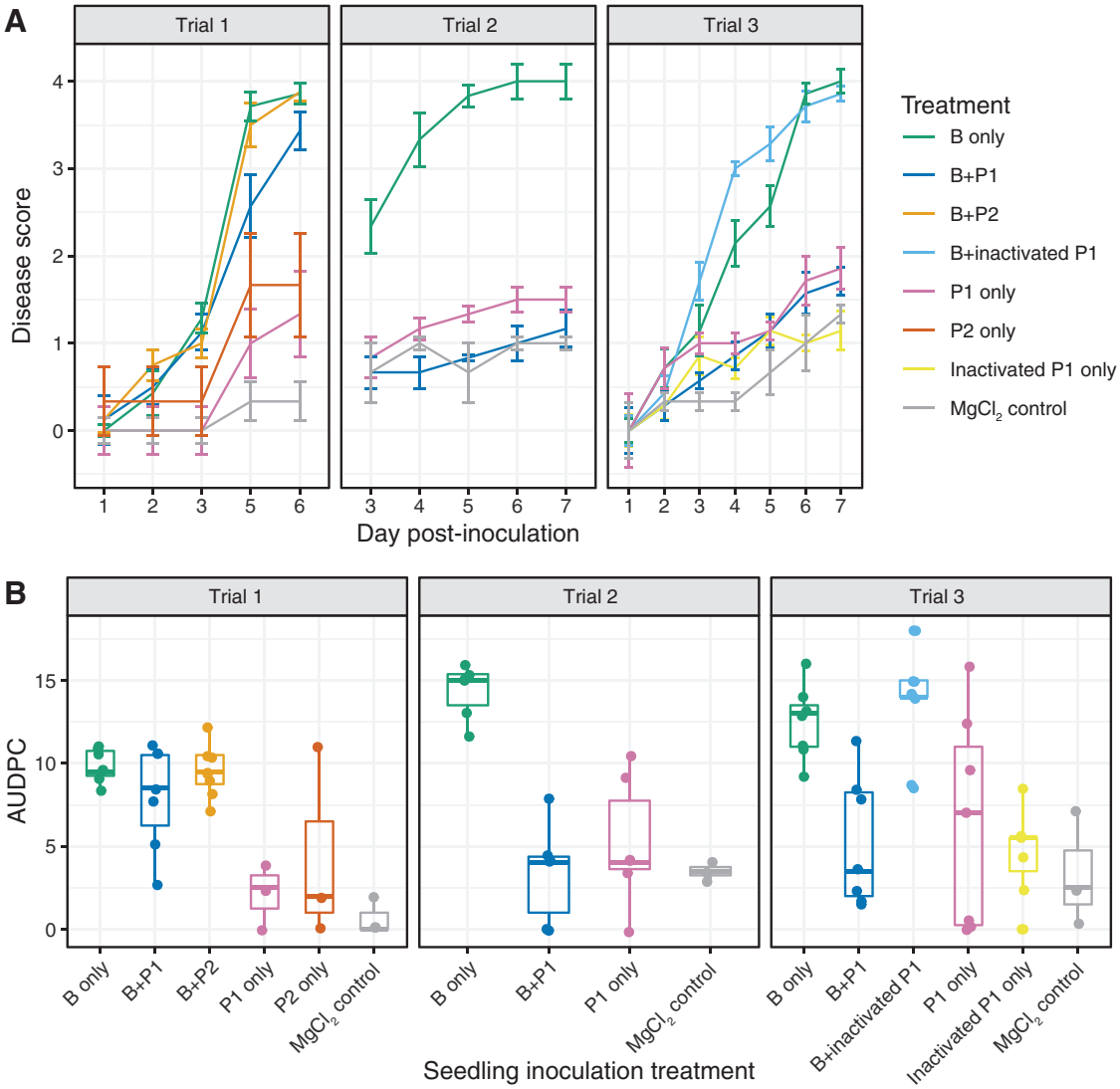

FIG. 2. Disease progression curves and AUDPC in all trials. Treatment abbreviations are as follows: DC3000 only is "B only," DC3000 with phage FRS is "B+P1," DC3000 with phage SHL is "B+P2," DC3000 with autoclaved phage FRS is "B+inactivated P1," phage FRS only is "P1 only," phage SHL only is "P2 only," autoclaved phage FRS only is "Inactivated P1 only," and the magnesium chloride control is "$MgCl_2$ control." **(A)** Seedling disease scores by day postinoculation for the tested treatments in each trial. The line represents the mean score for the treatment, and error bars are ±1 SE, corrected for the within-subjects design. **(B)** Boxplots of AUDPC values for each treatment in each trial. The horizontal line within the box displays the median value, the box displays the interquartile range, and the whiskers represent ±1.5 times the interquartile range. Points outside the whiskers are possible outliers. AUDPC, area under the disease progression curve.

$p = 0.7205$; P2: $p = 0.99$), but disease scores were reduced by phage P1 in trials 2 and 3 (trial 2: $p = 0.016$; trial 3: $p = 0.0211$). Autoclaved phage did not reduce AUDPC ($p = 0.97$).

Active phages replicate on seedlings and reduce bacterial densities

We analyzed endpoint densities in two ways: first using ddPCR to quantify the absolute number of DNA copies that match our bacteria and phage probes, and then quantifying CFUs of bacteria. For ddPCR results, we included all samples in the initial test of a treatment effect on endpoint bacteria and phage densities (Fig. 3A). Treatment significantly impacted densities of bacteria and phage in all trials (all $p < 0.002$). Compared with bacteria-inoculated seedlings without phage, phage P1 reduced bacterial densities in trials 2 and 3 (trial 2: $p = 0.0039$; trial 3: $p = 0.015$), but autoclaved phage had no effect ($p = 1.00$). We tested whether phage replication occurred by comparing P1 densities in B+P1 and P1 only treatments, and found that B+P1 phage densities were significantly higher in all trials (trial 1: $p = 0.0167$; trial 2: $p = 0.0050$; trial 3: $p = 0.0014$). We then hypothesized that samples with the highest phage densities may have the lowest bacterial densities, which we tested using densities from the B+P1 treatment across all trials. Interestingly, we instead observed a significant positive correlation between endpoint bacteria and phage densities ($b = 0.64$, $t(16) = 5.37$, $p < 0.001$; Fig. 3C), suggesting that phage replication may be limited by bacterial density.

CFU results led to similar qualitative conclusions and were positively correlated with ddPCR data ($b = 0.22$, $t(46) = 2.33$, $p = 0.0241$; Supplementary Fig. S1). In the case of CFUs, statistical analysis only included treatments inoculated with bacteria (both in the presence and absence of phage), as all others were zero except for a single contaminated sample. In trial 1, there was no effect of phage on bacterial densities ($F(2, 19) = 0.16$, $p = 0.8563$), as already observed, but phage P1 reduced bacterial densities in trial 2 ($F(1, 10) = 21.33$, $p = 0.0010$; Fig. 3B). In trial 3, there was an overall main effect of treatment on bacterial densities ($F(2, 18) = 4.02$, $p = 0.0361$). B+P1 bacterial densities were lower than that of B+inactivated P1 ($p = 0.0497$), but were not significantly lower than B only samples (although marginally significant, $p = 0.0755$).

At the end of trial 3, we streaked colonies from the B+P1 populations to determine whether any colonies were phage resistant. None of the isolated colonies (total $n = 44$) were resistant, in line with previous observations from adult plants.[37]

Discussion

In this study, we tested a seedling-based method for screening phage-mediated reduction of disease symptoms and bacterial densities *in vivo*. In two of three trials, prophylactic application of a *P. syringae* phage reduced disease symptoms and endpoint bacterial densities. Phages replicated in all trials and final phage density positively correlated with bacterial density. We propose that this method could be used to screen for biocontrol candidates before large scale trials in agricultural settings.

Although we did not explicitly test the impact of applied MOI within a single trial, the different outcomes across trials offer some suggestion that dosing is important. Phage P1 replicated in all trials, but only reduced disease progression and pathogen densities in trials 2 and 3. In the first trial, we inoculated seedlings with a higher dose of bacteria and lower MOI than in the other two trials. The second and third trials were done with the same starting bacterial densities, but trial 3 had a much higher applied MOI (455:1 compared with ~8:1). Interestingly, we expected that this higher MOI would lead to greater bacterial reduction, but we instead found the opposite. Phages reduced bacterial densities in both trials, but in trial 2 there were some seedlings in which we recovered no live bacterial cells, which we did not observe in trial 3. One possible explanation is that the high concentration of phage in trial 3 could have resulted in some nonproductive adsorption events (as in the phenomenon ''lysis from without''), in which binding of multiple phages leads to cellular bursting without productive replication and with loss of adsorbed phages.[51,52] It is also possible that the high MOI imposed strong selection for phage resistance, but results from our streaking assays suggest that if any resistant mutants did evolve, they did not reach a high frequency. Optimal dosing for phage therapy is a nontrivial challenge, and the results from our third trial suggest that more phage is not necessarily better for this pathosystem.

Despite the active phage replication observed in the first trial, endpoint bacterial densities were not different from seedlings that had been inoculated with bacteria alone. This is not unexpected given that we sampled for bacterial density at a single time point. It is possible that phage replication and bacterial lysis occurred early after inoculation, and bacterial densities had sufficient time to rebound and reach carrying capacity in the seedlings. This pathogen first grows as an epiphyte on the leaf surface, and then grows in the internal spaces of leaves causing disease symptoms.[53] During the internal phase, *P. syringae* forms biofilms that may be limiting bacteria–phage contact rates.[54–56] If true, phage replication may be occurring largely in the initial stages of growth on the leaf surface. In addition, it is possible that *P. syringae* evolved resistance to the phage as we did not assay for resistance at the end of this first trial. However, the MOI (and essentially strength of selection for resistance) in the first trial was orders of magnitude lower than in trial 3, in which we did not identify resistant colonies. Prior study in this system also found that rates of phage resistance evolution were extremely low during experimental evolution *in vivo*.[37] Nevertheless, both limited internal replication and resistance evolution could be occurring in this system, and future study could test this by combining time series sampling for bacteria and phage densities with assays for phage resistance.

By quantifying both disease scores and bacterial density, we also investigated whether virulence (defined as severity of disease) had any qualitative relationship with endpoint pathogen density. The two trials where phages reduced pathogen density also had reduced AUDPC values compared with B only seedlings, which suggests that disease scores alone could be used to identify effective phages. This would eliminate the need for endpoint sampling, and further reduce the resources needed to screen for phage therapy candidates (although the relationship between disease progression and pathogen density should first be experimentally tested in any system of interest).

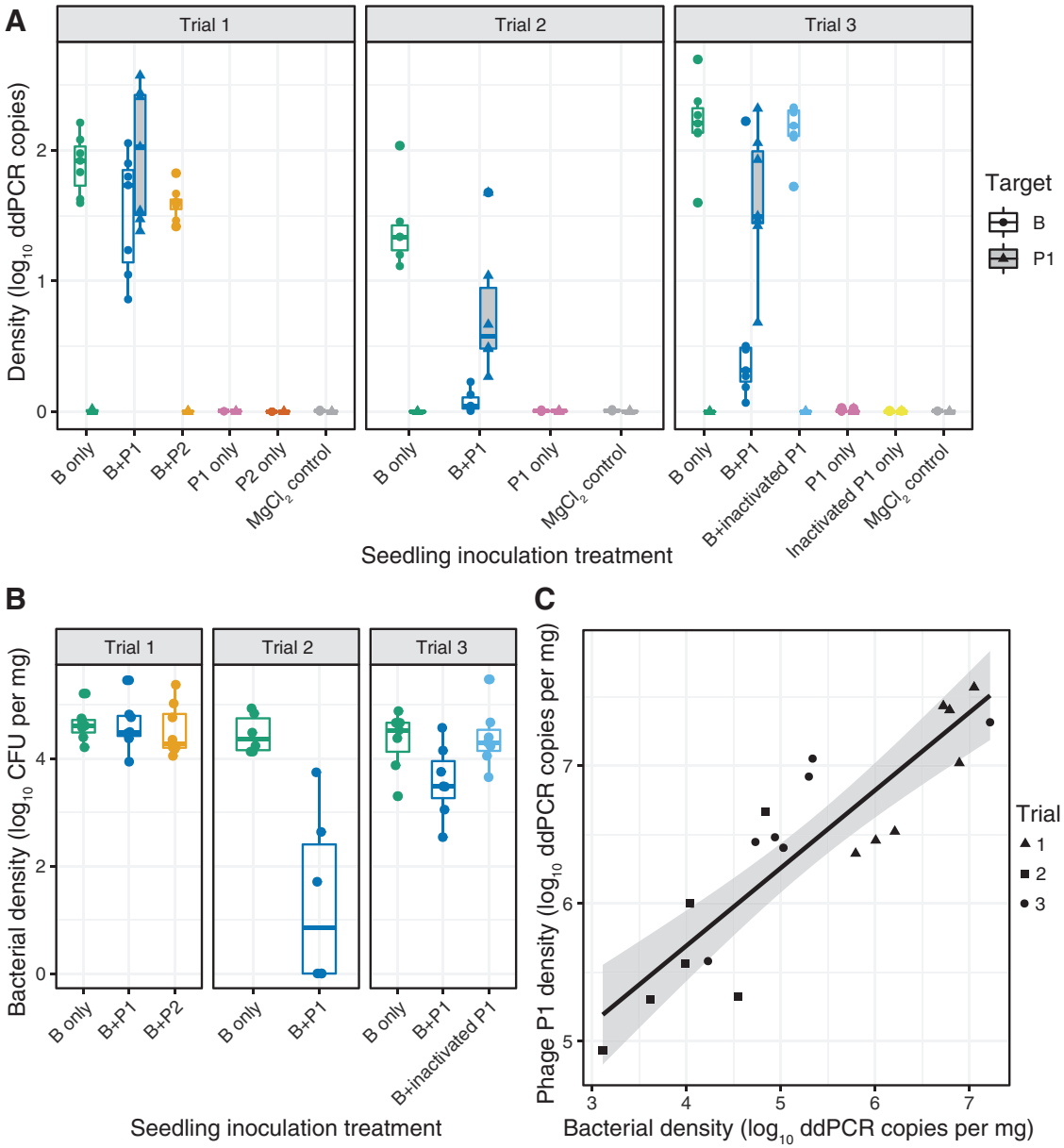

FIG. 3. Endpoint bacteria and phage densities quantified by CFUs and target DNA copies (by ddPCR). Treatment abbreviations are as follows: DC3000 only is ''B only,'' DC3000 with phage FRS is ''B+P1,'' DC3000 with phage SHL is ''B+P2,'' DC3000 with autoclaved phage FRS is ''B+inactivated P1,'' phage FRS only is ''P1 only,'' SHL phage only is ''P2 only,'' autoclaved phage FRS only is ''Inactivated P1 only,'' and the magnesium chloride control is ''$MgCl_2$ control.'' **(A)** Copies per microliter of DNA target (either bacteria or phage) in the ddPCRs, normalized by weight of each seedling, and $\log_{10}$ transformed. The left box in each treatment (circular points, white box) represents the bacterial density (''B''), and the right box (triangular points, gray shaded box) represents the phage P1 density (''P1''). Note that the bacteria and phage P1 probes were used for all samples, and phage P2 was not quantified using ddPCR. Also note that to avoid artificially inflating low value samples near the limit of detection, the *y*-axis in **(A)** differs from that of **(C)** (which is presenting a subset of relatively high concentration samples). The horizontal line within the box displays the median value, the box displays the interquartile range, and the whiskers represent ±1.5 times the interquartile range. Points outside the whiskers are possible outliers. **(B)** Bacterial density (in $\log_{10}$ CFU per milligram seedling) for treatments that had been inoculated with bacteria in each trial. **(C)** Correlation between bacterial density (in $\log_{10}$ ddPCR copies per milligram seedling) and phage P1 density (also in $\log_{10}$ ddPCR copies per milligram seedling) in each trial, exclusively for B+P1 samples. The solid black line represents the correlation of bacteria and phage P1 density across all samples, and the gray shaded region represents ±1 SE. Point shape corresponds to trial number. CFUs, colony forming units; ddPCR, droplet digital polymerase chain reaction.

Conclusion

This is a resource-efficient screening method for testing phage therapy candidates *in vivo*. Greenhouse and incubator space can limit the number of replicates in plant experiments, particularly when working with older plants. Using this highly controlled method, each replicate requires the incubation space of a single 15 mL conical tube, and phage effectiveness can be determined in <2 weeks. This method also minimizes the likelihood of bacteria–phage interactions occurring on off-target surfaces such as the water agar surface (which is more likely in studies using water agar plates). Importantly, however, field and greenhouse trials of phage effectiveness are critical components of effective therapy development. Age-related changes in plant immunity and phyllosphere tissue structures can impact bacteria–phage interactions, and seedling-based methods should act in concert with, but not replace, more agriculturally relevant studies.[57] We propose a model in which researchers first screen for possible phage therapy candidates on seedlings, and then test the impact of phage treatment on improving crop yield in greenhouse and field trials.

Authors' Contributions

C.A.H. and B.K. designed experiments, C.A.H. and A.J.S. performed experiments, C.A.H. analyzed results and wrote the initial draft of the article, all authors edited subsequent drafts of the article. All authors have reviewed and approved of the article before submission.

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Disclaimer

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Author Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary Figure S1

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