Genomic Characterization of Three Novel *Acidovorax* Phage Genera and Their Potential in Phage Biocontrol

Francisca Manuel Gaspar Vieira
francisca.vieira@tecnico.ulisboa.pt

Instituto Superior Técnico, Lisboa, Portugal

October 2021

Abstract

Bacterial black spot, caused by *Acidovorax valerianellae*, is responsible for significant yield losses in lamb's lettuce (*Valerianella locusta*) in many producing countries, especially in Europe. To date, no resistant varieties of *V. locusta* are available that effectively control the disease under field conditions. Moreover, concerns over the environmental impact of chemical pesticides and the development of bacterial resistance to antibiotics has urged the discovery of new approaches for disease management. Bacteriophage-based biocontrol has been suggested as a sustainable and natural alternative strategy to combat bacterial pathogens. In this study, novel phages infecting *A. valerianellae* and *A. cattleyae*, Alfacinha1, Alfacinha3, Acica and Aval, were isolated and characterized, being representative of three new phage genera. Aval and Acica phages revealed genomic features characteristic of temperate lifestyle, encoding toxins likely associated with lysogenic conversion, which is in sharp contrast to Alfacinha1 and Alfacinha3 phages, that displayed a lytic lifestyle. Alfacinha3 was selected for application as a biocontrol agent during seed steeping. It could achieve an 87% reduction in bacterial concentration on artificial infested seeds, and an increase in germination rate from 58.9% to 93.3%. Additionally, after 22 days of growth, the infected seedlings had a dramatic reduction in vigor index, whilst the phage-treated ones had a vigor index similar to the negative control, reinforcing the ability of bacteriophages to effectively reduce disease progression. This study shows how genomic analyses represent an essential route to ensure safe phage application and demonstrates the potential of a phage-based biocontrol strategy against *A. valerianellae*.

Keywords: *Acidovorax*, Bacteriophages, Biocontrol, Lamb's lettuce, Genome Analysis

1. Introduction

Several predictions showed that global crop production is not growing accordingly with the projected demands from rising population, diet transitions, and increasing biofuels consumption [1, 2]. In order to boost agricultural yields, limiting factors need to be minimized. In this respect, bacterial plant diseases place major constraints on crop production, accounting for significant annual losses up to 40% on a global scale, according to the FAO [3]. However, the identification and deployment of disease management solutions for bacterial diseases still remain a formidable challenge.

Acidovorax spp. are prominent phytopathogenic bacteria able to cause disease in a wide range of economically relevant crops such as cucurbits, cereal crops and sugarcane, among others [4]. One of its pathovars is *Acidovorax valerianellae*, the causal agent of bacterial black spot in lamb's lettuce (*Valerianella locusta*). It was first reported in western France fields in 1991 and since then became widespread in several other countries in Europe, being responsible for economic losses of at least 10% every year [5]. Typical disease symptoms appear as black spots on cotyledons, leaves, petioles and stems, that may in a later stage also coalesce into blights, reducing significantly corn-salad quality, making the affected batches unmarketable. Transmission by contaminated seeds and soil are discussed as major infection sources, and the pathogen is known to persist in seeds and plant debris in the soil up to 39 days after harvest of a diseased crop [6]. Additionally, bacteria can also enter the host leaf tissue through natural openings, such as stomata, or wounds after spread via splashing water and wind driven rain [7]. It is particularly difficult to control lamb's lettuce black spot in the field once an outbreak occurs. To date, no resistant varieties of *V. locusta* or chemicals are available that effectively control the disease. Nevertheless, significant efforts to seek for seed treatments have been made [8, 9].

As a result of the recent restrictions on general antibiotics and chemicals to ensure public health and to limit the occurrence of resistant strains, the application of bacteriophages in biocontrol has emerged as an alternative strategy. *Dickeya*, *Pectobacterium*, *Xanthomonas*, *Erwinia amylovora* and *Ralstonia solanacearum* are among the most common crop pathogens where phage biocontrol has been studied and has shown promising outcomes [10]. However, despite the growing evidence of the benefits of phage application in several plant diseases in different crops, phage research resulted in a limited number of commercial phage-based products for agricultural use. The Agriphage product line from Omnilytics, consisting of four commercial bacteriophage cocktails, addresses bacterial speck and spot disease in tomato and pepper, bacterial canker in tomato, fire blight in apple and pear trees and citrus canker in citrus trees [11]. XylPhi-PD, developed by Otsuka Pharmaceutical, contains bacteriophages infecting *Xylella fastidiosa*, the causal agent of Pierce disease of grape [12]. In Europe, the availability of phage-based products on the market is more restricted. The Hungarian company Enviroinvest was authorized to locally sell a phage cocktail, Erwiphage, for the control of fire blight of apple trees, caused by *Erwinia amylovora* [13], and APS biocontrol developed a a postharvest bacteriophage-based wash solution for potatoes tubers, Biolyse, to prevent soft rot disease caused by *Enterobacteriacea* [14].

Phage biocontrol studies in *Acidovorax* pathogens have hardly been reported, being limited to two bacteriophages infecting *Acidovorax citrulli*, ACP17 and ACPWH, isolated and fully characterized by Rahimi-Midani and colleagues. Their applicability in the control of bacterial fruit blotch in cucurbit crops was further demonstrated by both seed coating [15] and soil-based [16] plant assays. Phages infecting *Acidovorax valerianellae* have not yet been described. Therefore, this study focuses on the isolation of novel phages infecting this pathogen and their extensive characterization. A seed bioassay was also further performed to evaluate the potential in biocontrol of bacterial black spot in lamb's lettuce seeds.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

The bacterial strains used in this study, supplied by the Institute for Agricultural and Fisheries Research (ILVO), are listed in Table 1.

The bacteria were grown at 25ºC in Lysogeny Broth with medium salt concentrations (LB_{ms}) (10g/L Trypton (Neogen), 5 g/L yeast extract (Neogen) and 1.5 g/L NaCl (Acros Organics)), while shaking at 200 rpm. LB_{ms} was supplemented with 1.5% agar (bacteriological agar (Neogen)) for plating and with

Table 1: *Acidovorax* strains used in this study, their year of isolation, geographical origin and their phage sensitivity. [a] GBBC: Culture collection of plant pathogenic bacteria at ILVO; CFBP, Collection Française de Bactéries Phytopathogènes; LMG, Belgian Coordinated Collections of Microorganisms at the Laboratory of Microbiology of Ghent University with [T] as type strains.

Species	Strain[a]	Origin	Year	Alfacinha1	Alfacinha3	Aval	Acica
Acidovorax anthuri	CFBP 3232[T]	Martinique	1991	-	-	-	-
Acidovorax oryzae	CFBP 2426[T]	Japan	1963	-	-	-	-
Acidovorax citrulli	LMG 5376[T]	USA	1977	-	-	-	-
Acidovorax cattleyae	LMG 5286[T]	USA	1961	-	-	-	-
Acidovorax cattleyae	GBBC 705	Belgium	2000	+	-	-	+
Acidovorax cattleyae	GBBC 1100	Belgium	2001	-	-	-	-
Acidovorax cattleyae	GBBC 1148	Belgium	-	+	-	-	+
Acidovorax cattleyae	GBBC 1149	Belgium	-	+	-	-	+
Acidovorax cattleyae	GBBC 1303	Belgium	-	+	-	-	+
Acidovorax valerianellae	CFBP 6945	France	2006	-	+	-	-
Acidovorax valerianellae	GBBC 3037	Belgium	2015	+	-	-	-
Acidovorax valerianellae	GBBC 3038	Belgium	2015	-	-	-	-
Acidovorax valerianellae	GBBC 3039	Belgium	2015	+	-	-	-
Acidovorax valerianellae	GBBC 3042	Belgium	2015	-	-	-	-
Acidovorax valerianellae	GBBC 3043	Belgium	2015	-	-	+	-
Acidovorax valerianellae	GBBC 3129	Belgium	2016	+	+	-	-
Acidovorax valerianellae	GBBC 3161	Belgium	2016	-	+	-	-
Acidovorax valerianellae	GBBC 3208	Belgium	2017	+	-	-	-
Acidovorax valerianellae	GBBC 3209	Belgium	2017	-	+	-	-
Acidovorax valerianellae	GBBC 3340	Belgium	2019	-	-	-	-
Acidovorax valerianellae	GBBC 3341	Belgium	2019	-	-	+	-
Acidovorax valerianellae	GBBC 3342	Belgium	2019	-	+	-	-
Acidovorax valerianellae	GBBC 3353	Belgium	2019	+	+	-	-
Acidovorax valerianellae	GBBC 3354	Belgium	2019	-	-	-	-
Acidovorax valerianellae	GBBC 3355	Belgium	2019	-	-	-	-
Acidovorax valerianellae	GBBC 3356	Belgium	2019	-	-	-	-
Acidovorax valerianellae	GBBC 3357	Belgium	2019	+	+	+	-
Acidovorax valerianellae	GBBC 3358	Belgium	2019	-	-	-	-

0.5% agar for agar overlays. For long term storage at -80°C, cell stocks were prepared by adding glycerol (Acros Organics) to an overnight culture to a final concentration of 20%.

2.2. Bacteriophage Isolation, Amplification, and Purification

Phages were isolated from soil samples received from Proefcentrum voor de Groenteteelt (PCG), Proeftuin Sint-Katelijne-Waver (PSKW) and Inagro, from Flanders, Belgium. To enrich for phages, overnight cultures of all bacterial strains (Table 1) were grown in 1 mL LB_{ms} at 25°C in 96-deep-well plates and around 1 g of each soil sample was added in each well. After overnight incubation, 1 drop of chloroform was added in each well and incubated for 1h. The mixture was then centrifuged (30min, 3000rpm, 4°C), using a Sorvall Legend RT+ centrifuge (Thermo Scientific), and 3 µL of the supernatant was spotted on a soft agar layer that contained the bacterial host. Lysis zones were picked up with sterile toothpicks and suspended in 100 µL phage buffer (10 mM Trizma base (Sigma Aldrich); 10 mM $MgSO_4$ (Sigma Aldrich); 150 mM NaCl (Acros Organics); pH 7.5). These suspensions were plated by pooling 250 µL overnight bacterial host culture, 100 µL phage suspension and 4 mL LB overlay agar. After overnight incubation at 25°C, single plaques were picked up again. Three successive single plaque isolations were performed to achieve pure phage isolates.

Phages were amplified by infecting a liquid culture (in LB supplemented with 10 mM $CaCl_2$ and 5 mM $MgSO_4$) of the respective bacterial host at Optical Density at 600 nm (OD_{600}) of 0.3 with a multiplicity of infection (MOI) of 0.01. For Alfacinha1 and Aval the bacterial host used for amplification was GBBC 3357, while for Alfacinha3 was GBBC 3161 and for Acica was GBBC 1148. After overnight incubation, the supernatant was filtered over a 0.45 µm pore size filter (Millex-HV; Merck Millipore Ltd.). To obtain a phage stock for downstream experiments, polyethylene glycol (PEG_{8000}) (Acros Organics) was added to the filtered phage lysate to a final concentration of 30% v/v. After overnight incubation at 4°C, phages were precipitated by centrifugation (30 min, 4000 rpm, 4°C) and the pellet was dissolved in 2 mL phage buffer.

2.3. Electron Microscopy

Transmission electron microscopy (TEM) pictures were made by Dr. Marta Vallino (IPSP-CNR, Turin). In short, the phage suspensions adsorbed for 3 min on carbon and formvar-coated copper-palladium grids, which were then rinsed several times with water. The grids were negatively stained with aqueous 0.5% uranyl acetate and the excess fluid was removed with filter paper. Observations and photographs were made with a Philips CM10 transmission electron microscope (TEM) (Eindhoven, The Netherlands) at 80 kV. Micrograph films were developed and digitally acquired at high resolution with a D800 Nikon camera. Finally, the images were trimmed and adjusted for brightness and contrast using the Fiji software [17].

2.4. Host Range Analysis

To test the susceptibility of all the *Acidovorax* strains (Table 1) to each phage the double agar overlay method was used, by adding 250 µL of an overnight culture to 4 mL of LB soft agar and poured on top of an LB agar plate. After that, 3 µL of each phage (at least 10^6 PFU/mL) were spotted onto the solidified overlays. After overnight incubation at 25°C, the plates were examined for plaques.

2.5. Adsorption and Infection Curves

In adsorption assays, the host strain GBBC 3161 was grown to an OD_{600} of 0.3 and infected with Alfacinha3 at MOI of 0.01. Immediately after infection, a 200 µL sample was taken and transferred into a Zymo-Spin IC column (Zymo Research) in a pre-cooled eppendorf tube and centrifuged for a few seconds. The filtered suspension, kept on ice, was titrated to determine the amount of non-adsorbed or reversibly adsorbed phages. This was repeated after 1, 5 and 10 minutes.

Killing curves were established for the strain GBBC 3161 infected with Alfacinha3 at MOIs of 0.1, 1 and 10. The bacterial culture was initially infected at OD_{600} of 0.3, and monitored every 10 min for 2h and compared with that of an uninfected culture. OD_{600} results are the average of three independent biological repeats.

2.6. DNA Extraction and Sequencing

Phage DNA was extracted from a high-titer lysate (minimum of 10^8 PFU/mL). 1 µL of DNaseI (ThermoFisher Scientific) and 1 µL of RNaseA (ThermoFisher Scientific) were added to 10 µL of the phage stock. After incubation at 37°C for 30 minutes, 4 µL of EDTA (Acros Organics), 5 µL of SDS 10% (Acros Organics) and 1 µL of ProteinaseK (ThermoFisher Scientific) were added, and then incubated at 56°C in a thermal bath for 45 minutes. The Kit DNA Clean & Concentrator™-5 (Zymo Research) was used to purify the phage DNA following the manufacturer's instructions. The DNA was sequenced using Illumina MiniSeq platform at the Laboratory of Gene Technology, KU Leuven. A library was prepared using the Nextera™ Flex DNA Library Kit for each sample, according to the manufacturer's guidelines. The quality of each library preparation was controlled using an Agilent Bioanalyzer 2100. All library preps were equally pooled and sequenced using a MiniSeq Mid Output flowcell (300 cycles; 2*150 bp reads). The reads were trimmed with the Trimmomatic tool (v0.36.5), using standard settings with the addition of an initial ILLUMINA CLIP step to remove the Nextera adapters [18]. Next, the quality of the reads was assessed using the FastQC tool (v0.11.8) [19].

2.7. Data Processing and Analysis

The raw read data was processed (assembly and annotation) using online tools on the public servers of Galaxy (v21.05) [20] and PATRIC (v3.6.9) [21]. The reads were assembled using SPAdes algorithm [22]. The assembled contigs were visualized and their quality was assessed using Bandage (v0.8.1) [23]. Bowtie2 (v2.4.2) was used for the alignment of the sequenced reads and assembled sequence[24]. The automated annotation was manually curated by verifying the translated ORFs in a BLASTp analysis ((National Centre for Biotechnology Information (NCBI)) [25] against the non-redundant GenBank protein database [26]. The viral proteomic tree was generated with the online ViPTree server (v1.9) [27] (accessed in April 2021), and the intergenomic distances/similarities amongst the related viral genomes were computed using VIRIDIC web tool [28]. Easyfig (v2.2.2) [29] was used to create linear comparison figures of multiple genomes and BLAST comparisons between multiple genomic regions.

2.8. Seed Bioassay *in vitro*

Valerianella locusta seeds - Groene van Cambrai (Aveve) were first sterilized by suspending and shaking them for 7 minutes in a 1% NaClO solution. Next, the seeds were rinsed three times with sterile mQ water and left to dry under a laminar flow. The surface sterility of the seeds was tested on LB_{ms} agar plates. The seeds were infected with GBBC 3161 with OD_{600} = 0.15 (around 10^8 CFU/mL) and incubated for 1h30, while shaking using the HulaMixer™ Sample Mixer (Thermo Scientific) at 25 rpm and room temperature. Following seed drying, Alfacinha3 phage solution was added to the seeds with a concentration of 10^9 PFU/mL and shaked overnight using the HulaMixer™ Sample Mixer at 25 rpm at 16°C. The seeds were crushed and suspended in phage buffer. The bacterial concentrations were quantified by plating them on LB_{ms} agar plates. The quantification of phages was done by using soft agar overlay method. Overall, three independent repeats were performed for each one of the 4 different conditions (negative control, phage only, bacteria only and bacteria plus phage).

2.9. Seedlings Bioassay *in vitro*

Similarly as previously described, lamb's lettuce seeds (Aveve) were surface sterilized, infected (OD_{600} = 0.15) and phage primed. After priming, the seeds were dried under a laminar flow. Using sterilized tweezers, 30 seeds per condition were then sown onto plant growth medium, 1/4 MS Agar (1.1 g/L Murashige and Skoog basal medium (Sigma Aldrich); 15 g/L bacteriological agar (Neogen)), in each plate. The plant growth medium enclosed half of the plates so that the seedling started growing parallel to the plate bottom. Furthermore, the plates were sealed with Parafilm (Sigma-Aldrich) to keep the moisture in, and set vertically under a lamp providing light for 16 hours and 8 hours of darkness, in a room maintained at 16°C. For each condition, three independent repeats were performed, and so, 90 seeds of each condition were sown in total. After 22 days, the shoot and root length of each seedling was measured using a caliper. Statistical analyses were performed with JMP Pro 15. Multiple non-parametric Wilcoxon comparison tests were performed at a significance level of 0.05. For statistical data visualization, such as the design of the boxplots, Seaborn Python library (v0.11.1) was used [30].

3. Results and Discussion

3.1. Isolation of Novel Bacteriophages

Phages were first isolated from soil samples taken in Flanders (Belgium) from infected lamb's lettuce beds. Using an enrichment, four novel phages were discovered infecting *A. valerianellae* and *A. cattleyae*, named Alfacinha1 and Alfacinha3 (*Alfacinha* is the Portuguese word for "little lettuce", with lettuce being the main target of *A. valerianellae* and the focus of this study; *Alfacinha* is also popularly used to designate the natives of Lisbon, including the author of this work), Aval (referring to *Acidovorax valerianellae*) and Acica (referring to *Acidovorax cattleyae*). The isolate of Alfacinha1 was found by enriching soil samples with *A. valerianellae* strain GBBC 3357, Aval with GBBC 3043, Alfacinha3 with GBBC 3161 and Acica with GBBC 1148. These strains were used to optimize phage amplifications, with the exception of Aval, that was amplified with GBBC 3357.

Transmission Electron Microscopy images (Figure 1) showed that the 4 phages exhibited icosahedral heads and long, contractile tails, typical of the myovirus morphology. Interestingly, the tail of Alfacinha1 exhibited convoluted fibers, being therefore clearly distinguishable from the other phages.

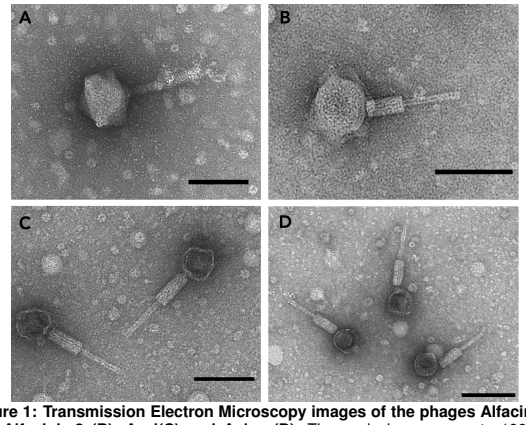

Figure 1: Transmission Electron Microscopy images of the phages Alfacinha1 (A), Alfacinha3 (B), Aval(C) and Acica (D). The scale bar represents 100 nm. Phages negatively stained with 0.5% uranyl acetate.

The images of Aval and Acica (Fig.1C and 1D) showed contracted tail sheaths and empty capsids, indicating a previous ejection of the DNA, and so, revealing the presence of several inactive phages in the stock. That suggests that the stock, after the removal of the bacterial cells by filtration, or even after PEG purification, still contain molecules that act as receptors to the phages, allowing them to bind and eject the DNA but without producing any progeny. Indeed, PEG precipitation has been shown to not completely remove endotoxins [31], typically requiring downstream implementation of other endotoxin removal techniques.

3.1.1 Host Range

To investigate the specificity of the phages, a host range analysis was performed (Table 1). Aval has the narrowest host range, limited to three *A. valerianellae* strains, while Alfacinha1 has the broadest one, being able to infect not only strains of the *A. valerianellae* species but also strains from the *A. cattleyae* species. Alfacinha3 infects seven different strains of *A. valerianellae* and Acica infects solely *A. cattleyae*. The strain GBBC 3357 could be infected by all three *A. valerianellae* phages, Alfacinha1 and 3 and Aval. In total, 57% of the collection of *Acidovorax* strains could be infected by at least one of the phages. *Acidovorax anthuri, oryzae* and *citrulli* strains were not infected by any of the isolated phages.

Within the four novel phages, the host range analysis revealed diverse specificities. Distinct host ranges might be explained by genomic variations at different infection stages, such as the recognition of different host receptors during surface-adhesion, different adaptations to evade the host intra-cellular defense systems, or for instance by lysing different cell-wall structures to release progeny [32]. Nevertheless, no specific mechanism could be identified to explain the observed host range differences. To provide further insight about these differences, a knock-out library of the host strains using transposon mutagenesis could be used in future studies to assess which genes determine the infection mechanism in each phage.

3.2. Genome Analysis

The genomes of all four phages were sequenced and assembled. Information about the genome characteristics of the phages is summarized in Table 2.

Table 2: Summary of the main genomic characteristics of the four phages Aval, Alfacinha1, Alfacinha3 and Acica.

Phage name	Genome length (bp)	GC content (%)	#ORFs
Aval	39.584	66.1	48
Alfacinha 1	40.274	65.2	53
Alfacinha 3	40.526	65.0	53
Acica	37.472	67.5	49

The viral taxonomy of the novel phages and their similarity with other known bacteriophages was investigated by generating a proteomic tree (Figures 2 and 3), according to a ViPTree analysis [27]. Based on these results, a VIRIDIC analysis [28] allowed to compute the pairwise intergenomic similarities amongst the most related viral genomes.

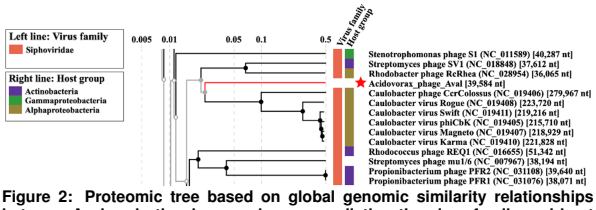

Figure 2: Proteomic tree based on global genomic similarity relationships between Aval and other known phages, predicting the virus family and host group. Only the section with the most related phages of the tree is presented in this figure. Aval is indicated with a red star. Generated in ViPTree server.

These analyses showed that Aval clustered together with phages of the *Siphoviridae* family, mainly with *Caulobacter* phages. However, it does not belong to any specific cluster and show less than 5% of similarity with the other phages, thus representing a novel species and genus according to current ICTV guidelines [33].

The other three phages, Alfacinha1, Alfacinha3 and Acica, clustered together in the proteomic tree with phages of the *Myoviridae* family (Figure 3), that is in accordance with the morphology shown in the TEM images (Figure 1). They represent a single cluster, sharing low homology with other phage genomes in the database, with a maximum similarity of about 14% with the *Ralstonia* phages phiRSA1 and RSY1. Therefore, they can be considered as novel phages, which should be placed in a new taxonomic group, belonging to the *Peduovirinae* subfamily, such as the majority of the most related phages.

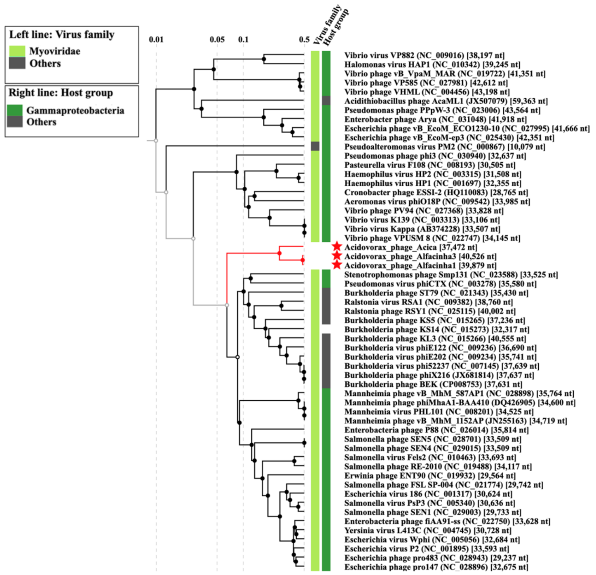

Figure 3: Proteomic tree based on global genomic similarity relationships between Acica, Alfacinha1, Alfacinha3 and other known phages, predicting the virus family and host group. Only the section with the most related phages of the tree is presented in this figure. Alfacinha1, Alfacinha3 and Acica are indicated with red stars. Generated in ViPTree server.

Within their cluster, Alfacinha1 and Alfacinha3 present higher similarity between each other (97.5%) than with Acica (57%). Thus, according to the current ICTV guidelines [33], two new different genera should be proposed, e.g. 'Alfacinhavirus' and 'Acicavirus'. It is worth noting that, with the new taxonomic proposal of ICTV, their subfamily will be reclassified up to family level [34], and so these two novel phage genera will potentially form a new subfamily.

To explore the genome organization of the new four phages and to compare it with other known related phages, their genomes were annotated using Patric [21] and manually curated. The encoded ORFs, ranging from 48 to 53 (Table 2) were identified, and some functions could be assigned by verifying similarity at the protein level by Blastp analysis. In Figure 4 can be visualized the genome maps of Aval (A) and of Alfacinha1, Alfacinha3 and Acica (B) along with their comparison to the genomes of the *Ralstonia* phages phiRSA1 (accession number: NC_009382.1) and RSY1 (accession number: NC_025115.1).

The genome of Aval showed an organization of three main modules (Fig.4A), beginning with DNA-binding, followed by DNA packaging and structural modules. In the first module, genes related with lysogenic activity were identified, namely encoding the transcriptional activator CII (gp14), that determines if the phage will incorporate the genome or follow the lytic cycle, and the integrase (gp1) that mediates the incorporation in the host's genome, upon activation by CII [35]. In the same module, gp6, encoding a partitioning protein, shows an alternative ability to maintain a temperate lifestyle, by replication as an extrachromosomal prophage [36]. It is interesting to notice the simultaneous presence of these two different ways of prophage maintenance in Aval's genome, since genes with partitioning functions are usually present in temperate phages as a replacement of the integration cassette [36]. Additionally, the encoded DNA methyltransferase (gp44) might be complementing this 'double ability' to generate and maintain stable lysogens by protecting the phage from host's restriction endonucleases, as has been shown in various lytic and lysogenic phages [37]. If, on the one hand, the phage tries to protect itself from its own host, on the other hand it is also conferring it a competitive advantage by turning the host more virulent through lysogenic

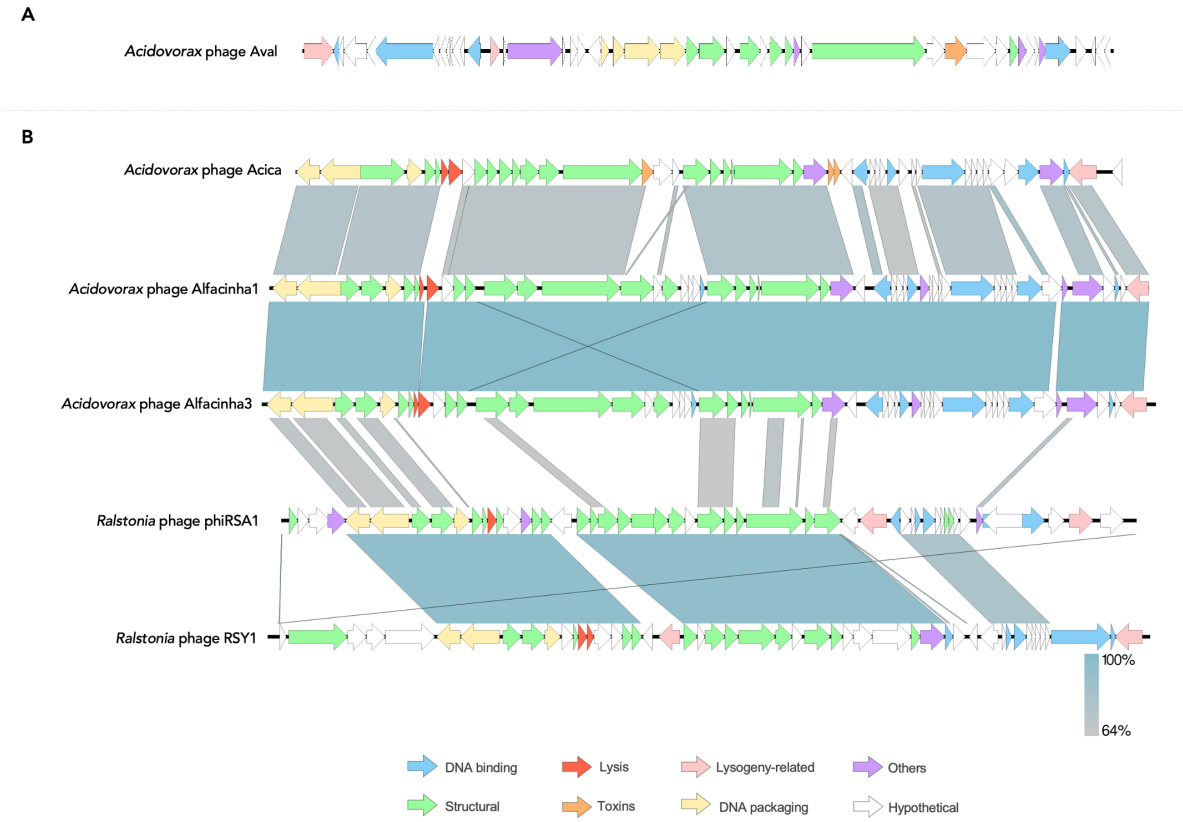

Figure 4: Genome map of the *Acidovorax* phages Acica, Alfacinha1 and Alfacinha3 and of the *Ralstonia* phages phiRSA1 and RSY1. The arrows indicate predicted ORFs and the direction of their translation: in white—encoding hypothetical proteins, blue-encoding DNA associated proteins, green-encoding structural proteins, red-encoding lysis related proteins, orange-encoding toxins, yellow—encoding DNA packaging proteins, pink-encoding lysogeny related proteins and purple-encoding other proteins. Generated with EasyFig.

conversion. Indeed, several bacteriocins, such as the encoded pyocin (gp36), have been discovered in temperate phages, providing evolutionary benefits to the infected host in intraspecies competition [38], once they can be deployed to kill the bacterial neighbors.

Given that several genomic indicators pointed for a temperate infection cycle, a phenotypic test was performed with Aval in two different hosts to screen for lysogenic activity. The identification of spontaneous phage release from its lysogenized host and gain of resistance confirmed Aval's temperate lifestyle. This could explain the highly specific and narrow host range of this phage, including only three out of nineteen strains of *A. valerianellae*. As the host range analysis was only based on the successful production of plaques, the infection by a temperate phage might not be detected, since it can also integrate the host's genome without lysing the bacteria. On the other hand, just as there is the possibility of Aval to integrate into the genome, there may also be another prophage already integrated in the same host. Since there is still no information about the genome of the strains in collection, it can be possible that they contain integrated prophages providing immunity to their host against a superinfection. Indeed, the ability to cause homoimmunity was also verified in Aval.

A different genome organization was found in the other three phages (Fig.4B), similar to the one of the *Ralstonia* phages phiRSA1 and RSY1. It is comprised of four main modules beginning with DNA-packaging, followed by a lysis-related region, the structural module and ending with the DNA-binding module. The phages Alfacinha1 and Alfacinha3 did not show any encoded toxins or other known proteins associated with virulence. In sharp contrast, the phage Acica revealed the presence of the BrnT/BrnA toxin-antitoxin system (gp27 and gp28), that has been reported to regulate stress adaptation and persistence during antimicrobial treatment [39]. In more detail, in response to various environmental stressors, such as low pH and oxidative stress, transcription of the toxin induces a bacteriostatic condition, in which the cells are still viable but unable to proliferate. This condition can be fully reversed by expression of the cognate antitoxins, thereby protecting the cells from long-term starvation. Additionally, Acica encodes a rearrangement hotspot (RHS) repeat (gp17). Despite the functions of this gene family are not yet well understood, it has been reported to be associated with toxin domains, which can be deployed to inhibit the growth of neighboring cells [40].

The toxin systems found in both Acica and Aval genomes likely contribute to a strong positive selection of their hosts, thus providing a competitive and evolutionary advantage. Therefore, both phages are not adequate as biocontrol agents. Alfacinha3, on other hand, showed to infect specifically *A. valerianellae* strains and produced clear plaques, thus displaying a lytic lifestyle. It was easily amplified in high concentrations and was able to fully lyse the bacterial cultures. Since no toxins or virulence factors were found in its genome, it was selected for further investigation in phage-based biocontrol, including microbiological and seed bioassays.

3.3. Adsorption and Infection Curves

An adsorption assay (Figure 5) was performed with Alfacinha3 at MOI of 0.01 to assess the speed of irreversible adsorption of the phage particle to the host cell (*A. valerianellae* GBBC 3161) and the efficacy of this process.

After one minute, 64.3% of the phage particles were adsorbed to the host cell and after ten minutes more than 77.3%. Under these circumstances, this gives an adsorption constant, described by Equation 1 [41], with B representing the bacterial titer, t the time, P_0 the initial phage titer and P the phage titer after time t, of 3.52 x 10^{-9} mL/min after one minute.

$$k = \frac{2.3}{B * t} * \log_{10} \frac{P_0}{P} \quad (1)$$

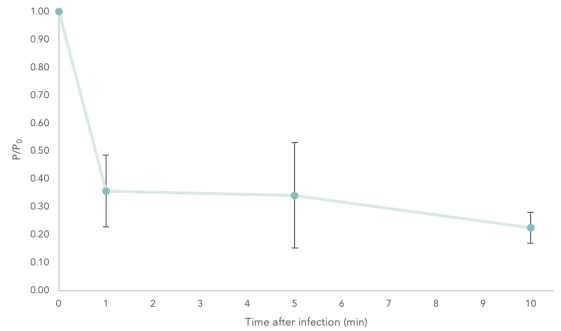

Figure 5: Adsorption curve of Alfacinha3 to the host strain *A. valerianellae* GBBC 3161, with MOI=0.01. The ratio of non-adsorbed phages (P) to the initial titer (P_0) is followed through time. Error bars indicate the standard deviation and are based on three independent repeats.

Upon comparison of the adsorption constant to other *Myoviridae* phages, Alfacinha3 adsorption was slower than that of the *Dickeya* phage LIMEstone2 (2.1 x 10^{-8} mL/min) [42] the *Pseudomonas* phage KIL3 (7.5 × 10^{-9} mL/min) [43], but still faster than what was reported for the phage T4 (2.4 × 10^{-9} mL/min) [41]. On the other hand, when comparing the total amount of phages irreversibly adsorbed to the host cell after ten minutes, Alfacinha3 shows lower numbers (77.3%), than, for instance, the phages LIMEstone2 and KIL3 where more than 99% of the phages are adsorbed after ten minutes.

To assess the speed of the infection and cell lysis process, exponentially growing cultures of *A. valerianellae* GBBC 3161 were infected with the phage Alfacinha3 at different MOIs, and the variation in optical density (OD_{600}) during growth was monitored through time (Figure 6).

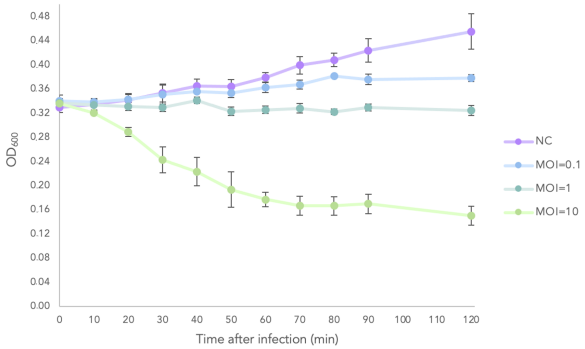

Figure 6: Infection curves of *A.valerianellae* GBBC 3161 infected with phage Alfacinha3 at different multiplicities of infection (MOI). The variation in optical density (OD_{600}) during growth of *A.valerianellae* GBBC 3161 with different concentrations of phage is followed through time. The negative control is indicated with lilac (●), MOI 0.1 with blue (●), MOI 1 with turquoise (●) and MOI 10 with light green (●). Error bars indicate the standard deviation and are based on three independent repeats.

When comparing the optical density of the infected bacterial cultures with the negative control, it is observed that after 40 minutes, a decrease in growth is detected with all the MOIs, demonstrating the virulence of the phage. Noteworthy, a steeper decline reaching an OD_{600} of almost 0.1 is observed with MOI 10 within 120 minutes.

No bacterial resistance could be detected within this time frame, however a study of the interaction dynamics between the phage and the host in a more long-term, along with the evaluation of the emerging phage-resistant mutants, could be useful to get insight into the possible mechanisms and whether they have an altered effect on virulence.

3.4. Seed Bioassay

Seed treatment has been proposed as a control strategy for *A. valerianellae*, since transmission by contaminated seeds is one major infection source. The use of bacteriophages could provide several advantages when compared with the available seed treatments, as phages are able to remain infective for long periods around the seeds, even after germination, and represent a more selective and ecologically sustainable strategy. The use of phage-coated seeds has been previously reported to be an effective approach in the biocontrol of another *Acidovorax* species, *A. citrulli*, being able to reduce the development of bacterial fruit blotch (BFB). Indeed, the treatment showed to increase the germination rate of watermelon infested seeds from 55% to 88% and the plant survival rate after three weeks from 15% to 100% [15]. As such, in this study, a seed bioassay was performed to test the efficacy of the Alfacinha3 phage in the control of *A. valerianellae* on seeds. Lamb's lettuce seeds were first inoculated with *A. valerianellae* strain GBBC 3161 (10^8 CFU/mL) and then primed with the phage Alfacinha3 (10^9 PFU/mL). The final concentration of phage and bacteria per gram of seeds was determined after phage incubation overnight (Figure 7).

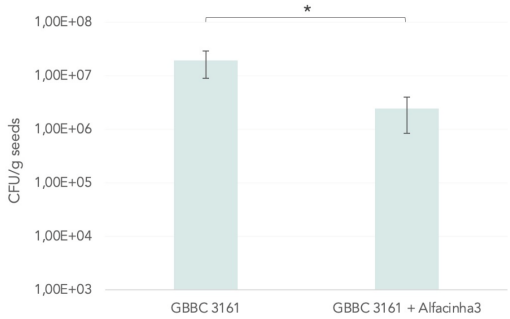

Figure 7: Bacterial concentration on the seeds for the four different conditions: Negative control, bacteria only (infected with *A.valerianellae* strain GBBC 3161 at 10^8 CFU/mL), phage only (primed with Alfacinha3 at 10^9 PFU/mL) and bacteria plus phage (GBBC 3161 + Alfacinha3). Error bars indicate the standard deviation and are based on three independent repeats. Statistical support is based on T-test, showing significant difference ($p<0.05$)

A reduction of 87% of the bacterial concentration inside the seeds could be achieved. In Figure 8, the seedlings are visualized after growing over 22 days under four different conditions, A- negative control, B- infected with bacteria, C- primed with phage and D- infected with bacteria and primed with phage.

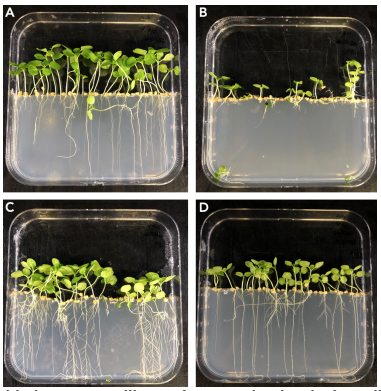

Figure 8: Lamb's lettuce seedlings after germination in four different conditions: A) negative control; B) bacteria only (infected with *A.valerianellae* strain GBBC 3161 at 10^8 CFU/mL); C) phage only (primed with Alfacinha3 at 10^9 PFU/mL) and D) bacteria plus phage (GBBC 3161 + Alfacinha3).

A clear difference can be observed between the infected seedlings (Fig.8B) and the other conditions, showing lower germination and poor growth. The germination rate, the shoot and root length were then measured, and the vigor index was calculated by the product of the shoot and root lengths with the percentage of seed germination. The main parameters are summarized in Table 3. The distribution of the shoot (A) and root (B) length data for each condition is presented in the box plots of Figure 9.

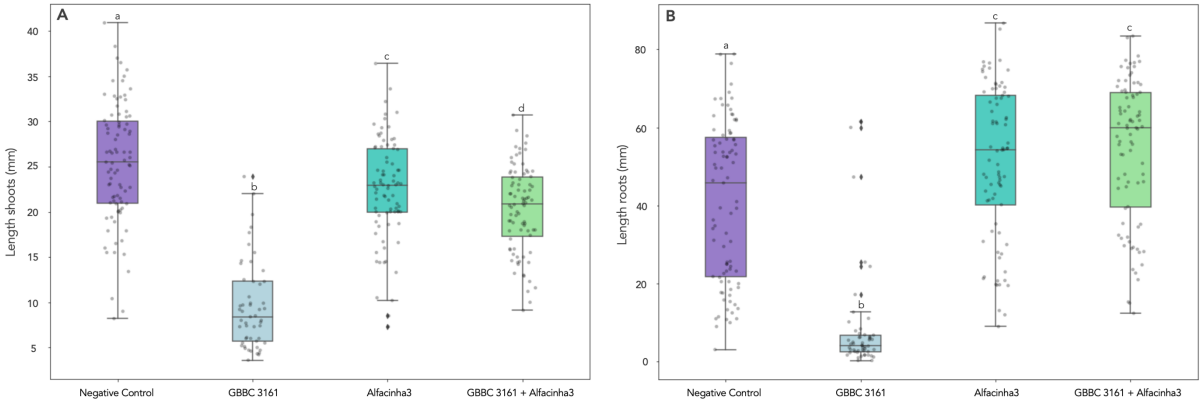

Figure 9: Measurements of the shoot and root length after germination in four different conditions: Negative control; bacteria only (infected with *A.valerianellae* strain GBBC 3161 at 10^8 CFU/mL); phage only (primed with Alfacinha3 at 10^9 PFU/mL) and bacteria plus phage (GBBC 3161 + Alfacinha3). The results are based on three independent repeats, each one using 30 seeds per condition. Statistical relevance is represented by the connecting letters based on the nonparametric comparisons performed with Wilcoxon method (p=0.05).

Table 3: Measurements of seedlings grown for 22 days from each condition: Negative control, bacteria only (strain GBBC 3161), phage only (Alfacinha3) and bacteria plus phage (GBBC 3161 + Alfacinha3). The vigor index was calculated by the product of the shoot and root lengths with the percentage of seed germination. The results are based on three independent repeats, each one using 30 seeds per condition. Statistical support is given for the vigor index parameter by the connecting letters (^{a} and ^{b}), based on the comparisons performed with Wilcoxon method (p=0.05).

	Mean shoot length (mm)	Mean root length (mm)	Germination rate (%)	Vigor index
Negative Control	25.4	40.9	96.7	1044.1 ^{a}
GBBC 3161	9.7	8.5	58.9	62.1 ^{b}
Alfacinha3	22.5	50.7	90.0	1085.6 ^{a}
GBBC 3161 + Alfacinha3	20.1	54.2	93.3	1054.3 ^{a}

The results demonstrate that the addition of the phage Alfacinha3 to the *A. valerianellae* infected seeds increased the germination rate from 58.9% to 93.3% and resulted in a dramatic rise in the vigor index. The infected seedlings revealed an abnormal growth, with a marked reduction in shoot (Fig.9A) and root (Fig.9B) length, in contrast to the phage-treated ones, which did not develop symptoms and were able to grow almost similarly to the non-infected plants. This demonstrates that the phage could significantly reduce the development and progression of the disease.

Interestingly, the plants whose seeds were phage-primed revealed significant differences in morphology. Despite shorter shoots they presented considerably longer and more developed roots, with increased lateral root density (as verified in Fig. 8C and 8D). This indicates that the interaction with the phage solution is inducing a plant response. Plants are known to respond to a variety of stimuli, including PAMPs (pathogen-associated molecular patterns), such as lipopolysaccharides, peptidoglycans and bacterial flagellin, that trigger a defense response to protect the plant from invading pathogens [44]. The detection of quorum sensing (QS) signaling molecules of microorganisms, including amino acids, fat derivatives, and other organic compounds has also been reported to induce a plant response. For instance, a recent study showed that diketopiperazines (QS signal molecules) promoted lateral root development and root hair formation in *Arabidopsis thaliana* by enhancing the polar transport of the plant hormone auxin from the shoots to the roots. This led to the accumulation of auxin at the root tip, that in turn, accelerated root growth [45]. Therefore, it is hypothesized that the phage stock used in the seed coating still contains bacterial compounds that cause a plant response. The bacterial compounds could include endotoxins, such as LPS, that could not be completely removed by PEG precipitation [31], or QS signaling molecules, that induce hormone signaling, promoting the specific growth and development of the root. As such, prior to phage application, additional purification steps should be applied to avoid harming plant shoots growth. Alternative methods to PEG precipitation, including combinations of dead-end filtration, cross-flow filtration and affinity chromatography have also been suggested [31].

4. Conclusions and Future Perspectives

The growing human population, along with the limited area of cultivable land, requires an increase of current crop yields. However, plant pathogenic bacteria represent a key limiting factor. Issues concerning resistance towards the existing treatments are prominent, and therefore, there is a great need for long-term and safe alternatives. In this regard, bacteriophages are hypothesized as a potential solution to control plant diseases, and so, their applicability to combat *Acidovorax valerianellae*, the causing agent of lamb's lettuce black spot, was further investigated in this research work.

In this work, the first phages infecting *A. valerianellae* and *A. cattleyae*, Alfacinha1, Alfacinha3, Acica and Aval were isolated and characterized. Based on their overall genome sequence, they showed to be representative of three novel genera, 'Alfacinhavirus', 'Acicavirus' and 'Avalvirus', revealing significant genomic differences to other known phages. While the Alfacinha phages displayed a lytic lifestyle, Acica and Aval encoded toxin systems, typically correlated with a temperate lifestyle, and possibly associated with lysogenic conversion. These genes can also be passed on to other bacteria via horizontal gene transfer, and consequently select for more virulent and competitive hosts. Nevertheless, further investigation would be required to clarify the exact contribution or impact of these phages in the evolution and virulence of the *Acidovorax* strains. Alfacinha3 was selected for a bioassay, in which its potential in the biocontrol of *A. valerianellae* in lamb's lettuce seeds was demonstrated. The examination of the practical applicability of the phage in different settings, would help to improve and optimize a phage-based biocontrol strategy. Indeed, despite Alfacinha3 has proven to prevent the progression of the disease at an early stage, it should also be able to curb it at a later stage, when transmission can occur from contaminated soil or when the pathogen enters the plant foliage. Therefore, further research should consider soil-based phage delivery and include the evaluation of the vascular uptake of phages and their translocation to the leaves. Following the concentration of phages over time could also unravel their long-term protective effect based on different application strategies, and assess the need of protective formulations.

In the future, more attention should be provided to sustainable pest control, in order to boost crop yield with a more rational application of resources. Phage-based products could, for instance, be implemented along with optimized cultural practices, such as vertical and smart farming techniques, that enhance land use efficiency and integrate robotics, machine learning and sensor-based technologies to exploit early disease detection and precise biocontrol. Bacteriophages, as naturally occurring bacterial predators, can, in turn, play a revolutionary role in a more sustainable future for crop production.

References

[1] D. K. Ray, N. D. Mueller, P. C. West, and J. A. Foley, "Yield Trends Are Insufficient to Double Global Crop Production by 2050," *PLoS ONE*, vol. 8, no. 6, 2013.

[2] FAO, "The future of food and agriculture – Alternative pathways to 2050," 2018. Rome. 224 pp. Licence: CC BY-NC-SA 3.0 IGO.

[3] Food and A. O. of the United Stations, "Plant health 2020." `http://www.fao.org/plant-health-2020/about/en/`. Accessed: 2021-07-10.

[4] S. Burdman and R. R. Walcott, *Plant-Pathogenic Acidovorax Species*. The American Phytopathological Society, 2018.

[5] K. Thiele, K. Smalla, S. Kropf, and F. Rabenstein, "Detection of Acidovorax valerianellae, the causing agent of bacterial leaf spots in corn salad [Valerianella locusta (L.) Laterr.], in corn salad seeds," *Letters in Applied Microbiology*, vol. 54, no. 2, pp. 112–118, 2012.

[6] C. Grondeau, C. Manceau, and R. Samson, "A semiselective medium for the isolation of Acidovorax valerianellae from soil and plant debris," *Plant Pathology*, vol. 56, no. 2, pp. 302–310, 2007.

[7] C. Manceau, E. Charbit, M. Lecerf, and P. Portier, " CHAPTER 9: Acidovorax valerianellae: Bacterial Black Spot of Lamb's Lettuce ," in *Plant-Pathogenic Acidovorax Species*, Bacteriology, pp. 121–130, The American Phytopathological Society, jan 2018.

[8] H. J. Schärer, M. Schnueriger, V. Hofer, J. Herforth-Rahmé, and M. Koller, "Effect of different seed treatments against seed borne diseases on corn salad," in *Acta Horticulturae*, vol. 1164, pp. 33–38, 2017.

[9] J. Herforth-Rahmé, J. G. Fuchs, V. Hofer, M. Schnueriger, H. J. Schärer, and M. Koller, "Bioseedling: A chain approach to the production of healthier seeds and seedlings of Lamb's lettuce Valerianella locusta," in *Acta Horticulturae*, vol. 1164, pp. 39–45, 2017.

[10] C. Buttimer, O. McAuliffe, R. P. Ross, C. Hill, J. O'Mahony, and A. Coffey, "Bacteriophages and bacterial plant diseases," *Frontiers in Microbiology*, vol. 8, no. JAN, 2017.

[11] OmniLytics Inc., "AgriPhage." `https://www.agriphage.com`. Accessed: 2021-07-18.

[12] A&P Inphatec, "XylPhi-PD." `https://inphatec.com/xylphi_pd`. Accessed: 2021-09-19.

[13] Enviroinvest Zrt., "Erwiphage." `https://www.erwiphage.com`. Accessed: 2021-07-18.

[14] APS Biocontrol Ltd., "Biolyse." `https://www.apsbiocontrol.com/products`. Accessed: 2021-07-18.

[15] A. Rahimi-Midani, J. O. Kim, J. H. Kim, J. Lim, J. G. Ryu, M. K. Kim, and T. J. Choi, "Potential use of newly isolated bacteriophage as a biocontrol against Acidovorax citrulli," *Archives of Microbiology*, vol. 202, no. 2, pp. 377–389, 2020.

[16] A. Rahimi-Midani and T. J. Choi, "Transport of phage in melon plants and inhibition of progression of bacterial fruit blotch," *Viruses*, vol. 12, no. 4, 2020.

[17] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, and A. Cardona, "Fiji: An open-source platform for biological-image analysis," 2012.

[18] A. M. Bolger, M. Lohse, and B. Usadel, "Trimmomatic: A flexible trimmer for Illumina sequence data," *Bioinformatics*, vol. 30, no. 15, pp. 2114–2120, 2014.

[19] S. Andrews, F. Krueger, A. Seconds-Pichon, F. Biggins, and S. Wingett, "FastQC. A quality control tool for high throughput sequence data. Babraham Bioinformatics," 2015.

[20] V. Jalili, E. Afgan, Q. Gu, D. Clements, D. Blankenberg, J. Goecks, J. Taylor, and A. Nekrutenko, "The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2020 update," *Nucleic Acids Research*, vol. 48, no. W1, pp. W395–W402, 2021.

[21] J. J. Davis, A. R. Wattam, R. K. Aziz, T. Brettin, R. Butler, R. M. Butler, P. Chlenski, N. Conrad, A. Dickerman, E. M. Dietrich, J. L. Gabbard, S. Gerdes, A. Guard, R. W. Kenyon, D. MacHi, C. Mao, D. Murphy-Olson, M. Nguyen, E. K. Nordberg, G. J. Olsen, R. D. Olson, J. C. Overbeek, R. Overbeek, B. Parrello, G. D. Pusch, M. Shukla, C. Thomas, M. Vanoeffelen, V. Vonstein, A. S. Warren, F. Xia, D. Xie, H. Yoo, and R. Stevens, "The PATRIC Bioinformatics Resource Center: Expanding data and analysis capabilities," *Nucleic Acids Research*, vol. 48, no. D1, pp. D606–D612, 2020.

[22] A. Bankevich, S. Nurk, D. Antipov, A. A. Gurevich, M. Dvorkin, A. S. Kulikov, V. M. Lesin, S. I. Nikolenko, S. Pham, A. D. Prjibelski, A. V. Pyshkin, A. V. Sirotkin, N. Vyahhi, G. Tesler, M. A. Alekseyev, and P. A. Pevzner, "SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing," *Journal of Computational Biology*, vol. 19, no. 5, pp. 455–477, 2012.

[23] R. R. Wick, M. B. Schultz, J. Zobel, and K. E. Holt, "Bandage: Interactive visualization of de novo genome assemblies," *Bioinformatics*, vol. 31, no. 20, pp. 3350–3352, 2015.

[24] B. Langmead and S. L. Salzberg, "Fast gapped-read alignment with Bowtie 2," *Nature Methods*, vol. 9, no. 4, pp. 357–359, 2012.

[25] S. F. Altschul, J. C. Wootton, E. M. Gertz, R. Agarwala, A. Morgulis, A. A. Schäffer, and Y. K. Yu, "Protein database searches using compositionally adjusted substitution matrices," *FEBS Journal*, vol. 272, no. 20, pp. 5101–5109, 2005.

[26] L. A. Kelley and M. J. Sternberg, "Protein structure prediction on the web: A case study using the phyre server," *Nature Protocols*, vol. 4, no. 3, pp. 363–373, 2009.

[27] Y. Nishimura, T. Yoshida, M. Kuronishi, H. Uehara, H. Ogata, and S. Goto, "ViPTree: The viral proteomic tree server," *Bioinformatics*, vol. 33, no. 15, pp. 2379–2380, 2017.

[28] C. Moraru, A. Varsani, and A. M. Kropinski, "VIRIDIC—A novel tool to calculate the intergenomic similarities of prokaryote-infecting viruses," *Viruses*, vol. 12, no. 11, 2020.

[29] M. J. Sullivan, N. K. Petty, and S. A. Beatson, "Easyfig: A genome comparison visualizer," *Bioinformatics*, vol. 27, no. 7, pp. 1009–1010, 2011.

[30] M. Waskom, "Seaborn: Statistical Data Visualization," *Journal of Open Source Software*, vol. 6, no. 60, p. 3021, 2021.

[31] T. Luong, A. C. Salabarria, R. A. Edwards, and D. R. Roach, "Standardized bacteriophage purification for personalized phage therapy," *Nature Protocols*, vol. 15, no. 9, pp. 2867–2890, 2020.

[32] P. A. de Jonge, F. L. Nobrega, S. J. Brouns, and B. E. Dutilh, "Molecular and Evolutionary Determinants of Bacteriophage Host Range," *Trends in Microbiology*, vol. 27, no. 1, pp. 51–63, 2019.

[33] E. M. Adriaenssens and J. Rodney Brister, "How to name and classify your phage: An informal guide," *Viruses*, vol. 9, no. 4, 2017.

[34] D. Turner, A. M. Kropinski, and E. M. Adriaenssens, "A roadmap for genome-based phage taxonomy," *Viruses*, vol. 13, no. 3, 2021.

[35] D. L. Court, A. B. Oppenheim, and S. L. Adhya, "A new look at bacteriophage λ genetic networks," *Journal of Bacteriology*, vol. 189, no. 2, pp. 298–304, 2007.

[36] R. M. Dedrick, T. N. Mavrich, W. L. Ng, J. C. Cervantes Reyes, M. R. Olm, R. E. Rush, D. Jacobs-Sera, D. A. Russell, and G. F. Hatfull, "Function, expression, specificity, diversity and incompatibility of actinobacteriophage parABS systems," *Molecular Microbiology*, vol. 101, no. 4, pp. 625–644, 2016.

[37] J. Murphy, J. Mahony, S. Ainsworth, A. Nauta, and D. van Sinderen, "Bacteriophage orphan DNA methyltransferases: Insights from their bacterial origin, function, and occurrence," *Applied and Environmental Microbiology*, vol. 79, no. 24, pp. 7547–7555, 2013.

[38] A. Dragoš, A. J. Andersen, C. N. Lozano-Andrade, P. J. Kempen, Á. T. Kovács, and M. L. Strube, "Phages carry interbacterial weapons encoded by biosynthetic gene clusters," *Current Biology*, 2021.

[39] B. E. Heaton, J. Herrou, A. E. Blackwell, V. H. Wysocki, and S. Crosson, "Molecular structure and function of the novel BrnT/BrnA toxin-antitoxin system of Brucella abortus," *Journal of Biological Chemistry*, vol. 287, no. 15, pp. 12098–12110, 2012.

[40] S. Koskiniemi, J. G. Lamoureux, K. C. Nikolakakis, C. T. De Roodenbeke, M. D. Kaplan, D. A. Low, and C. S. Hayes, "Rhs proteins from diverse bacteria mediate intercellular competition," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 17, pp. 7032–7037, 2013.

[41] L. M. Kasman, A. Kasman, C. Westwater, J. Dolan, M. G. Schmidt, and J. S. Norris, "Overcoming the Phage Replication Threshold: a Mathematical Model with Implications for Phage Therapy," *Journal of Virology*, vol. 76, no. 11, pp. 5557–5564, 2002.

[42] E. M. Adriaenssens, J. van Vaerenbergh, D. Vandenheuvel, V. Dunon, P. J. Ceyssens, M. de Proft, A. M. Kropinski, J. P. Noben, M. Maes, and R. Lavigne, "T4-related bacteriophage LIMEstone isolates for the control of soft rot on potato caused by 'Dickeya solani'," *PLoS ONE*, vol. 7, no. 3, 2012.

[43] S. Rombouts, A. Volckaert, S. Venneman, B. Declercq, D. Vandenheuvel, C. N. Allonsius, C. Van Malderghem, H. B. Jang, Y. Briers, J. P. Noben, J. Klumpp, J. Van Vaerenbergh, M. Maes, and R. Lavigne, "Characterization of novel bacteriophages for biocontrol of bacterial blight in leek caused by Pseudomonas syringae pv. porri," *Frontiers in Microbiology*, vol. 7, no. MAR, 2016.

[44] A. K. Grennan, "Plant response to bacterial pathogens. Overlap between innate and gene-for-gene defense response," *Plant Physiology*, vol. 142, no. 3, pp. 809–811, 2006.

[45] L. Yin, X. Chen, Q. Chen, D. Wei, X.-Y. Hu, and A.-Q. Jia, " Diketopiperazine Modulates Arabidopsis Thaliana Root System Architecture by Promoting Interactions of Auxin Receptor TIR1 and IAA7/17 Proteins ," *Plant and Cell Physiology*, sep 2021.