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Combinations of Echinacea (*Echinacea purpurea*) and Rue (*Ruta gravolens*) Plant Extracts with Lytic Phages: A Study on Interactions

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Abstract: The use of combined biocontrol strategies to combat bacterial-related issues is an increasingly popular approach. Therefore, a novel investigation was performed, where interactions of lytic bacteriophages (MS2, T4 and phi6) and methanolic plant extracts (*Echinacea purpurea* (EP) and *Ruta graveolens* (RG)) in the bacterial environment have been examined to understand their application potential and limitations. Due to the complexity of these interactions, many up-to-date techniques were used (microdilution method, phage extract coincubation assay, static interactions synographies and dynamic growth profile experiments in a bioreactor). As a result of our study, antagonism interactions were observed: EP and RG extracts showed antiphage and bacterial stimulating activity. Effects caused by low extract concentrations on microorganisms depended on the species of phage and bacteria, while high concentrations suppressed bacterial lysis in general. Moreover, interactions observed in the static environment differed from those performed in a dynamic environment, showing the importance of performing multiple analyses when investigating such complex mixtures.

Keywords: bacteriophage; phi6; MS2; T4; *E. coli*; *P. syringae*; plant extract; *Echinacea purpurea*; *Ruta graveolens*

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1. Introduction

Herbal preparations, including those using plant extracts of echinacea (*Echinacea purpurea*) and rue (*Ruta graveolens*), are widely known and increasingly used due to their significant healing properties, although little is known about their mode of action [1,2]. *E. purpurea* is a perennial plant native to eastern North America that belongs to the *Asteraceae* family. The plant is widely known for its medicinal properties, being one of the most used medicinal plants for its immunostimulant properties. This plant species is considered a safe herbal medicine, thus, it is usually used through extracts of the dried aerial parts or roots. The most common phytochemicals in *E. purpurea* are alkamides, polysaccharides, lipoproteins, betaine, sesquiterpenes, polyacetylene, saponins and phenolic compounds, responsible for its biological properties [2]. In turn, *R. graveolens* is widely distributed in various geographical regions of Afro-Asian countries. This plant is cultivated as a decorative plant and is used in traditional medicine for treating many disorders. All parts of the plant contain the active compound, but they are mostly found in leaves [1]. *R. gravolens* represents plants of the *Rutaceae* family, which are a big repository of secondary metabolites responsible for their aroma (terpenes), pigmentation (quinones and tannins) and flavour (terpenes). The extracts of these plants are a mixture of various compounds, such as terpenoids and phenols, to which antiseptic, antifungal, antioxidant and antitumoral properties are attributed [3,4]. Particular importance is attached to such botanical extracts in relation to their antimicrobial effects against certain pathogenic bacteria, where it is uncertain whether

they have any other effects on select groups of microorganisms [5–7]. It is worth knowing the effects of such mechanisms of action, as combinations of naturally antimicrobial plant extracts with other active agents of similar effectiveness may be more effective than those used as individual therapies [7]. Antimicrobial agents that can potentially be combined with plant extracts to create more effective preparations include bacteriophages (phages), which are currently getting more popular. Phages are viruses specific to bacterial cells known for over hundred years, which can successfully supplement or even (in some cases) replace traditional methods of prevention and treatment of bacterial diseases, especially those caused by strains which are found to be resistant to routinely used antimicrobial drugs [8]. The biochemical complexity of the molecules that make up the phage virion, their diversified size (expressed in the nanometric scale), the electric charge and hydrophobicity make bacteriophages interact with other materials in various ways. Those include different types of interactions with stimulating or antimicrobial effects, e.g., by altering phage activity as a result of a synergistic or antagonistic effect [9,10]. It should also be remembered that inhibition of bacteriophage proliferation or "antiphage activity test" is a sensitive, rapid and low-cost prescreening test, which can be applied to reliably detect pharmacological activity [11]. Moreover, bacteriophages have significant advantages, such as easy handling and cultivating in standard laboratory media. Therefore the employment of phages is being also propose to serve as eukaryotic cell virus surrogates [12–14].

Therefore, in the current study, interplays between lytic bacteriophages and plant extracts of echinacea (*Echinacea purpurea*) and rue (*Ruta graveolens*) have been investigated for their possible interactions in the bacterial environment, including possible antagonistic effects. To the best of our knowledge, this is the first work describing such phenomena.

2. Materials and Methods

2.1. Bacterial Hosts and Bacteriophages

Bacteriophages and their bacterial hosts were purchased from the German Collection of Microorganisms and Cell Cultures GmbH (Deutsche Sammlung von Mikroorganismen und Zellkulturen; DSMZ). In this study, *Pseudomonas syringae* (DSM 21482) with phage phi6 (DSM 21518), *Escherichia coli* (DSM 5695) with phage MS2 (DSM 13767) and *Escherichia coli* (DSM 613) with phage T4 (DSM 4505) were used. Chosen lytic phages were selected based on their different features (Table 1).

Table 1. Bacteriophage characteristics.

Bacteriophage	Family	Size	Genome	Features
Phi6 (Φ6)	*Cystoviridae*	~80–100 nm	dsRNA	Enveloped (lipid membrane), medium size, no tail
MS2	*Leviviridae*	~23–28 nm	ssRNA	Non-enveloped, small size and genome, no tail
T4	*Myoviridae*	~120–200 nm/86–90 nm	dsDNA	Non-enveloped, relatively big, contractile tail

Bacterial revival and phage propagation were conducted similarly, as described previously [15]. All strains were stored in TSB (trypticase soy broth) medium (BioMaxima, Lublin, Poland) with 10% (vol/vol) glycerol at −20 °C. The bacteria were revived on Luria–Bertani (LB agar) (BioMaxima, Lublin, Poland) for bacterial plate stocks by streaking glycerol stocks onto agar plates (plate incubation: 24 h at 37 °C for *E. coli* strains; 48 h at 28 °C for *P. syringae* strains). For bacteriophage amplification, several colonies from bacterial plate stock were used to inoculate 50 mL of LB, and were incubated (as described above) with shaking (120 rpm) in an orbital rotating shaker (Shaker–Incubator ES-20, BioSan, Józefów, Poland) to reach OD_{600nm} = 0.2. Optical density values were measured using an Infinite 200 PRO NanoQuant microplate reader (Tecan, Männedorf, Switzerland). Then, phage was added, and samples were further incubated until the lysis occurred. For MS2 and T4 lysate purification, chloroform was added (10%, vol/vol) and the samples were

vortexed for 5 min, and then centrifuged (Eppendorf Centrifuge model 5810 R, Hamburg, Germany) at 5000 rpm for 25 min at 4 °C. The supernatant was collected immediately. For phi6 lysate purification, samples were firstly centrifuged (5000 rpm, 15 min, 4 °C) and then sterilized by filtration (PES filter, 0.22 μm). Lysates were stored at 4 °C for further use. Phage activity and titres were tested by a double-overlay agar plaque assay [16].

2.2. Plant Extract Preparation

Preparation of herb extracts following the methanol extraction method was carried out as proposed in our previous work [17,18], with some modifications. Of the dried herbs (aerial parts of *Echinacea purpurea* (L.) Moench and *Ruta graveolens*; Flos, Mokrsko, Poland), 50 g was placed in the glass bottle and 70% aqueous methanol (MeOH) was added to obtain 100 mL volume. Next, the samples were placed in a shaker (Ika, Staufen im Breisgau, Germany) and extracted for 2 h at 70 °C with 150 rpm. The crude methanol extracts were filtered through a Büchner funnel, equipped with a cellulose filter. The extracts were then concentrated by evaporation at 50 °C to obtain aqueous solutions. After the evaporation of methanol, the samples were filter-sterilized (PES, 0.22 μm) and then used for further experiments. In order to maintain a relatively low viscosity (the viscosity of the extracts was 16 s) to prevent the later formation of cell agglomerates in the samples (uneven distribution), the solutions were not concentrated any more. At this stage, the *Echinacea purpurea* extract was marked as "EP" and the *Ruta graveolens* extract as "RG". Additionally, the dry mass of each extract was determined via a moisture analyser (Radwag, Puszczykowo, Poland). For the following experiments, different stock solutions (two-fold serial dilutions) of the extracts were diluted in sterile, deionized water, and kept at –20 °C until further analysis.

2.3. Plant Extract Studies on Bacterial Cells

To analyse the influence of plant extracts on bacteria, a 96-well microplate dilution protocol was carried out. For the microplate method, modified minimal inhibitory concentration (MIC) determination was used [19]. Several colonies from bacterial plates were used to inoculate Falcon tubes with 30 mL of LB and were incubated (*E. coli*: 37 °C; *P. syringae*: 28 °C) with shaking (120 rpm) in an orbital rotating shaker until they reached OD_{600nm} = 0.2. Two-fold serial dilutions of extracts (50 μL; 50–0.003%) were added into 96-well polystyrene flat-bottomed plates, and then bacterial suspensions (50 μL) were also added. For the positive control (bacteria growth control), sterile deionized water was used. Samples were then incubated for 24 h at temperatures appropriate for the tested bacteria. Optical density values were measured using an Infinite 200 PRO NanoQuant microplate reader (Tecan, Männedorf, Switzerland). The experiment was conducted in triplicate.

2.4. Phage-Extract Coincubation Assay

In order to test direct phage–extract interactions, namely the influence of extracts on bacteriophage plaque-forming ability and titre, a coincubation assay was carried out [10]. Briefly, 1 mL of phage lysates (phi6 and T4: 10^8 PFU/mL; MS2: 10^9 PFU/mL) were added into wells of 12-well flat-bottomed polystyrene plates. Then, extracts were added (1 mL) to reach the final concentrations of 50–0.049% (two-fold serial dilutions). Phage lysate and extract-free deionized water was used as a positive control. Samples were incubated at room temperature (20 °C) for 24 h, without light access. Then, samples were collected and titrated in the TM buffer (50 mM Tris-HCl, 10 mM $MgSO_4$ at pH 7.5) by spotting 3 μL of each dilution (10-fold) onto an LB plate that was already coated with a top agar layer (0.7%), mixed with overnight bacterial culture (double-layer agar technique). The experiment was conducted in triplicate.

2.5. Phage-Extract Synographies

Phage–extract interaction stoichiometries were performed as described elsewhere [20], with minor modifications. To prepare the testing cultures, 5 mL of the overnight culture was diluted in LB, in order to achieve OD_{600nm} = 1 (approx. 1×10^9 CFU/mL). Then, 100 μL

of the bacterial suspension was inoculated into each well of the 96-well flat-bottomed plates that were previously coated with varying concentrations of phages (50 µL, final 10^2–10^8 PFU/mL) and extracts (50 µL, final 25–0.049%), the checkerboard of phage and extract (Figure 1). Plates were incubated for 18 h (*E. coli*: 37 °C; *P. syringae*: 28 °C) and the OD_{600nm} values were measured using an Infinite 200 PRO NanoQuant microplate reader. Afterwards, in order to test the bacterial metabolic activity, a resazurin assay was carried out, by adding the dye to the samples in the wells (final 1 mg/mL). Plates were then further incubated (*E. coli* for 3 h, *P. syringae* for 4.5 h) without light access, and then fluorescence measurements were performed using a fluorescent plate reader (Synergy HTX, BioTek, Winooski, VT, USA) at 540 nm excitation and 590 nm emission. The experiment was conducted in triplicate.

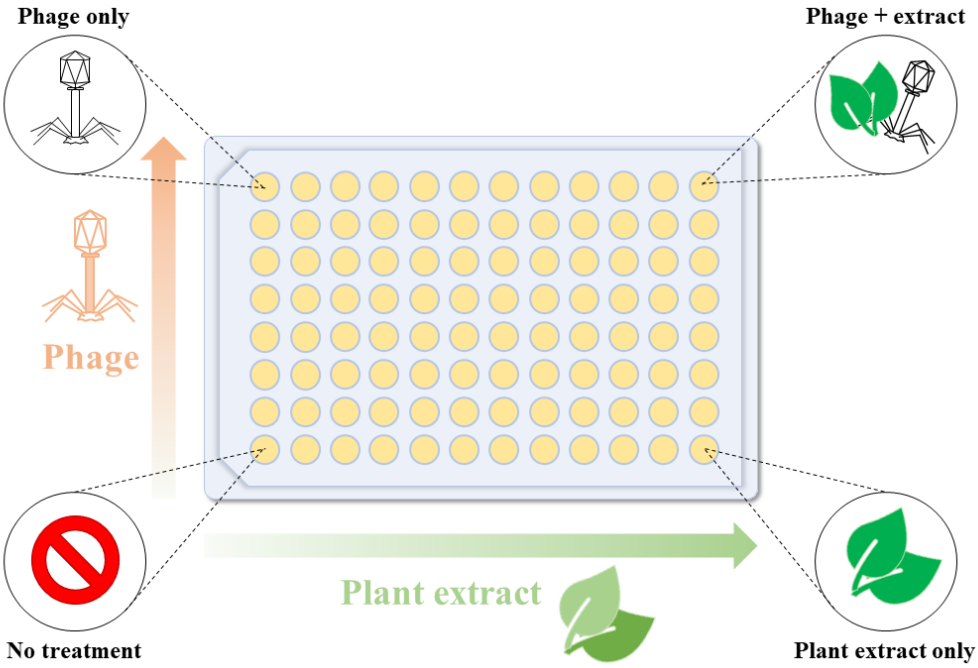

Figure 1. Synogram showing the distribution of phage–extract combinations against the bacterial inoculum.

2.6. Phage Infection and Lysis Profile Experiments

Based on results from the static (no mixing) synogram experiment, we chose combinations in which interesting phenomena were present (increased bacterial activity in resazurin assay with simultaneous OD measures showing a reduction in bacterial biomass), in order to analyse the influence of combined treatment of phage–extracts on bacterial host growth rate in real time, in a dynamic environment (mixing). Proper controls for result comparison (phage + extract maximal dose, extract maximal dose, bacterial growth control) were also applied (Table 2).

To keep the experimental assumptions of the synogram test, overnight bacterial host cultures were diluted in LB, in order to achieve OD_{600nm} = 1. Then, 5 mL of the bacterial suspensions were transferred into Falcon tubes. Afterwards, 2.5 mL of phage lysates were added (final titre 10^8 PFU/mL), along with 2.5 mL of particular plant extract concentrations, in order to obtain the chosen final concentrations (e.g., 100% extract was added to obtain a final concentration of 25%). For extract-alone treatments, instead of phage, 2.5 mL of sterile deionized water was added. For the host bacterial growth control, 5 mL of sterile deionized water was added to 5 mL of bacterial suspension. Samples were then incubated (*E. coli* at 37 °C, *P. syringae* at 28 °C) for 16 h, 150 rpm and real-time OD_{850nm} values were measured using BioSan bioreactors (BS-010160-A04, BioSan, Riga, Latvia).

Table 2. Chosen treatment combinations for real-time assessment of bacterial growth. EP: *Echinacea purpurea* extract; RG: *Ruta graveolens* extract.

Bacterial Host	Phage	Phage Titre	Extract	Extract (%)	Treatment Type
P. syringae DSM 21482	Phi6	10^8	EP	25	Phage + extract
P. syringae DSM 21482	-	-	EP	25	Extract
P. syringae DSM 21482	Phi6	10^8	EP	6.25	P+E+B combination *
P. syringae DSM 21482	Phi6	10^8	EP	0.049	P+E+B combination *
P. syringae DSM 21482	-	-	-	-	Growth control
E. coli DSM 5695	MS2	10^8	EP	25	Phage + extract
E. coli DSM 5695	-	-	EP	25	Extract
E. coli DSM 5695	MS2	10^8	EP	6.25	P+E+B combination *
E. coli DSM 5695	MS2	10^8	EP	0.049	P+E+B combination *
E. coli DSM 5695	-	-	-	-	Growth control
E. coli DSM 613	T4	10^8	EP	25	Phage + extract
E. coli DSM 613	-	-	EP	25	Extract
E. coli DSM 613	T4	10^8	EP	6.25	P+E+B combination *
E. coli DSM 613	T4	10^8	EP	0.049	P+E+B combination *
E. coli DSM 613	-	-	-	-	Growth control
P. syringae DSM 21482	Phi6	10^8	RG	25	Phage + extract
P. syringae DSM 21482	-	-	RG	25	Extract
P. syringae DSM 21482	Phi6	10^8	RG	0.78	P+E+B combination *
P. syringae DSM 21482	-	-	-	-	Growth control
E. coli DSM 5695	MS2	10^8	RG	25	Phage + extract
E. coli DSM 5695	-	-	RG	25	Extract
E. coli DSM 5695	MS2	10^8	RG	3.125	P+E+B combination *
E. coli DSM 5695	-	-	-	-	Growth control
E. coli DSM 613	T4	10^8	RG	25	Phage + extract
E. coli DSM 613	-	-	RG	25	Extract
E. coli DSM 613	T4	10^8	RG	0.78	P+E+B combination *
E. coli DSM 613	-	-	-	-	Growth control

* P+E+B combination: phage, extract and bacterial combination in which interesting phenomena were detected.

2.7. Scanning Electron Microscope (SEM) Visualization

SEM micrographs were performed as an additional test in order to visualize bacterial counts and fitness during lysis profile experiments. *P. syringae* and phage phi6, along with the lowest concentration of the EP extract (0.049%), have been selected for this assay to confirm that the presence of the extract does not affect the phage lysis process. Phage phi6 with *P. syringae* was used as a lysis control. Briefly, lysis profile experiments were repeated on a smaller scale in Eppendorf tubes, in a final volume of 1 mL. After 16 h of incubation at 28 °C, the samples were moved to room temperature (21 °C). Carbon-coated copper grids (400 mesh) were then immersed in the liquid samples and the cells were allowed to adhere for 30 min. Then, grids were taken out and the excess liquid was removed with a paper sheet. After the grids had dried, the samples were fixed for 18 h at 4 °C (2% glutaraldehyde in a 0.1 M sodium cacodylate (NaCac), pH 7.4). Next, samples were washed with 0.1 M sodium cacodylate and subsequently dehydrated in serial concentrations of ice-cold (–20 °C) methanol (10%, 20%, 40%, 60%, 80% and 100%) at 1 h intervals. The samples were then placed on a Petri dish and coated with a thin layer of gold in a sputter coater at room temperature (Quorum Technologies Q150R S, Laughton, East Sussex, UK). Then, the samples were viewed under scanning electron microscope (SEM). The microscopic analysis was carried out using a Vega 3 LMU microscope (Tescan, Brno-Kohoutovice, Czech Republic) and the test was performed at 25 °C, with a tungsten filament and an accelerating voltage of 10 kV.

2.8. Statistical Analysis

A one-way (plant extract antimicrobiological studies) and a two-way (phage–extract coincubation assay) ANOVA were used to statistically analyse the results, along with the

Dunnett's multiple comparisons test. Differences were considered significant at $p \leq 0.05$. All statistical analyses were carried out using GraphPad Prism 8.01 (GraphPad Software, San Diego, CA, USA). All data are presented as the mean with standard deviation (SD).

3. Results

3.1. Antibacterial Activity of Plant Extracts

The tested aqueous plant extracts were characterized by a dry mass of 14.9476% (149,476 g/L) for EP and 22.9407% (229,407 g/L) for RG. In order to test the effects of the extracts on bacterial cells, a microdilution assay was performed.

The addition of plant extracts to the bacterial cultures cultivated in a static environment resulted in mixed effects (Figure 2). In general, the EP extract resulted in an increased bacterial biomass of *E. coli* DSM 613, whereas for *E. coli* DSM 5695, the stimulating effect was present up to a concentration of 1.56%. Lower concentrations did not influence the bacterial growth or, in minor cases, inhibited it (0.097% and 0.003%) The highest concentrations of the EP extract (50% and 25%) did not alter the growth of *P. syringae* DSM 21482, while lower concentrations generally showed inhibitory potential (Figure 2A). RG extract also showed a stimulating effect on *E. coli* DSM 613 cells in the majority of the tested concentrations, except for the highest concentration (50%), which showed an inhibitory effect, and the concentrations of 12.5%, 6.25% and 3.125%, which did not influence bacterial growth. Similar effects were observed for the RG extract and *E. coli* DSM 5695, where the majority of the tested concentrations increased the bacterial biomass, except for two highest concentrations (50% and 25%) and the lowest concentration (0.003%), that did not alter bacteria multiplication compared to the control. The addition of the RG extract to *P. syringae* DSM 21482 cells led to the most varied results: concentrations of 50%, 12.5%, 6.25%, 3.125%, 0.19% and 0.003% were characterized by an inhibitory potential, while the concentrations of 25%, 1.56%, 0.78%, 0.097%, 0.049%, 0.024% and 0.012% did not affect *P. syringae* cells. However, a stimulating effect was also present and observed for two of the tested concentrations of the RG extract (0.39% and 0.006%) (Figure 2B).

3.2. Coincubation Assay

Coincubation of the tested plant extracts and bacteriophages in a static environment showed varied results (Figure 3). The addition of EP extract did not significantly influence MS2 phage counts, with the exception of two concentrations (12.5% and 1.56%) which resulted in a phage-stimulating effect. Phi6 phage in combination with the highest concentrations of the EP extract (50%, 25% and 12.5%) caused a phagicidal effect, where no phage plaques were present. Lower EP concentrations did not affect the phage counts. The combination of the EP extract with T4 phages also did not significantly alter phage plaquing fitness (Figure 3A). Similarly to the EP extract, the RG extract did not significantly influence MS2 phage counts, with the exception of two concentrations (0.19% and 0.097%) which stimulated the number of phage plaques, and one concentration (3.125%) that negatively affected phage counts. The addition of the RG extract to the phi6 phage lysate caused a significant phagicidal effect for most of the tested concentrations, with the exception of the two lowest concentrations (0.097% and 0.049%). When the RG extract was incubated with T4 phage, a significant drop in phage count was observed for three of the tested concentrations (6.25%, 1.56% and 0.097%), whereas other concentrations had no effect on the T4 phage (Figure 3B).

3.3. Phage–Extract Interaction Stoichiometries

For most of the tested samples, a darkening of the sample was present due to the dark colour of the concentrated extract. This was taken into account when analysing the results: the extract optical density background was removed from the data for a clear interpretation of the results.

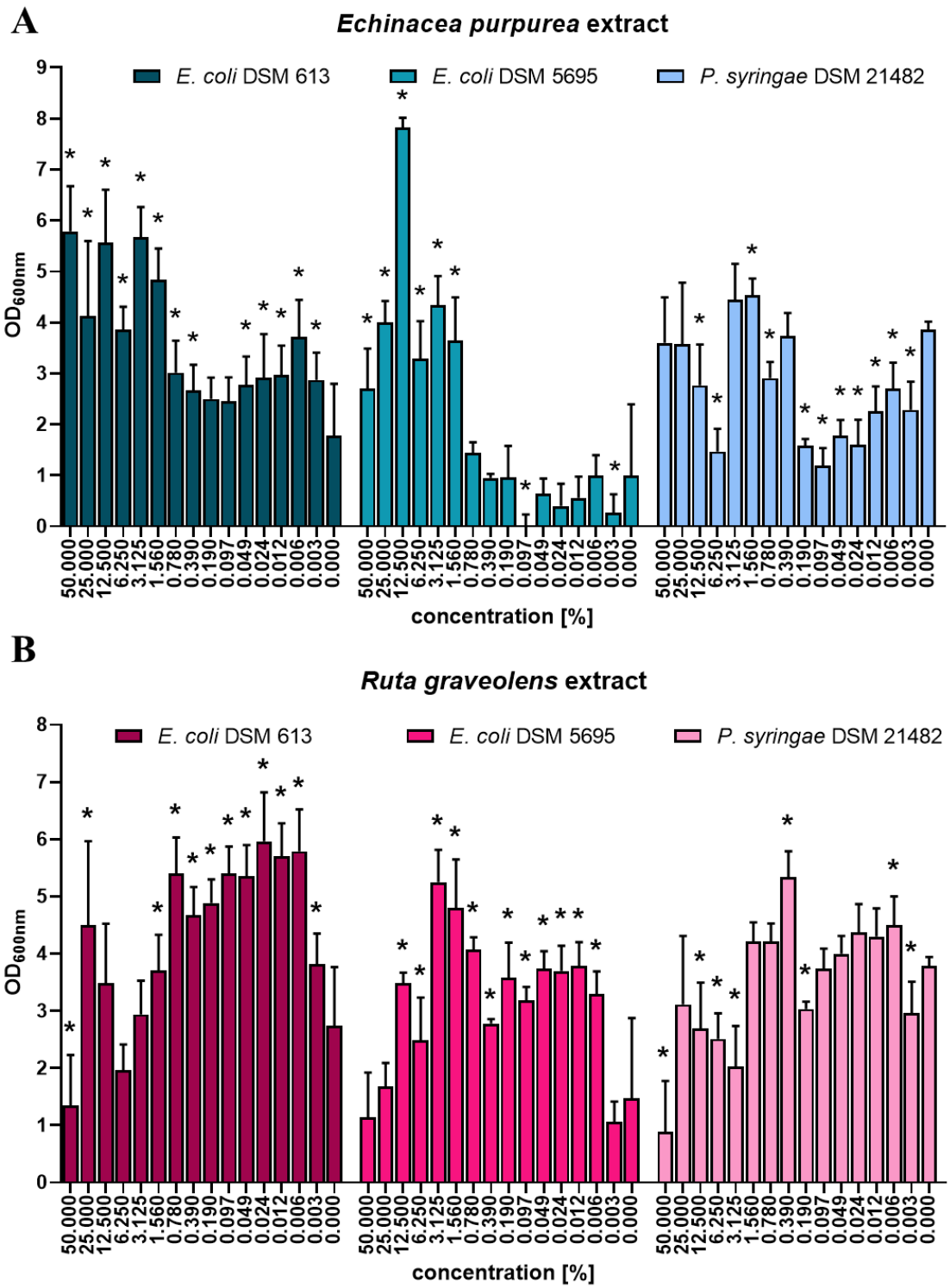

Figure 2. Bacterial biomass-altering activity of the tested plant extracts, measured after 24 h of incubation. *Echinacea purpurea* extract activity assessed by the microdilution method, (**A**) and *Ruta graveolens* extract activity assessed by the microdilution method (**B**). Error bars represent standard deviation (SD) between the samples. The means sharing the star asterisk are significantly different from the control (extract concentration 0%) at $p \leq 0.05$.

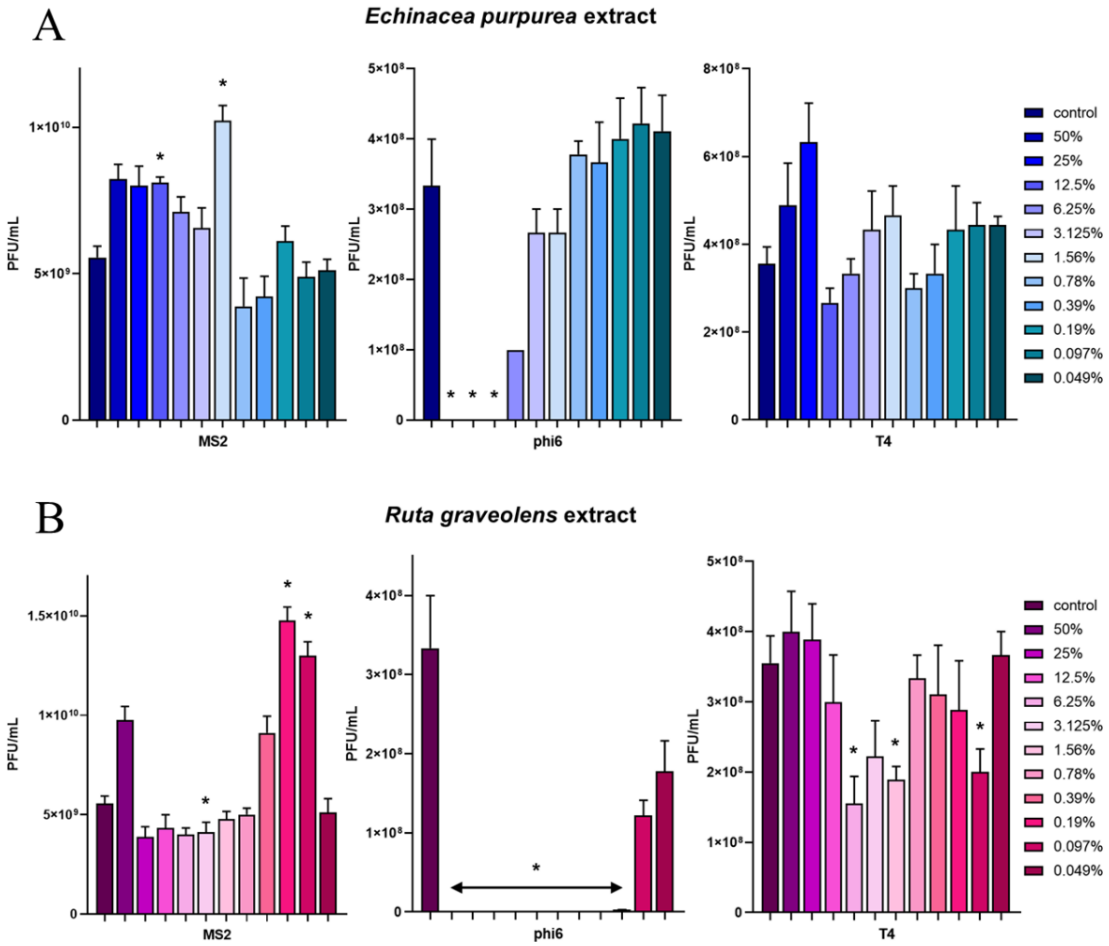

Figure 3. Phage titres (MS2, phi6 and T4) after 24 h exposure to different concentrations of plant extracts: results after coincubation of phages with *Echinacea purpurea* extract, (**A**) and after coincubation of phages with *Ruta graveolens* extract (**B**). Error bars represent standard deviation between the samples. The means sharing the star asterisk are significantly different from the control at $p \leq 0.05$.

The synogram experiment revealed complex relationship interactions between the tested extracts and bacteriophages within their bacterial hosts (Figure 4). Interestingly, in most of the cases, the highest extract concentration (25%) clearly caused an increased growth of the bacterial biomass; however, the cells were no longer active after 24 h of incubation in the stationary environment (Figure 4A′,A″,B–B″). This may indicate increased cell proliferation in the initial growth stage and earlier achievement of the stationary phase due to the presence of the extracts, regardless of the added amount of phage. Specific interactions between the tested elements were also observed. Two concentrations of the EP extract (3.125% and 1.56%) caused an increased growth of *P. syringae* cells when phi6 phage was not added. However, in most combinations, the addition of phage decreased cell activity at varying degrees: the concentrations of 6.25% and 0.049% in combination with a high dose of phage (10^8 PFU/mL) caused the cells to decrease their activity by half (48.9% of the control) or remained at a level similar to the control (89.9% of the control), respectively (Figure 4A). The EP extract influenced *E. coli* DSM 5695 by increasing bacterial activity in majority of the combinations, regardless of the concentration and amount of the phage added (measured at 21 h of incubation), even when the OD measures showed almost complete reduction of the bacterial biomass (measured at 18 h of incubation) (e.g., EP in concentrations of 6.25% and 0.049%: highest and lowest concentration where the phenomenon was observed, respectively) (Figure 4A′). The EP extract showed a similar influence on *E. coli* DSM 613 cells as on *P. syringae*, however, the cell activity was more phage and extract dose-dependent. Here, the phenomenon of the OD bacterial biomass reduction

in combination with varying bacterial activity was also observed (EP 6.25% = 39.1% activity compared to the control; EP 0.049% = 112.2% activity compared to the control), while high doses of the phage were applied (10^8 PFU/mL) (Figure 4A''). The addition of the RG extract to *P. syringae* cells resulted in a general reduction of the biomass at concentrations below 0.78%, along with a loss of cell activity. Interestingly, the concentration of 0.78% without the addition of phage caused avisible rise in the bacterial activity, while with the addition of phi6 (10^8 PFU/mL), the activity of the *P. syringae* cells dropped almost by half (55.6% compared to the control) (Figure 4B). When the RG extract was combined with *E. coli* DSM 5695, an overall increase in biomass was observed, while the cell activity levels dropped compared to the control. The exception was the concentration of 3.125% in combination with the phage (10^8 PFU/mL of MS2), where the OD values decreased to 59.9% of the control, with the cell activity being 37.8% compared to the control (Figure 4B'). When *E. coli* DSM 613 was tested, a similar outcome was observed, but the amount of bacterial biomass decreased at the concentrations of RG below 0.78%, with a simultaneous decrease in the activity of bacterial cells. The biggest decrease in the OD values was detected at the concentration of 0.78% (with the T4 phage addition of 10^8 PFU/mL), while *E. coli* DSM 613 activity was 23.1% compared to the control (Figure 4B'').

3.4. Bacteriophage Lytic Performance

For the phage lysis ability test in real time, bacteriophage performance against bacteria was tested in the presence of selected extracts at different concentrations, assessed by the changes in optical density measurements when the samples were cultivated in the bioreactors with mixing (150 rpm) (Figure 5). The extract concentrations were chosen based on the results from synogram experiment (combinations in which the above-described phenomena were present, along with maximal treatment doses as the controls). Experiment time was also shortened (16 h), as the synogram experiment (>18 h) revealed unactive cells. For most of the tested samples, the darkening of the sample was present due to the dark colour of the concentrated extract. This was taken into account when analysing the data: the extract optical density background was removed from the curves for a clear interpretation of the results.

The tested extracts influenced bacterial cells and phage activity in a varied manner; however, some dependencies were noted (Figure 5). In general, high concentrations (25%) of the tested extracts resulted in a stimulating effect on bacterial cells, often cancelling the lytic effect of the phages. The combination of *P. syringae*, phage phi6 and EP at concentrations of 25% and 6.25%, resulted in growth curves similar to the control bacterial curve: no phage lysis was observed. However, the treatment combination when the EP concentration was 0.049% led to the phi6 lysis curve to be the same as the control lysis curve (only phage and bacteria) (Figure 5A). Mixtures of the MS2 phage and 25% EP extract initially resulted in a similar, though slightly worse, lysis of *E. coli* DSM 5695 at 2 h to 4 h incubation. After this time, there was a clear rebound of the curve, suggesting increased bacterial proliferation. The reduction in the EP concentration to 6.25% also led to the bacterial lysis curve being weakened, but similar to the control lysis curve; however, the lysis started quicker (after 1 h vs. 2 h for the control), and later on, it also ended quicker (after 1.5 h vs. 3 h for the control). The EP concentration of 0.049% resulted in almost identical lysis curve as in the control lysis sample, except the low extract concentration also quickened the lysis visibly (Figure 5A'). When T4 phage was present, the high concentration of the EP extract (25%) eliminated the phage lytic effect after the first 2 h of incubation, simultaneously increasing the proliferation of *E. coli* DSM 613 cells. After 2 h, the sample optical density (proliferated bacterial biomass) started to drop very slowly. The EP concentration of 6.25% resulted in a very similar growth curve as the 25% concentration, until 6 h of incubation. After that time, a visible drop in the OD was observed, probably caused by the T4 phage activity. Finally, when 0.049% of the EP extract was added to the bacteria–phage sample, after the first 10 h of incubation, the curve was fairly similar to the control bacterial growth curve, meaning that the bacterial proliferation was only slightly altered. Then, after 10 h, a visible drop in

the OD was observed, as it was in the EP 6.25% sample, suggesting the occurrence of phage activity (Figure 5A″). When the RG extract was used (25%) along with the phi6 phage, a very high proliferation rate of *P. syringae* was observed after 3.5 h of incubation. After 10 h, the growth curve stabilised, suggesting that the bacterial stationary phase was reached (Figure 5B). The reduction in the RG concentration to 0.78% led to the observable lysis of the phi6 phage. However, the lysis was delayed, since it started after 5 h of incubation (control lysis started after 2 h) and also ended later in time (after 8 h, compared to the control that ended after 3.5 h), reaching the final OD values that were almost the same as that of the lysis control (Figure 5B(II)). When the RG extract (25% and 3.125%) was mixed with the MS2 phage and added to the *E. coli* DSM 5695 cells, initially no bacterial cell growth (and even a slight decrease in OD) was observed in the first 2 h of incubation. Afterwards, the *E. coli* proliferation boost was detected first, lasting until 4.5 h of incubation. After this time, bacterial cells continued multiplying at a slower rate, reaching only slightly higher OD values compared to the control bacterial growth curve (Figure 5B′). The highest tested RG concentration (25%) mixed with the T4 phage caused the *E. coli* DSM 613 growth curve to be similar to the control growth curve, stimulating cell multiplication slightly more. When RG 0.78% was used, bacterial proliferation was visibly weakened and, interestingly, a drop in OD values was noticed after 11 h of incubation (Figure 5B″).

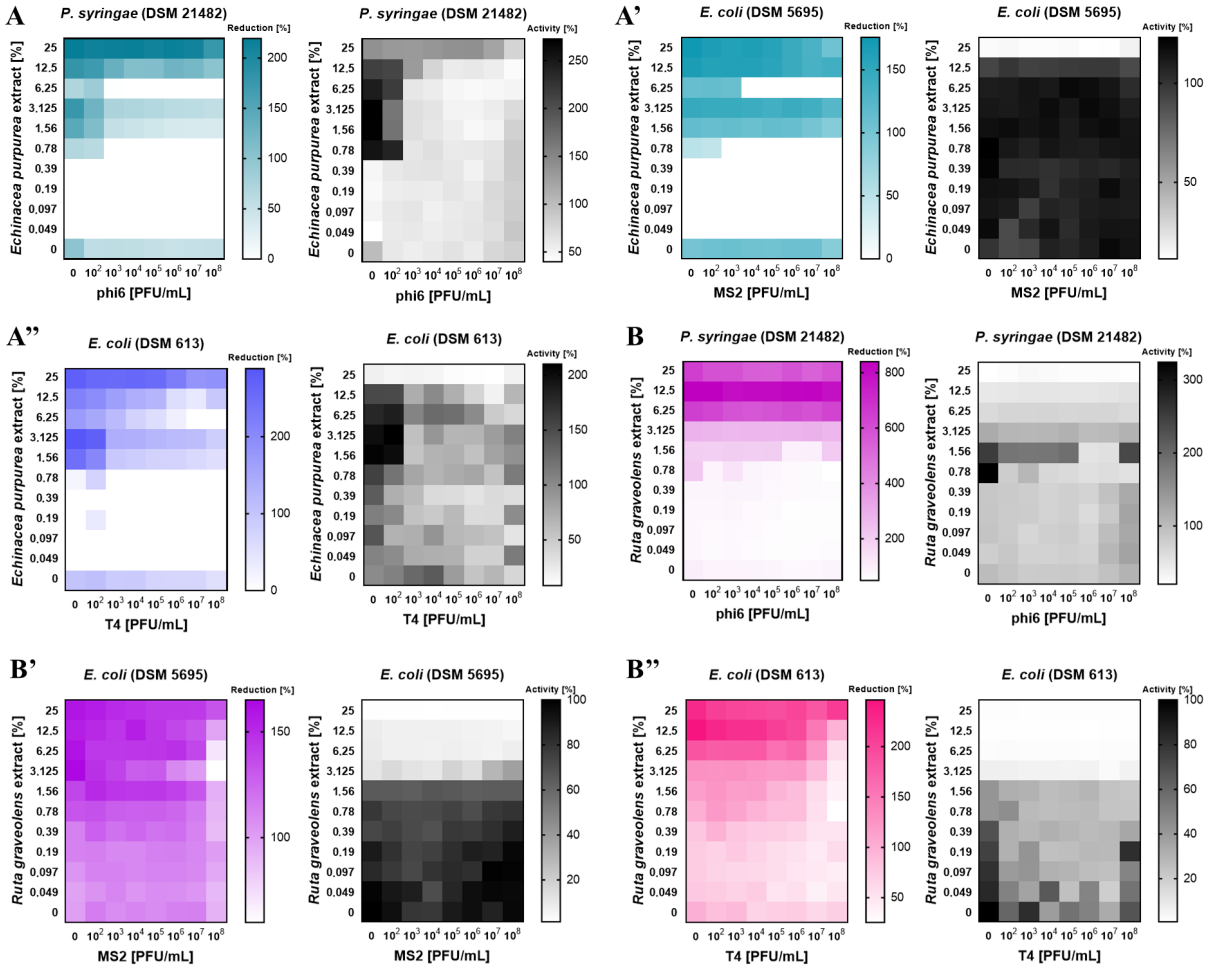

Figure 4. Effects of phage–extract combinations on host bacterial growth. Coloured heatmaps represent OD_{600nm} measurements, and black and white heatmaps represent corresponding fluorescence measurements. Combined treatment of phages with the *Echinacea purpurea* extract (**A**–**A″**), combined treatment of phages with the *Ruta graveolens* extract (**B**–**B″**). Synograms (t = 24 h) represent the mean reduction (% of the control) or activity (% of the control) percentage of each treatment from the three replicates.

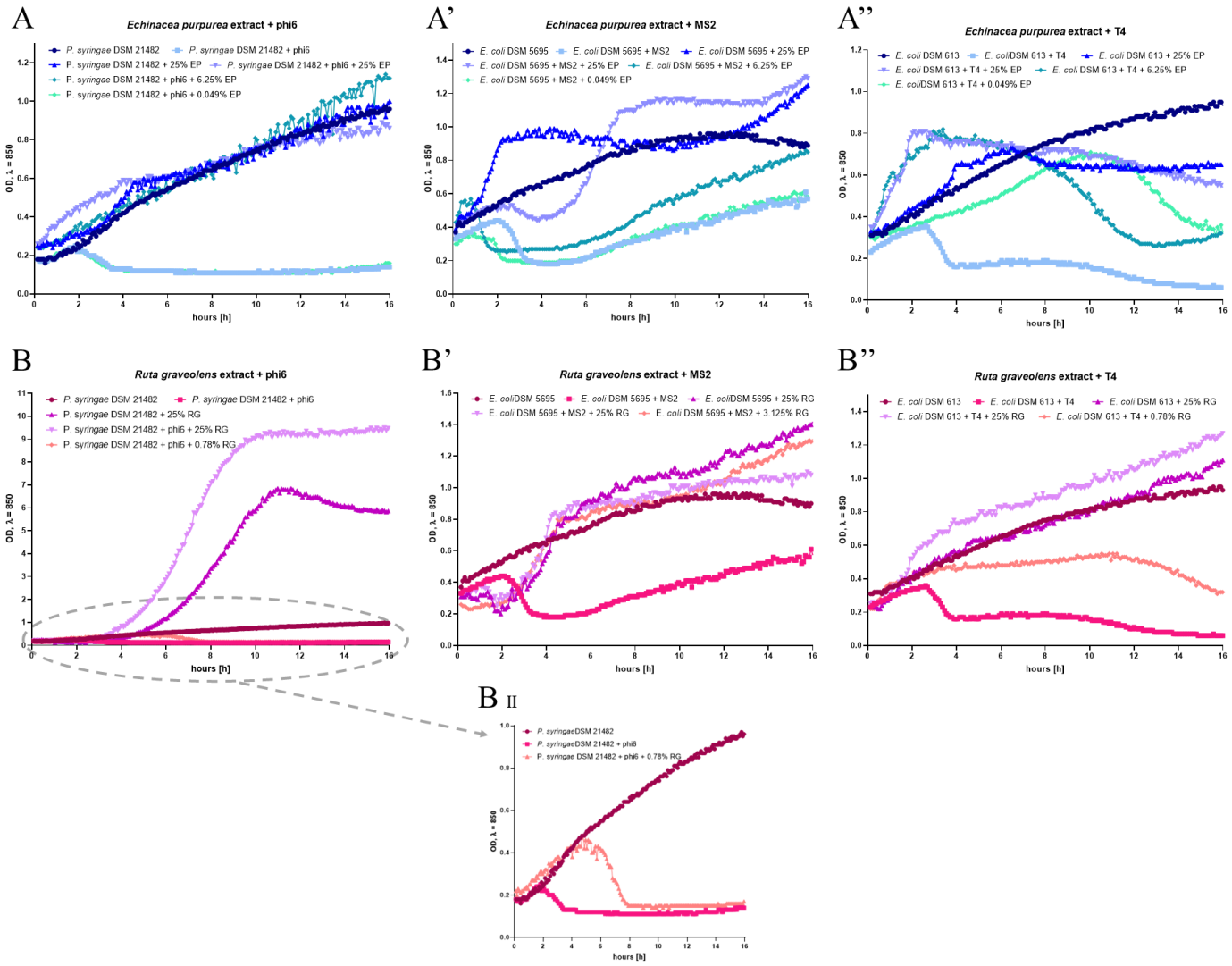

Figure 5. Bacteriophage lytic kinetics with different extract concentrations, chosen in the synogram experiment. Combined treatment of phages with the *Echinacea purpurea* extract (phi6 (**A**); MS2 (**A'**); T4 (**A''**)), combined treatment of phages with the *Ruta graveolens* extract (phi6 (**B**); MS2 (**B'**); T4 (**B''**); phi6 exported results of the chosen curves from the B graph (**B**(**II**))).

3.5. Microscopic Imaging

After the bioreactor lysis experiment was performed, SEM micrographs were carried out as an additional test, in order to visualize bacterial counts and fitness. *P. syringae* and phage phi6, along with the lowest concentration of the EP extract (0.049%), have been selected for this assay, and it was confirmed that the presence of the extract does not affect the bacterial cells and in consequence, the phage lysis process (Figure 6A). Compared to the control, when phage phi6 with *P. syringae* was used (Figure 6B), the addition of the EP extract did not visibly influence bacterial cells: the cell count was similar along with *P. syringae* morphology, suggesting an unchanged bacterial fitness (Figure 6).

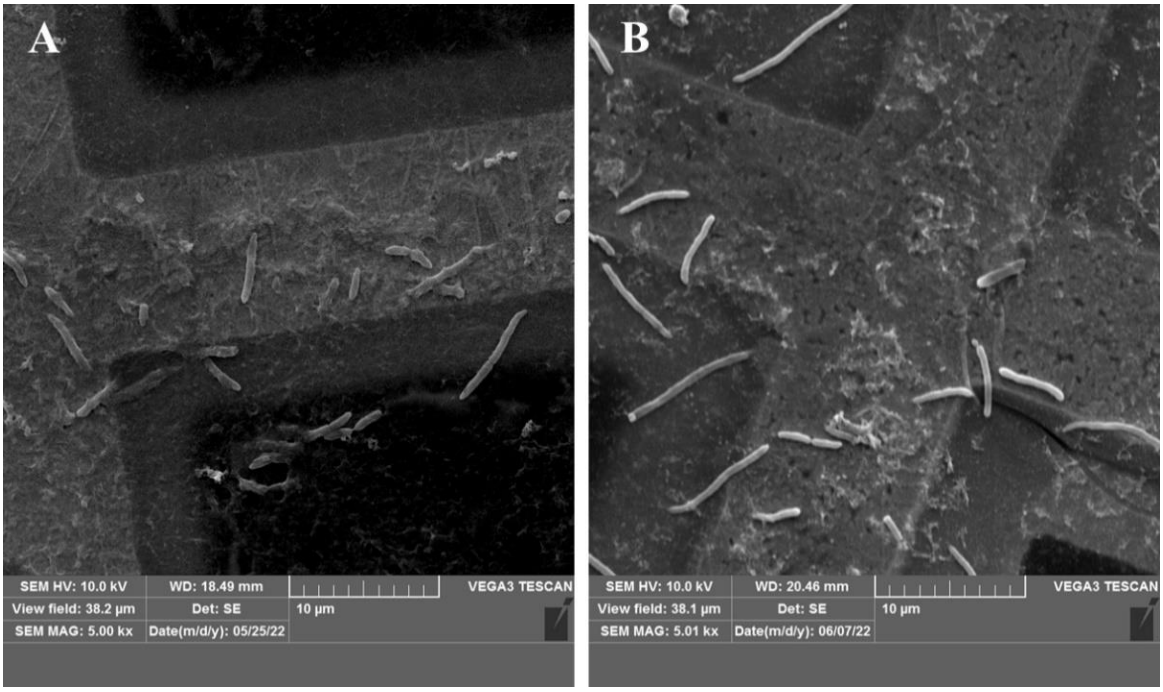

Figure 6. SEM micrographs of the mixtures of bacteria *P. syringae* and phage phi6 (**A**), compared to the same mixture of *P. syringae* and phi6, but with the addition of 0.049% *Echinacea purpurea* extract (**B**).

4. Discussion

Herbs such as *E. purpurea* and *R. graveolens* are commonly used for medical purposes, since they have many beneficial effects for humans [21–24], but also animals, for which these herbs can serve as feed additives or alternative feed source [25–27]. It has also been shown that phages can be used as feed additives to reduce bacteria in animal preslaughter, without negatively impacting microbial communities [28]. Therefore, in our work, we tested the possible interactions of *Echinacea purpurea* (L.) Moench (EP) and *Ruta graveolens* (RG) extracts and lytic phages as antibacterial agents (which can also serve as models of eukaryotic viruses) in the environment of bacteria. For this study, we have chosen well-known and studied phage models (phi6, MS2 and T4) from a certified collection, so the result interpretation will not be additionally complicated by using wild, unknown phages. Moreover, the selected phages were characterized by different sizes and specific features (Table 1) to ensure model diversity. Additionally, phage-matching bacterial hosts functioned as pathogens related to fauna and flora diseases (*P. syringae* and *E. coli*).

EP and RG methanol extracts revealed diverse and multidimensional interactions with the mixtures of phages and their bacterial hosts. In order to explain these interactions, it is necessary to draw general conclusions from the performed experiments and propose hypotheses explaining the results.

The lysis profile experiment of the EP extract, at the concentration of 25% in combination with the MS2 phage and *E. coli*, resulted in limited phage lytic activity at the begging of the curve, then stimulating the growth of bacteria. The 6.25% concentration caused weakening of the phage lysis, and 0.049% generated similar lysis outcomes compared to the control lysis. These concentrations did not influence phage activity in the coincubation test, but stimulated the host cells during a modified MIC test (static conditions/no mixing), therefore the presented results are a consequence of the extract's concentration-dependent influence on bacteria that was more pronounced than the phage lytic activity. When the phi6 phage and *P. syringae* were tested, the EP concentrations of 25% and 6.25% resulted in the lack of phage lysis (curves comparable to the control bacterial growth), while at 0.049%, the lysis curve was the same as the lysis control. The highest tested concentration (25%) also showed phagicidal activity, therefore the curve assay effect is the result of the

extract influence on the phage. Since no phage killing effect was present in the coincubation test at the lower EP concentrations, the middle concentration (6.25%) is the result of the only extract effect on bacterial cells (or mixing could turn this concentration phagicidal), which disappeared at a low concentration of 0.049%. The lack of EP 0.049% effect was also additionally proven by the SEM micrographs. When mixtures of EP extract with the T4 phage and *E. coli* were studied, the highest EP concentration (25%) caused initial bacterial stimulation, followed by a slow decrease in the *E. coli* proliferation after 6 h of incubation. The 6.25% EP extract resulted in a similar outcome, however the drop in bacterial biomass after 6 h was more pronounced. When the concentration of 0.049% was used, the curve was initially similar to the control growth curve, and after 10 h of incubation, a gradual decrease in the cell density was observed. The tested concentrations did not have any influence on the phage T4 in the coincubation assay, and it stimulated bacterial growth in the modified MIC assay; therefore, these results are again explained through the extract concentration-dependent stimulating influence on bacteria, that is stronger than the phage lytic activity. Moreover, the effect is also time-dependent, as it fades away with time, allowing for phage lysis afterwards.

Our hypotheses of EP extract bacterial stimulating effects are in line with the results of other authors, where the activity of *E. purpurea* against bacterial cells varied. The general antibacterial properties of *E. purpurea* are detected mainly for their ethanol extracts [29]. Nevertheless, in the work, six different echinacea commercial ethanol extracts were analysed, including extracts from *E. purpurea* roots and *E. purpurea* aerial parts, and *E. coli* was a species relatively insensitive to those extracts: no bacterial reducing effect was detected [6]. When the plant activity against Gram-negative bacteria was observed, hydroethanol extracts of the aerial parts of *E. purpurea* were used, significantly *inhibiting E. coli* growth [30]. At the same time, the work of other authors highlights that even in commercial hydroalcoholic echinacea extracts (roots of *Echinacea purpurea* L. Moench; roots, leaves, flowers and seeds of *E. purpurea*; aerial parts and roots of *E. purpurea*; and roots of *E. angustifolia*), obtained from certified trading houses, there is a difference in the extracts' properties [31]. That points out the differences of the plant activity against bacteria, that seems to be associated with the type of extraction and parts of the plant used in the experiments. As performed in our work, when the methanolic extract of *E. purpurea* aerial parts was tested, there was no evidence of microbicide activity against *E. coli*, even at the highest tested concentration of 20 mg/mL [2] (highest tested concentration in our work: 25%, 37.4 g/L). Similar to our studies, in which the stimulating effect of EP was detected, it has been shown that echinacea supplementation (dietary supplement; aerial and root parts of the plant; patented extraction method) can stimulate bacteria, namely the selected groups of human gastrointestinal tract microbiota [5]. Moreover, studies have shown that *E. purpurea* also possesses antiviral activity [21,32–34] that could support our findings on the EP extract antiphage activity.

The lysis profile experiments of the RG extract at the concentrations of 25% and 3.125% with the MS2 phage and *E. coli* generally resulted in slightly increased biomass production compared to the control bacterial growth. At the coincubation test, RG 25% did not influence phage viability, whereas RG 3.125% caused a drop in MS2 counts. A modified microdilution assay in static conditions showed that RG 25% did not alter bacterial growth, whereas RG 3.125% stimulated *E. coli*. Therefore, the presented results concerning the higher extract concentration are a consequence of introducing mixing to the experimental environment, indicating that RG 25% could have become bacteria-stimulating and/or phage-inhibitory (as it was detected for RG 3.125%). That statement is additionally confirmed by the control curve of *E. coli* + RG 25%, which is also characterized by a higher biomass production compared to the control *E. coli* growth. The stimulating effect of the RG extract was also observed in the combination of *P. syringae* and phage phi6, where RG 25% caused greatly enhanced bacterial proliferation. When RG 0.78% was used, phage lysis was present, but delayed by approximately 3 h. Coincubation assay revealed that both RG concentrations showed phagicidal activity, whereas a modified MIC test demonstrated a lack of their

influence on bacteria. These interactions concerning RG 25% are again explained by both the phage-inhibitory and bacteria-stimulating (gained in the mixing environment) RG activity, since bacteria + extract control (*P. syringae* + RG 25%) also showed pronounced bacteria-stimulating effect. The results observed for RG 0.78% are therefore the effect of reducing the stimulating influence of the extract by lowering its concentration and simultaneously abolishing the phagicidal activity of the extract in a dynamic environment. When mixtures of RG with T4 phage and *E. coli* were studied, the RG concentration of 25% resulted in slight bacterial stimulation, whereas RG 0.78% caused weakened lysis, that was also extended in time. These concentrations did not influence phage viability in the previous experiment, while they positively altered bacterial growth in the modified MIC test. Here, the results for RG 25% can be explained by the bacteria-stimulating effect of the extract and probable gain of phage-inhibitory activity in the mixing environment. Therefore, when the RG concentration was lower (0.78%), the extract phage-inhibitory influence was weaker, allowing for limited lysis, so the effect was also concentration-dependent.

Unfortunately, there is limited knowledge on *Ruta graveolens* activity on Gram-negative bacterial cells performed with modern experimental methods. Moreover, the tendency of differentiated plant activity on bacteria associated with the type of extraction and used plant parts is also observed. The study in which antibacterial activity of ethanol, methanol, chloroform and distilled water extracts of *R. graveolens* leaves was tested by the disk diffusion methods, the methanol and chloroform extracts recorded better antibacterial activity than the ethanol extract, and the water extract did not exert any activity. Moreover, the authors also point out that the phytochemical analysis of different solvent extracts show considerable change in the nature of the chemicals [35]. Other authors demonstrate that aquatic, ethanolic and methanolic extracts likely of the whole *R. graveolens* plant (not specified in the paper) have been almost equally effective against *E. coli* (25 g/L MIC aquatic extract; 12.5 g/L MIC ethanolic extract; 25 g/L MIC methanolic extract) and *P. aeruginosa* (25 g/L MIC aquatic extract; 25 g/L MIC ethanolic extract; 25 g/L MIC methanolic extract). The experiments were performed using disk diffusion and well diffusion methods [36]. Another study designed to screen various solvent extracts of *Ruta graveolens* used leaves, stems and seeds of the plant, extracted by four different solvents, receiving ethanolic, methanolic, chloroform and aqueous extracts. In this work, the disk diffusion method showed that *B. subtilis* demonstrated a high zone of inhibition (20 mm) at 200 mg/mL of methanolic extract, but *E. coli* and *C. tropicalis* did not show any zone of inhibition against any extract [37]. The lack of *R. graveolens* plant extract activity against *E. coli* was also found in the work of other authors, where methanol, petroleum ether, ethyl acetate and water–methanol extracts of the plant aerial parts were tested, also using the disk diffusion method [38]. This phenomenon was also proven in the works of other authors, in which hydro and hydroalcoholic extracts of *Ruta graveolens* (tested by the disk diffusion method and serial microdilution method) did not show an inhibitory effect on the growth of studied bacteria (i.a. *E. faecalis*, *E. coli*, *K. pneumoniae*, *S.* Typhi) up to a concentration of 5 mg/mL [39]. Bearing in mind the reports showing the lack of antibacterial effect of the *R. graveolens* extract concluded from outdated research methods, the stimulating effect of RG presented in our work is possible. Moreover, it has been reported that *R. graveolens* compounds show stimulating activity on the growth of fungi in a concentration-dependent manner, when tested using a 96-well microtiter assay [40]. Even if there are no papers describing the influence of *R. graveolens* on bacteriophages, the antiviral activity of the plant was confirmed on acyclovir-resistant HSV-1 [41], which can support our findings regarding the RG antiphage activity.

Currently, the literature describes the simultaneous application of phages and plant extracts or the effects of extracts on phages at a limited degree, and even fewer works describe their interactions in the environment of bacterial hosts. Previous works have mainly focused on the plaque-forming ability of the phages after their contact with plant extracts [11,42–44]. However, there are some findings describing more complex interactions. One shows the influence of a crude acetone extract of the pit of date palm (*Phoenix dactylifera*

L.) on the lytic *Pseudomonas* phage ATCC 14209-B1 by changes in the absorbance. It was found that the extract showed the ability to inhibit the infectivity of the phage and completely prevent bacterial lysis. Similarly to our work, authors observed that the higher the concentration, the bigger the phage inhibition [45]. However, a limited number of extract concentrations were used, and no information of the extract influence on bacteria alone was present. In another work, aqueous extract of *E. amoenum* dried flower was used, and the antiviral activity against *S. aureus* bacteriophage 3C was determined by the agar overlay method and one-step growth experiment. Antibacterial activity was determined by the agar well diffusion and minimum inhibitory concentration methods. The extract also showed concentration-dependent antiviral activity and reduced the yield of phage [46]. However, since a subinhibitory concentration of the extract was used in the extract–bacteria–phage tests, the observed effects could also be due to the influence of the extract on bacterial cells to some extent. The importance of performing multiple tests in order to understand such complex interactions (that can change in different environments) was noticed in the study of other authors. The influence of phenolic compounds from pomegranate peels (Ps), grape seeds (GSs) and black cumin (NS) on *S. aureus* phages was investigated by examining the effect of phenolic extracts on phage plaque sizes, and later calculating phage titers and bacterial counts from the mixed culture (phages–compounds–bacteria) experiment. It was found that only the NS extract had a positive effect on phage activity by increasing the phage plaque size; however, the same effect was not reflected in phage titers in a liquid medium. Overall, no synergistic effect was observed in liquid media experiments. Moreover, it was found that P and GS extracts inhibited phage activity, and the observed interactions were generally found to be more closely related to antagonism, similarly to what we found in our work [47]. Finally, comparably to some findings in our study, it was observed that plant extracts in combination with virulent phages can significantly reduce the bacterial concentration compared to untreated and extract-treated controls up to 6 h, but this reduction did not extend further (decreased over time). Moreover, in most cases, the phage and extract combinations did not significantly reduce the bacterial content compared to phages alone [7].

In our work, we also hypothesise that mixing itself can be responsible for some of the observed effects of the EP and RG plant extracts (by changing the environment conditions). It is worth noting that in our previous research, we also observed the stimulation of bacteria by plant extracts after introducing the mixtures into the dynamic (mixing) environment of a bioreactor [18]. This phenomenon can simply be explained by the enhanced physical contact of the extract molecules, bacterial cells and phages caused by mixing, which consequently enhances the interactions in the mixture that could not be observed in static conditions. However, a thorough understanding of this phenomenon may require further research.

5. Conclusions

This is a first study describing interactions between the echinacea and rue plant extracts in combination with phages, in a bacterial host milieu. The effects of low concentrations of *E. purpurea* and *R. gravolens* methanol extracts, in a dynamic environment (mixing bioreactor) on the course of phage lysis (phage activity), depends on the species of the phage and bacterial host. High concentrations of the extracts can eliminate the phage lytic activity to a large extent by exerting a stronger stimulating effect on bacterial cells, however, gaining antiphage activity of those concentrations in a dynamic environment is also possible. Moreover, in some cases, extracts can affect the phage activity itself, regardless of the phage and host species. The interactions of phage–extract factors against bacteria in a static environment are often different than in a dynamic environment; therefore, many varied experiments should be performed, especially when examining multifactorial mixtures. Further studies are needed to understand the basics of the interactions between phages and plant extracts for the possible future use of the phage–extract combinations for biocontrol purposes.

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