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Characterization of bacteriophages as biocontrol agents against crown gall disease in Egypt

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Crown gall is a devastating disease with severe economic impacts, particularly in nurseries cultivating mango and other stone fruit trees. Virulent strains of *Agrobacterium tumefaciens* cause the disease. In this study, water samples were collected from agricultural drains in Beni-Suef, Egypt, to isolate bacteriophages. A bacteriophage was successfully isolated using *A. tumefaciens* strain AUMCB-455, a known crown gall pathogen. Genome characterization and electron microscopy were employed to identify the virus, which was assigned as vB_AgrP-B1 and classified to the family Podoviridae. The bacteriophage remained active at 65°C and optimal at pH 7, with a dilution endpoint of 10^{-11}. Plaque formation was influenced by various salts, sugars, and heavy metals at different concentrations (10 mM, 1 mM, 0.1 mM); salts either enhanced or inhibited plaques, sugars increased plaque numbers, while heavy metals suppressedF plaque formation. In a controlled laboratory assay, the application of vB_AgrP-B1 to infected carrot tuber discs effectively inhibited the formation of crown galls caused by *A. tumefaciens* AUMCB-455. These findings highlight the potential of vB_AgrP-B1 as a biocontrol agent for managing crown gall disease.

Keywords: *Agrobacterium tumefaciens*, biocontrol agent, carrot tuber assay, crown gall disease, Podoviridae

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INTRODUCTION

Crown gall, caused by *Agrobacterium tumefaciens*, is a serious plant disease that affects a wide range of crops, leading to substantial agricultural losses worldwide. The disease manifests tumor-like growths, primarily on dicotyledonous plants, which severely compromise the productivity of high-value crops, including fruit trees, vines, and ornamental plants. Traditional chemical treatments and non-pathogenic strains offer limited success (Kawaguchi, 2022).

Agrobacterium tumefaciens is a Gram-negative, rod-shaped, aerobic, and motile bacterium within the Rhizobiaceae family. Unlike some other bacterial species, *A. tumefaciens* does not form endospores. It infects plants through wounds, causing tumor formation on a range of hosts, including dicots, certain monocots, and some gymnosperms. The bacterium's optimal growth temperature is 28°C. Both *A. tumefaciens* and the nonpathogenic *A. radiobacter* can metabolize essential carbon sources and grow in low-salt media, with ammonium salts and nitrates serving as suitable nitrogen sources. In contrast, *A. rhizogenes* and *A. rubi* exhibit different nitrogen utilization patterns (Wise et al., 2006).

Pathogenic strains of *A. tumefaciens* are prevalent in soils, especially in the rhizosphere of plants. Environmental factors, such as soil moisture retention in loamy soils and an acidic pH, have been associated with the predominance of virulent strains over nonvirulent strains (Marashi, 2000). The pathogenicity of *A. tumefaciens* is largely attributed to the tumor-inducing (Ti) plasmid, which harbors the transferred DNA (T-DNA) and virulence (vir) genes responsible for initiating crown gall formation (Watson et al., 1975).

Bacteriophages, viruses that specifically target bacteria, are gaining recognition as an alternative to conventional antibacterial agents for managing bacterial plant diseases. Phages are highly host specific, capable of evolving in response to bacterial mutations, and environmentally friendly. They are cost effective to produce and naturally abundant in ecosystems, including soils and the rhizosphere, where they contribute to bacterial population regulation (Gordillo Altamirano and Barr, 2019; Stone et al., 2019). Their specificity and self-limiting nature make phages an attractive option for controlling *Agrobacterium* infections without affecting plant health.

Phages have shown effectiveness in controlling bacterial plant diseases, such as blast disease in rice caused by *Xanthomonas oryzae* pv. *oryzae* (Nazir et al., 2021) and bacterial wilt in tomatoes caused by *Ralstonia solanacearum* (Bhunchoth et al., 2015). Additionally, studies have established a strong correlation between environmental factors, such as soil moisture, pH, and organic matter availability, and the composition of phage communities, which are particularly abundant in the rhizosphere (Williamson et al., 2005; Pratama and Van Elsas, 2018; Dion et al., 2020; Chevallereau et al., 2022; Muscatt et al., 2022, Thiena et al., 2024).

Phages thrive in diverse environments, ranging from freshwater and saltwater to extreme habitats,

including hot springs (up to 76°C) and polar regions (Filippova et al., 2016). In many ecosystems, phages often outnumber bacterial populations, sometimes by more than tenfold (Chevallereau et al., 2022; Elois et al., 2023). While some phages exhibit broad host ranges, others, such as *Agrobacterium* phage Atu_ph07, are more specific, targeting particular *A. tumefaciens* strains (Attai et al., 2018).

The application of bacteriophages has garnered significant interest across various fields, including agriculture, medicine, veterinary care, public health, water treatment, and food industry (Sohail et al., 2020; Elfadadny et al., 2024). Given their ability to specifically target bacterial pathogens, phages represent a promising and sustainable approach to controlling crown gall disease.

This study aims to isolate and characterize bacteriophages from agricultural environments in Egypt and assess their potential as biocontrol agents against *A. tumefaciens*. By addressing the limitations of conventional methods, this research seeks to develop sustainable strategies that can mitigate the economic and ecological impact of crown gall disease.

MATERIALS AND METHODS

Bacterial Strains

The bacteriophage was isolated using *Agrobacterium tumefaciens* strain AUMCB-455. The bacterial strain's sequencing data has been deposited in GenBank under accession number ON600994. The strain was preserved at -20°C in 50% glycerol (v/v) for long-term storage. For the host range analysis (spot test), *Agrobacterium fabrum* strains were obtained from the Microbiology Lab at Faculty of Agriculture, Beni-Suef University. These strains have been deposited in GenBank with accession numbers PQ253061 and PQ269222. Additionally, *Agrobacterium tumefaciens* strain was sourced from the Agricultural Research Center in Dokki, Cairo, Egypt.

Isolation and Enrichment of Bacteriophages

Four wastewater samples were collected in polyethylene bags during winter 2023 from agricultural water drains with a pH of 7.8 in Beni-Suef, Egypt. The samples were filtered through filter paper to remove larger particles, following the method of Attai and Brown (2019) with minor modifications. Triplicates of 10 mL of water samples were transferred into 250 mL Erlenmeyer flasks containing 50 mL of nutrient broth and inoculated with 10 mL of a 24-hour-old bacterial culture. The flasks were incubated for 24 hours in a shaking incubator at 30–35°C. Each flask received 1 mL of chloroform and was then incubated on an orbital shaker for 30 minutes at room temperature. The suspensions were centrifuged at 9700 x g for 10 minutes to remove bacterial cells. The supernatants were collected, sterilized by syringe filtration through 0.45 µm filters, and stored at 4°C in the dark until further use. Bacteriophages were subsequently isolated using the plaque assay method, as described by Łoś et al. (2008).

Purification and Enumeration of Bacteriophages

The bacteriophage filtrate was serially diluted (10^{-1} to 10^{-10}) using SM buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, and 0.01% gelatin (v/w)) before being applied in the double-layer assay, following the protocol of Ravensdale et al. (2007) with modifications. The assay involved a soft agar overlay, where 9 mL of soft nutritional agar (comprising 500 µL of an overnight bacterial culture, 500 µL of phage filtrate, and 8 mL of soft agar mixed with 0.2 g/L $MgCl_2$) was applied over a bottom layer of 15 mL nutrient agar. The plates were incubated at 30–35°C for 24 hours and then examined for plaque formation. When plaques were observed, a sterilized pipette tip was used to isolate a single plaque, which was transferred to 1 mL of sterile SM buffer. To remove bacterial cell debris, the tube was vortexed and then centrifuged at 11,000 × g for 15 minutes at 10°C, as described by Tovkach et al. (2012). The supernatant was filtered through a 0.45 µm syringe filter. The filtrate was then serially diluted elevenfold, and the plaque assay was repeated several times to ensure the isolation of single plaques of the phage(s). The purified bacteriophages were stored in SM buffer at 4°C in the dark.

Transmission Electron Microscopy

A drop of bacteriophage lysate was adsorbed onto a carbon-coated copper grid and allowed to air-dry before being negatively stained with 3% uranyl acetate. The sample was then examined using a JEOL JEM-1010 transmission electron microscope, operating at 80 kV, at the Regional Center of Mycology and Biotechnology (RCMB) at Al-Azhar University, Cairo, Egypt.

Extraction of Phage Nucleic Acid

Phage nucleic acid was extracted by adding 500 µL of concentrated phage lysate, 20 µL of 10% SDS, and 1 µL of 20 mg/mL proteinase K in a clean, sterile Eppendorf tube. The mixture was incubated at 56°C for 1 hour. Following incubation, an equal volume of phenol was added to the mixture, which was then

thoroughly mixed and centrifuged for 5 minutes at 6050 × g at room temperature. The aqueous supernatant was transferred into a new Eppendorf tube, and an equivalent volume of chloroform was added. After inverting and mixing, the mixture was centrifuged again. The resulting supernatant was moved into another new Eppendorf tube, and it was then mixed with two volumes of frozen ethanol. After that, the mixture was incubated at -20°C overnight. The next day, the sample was centrifuged at maximum speed for 20 minutes. The supernatant was discarded, and 70% ethanol was added to the pellet. After centrifugation at maximum speed for 2 minutes, the ethanol was removed, and the pellet was air-dried for 15 to 30 minutes. Finally, the dried pellet was dissolved in 20 µL of TAE buffer (AlKhazindar et al., 2016).

Identification of Phage Nucleic Acid Type

To determine the type of nucleic acid in the phage, 5 µL of isolated nucleic acid from each sample was prepared. One sample was treated with DNase (1 µL) and the other with RNase (1 µL). The mixtures were incubated at 37°C for two hours to facilitate enzymatic digestion. Following digestion, electrophoresis was performed on a 0.8% agarose gel in 1X TAE buffer. Since this experiment is designed to distinguish between DNA and RNA rather than determine the fragment size, the presence or absence of bands after enzymatic treatment was used to confirm the nucleic acid type. The gel was stained with ethidium bromide (0.5 µg/mL) and visualized using a UV trans-illuminator (λ = 254 nm). Images were captured with a Canon digital camera. The nomenclature for the isolated bacteriophage was done according to Kropinski et al. (2009).

Host Range

Three bacterial strains were used to determine the host range of the identified bacteriophage. The spot test was performed on overnight bacterial cultures following the method of Elhalag et al. (2018) with minor modifications. For the spot test, 200 µL of a 24-hour-old bacterial culture, with a biomass count of 10^8 CFU/mL, was added to 3 mL of soft agar, gently mixed, and poured onto the surface of nutrient agar plates. A 10 µL drop of phage suspension (2.5×10^{11} PFU/mL) was spotted onto the plates, which were then incubated at 30–35°C overnight. Lysis zones were monitored for 48 hours to assess the effectiveness of phage-induced lysis.

Physical Properties

Dilution End Point (DEP): A serial dilution of tenfold (10^{-1} to 10^{-10}) of the isolated bacteriophage was prepared. The dilution endpoint was determined using a double-layer assay, identifying the last dilution that yielded no distinct plaques. This assessment was performed duplicate, with each experiment conducted in triplicate.

Thermal Inactivation Point (TIP): The TIP was determined following the protocol of Holmes (1956) with slight modifications. Phage lysate was incubated at various temperatures (5°C, 15°C, 25°C, 35°C, 45°C, 55°C, and 65°C) for one hour. After incubation, a double-layer assay was conducted to quantify plaque formation after 24 hours, identifying the temperature at which the bacteriophage lost its activity. This test was performed in duplicate, with each trial executed in triplicate.

Effect of pH: To evaluate the effect of pH on phage activity, the phage lysate was incubated in SM buffer at pH values ranging from 1 to 12 at 5°C for 24 hours. The impact of pH was assessed by performing a double-layer assay to count the number of plaques, as described by Holmes (1956). This experiment was conducted in duplicate, with each trial performed in triplicate.

Effect of Salts, Sugars, and Heavy Metals on Bacteriophage Activity

The influence of various salts, sugars, and heavy metals on bacteriophage vB_AgrP-B1 activity was evaluated following the method of Paunikar et al. (2012). In all experiments, phage filtrate (500 µL) and an overnight bacterial culture of *Agrobacterium tumefaciens* AUMCB-455 (500 µL) were mixed in soft agar tubes containing the test compounds at final concentrations of 10 mM, 1 mM, and 0.1 mM. The mixtures were then poured onto sterile 9 cm Petri dishes with a presolidified nutrient agar base and incubated at 30–35°C for 24 hours. A control plate without treatment was included for comparison. For the salt experiment, monovalent (NaCl, KCl), divalent ($CaCl_2$, $MgCl_2 \cdot 6H_2O$), and trivalent ($FeCl_3$) chloride salts were tested. The sugar experiment included monosaccharides (fructose, glucose) and disaccharides (lactose, sucrose). The heavy metal experiment examined the effects of cobalt chloride ($CoCl_2$), copper chloride ($CuCl_2$), mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$), and zinc sulfate ($ZnSO_4$). The number of plaques was counted and compared to the untreated control to assess the impact of each compound. The dilution factor employed was 10^{-6}.

Small-Scale Application of Phage Therapy

Carrot tubers were surface sterilized with 70% ethanol, then sliced into 0.5 cm discs, and placed on sterilized 12 cm Petri dishes containing wet Whatman No. 1 filter paper. Phage lysate (250 µL) was applied to the carrot discs and allowed to incubate for 30 minutes, followed by the addition of an equal volume of bacterial inoculum from a 24-hour-old *Agrobacterium tumefaciens* AUMCB-455 culture. The positive control received 500 µL of bacterial suspension, while the negative control was treated with 500 µL of SM buffer. All plates were incubated at room temperature in the dark for 3-4 weeks, with the filter paper maintained in a moist condition using sterile water. After the incubation period, the plates were examined to assess the ability of the isolated bacteriophage to protect the carrot discs from crown gall formation. The experiment was conducted in triplicate with duplicate plates for each treatment.

RESULTS

Bacteriophage Isolation

The isolation of bacteriophages yielded a range of plaque morphologies and sizes. The isolate selected for subsequent analysis, characterized by its distinct features, is shown in Figure 1.

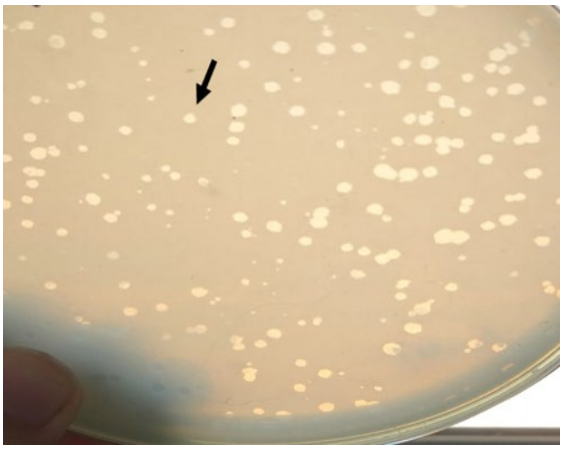

Figure 1. Plaques formed by isolated bacteriophages infecting *Agrobacterium tumefaciens* AUMCB-455.

Bacteriophage Purification and Characterization

The selected bacteriophage isolate was purified and labeled as Ph1. It displayed clear halo zone plaques with an average diameter of 0.2 cm, as shown in Figure 2.

Figure 2. Morphology of the purified phage (Ph1).

Transmission Electron Microscopy

Examination of the bacteriophage Ph1, following negative staining with 3% uranyl acetate, using TEM revealed that it possesses an icosahedral head with a diameter of 161 nm, as well as a short neck measuring 88.6 nm in length and 19.1 nm in width, as demonstrated in Figure 3.

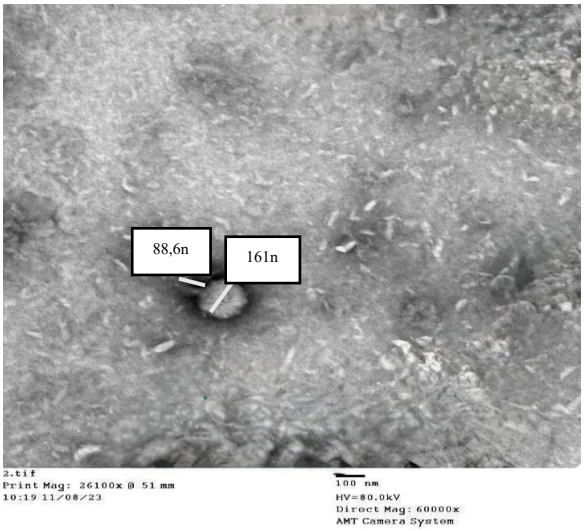

Figure 3. Transmission electron microscopy (TEM) image of the bacteriophage Ph1, negatively stained with 3% uranyl acetate.

Identification of Phage Nucleic Acid Type

The nucleic acid composition of the bacteriophage was determined through enzymatic digestion with DNase and RNase. DNase effectively degraded the phage nucleic acid, while RNase showed no effect, confirming that DNA is the genetic material, as shown in Figure 4.

Bacteriophage Nomenclature

The nomenclature of the bacteriophage was established based on a comprehensive analysis of its

nucleic acid type and morphological features observed under transmission electron microscopy (TEM). The enzymatic digestion with RNase and DNase conclusively identified the phage as possessing a DNA genome. Morphologically, the bacteriophage exhibited characteristic features consistent with members of the Podoviridae family, notably its short, noncontractile tail structure. Based on these findings, the bacteriophage was classified within the Podoviridae family (P). Consequently, the bacteriophage was designated as vB_AgrP-B1, following standard viral classification protocols that incorporate both the nucleic acid type and morphological characteristics.

Host Range

The isolated bacteriophage demonstrated lytic activity against both *Agrobacterium fabrum* and *Agrobacterium tumefaciens*, as shown by clear plaque formation in the double-layer assay (Figure 5). This indicates the bacteriophage's ability to infect and lyse the bacterial cells, supporting its potential as a biocontrol agent.

Physical Properties

Dilution End Point: Phage vB_AgrP-B1 was subjected to serial dilution up to 10^{-11}, as detailed in Table 1. At concentrations ranging from 10^{-1} to 10^{-5}, the plaque count was too numerous to quantify. Plaque formation became countable at a dilution of 10^{-6} and progressively diminished with increasing dilution. The dilution endpoint, where no plaques were detectable, was reached at 10^{-11}.

Thermal Inactivation Point: The stability of phage vB_AgrP-B1 was evaluated at different temperatures to determine its thermal inactivation point. The phage retained its viability up to 55°C, but beyond this temperature it lost its infectivity, as shown in Figure 6.

Effect of pH: The phage vB_AgrP-B1 exhibited optimal infectivity within a pH range of 7 to 8. A marked reduction in infectivity was observed under both acidic and alkaline conditions, as shown in Figure 7.

Effect of Different Salts, Sugars, and Heavy Metals on the Plaque Formation of vB_AgrP-B1

The influence of various salts, sugars, and heavy metals on the plaque formation of bacteriophage vB_AgrP-B1 was assessed at different concentrations (0, 0.1 mM, 1 mM, and 10 mM). The key findings are summarized in Table 2.

Effect of Salts on Plaque Formation: As shown in Figure 8, NaCl had no significant effect on phage

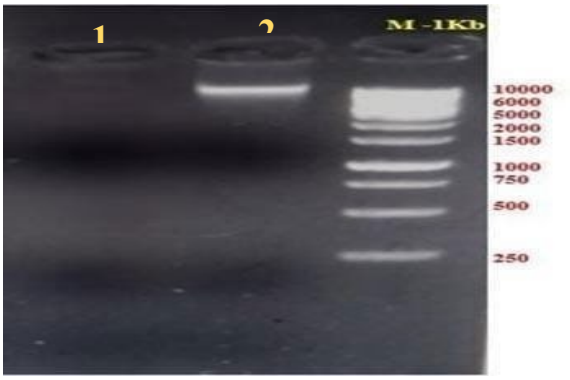

Figure 4. Agarose (1%) gel electrophoresis of the bacteriophage (Ph1) nucleic acid composition, determined by enzymatic digestion with DNase and RNase. Lane 1: DNase digestion of genomic DNA. Lane 2: RNase digestion of genomic DNA. M: marker (1 kb).

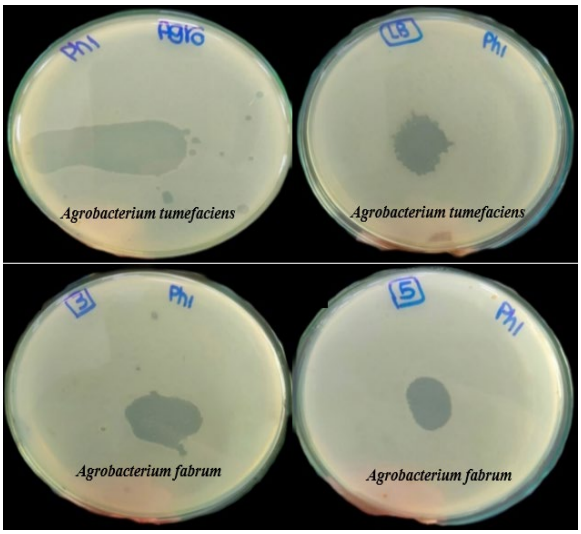

Figure 5. Lytic activity of vB_AgrP-B1 (Ph1) against different bacterial strains causing crown gall disease. The bacteriophage demonstrated clear plaque formation in the double-layer assay, indicating its ability to infect and lyse *Agrobacterium tumefaciens* (A, B) and *Agrobacterium fabrum* (C, D), supporting its potential as a biocontrol agent.

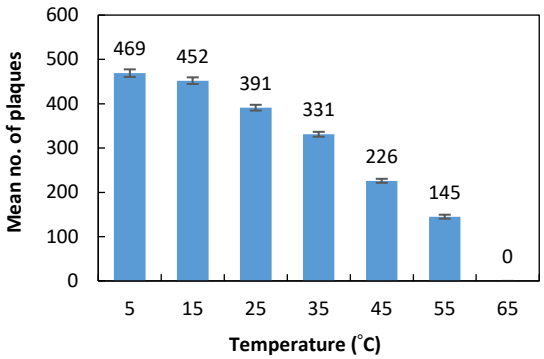

Figure 6. The thermal inactivation point (TIP) of vB_AgrP-B1 showing the retained at 55°C and the thermal inactivation point at 65°C.

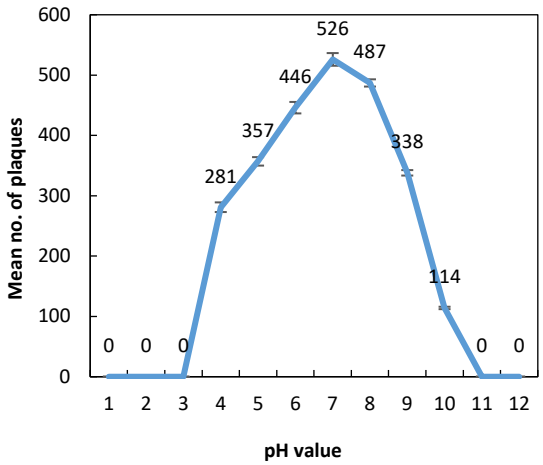

Figure 7. The effect of different pH ranges on the stability and infectivity of phage vB_AgrP-B1 showing the optimum activity at pH 7 with a decrease in plaque numbers under both acidic and alkaline conditions.

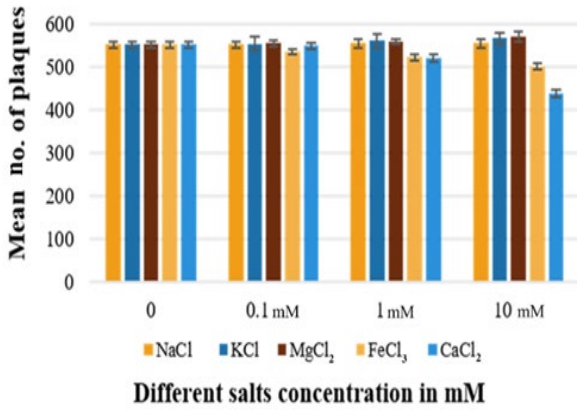

Figure 8. The effect of various salts (NaCl, KCl, $MgCl_2$, $CaCl_2$, and $FeCl_3$) on the activity of bacteriophage vB_AgrP-B1. The phage infectivity was evaluated at concentrations 0.1 mM, 1 mM, and 10 mM.

Table 1. Dilution end point (DEP) assay of phage vB_AgrP-B1. The table shows the number of plaques formed at serial phage dilutions.

Phage dilution	Number of plaques
10^{-1}	Uncountable
10^{-2}	Uncountable
10^{-3}	Uncountable
10^{-4}	Uncountable
10^{-5}	Uncountable
10^{-6}	544
10^{-7}	131
10^{-8}	22
10^{-9}	3
10^{-10}	1
10^{-11}	0

activity, with plaque counts comparable to the control. In contrast, KCl and $MgCl_2$ enhanced plaque formation, particularly at higher concentrations (1 mM and 10 mM). $CaCl_2$ exhibited strong inhibition, significantly reducing plaque counts at these same concentrations. $FeCl_3$ had a slight inhibitory effect, with fewer plaques observed at 10 mM.

Effect of Sugars on Bacteriophage Activity: The addition of sucrose, glucose, and lactose resulted in a marked increase in plaque formation at all tested concentrations (Figure 9). Fructose showed a similar effect at 1 mM and 10 mM, but no notable change was observed at 0.1 mM.

Effect of Heavy Metals on Plaque Formation: As shown in Figure 10, $HgCl_2$ and $AgNO_3$ completely inhibited plaque formation at 10 mM and 1 mM, with a strong reduction even at 0.1 mM. $CoCl_2$, $CuCl_2$, and $ZnSO_4$ demonstrating a concentration-dependent inhibitory effect, with higher concentrations leading to a more pronounced reduction in plaque counts. The results indicate that certain salts (KCl, $MgCl_2$) and sugars (sucrose, glucose, and lactose) enhance bacteriophage infectivity, while others ($CaCl_2$, $FeCl_3$, and all tested heavy metals) significantly inhibit plaque formation in a concentration-dependent manner.

Application of Phage Therapy in the Lab

Carrot slices treated with phage lysate exhibited significantly reduced crown gall formation compared to the positive control (bacteria only), which showed extensive crown gall development (Figure 11). The negative control, treated with SM buffer only, also displayed a similar level of crown gall formation as the positive control, confirming that the phage treatment was responsible for the observed protective effect.

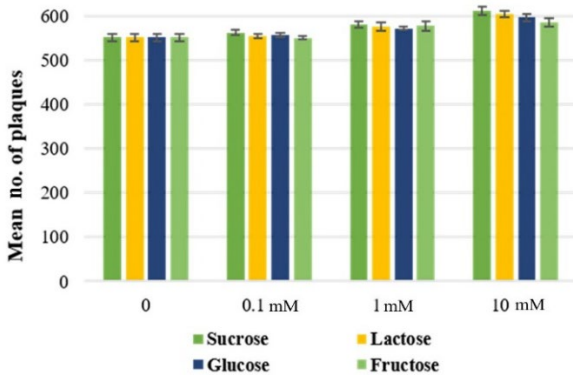

Figure 9. The effect of different sugar concentrations on bacteriophage vB_AgrP-B1. The determination of sucrose, lactose, glucose, and fructose at concentrations of 0.1 mM, 1 mM, and 10 mM on phage activity.

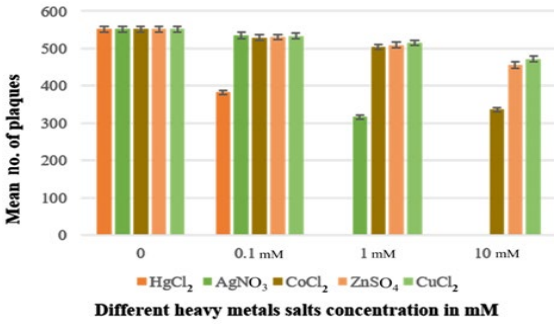

Figure 10. The effect of various heavy metal salts on the plaque formation of bacteriophage vB_AgrP-B1. The impact of different heavy metal salts ($HgCl_2$, $AgNO_3$, $CoCl_2$, $ZnSO_4$, and $CuCl_2$) on phage activity was assessed at concentrations of 0.1 mM, 1 mM, and 10 mM.

Figure 11. Application of phage vB_AgrP-B1 in the control of crown gall on carrot slices. Slice (a) shows carrot slices infected with *Agrobacterium tumefaciens*, exhibiting crown gall formation. Slice (b) shows carrot slices treated with SM buffer, also displaying crown gall formation. Slice (c) shows carrot slices treated with phage vB_AgrP-B1, with no crown gall formation.

Table 2. Effect of salts, sugars, and heavy metals on bacteriophage vB_AgrP-B1 plaque formation

Compound	Effect on plaque formation	Notable observations
NaCl	No significant effect	Plaque counts like control
KCl	Increased plaque formation	Higher counts at 1 mM and 10 mM
$MgCl_2$	Enhanced phage activity	Concentration-dependent increase
$CaCl_2$	Strong inhibitory effect	Significant reduction at 1 mM and 10 mM
$FeCl_3$	Slight inhibition	Decreased plaque numbers at 10 mM
Sucrose, glucose, lactose	Enhanced plaque formation	Significant increase at all concentrations
Fructose	Moderate increase	No effect at 0.1 mM, enhancement at higher concentrations
$HgCl_2$, $AgNO_3$	Complete inhibition	No plaques at 10 mM and 1 mM, strong reduction at 0.1 mM
$CoCl_2$, $CuCl_2$, $ZnCl_2$	Dose-dependent inhibition	Greater reduction at higher concentrations

DISCUSSION

Crown gall disease, caused by *Agrobacterium tumefaciens*, is a significant bacterial infection that affects a wide range of plants, especially fruit trees (Habbadi et al., 2023). Although the genus *Agrobacterium* was originally believed to primarily consist of phytopathogenic species, it has since been recognized to include both pathogenic and non-pathogenic strains, positioning it uniquely within the Rhizobiaceae family (Naranjo et al., 2023).

The aim of the current study was to isolate bacteriophages capable of targeting *Agrobacterium tumefaciens*, thereby reducing the economic losses caused by crown gall disease in fruit cultivation. Water sources have been widely recognized as abundant reservoirs for bacteriophages, with numerous studies documenting phage isolation from wastewater (Arivo et al., 2016), seawater (Li et al., 2016), freshwater (Novianty and Budiarti, 2014), pond water (Madsen et al., 2013), and even extreme environments such as geothermal waters (Nagayoshi et al., 2016). Ramadhan et al. (2022) isolated bacteriophages from agricultural drainage water, like the source used in the present study. The isolation and purification procedures employed in our research, including the use of a double-layer agar assay to obtain clear visible plaques, were consistent with established methods for phage isolation (Lillehaug, 1997).

In this study, a bacteriophage was successfully isolated from water samples collected from agricultural water drains in Beni-Suef, Egypt. This phage, designated Ph1, was characterized using transmission electron microscopy, revealing an icosahedral head and a tail structure. Genomic analysis confirmed that this phage possesses a DNA genome and belongs to the Podoviridae family. Our results are consistent with those of Fortuna et al. (2023), who isolated an *Agrobacterium* phage named OLIVR, also classified as a podovirus. The morphological characteristics observed in their study closely resemble those of our isolated bacteriophage.

Supporting previous research, our findings demonstrate that bacteriophages can be effectively utilized to control crown gall disease. For example, Attai and Brown (2019) isolated *Agrobacterium tumefaciens* phage (Atu_ph08) from wastewater, including agricultural runoff, and Sabri et al. (2024) identified a high-lysis phage (PAT1) from wastewater

that demonstrated potent activity against *A. tumefaciens*. These studies highlight the potential of bacteriophages as biocontrol agents against plant pathogenic bacteria.

In our study, we tested the bacteriophage vB_AgrP-B1 for its ability to infect various *Agrobacterium* strains responsible for crown gall disease. The isolated phage exhibited thermal lability, maintaining stability up to 55°C, and showed optimal activity at 5°C, with plaque formation significantly reduced at temperatures exceeding 35°C. Optimal phage activity was observed at neutral pH, with significant declines in plaque numbers under acidic (pH < 4) and alkaline (pH > 10) conditions. These findings are in accordance with Ramadhan et al. (2022), who noted that phage adsorption to host bacterial cells is influenced by pH, likely due to changes in the protein capsid's charge. Temperature and pH can also impact phage infectivity, leading to lipid dissolution and structural degradation (Nagayoshi et al., 2016).

Additionally, we evaluated the effects of various salts (sodium chloride, potassium chloride, calcium chloride, magnesium chloride, and ferric chloride) on the activity of vB_AgrP-B1. Sodium chloride did not affect phage activity, consistent with the findings of Czajkowski et al. (2014). In contrast, potassium and magnesium chlorides significantly enhanced phage activity, as previously reported by Abdel-Aal et al. (2024), who suggested that K^+ ions may enhance phage absorption and penetration. Similarly, Mg^{++} ions were found to promote phage growth (Tucker, 1961). However, calcium chloride and ferric chloride reduced phage activity, corroborating studies by Roslycky et al. (1963) and Rowatt (1984), which reported that Fe^{3+} ions negatively impact bacteriophage function and Ca^{++} ions inhibit phage inactivation in saline solutions.

We also examined the impact of monosaccharides (fructose and glucose) and disaccharides (lactose and sucrose) on phage activity. All tested sugars significantly enhanced phage activity, as evidenced by increased plaque numbers. This supports the findings of Cormier and Janes (2014), who demonstrated that sugars serve as both carbon sources and essential building blocks for the synthesis of key cellular components, thus promoting bacterial growth and enhancing phage propagation.

The effects of heavy metals ($HgCl_2$ and $AgNO_3$) were also assessed, revealing strong inhibitory effects on plaque formation, particularly at higher concentrations. This can be attributed to the toxic effects of Hg^{++} ions, which cause protein deformation and precipitation (Patra et al., 2004), and Ag^+ ions, which interact with thiol groups on proteins and disrupt DNA replication (Paunikar et al., 2012). Similarly, $CoCl_2$ reduced plaque counts, likely due to Co^{++} ions' involvement in enhancing viral transformation (Casto et al., 1979). Zinc ($ZnSO_4$) and copper ($CuCl_2$) also diminished phage activity at higher concentrations, with copper compounds generating reactive oxygen species (ROS) that degrade phage proteins and DNA (Abdel-Aal et al., 2024).

In laboratory applications, the bacteriophage vB_AgrP-B1 demonstrated a significant protective effect against crown gall disease caused by *A. tumefaciens* AUMCB-455. These findings emphasize the potential of bacteriophage vB_AgrP-B1 as an effective biocontrol agent for crown gall disease in controlled laboratory settings. This supports the viability of phage therapy as an alternative approach for managing *A. tumefaciens*-induced diseases in agricultural applications.

However, despite these promising results, several challenges must be considered for field applications. Environmental factors such as UV radiation, fluctuating temperatures, and microbial competition may reduce phage efficacy. UV exposure can rapidly degrade phage particles, limiting their longevity on plant surfaces (Gdanetz et al., 2024). Additionally, soil and water conditions may affect phage adsorption and infectivity, potentially reducing their biocontrol efficiency. Another major concern is the emergence of phage-resistant bacterial strains, which could diminish the long-term sustainability of phage therapy. Addressing these limitations through protective formulations (e.g., encapsulation) and developing phage cocktails with diverse host ranges could enhance the effectiveness of bacteriophage-based biocontrol strategies in real-world agricultural settings.

CONCLUSION

In conclusion, we successfully isolated the bacteriophage vB_AgrP-B1 from agricultural drainage water in Egypt, demonstrating its efficacy against *Agrobacterium* strains. Transmission electron microscopy allowed the classification of the phage within the Podoviridae family based on its morphological features. Our study also highlighted the phage's stability across a broad pH range and temperature, as well as its interactions with various salts, sugars, and heavy metals. Notably, the isolated

phage effectively prevented crown gall disease on carrot slices, suggesting its potential as a biocontrol agent for bacterial plant diseases. Future research should focus on exploring the application of phage therapy under field conditions, addressing potential challenges such as phage inactivation due to temperature fluctuations and UV radiation exposure (Gdanetz et al., 2024). Future work should explore phage efficacy under field conditions, considering environmental factors like soil composition and microbial competition. As suggested by Czajkowski et al. (2014), applying phages to seedlings prior to transplantation may enhance their effectiveness in managing crown gall disease.

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