

REVIEW

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Challenges of bacteriophages application in controlling bacterial plant diseases and how to overcome them

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Abstract

Due to the emergence of antibiotic-resistant bacteria in agricultural sector, controlling bacterial plant diseases using antibiotics has become challenging. Researchers have turned to alternative methods, such as using bacteriophages as a biocide for plants instead of antibiotics, to control pathogenic bacterial plant diseases. However, the application of bacteriophages as a biocide in agriculture faces several challenges that may impede its success. In this review article, we discuss the various issues that could lead to the failure of its application. We also propose solutions to address each problem to increase awareness and familiarity before implementing the method to better ensure its success.

Keywords Virus, Bacteriophage, Phage therapy, Biocontrol, Plant diseases, Challenges

Background

Viruses are microscopic, obligate intracellular parasites that replicate and propagate by exploiting the host cell machinery. Therefore, they depend entirely on their host cells for survival [1]. Viruses cannot survive for long periods outside their host cells, which means their life cycle is entirely dependent on their hosts [2]. Due to their tiny size and complete dependence on host cells, viruses can infect a wide range of organisms, including bacteria, plants, and mammals [3]. Bacteriophages, or phages, are the most abundant viruses on Earth. They infect bacteria and archaea [4], and the term bacteriophage is a combination of two words: "bacterio," derived from "bacteria," and "phage," derived from the Greek "phagein," meaning "to devour" [5]. Since phages display high specificity for infecting and destroying bacteria, they are present wherever bacteria are found and have been utilized to eliminate pathogenic bacteria [6]. Bacteriophages are

generally classified into two categories: virulent phages and temperate phages [7]. In recent years, there has been growing interest in using bacteriophages to control plant diseases caused by bacterial pathogens. Several studies have demonstrated the effectiveness of bacteriophages in controlling plant diseases. For example, a study conducted by Abuladze et al. (2008) showed that applying bacteriophages reduced the severity of bacterial spot disease in tomato plants [8]. Despite the potential benefits of using bacteriophages for plant disease control, some challenges still need to be addressed. This review article aims to summarize the challenges that may hinder successful phage applications and provide solutions to overcome these challenges.

The mechanism of interaction between phage and bacteria (Fig. 1):

A - The first step of infection is: Adsorption of phage to the bacterium surface

Infection of bacterial cells by virulent or temperate phages begins with the phage adsorption organelle binding to bacterial-specific receptors on the bacterial

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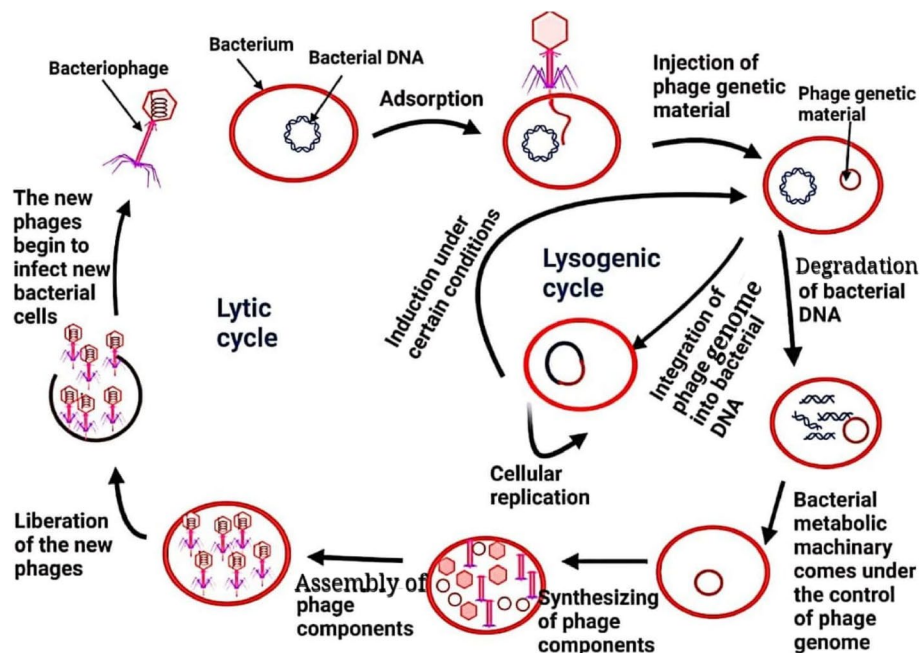


Fig. 1 Mechanism of interaction between bacteriophage and bacteria. "Figure created with <http://biorender.com>"

surface. The bacterial and viral attachment sites are different. Examples of cell surface components that can serve as phage receptors include proteins, lipopolysaccharides in gram-negative bacteria, and peptidoglycan and teichoic acid in gram-positive bacteria. It is not uncommon for two or more phages to detect the same receptor site on the same bacterium; a small number of phages may even be capable of binding to two or more different receptors [9].

In addition to the secondary function that a phage utilizes, the receptor site on the bacterial surface performs primary functions. Among these primary functions is the transport of nutrients (such as vitamins, sugars, and amino acids) by numerous proteinaceous receptor sites and other receptors associated with organelles of specific functions, such as flagella and conjugative pili [10].

Initially, the phage adsorption organelle binds reversibly to the bacterial receptors. The bacteriophage can be separated while retaining its infectious properties, such as through diluting the reaction mixture or bactericidal killing. The process that leads to the penetration of the phage's genetic material into the bacterial cell ultimately renders the relationship irreversible [11].

B - The second step of infection: Injection of phage genetic material into the bacterial cells

After attaching the phage adsorption organelle to the bacterial receptor, the phage begins inserting its genetic

material into the cytoplasm. This occurs due to the tail sheath's contraction, which functions as a hypodermic needle to inject the phage genome into the cell membrane and wall. Only the genetic material of the phage enters the bacteria; the rest remains outside [12].

C - The third step of infection

This step differs in virulent phages from temperate phages. Therefore, we discuss this step in both types of phages separately as follows:

– The third step of infection in virulent phage

After the phage genetic material enters the bacterial cytoplasm, the phage synthesizes virus-encoded endonucleases to degrade the bacterial chromosome [13]. Subsequently, the phage genome takes over the bacterium's metabolic machinery, converting it into a "factory" for manufacturing phage components, including capsomeres, sheath, base plates, tail fibers, and phage enzymes. Once the phage components' synthesis is complete, they assemble to form new phages called progeny phages [9]. Phage proteins like holin or lysozyme then disrupt the bacterial cell wall to release the new phages, which ultimately infect new cells [14, 15].

– The third step of infection in temperate phage

After entering the bacterial cytoplasm, the genetic material of a phage integrates into the bacterial chromosome and becomes a prophage, which makes the bacterium a lysogen. Lysogeny is the process by which a temperate phage infects a bacterium. During reproduction, as the bacterium replicates its chromosome, it also replicates the phage's DNA and transfers it to new daughter cells [16]. The presence of a phage genome in the bacterial chromosome can alter the bacterium's phenotype by introducing additional genes, such as toxin genes, which can increase bacterial virulence. This alteration in the host phenotype is called "lysogenic conversion" or "phage conversion".

In certain bacteria, such as *Vibrio cholera* and *Clostridium botulinum*, the absence of the prophage results in decreased virulence. The phages that infect these bacteria carry toxin genes in their genomes which, when expressed, increase the host's pathogenicity. Phage-encoded toxins can induce paralysis in *Clostridium botulinum* and severe diarrhea in *Vibrio cholera* [16–19].

During lysogeny, the prophage persists in the bacterial chromosome and replicates as a unit without destroying the bacterial cell. However, under certain conditions, lysogenic phages can be induced to follow a lytic cycle and undergo lysogeny in a newly infected cell [16].

1 - Application of viruses in phage therapy

The use of phages in the treatment of bacterial diseases

Phages are becoming increasingly popular as alternative biocontrol agents for controlling microbial resistance [20]. Phage therapy is the use of phages to treat infections caused by pathogenic bacteria [21]. Phages are highly effective in antimicrobial phage therapy because they

have the ability to specifically identify, bind to, multiply within, and lyse bacterial cells. Bacteriophages can effectively target both gram-positive and gram-negative bacteria that are resistant to antibiotics, and they typically act with a high degree of specificity [22].

There are several potential advantages to using phages in disease control

Phages offer numerous benefits, including their ubiquitous presence wherever bacteria exist and their ability to replicate as long as bacteria are present. Additionally, phages possess high specificity to infect target bacteria without harming others, are non-toxic to eukaryotic cells, can be easily and inexpensively prepared and produced, and can be stored for months under preservation conditions without significant loss in titer [23]. Phages are an environmentally friendly biocide that can eliminate antibiotic-resistant bacteria [24], and can also be used to produce crops free of chemical pesticides, which are increasingly in demand among consumers [24, 25].

Challenges that may hinder successful phages application for plant disease control and how these challenges can be overcome (Table 1):

(1) Inactivation of phages by UV irradiation from the sun

UV radiation can damage the DNA of phages, potentially inhibiting DNA replication. To mitigate this issue, phages can be applied after sunset to reduce the damaging effects of UV [26]. Research has demonstrated that the application of phages onto tomato leaves during the evening hours leads to an extended duration of phage persistence within the phyllosphere. This prolonged

Table 1 Challenges that may hinder successful phages application and how they can be overcome

Challenges that may hinder successful phages application	How they can be overcome
1) Inactivation of phages by UV irradiation	- Using a protective formulation that protects phages against UV - Applying phages after sunset
2) Poor persistence of phages in the rhizosphere and phyllosphere	- Accompanying phages by a viable host - Avoiding daylight during phages application
3) Possibility of lysogens or pseudo lysogen production	- Using only lytic phages
4) The difficulty of eliminating every member of a particular bacterial genus or species due to the narrow host range for phages	- Development of phage cocktails
5) Instability of phages survival under improper storage conditions	- Keeping phages refrigerated and protected from light - Production of stable bio formulation and conversion of phage formulation from liquid to powder state
6) Possible development of phage resistance in the bacterial host	- Development of phage cocktails
7) The inability of phages to disperse or interact with their target bacteria when there is a lack of moisture on the leaf surface	- Applying phages when free moisture is expected to stay on the leaves (such as dew or rain)
8) The difficulty of applying phages evenly over large tree leaves	- Applying phages via tree vascular system

persistence provides phages with increased opportunity to infect and eliminate their bacterial targets [26].

Alternatively, a formulation that shields the phages from UV radiation can be used. Born et al. (2015) examined a range of substances to determine their effectiveness in providing UV protection for phages. They found that natural extracts from carrot, red pepper, beetroot, casein, soy peptone, purified aromatic amino acids, astaxanthin, and tween 80 all provided UV protection, without inhibiting phage infection or stability [27]. Therefore, various compounds may enhance phage performance in the phyllosphere, with the essential criterion being their ability to absorb UV to reduce phage exposure. It has also been demonstrated that biodegradable polymers [28], Congo red dye [29], iron oxide particles in groundwater [30], ferric chloride coagulant, and humic acid [31] all have UV protection properties for phages.

According to Templeton et al. (2005), certain organic colloidal particles have the ability to protect phages from UV light, whereas inorganic kaolin clay particles do not offer such protection [31]. In a subsequent study by Templeton et al. (2006), it was observed that the UV inactivation of both phages in "raw" groundwater was significantly lower than in EDTA-preserved groundwater. This was attributed to the association of the phages with UV-absorbing iron precipitate particles. A phage elution technique confirmed that a considerable proportion of the phages that survived UV exposure were associated with particles [30].

Khalil et al. (2016) conducted a study which demonstrated that incorporating Poly- γ -glutamic acid (γ -PGA), a biodegradable polymer, into phage formulations yielded protection against UV damage, high temperatures, and extreme pH values. Additionally, these biodegradable polymers prolonged the persistence and viability of phages, thereby enhancing the efficacy of biocontrol using phages in comparison to non-formulated phages. The authors suggest that this protection may be attributed to physical shielding of the virus particle by γ -PGA, which reduces the levels of heat reaching the viral particles. Alternatively, the high amino acid content of γ -PGA may promote virion survival [28].

According to a recent investigation carried out by Wdowiak et al. (2023), it was revealed that Congo red, a dye commonly utilized in laboratory research, exhibits exceptional protective characteristics towards non-enveloped phages against extended exposure to UV radiation. In contrast, non-protected phages without Congo red were completely deactivated within one minute of UV irradiation. The dye functioned as a "molecular sunscreen," shielding phages from the damaging effects of UV radiation. This outcome presents a promising

solution for overcoming the issue of phage inactivation by UV radiation [29].

(2) Poor persistence or short-lived phages in the rhizosphere and phyllosphere

The region surrounding the roots of a plant is known as the rhizosphere [32]. The aerial parts of a plant above the ground are known as the phyllosphere [33]. Phages are poorly persistent in the rhizosphere due to several factors, including low rates of phage diffusion through the heterogeneous soil matrix, the ability of phages to become trapped in biofilms and reversibly adsorb to soil particles like clay, low soil pH, which can also render phages inactive, and physical refuges that prevent bacteria from contacting phages. Notably, a few phages survive due to low phage diffusion rates and high phage inactivation rates. The poor persistence of phages in the phyllosphere is attributable to environmental factors such as temperature, desiccation, and exposure to specific chemical pesticides such as copper bactericides on leaf surfaces [34].

These issues can be resolved if viable hosts and phages are present (propagating bacterium strains). A viable host can be an avirulent strain of the pathogenic bacterium being targeted or another naturally occurring bacterial strain in the environment. These bacteria may increase the likelihood of continued phage propagation to counteract expected losses from environmental factors such as sunlight and dehydration. Since they readily establish themselves in the phyllosphere and can compete with pathogenic bacteria, nonpathogenic epiphytes may be the best candidates for this role. In addition, nonpathogenic or attenuated phytopathogen strains, which no longer cause disease, can also be used for phage propagation. Since these bacterial strains are the same species as the bacterial pathogen, they will likely propagate most phages and exert some antagonistic effects [35]. According to Nagai et al. (2017), a study was conducted on broccoli plants infected with black rot disease to investigate the potential of using bacteriophages accompanied by avirulent bacterial species as a means of enhancing phage persistence on the leaf surface and improving the biocontrol of the disease. The results of the study indicated that this approach was successful in achieving these objectives [36]. It has also been reported that applying phages in combination with avirulent bacterial strains has proven to be effective in the biocontrol of tobacco wilt disease [37].

Additionally, avoiding direct sunlight during application has been observed to improve phage-based biocontrol. Delivering phages into tomato leaves in the evening causes them to remain longer in the phyllosphere, giving

them more opportunities to infect and destroy their bacterial hosts [38].

(3) Possibility of lysogen or psuedolysogen production

The persistence of the phage genome within a host cell can provide superinfection immunity, thereby reducing the biological efficacy of the phage and conferring additional characteristics on the target bacterium. For example, the phage RSS1, which is present in *Ralstonia solanacearum* in a persistent infective state, enhances the virulence of the bacterial host on tomato [39]. To avoid issues related to lysogeny, it is recommended to use only lytic (virulent) phages for biocontrol [24, 40]. According to the findings of Álvarez et al. (2019), three lytic phages were isolated from river water and found to exhibit activity against *R. solanacearum*, resulting in significant biocontrol efficacy against bacterial wilt disease. This study represents the first reported instance of successful biocontrol of *R. solanacearum* using single or combined bacteriophages delivered through irrigation water under conditions that mimic those of natural settings [41]. Thepa Magar et al. (2022) have conducted a recent study that examines the biocontrol potential of two isolated lytic phages against the invasion of *Ralstonia pseudosolanacearum*, the causative agent of bacterial wilt disease in tomato plants. The study found that treatment with either of the two phages alone or in combination resulted in a noteworthy decrease in the incidence of bacterial wilt [42]. There are also several studies that have utilized lytic phages and demonstrated their efficacy as a biocontrol agent against various plant diseases [43–48].

(4) Narrow bacterial host range for phages

Phages could demonstrate narrow host ranges as they are highly host-species-specific and commonly only can infect one bacterial species or even a subspecies [49]. The production of phage cocktails can resolve this issue [24]. The utilization of phage cocktails has demonstrated a broad spectrum of host range and effectiveness in the biocontrol of various bacterial plant diseases [50]. Iriate et al. (2012) conducted a study which demonstrated the efficacy of phage cocktail application in the biocontrol of *Xanthomonas perforans*, a causative agent of disease in tomato plants [51]. Similarly, Wang et al. (2019) conducted a separate study which confirmed the efficacy of phage cocktails in the biocontrol of tomato wilt disease [52]. According to a study carried out by Wei et al. (2017), the utilization of phage cocktails has proven effective in controlling potato bacterial wilt disease [53]. Also, there have been numerous studies that have applied phage

cocktails and reported successful biocontrol outcomes for various plant diseases [50, 54–58].

Cocktail phages may be effective against different strains of the same bacterial species. However, based on their lytic activities, their combined efficacy in killing target bacteria might exceed expectation. This phenomenon, which may be beneficial and valuable therapeutically, is known as synergy [59, 60]. According to Schmerer et al. (2014), such synergy can be achieved when one phage facilitates infecting the same bacterium for another phage. They isolated phages from sewage and observed that these phages caused a mucoid *E. coli* strain to produce numerous plaques. The combined activity of two phages, J8-65 (producing turbid plaques with a halo effect) and T7 (forming small plaques), increased the host bacterial killing effectiveness by 10–100 fold compared to each phage acting alone [60]. Understanding the possibility of achieving synergy can significantly improve the production of phage preparations for phage therapy, as it increases their potential efficacy [22].

(5) Instability of phage survival under improper storage conditions

The successful use of phages depends on their stability. Temperature is the most significant factor affecting phage stability since it affects phage proliferation, action, and preservation [61]. Other factors that affect phage stability include techniques used to make phage compositions, substances and components they contain, forms in which they are used, preservation conditions kept, and application methods [62]. The issue of phage instability under improper storage conditions can be resolved by keeping phages cold and protected from light. In this manner, phage cocktails can be stored for months without significant titer loss. Also, depending on bacteriophage type, they may be frozen with or without propagating bacterium [35].

An alternative approach for addressing the issue of phage instability due to inadequate storage conditions involves the production of a stable bioformulation, accompanied by the transformation of the phage formulation from a liquid to a powdered state. This method of preparation enables the phage formulations to withstand harsh environmental conditions for an extended duration [63]. A practical investigation was carried out by Leung et al. (2018) to evaluate the stability of bacteriophages in powder form during storage at ambient temperature. According to the findings of this study, spray dried bacteriophage powder can be successfully stored for up to one year with vacuum packaging at 4 °C and 20 °C [64]. Similarly, Chang et al. (2019) conducted a study to examine the storage stability of phage powder under

normal environmental conditions. As per the results of this study, dried phage powders are physically and biologically stable during long-term storage at surrounding temperature [65].

(6) Possible development of phage resistance in the bacterial host

Like with antibiotics, bacteria can develop resistance to phages through various mechanisms [39]. These defense mechanisms include changes in the bacterial surface receptors, such as CRISPR/Cas [66], mucous production [67], DISARM [68], lysogen production [69, 70], BREX [71], RM [72], and nine other novel systems [73]. This issue can be resolved through the production of phage cocktails that combine phages with narrow, wide, and/or host range mutant combinations [39, 40].

The utilization of phage cocktails has demonstrated a decrease in bacterial resistance development to bacteriophages, as well as their efficacy in controlling bacterial plant diseases [74, 75]. In a study conducted by Kim et al. (2022), it was reported that phage cocktails successfully inhibited the emergence of *P. carotovorum* strains resistant to phages. Additionally, the phage cocktails exhibited biocontrol properties against soft rot disease in napa cabbage [76].

In order to prevent and counteract the emergence of microbial resistance, several supplementary strategies have been proposed. These include the utilization of diverse therapeutic approaches in combination, the implementation of mutant phages derived from the wild type bacteriophage to regain activity against bacteria [77], and the isolation of novel or modified phages [78] that exhibit effectiveness against resistant microorganisms. Sieiro et al. (2020) have suggested the implementation of phage cocktails as a biocontrol agent in agriculture, in combination with the use of endolysins and antibiotics as part of an integrated approach to manage microbial infections and impede the emergence of resistant bacterial strains [79].

(7) Inability of phages to disperse or interact with their target bacterium when there is a lack of moisture on the leaf surface [80].

To ensure longer exposure time with the target bacteria, the issue can be resolved by applying phages during periods of extended free moisture on the leaves, such as during rainfall or when dew is present on the leaves at night and in the early morning [80].

(8) Potential difficulty in applying phages evenly over large tree leaves

Phages applied through the vascular system of the tree can resolve this issue. In addition, this type of phage application may facilitate the systemic delivery of the phages throughout the tree's vascular system and water flow [81].

Some additional applications that can exploit viruses in their applications

The use of phages in the treatment of diseases, as mentioned earlier, is one of the applications of viruses. However, viruses can also be employed in the following applications:

2 - Application of viruses in Nanotechnology

Viruses are excellent bio-nanomaterial due to having unique characteristics. Viruses are minuscule in size, modifiable, able to carry materials, and have high self-assembly precision. These distinctive properties contribute to their application in nanoscience and nanotechnology [82]. Indeed, many different viruses have been successfully applied in nanotechnology. For example, mammalian viruses have been applied in the medical field as vectors. Plant viruses can be produced easily and safely as they do not infect human or animal cells. In addition, plant viruses possess a symmetrical structure and biodegradability. Recently, nanomaterials have been used to transport active molecules and drugs into cancer cells using plant viruses. Therefore, plant viruses offer a novel and potent therapy against cancer. CPMV, TMV, and PVX are the viruses from which the most well-known viral vectors are generated [82–85].

3 - Application of viruses in vaccine delivery

Viruses such as adenovirus can be used as vectors to deliver vaccine antigens to target cells. Adenovirus is characterized by its ability to infect a wide range of hosts, induce high levels of transgene expression without integrating its genes into the host genome, and induce the host's innate immune responses via toll-like receptor-independent and toll-like receptor-dependent pathways. All these distinctive features of adenovirus have led to its utilization in many vaccine developments, including HIV vaccines [86, 87]. Another example of a virus exploited as a vaccine vector is the alphavirus. Alphaviral vaccine vectors have been explored in myriad applications for cancers, HIV, and the human parainfluenza virus. Alphavirus can create a proper environment for cross-priming vaccine antigens by inducing apoptosis in some cells [88–90].

4 - Application of viruses as a bioinsecticide

As a bioinsecticide, viruses can control insect pests, such as baculoviruses [91]. Using these viruses as bioinsecticides has several advantages, including low cost, practicality, environmental friendliness, potency against agricultural pests, safety for animals due to their inability to multiply within animal tissue despite their ability to penetrate it, and a wide variety of hosts [92]. In a practical study, 40 different viruses were used as bioinsecticides. The results indicated that these viruses were more effective than chemical insecticides at controlling insect pests. Baculoviruses control insect pests by penetrating insect cells, multiplying and replicating rapidly, and causing cytotoxicity in their hosts [82].

Conclusion

After reviewing this review article, we have come to the realization that the use of bacteriophages in agriculture to control bacterial plant diseases faces numerous challenges that may impede their successful application. However, we have also discussed potential solutions to overcome these obstacles. It is crucial to understand these problems and their resolutions before the implementation of bacteriophages in the agricultural sector to ensure their success.

In the future, we should explore the potential of viruses and their applications in various fields. Additionally, we must focus our attention on using these tools to combat global crises, such as the worldwide spread of antibiotic resistance.

Abbreviations

CPMV	Cowpea mosaic virus
TMV	Tobacco mosaic virus
PVX	Potato virus
RM	Restriction/Modification systems
BREX	Bacteriophage Exclusion system
DISARM	Defense island system associated with restriction-modification

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