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The Future of Phage-Mediated Biocontrol of Tomato Bacterial Diseases

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ABSTRACT

Phytopathogenic bacteria cause significant economic losses in tomato production. Tomato bacterial spot, speck, wilt and cancer disease agents are the most important phytopathogens that cause damage to tomatoes. Chemical methods have been generally used to control these diseases. However, the disadvantages of chemicals like development of resistance in bacterial strains, damage to non-target microorganisms and undesirable effects on the environment have increased the interest in alternative control strategies for sustainable agriculture. The use of bacteriophages, virus infecting bacteria, provides a remarkable alternative in controlling bacterial diseases of tomato. On the other hand, phage-mediated control strategies have three main limitations which are emergence of resistance in bacteria, stability during storage and persistence in the environment. The development of resistance can be mitigated or prevented using phage cocktails. In addition, encapsulation methods such as lyophilization (freeze-drying), emulsification and spray drying can be used for prolonging shelf life and increase the efficacy in field conditions. Studies on the use of phages against tomato bacterial diseases remained mostly as laboratory experiments, and except AgriPhage, a commercialized product, there is no product that can be used to treat diseases under field conditions. The use of eco-friendly products based on bacteriophages is very important for sustainable agriculture. This review compiled information on useful formulation of phage and phages identified in combating four tomato bacterial diseases which was determined as bacterial canker, bacterial speck, bacterial wilt and bacterial spot.

Keywords: Phytopathogenic bacteria, Bacteriophage, Formulation, Biocontrol, Tomato diseases

1 Introduction

Tomato, *Solanum lycopersicum* L. (Solanaceae), is one of the most widely grown vegetables in the world with an annual production of over 180 million tonnes [1]. China alone realizes 31% of the total tomato production, followed by America, India and Turkey are other important producing countries. The huge proportion of production can be adversely affected by biotic and abiotic stress that cause damage on all parts of the plant [2].

Microorganisms, which cause diseases during plant growth and post-harvest storage, are important biotic factors that cause economic losses. More than 60 pathogens including bacteria, fungi, viruses, and

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nematodes that cause disease in tomatoes have been reported [3].

Bacterial speck (caused by *Pseudomonas syringae* pv. *tomato*), spots (caused by *Xanthomonas perforans, X. gardneri, X. vesicatoria, X. euvesicatoria*), wilt (caused by *Ralstonia solanacearum, Clavibacter michiganensis* subsp. *michiganenesis*) in tomatoes are the most common diseases. Tomato production is influenced by stress, pests and disease negatively and diseases affect both root system and fruit. So, the diseases can suppress the plant growth and decrease the fruit yields and quality.

Antibiotics and copper-based compounds have been commonly used to combat diseases. Although the use of antibiotics is more limited than the copper-based compounds, they are also part of control strategies [4]. The excessive use of chemicals resulted in the emergence of resistant strains over time. Some countries have restricted or prohibited the use of copper-based compounds [5]. Instead of conventional methods, the use of bacteriophages is promising tools for bacterial plant diseases [6]. In agricultural applications, the development of protective formulations that will significantly reduce the adverse effects of environmental factors on phages is important for the stability and effectiveness of phages under field conditions. In addition, applications should be performed in the early morning or late evening hours for increasing the efficacy [7].

In this review, we present the current knowledge on the use of available control methods and bacteriophages in the management of tomato bacterial diseases. In addition, information is provided on how to increase the potential for the use of phages against plant pathogens and their formulation.

2 Bacterial Diseases of Tomato

Tomato, which is an economically important food crop, suffers from bacterial diseases that cause serious yield losses. The most common pathogens that cause disease in tomatoes are bacteria belonging to the genus *Pseudomonas, Ralstonia, Clavibacter* and *Xanthomonas.*

2.1 Bacterial Canker and Wilt Disease

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is a pathogenic bacterium that causes bacterial canker and wilt in tomatoes. *Cmm* is an encapsulated, non-motile, gram-positive and aerobic bacterium which infects not only tomato plant but also pepper, corn, peas, beans and onions. The disease was firstly reported in Michigan, USA in 1909 [8]. Even less than 1% of infected seeds may cause about 60-70% of crop loss in the field. Plant debris, tissues of plants and contaminated seeds allow bacteria to spread over long distances [9]. The pathogen can remain viable in the soil for 2-3 years. White, small and superficial spots that approximately 1-3 mm in diameter appears surface of green tomato fruit. Disease is transmitted by seeds and cause wiltering, stunting, reduced fruit yield and premature plant death [10,11].

2.1 Bacterial Wilt

Ralstonia solanacearum (*Rs*) is a plant pathogenic bacterium that causes bacterial wilt disease in tomatoes. The pathogen is a gram-negative, rod-shaped, non-encapsulated bacterium [12]. In the early stage of the disease, symptoms such as wilting and drying appear on the young leaves, while as the disease progresses, the infection may spread to the whole plant and cause death. These symptoms can sometimes occur during plant development and cause sudden wilting in healthy-looking plants. Also, among the common symptoms of this disease is stunting of plants. This soil and water borne pathogen penetrates the host via root and causes wilting [13,14].

2.2 Bacterial Speck

Pseudomonas syringae pv. *tomato* (*Pst*) is a gram-negative, pathogenic bacterium that causes bacterial speck disease, and it is one of the foliar diseases that cause serious economic losses in rainy weather. The disease was firstly reported in Taiwan by Okabe [15]. Small brown spots 1-2 mm in diameter are surrounded by yellow rings on the leaves. As the disease progresses, the lesions coalesce and burns occur, especially on the leaf margins. *Pst* reaches the intercellular spaces of leaves through natural openings such as stomata then it multiplies asymptomatically, infects the green tomato fruit, leaves and stems. Finally creating necrotic spots and delaying ripening [16].

2.3 Bacterial Spot

Initially, bacterial spot disease pathogen was defined as *Bacterium vesicatoria*. Later, the bacteria reclassified as *X. vesicatoria* according to their biochemical and physical properties [17]. *Pseudomonas gardneri* was the first bacterial spot disease pathogen in tomatoes [18]. Considering its physical and biochemical properties, *P. gardneri* was named *X. gardneri*, which is a synonym for *X. vesicatoria* [19]. Young et al. [20] named the causative agent *X. campestris vesicatoria* according to DNA homology, *X. campestris* pv. *vesicatoria* divided into two groups (*X. vesicatoria* and *X. axonopodis* pv. *vesicatoria*) [21]. As a result, Jones [22] reclassified the disease agents as four species according to serological and pathological tests. The causal agent of tomato bacterial spot is a complex of at least four species of Xanthomonas (*X. euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri*) [22] though recent studies suggest that *X. euvesicatoria* and *X. perforans* may be considered as a single species [23, 24]. Based on their virulence on a set of differential genotypes, four races (T1 in *X. euvesicatoria*, T2 in *X. vesicatoria*, T3 and T4 in *X. perforans*) have been identified [25].

3 Management of Bacterial Diseases in Tomato

To overcome the tomato bacterial diseases, several strategies such as field sanitation, using resistant species, crop rotation, using pathogen-free seeds and removal of infected plant debris have been used. However, these applications are not sufficient for disease management. Although using pathogen-free seeds is one of the most effective strategies, pathogen contamination during harvesting and planting is another significant problem. For example, seed coated with 1% hydrochloric acid and soil treatment with formaldehyde, decrease both the bacterial titer and symptom development, but are partially efficient against bacterial canker [26,27,28]. The application of chemical pesticides such as copper compounds, copper hydroxide and copper sulfate is the main method used to combat the bacterial tomato disease. Their usage gave successful results at the beginning, but the excessive use of copper and copper-based compounds has caused pathogens to develop resistant strains. Although the application of copper-based formulations before or after *Pst* infection reduced disease severity, the emergence of copper-resistant *Pst* strains has been observed [29,30]. In addition, chemical application does not reduce the symptoms of some diseases such as bacterial wilt (BW) Therefore, it is difficult to combat BW disease. However, validamycin A and validoxylamine which are plant activators that create systemic resistance on tomatoes have been used for management of BW disease. In addition to the development of resistant pathogens, the use of chemical pesticides creates environmental pollution and residue problems, so their use raises concerns. The unregulated and excessive uses of chemicals reduce biodiversity by destroying non-target microflora [31]. Also, accumulation of copper in the soil causes decrease in growth and fruit yield [32,33]. As a result, it is obvious that the use of chemicals in the management of tomato bacterial diseases does not sufficiently protect the plant. Thus, integrated control methods that will eliminate or reduce the effect of the diseases should be applied for a sustainable agriculture.

Some metabolites of microorganisms, alternative to conventional chemicals, can be used as biological control agents against soil-borne pathogens. Antimicrobial peptides such as hexapeptide KCM21 are effective against *Cmm* and *Pst* infections [34]. Metabolites of some beneficial *Pseudomonas* spp. protect the tomato plants from soil-borne pathogen *Pst* [35]. In addition, *Pseudomonas*, *Azotobacter, Azospirillum, Klebsiella, Alcaligenes, Enterobacter, Arthrobacter, Burkholderia, Bacillus*, and *Serratia* which are plant promoting bacteria not only promote plant growth but also decrease the disease level in tomato [36,37,38,39]. *Bacillus* and *Pseudomonas* treated seeds reduced the *Cmm* infection in the field [26,40]. Organic substances such as essential oil and extracts are also used to control plant pathogens on tomatoes. Pomegranate peel extract shows an antibacterial effect against *Pst* [41]. Similarly, *Albizia lebbeck* extract has protective properties on tomato plants against bacterial speck disease [42]. Also, seed coating with *Origanum vulgare* (oregano) essential oil protects the tomato plants against *Cmm* infection [43]. Another perspective is the use of phages which are a fast-expanding area with great potential to replace the chemical control.

4 Bacteriophages

Bacteriophage, also known as phage, are the viruses that specifically infect bacteria. These microorganisms, which are abundant in nature, have the potential to be used against plant pathogenic bacteria that cause damage to products with high economic value [44]. An important aspect is that phages are species and strain-specific, have rapid multiplication and effect in a short time, and are harmless to non-target organisms [45].

4.1. Bacteriophage Life Cycle

Phage propagations are classified lytic and lysogenic (temperate) for their life strategy. Life cycle of phages have four basic steps: transfer of nucleic acids into the host cell, expression and replication, assembly of virions, and release and transmission of new progeny phages. After their nucleic acid transfer to the host cell, the phage follows either the lytic or lysogenic pathway and completes its life cycle [46]. Lytic phages create phage components using host metabolism, lyses the cell and release new virions. Then released virions similarly infect other bacteria and the life cycle continues. Lysogenic phages infect the host cell, form an episome or integrate their nucleic acid into the bacterial genome and prophage is formed. The prophage replicates with the cell without lysis of the cell [47,48]. If the lysogenic cycle is induced by physical or chemical means, it can switch to the lytic cycle [49].

4.2. Bacteriophage-Based Biocontrol

The first trials of using phages as plant protection agents started in 1924. However, the easy and safe use of chemicals at that time reduced the interest in phage biocontrol. At the beginning of the 21st century, developments of biotechnology and notice the side effects of chemicals have increased the interest in phage biocontrol again [50]. The use of phages against plant pathogens are promising the future. The most important step in selecting phages to be used as biocontrol agents is whether is lytic or lysogenic. Phages to be used as biocontrol agents should be able to multiply lytically by infecting all strains of the target pathogen species [30]. Lysogenic phages are not suitable for use as biocontrol agents, as they are less effective, do not lysis the host cell, and have virulence genes that can make the pathogen more virulent [48,51].

4.3. Host Resistance Mechanisms

The most important point to be considered in bacteriophage applications is the resistant development of the bacterial hosts. Host bacteria have developed several strategies to prevent phage proliferation at various stages of the infection cycle. For example, the exopolysaccharide produced by *Erwinia amylovora* acts as a physical barrier, preventing the phage from recognizing the host receptor and protecting the bacteria against phage infection [52]. In addition, restriction modifications, CRISPR-CAS systems and abortive infection (Abi) defense systems exist among the strategies developed against phage invasion [53,54]. On the other hand, phages also develop strategies to continue their life cycle and overcome bacterial resistance development. Some phages specifically recognize extracellular polymers and degrade them with hydrolase or lyase enzymes [55]. Recently, the use of phage cocktails is another significant strategy to prevent bacterial resistance [56].

4.4. Phage Cocktails

Phage cocktails are used against various plant diseases as well as combat tomato bacterial diseases. Single point mutations in different receptors will not occur simultaneously, using more than one phage targeting different receptors can prevent the resistance problem [57,58, 59, 60]. Flathery et al. [61], tested four different *Xanthomonas campestris* pv. *vesicatoria* phages (at 10^8 pfu/mL concentration) in greenhouses and observed that disease symptoms were 40.5% in control (non-treated) and 0.9% in bacteriophage-treated plants. Balogh et al. [62] reported that the use of phage cocktails in the control of *Xanthomonas campestris* pv. *vesicatoria* prevented the progression of the disease in greenhouse and field conditions. However, one phage can increase the lytic capacity of the other phage by showing a synergistic effect, or it can decrease it by showing an antagonistic effect. For example, in the study conducted by Fujiwara et al. [63], the effectiveness of *R. solanacearum* phages was tested separately and in cocktail form, and it was seen that the phage applied alone was more effective than the cocktail.

4.5. Bacteriophage Formulation

For the successful application of the phages as a biocontrol agent, they need to be protected from the various factors that reduced their effectiveness in the fields. Phages are susceptible to adverse environmental factors like UV radiation, pH, temperature, low humidity, heavy rain and therefore decrease or disappear the efficacy can be observed when applied on aerial surfaces of the plants. Study performed on tomato leaves indicated that the UV irradiation drastically reduced the phage population [64]. Bacteriophage viability declines at day hours due to ultraviolet irradiation and low humidity on leaf surface [62]. The use of protective formulations increases bacteriophage persistence on tomato leaves surface, thus provides increasing stability and efficacy.

In these techniques, which include lyophilization, emulsification, spray drying and liposome encapsulation, phages are coated with certain stabilizing agents and protected from adverse environmental factors [65]. Also, the coating agent should allow the phage to separate from the complex for recognizing the host cell receptor.

Emulsification can be considered as an encapsulation method that can be used in phage formulations, but the difficulty of large-scale storage, prone to bacterial contamination and stored in a stable only in the refrigerator are the factors limiting its use. Spray drying is the conversion of a liquid substance into dried particle form by evaporation. During the drying process, factors such as air flow rate, drying temperature, initial concentration may affect the titer of the phage obtained. A low inlet air temperature (85°C at 300 liters per minute) seem to be the best parameters for phage survival. Also, at least 1010

pfu/ml of phage must be used in the processes because phage titer decreases during drying [66]. As with other encapsulation methods, excipients (trehalose, leucine,) contribute to phage stabilization [67,68]. After the lipids are dissolved in an organic solvent such as chloroform, the solvent is evaporated, and a dry lipid layer remains. The phage suspension is added to the dry lipid film, causing the dry lipid aggregates to swell and become fluid. With agitation, the lipid layers form heterogeneous multilayer liposomes [68]. The inability to control the encapsulation efficiency of the pages in liposomes may limit the use of liposome encapsulation. Among some encapsulation methods, lyophilization (freeze-drying) is an effective method that can increase bacteriophage stability up to 21 years [69]. Lyophilization provides facilitate of storage by bringing cultures into powder form to preserve the viability of microorganisms. It can be easily grown again in culture medium after long-term storage. The coating material to be selected in the lyophilization of phages should preserve the morphological structure of the phages and be biocompatible. Substances such as skim milk, gelatin, peptone, sodium glutamate, polyethylene glycol (PEG), glycerol and sugars (mannitol, sucrose and trealose) can be used as coating material. The use of sugars is thought to be the most suitable material for the phage stability in lyophilization [70]. Alvarez et al., [71] the stability of phages that can be used against the *Ralstonia solanacearum* after lyophilization was evaluated. Glucose, sucrose and trehalose were used as cryoprotectants in different concentrations. As a result of the study, the use of a high concentration of trehalose showed the most effective result in maintaining phage stability. Uses of formulation strategy, increase the potential of phages to be used as a biological control agent against tomato bacterial diseases. Balong et al. [62] tried unformulated and casecrete (protein polymer) formulated (mixed application only) phages against *Xanthomonas campestris* pv. *vesicatoria* in field condition. Phage formulated with casecrete reduced disease development by 43%. OmniLytics is the first company in USA to receive approval from the environmental protection agency (EPA) for the use of bacteriophages in agriculture. AgriPhage, a phage-based product, is a bactericide prepared for combating bacterial canker, speck and spot diseases in tomatoes and peppers [72]. Table also presents an overview of the most recent phage biocontrol studies about the bacterial tomato diseases under laboratory and field conditions.

Table 1: *Some important publications on bacteriophages application against bacterial disease of tomato (Since, 2000).*

Pathogen	*Bacteriophage(s)*	Strategy	Result	Reference
Ralstonia solanacearum	PE226 filamentous bacteriophage	Plaque assays	High efficacy on a wide range of plant pathogenic *Ralstonia solanacearum* strains.	[73]
	PE204	Simultaneous treatment of phage PE204 on tomato rhizosphere at10^8 pfu mL^{-1}	Complete inhibition of bacterial wilt disease	[74]
	vRsoP-WF2, *vRsoP-WM2* *vRsoP-WR*	Two applications with an irrigation system. Application at 10^9, 10^6 pfu/ml and its ten-fold dilution	Remarkable reduction of disease symptoms	[71]
	not specified	Phages application to soil in greenhouse at 10^6 pfu mL^{-1} and field application at 10^9 pfu mL^{-1}	Decrease in the incidence of disease by up to 80%	[75]
	ϕRSL1	*In planta* application at 10^{10} pfu per pot)	Rapid decrease in the host bacterial cell density	[63]
	ϕsp1	Plant bioassay on tomato seedlings at 10^8 pfu per pot	Highly host specific and effective in biofilm prevention	[76]
	φRSM3 filamentous phages	*In planta* application	Several cultural and physiological changes in host cells, especially loss of virulence.	[77]
	RsPod1EGY	Under greenhouse condition, soil treatment at 10^{11} pfu mL^{-1}	Completely suppression in disease symptoms	[78]
	J2 J2 + φRSB2	Soil application at 10^{10} pfu mL^{-1}	Reduction in the amount of the pathogen in contaminated soil and prevention wilting of infected tomato plants	[79]

	Six different phages	Application to the rhizosphere at 2.86×10^6 pfu mL^{-1} under planthouse conditions	Reduction in wilt incidence up to 20%.	[80]
Xanthomonas campestris pv. *vesicatoria*	Phage mixture	Greenhouse and field application with pregelatinized corn flour, skim milk and cassette formulation at 10^9 pfu mL^{-1}.	In greenhouse experiments, reduction in disease severity up to 30–62%. In field experiments, reduction in disease severity up to 12–43%.	[62]
	Phage cocktails (six different phage)	In field application, Combinations of the harpin protein, acibenzolar-S-methyl, and bacteriophages (application at 10^{10} pfu mL^{-1}).	Remarkable decrease in disease progression and improvement of fruit yield	[81]
	Four phage cocktails	Field and greenhouse treatment (application at 10^8 pfu mL^{-1}).	In field trials, phage application reduced disease severity by~17% In greenhouse-grown plants disease incidence reduced in two trials from 40.5% (control) to 0.9% and from 17.4% to2.7%	[61]
Xanthomonas euvesicatoria	Kϕ1, Kϕ2, Kϕ3, Kϕ4, Kϕ5, Kϕ6, Kϕ7, Kϕ8, Kϕ9	Plaque assays	Clear plaque formation on 59 *X. euvesicatoria* strains	[82]
Xanthomonas vesicatoria	SfXv124t/1, SfXv124t/2, SfXv124t/3	Plaque assays on different strains of *X. vesicatoria* at 10^6 pfu mL^{-1}	Clear plaque formation on some strain and no plaque on some strain	[83]
Clavibacter michiganensis subsp. *michiganensis*	CMP1 and CN77	Endolysin activity of phages in *Cmm*	High efficacy by endolysin on *Cmm* cells.	[84]

5 Conclusion

Sustainable strategies are needed to control bacterial diseases in agriculture. Bacteriophages have great potential to prevent these infections. However, the use of phages under field conditions is limited due to UV radiation, pH, temperature, low humidity, and heavy rain. Formulation studies need to be developed to overcome these limitations. Future studies should focus on the possibility of encapsulating bacteriophages, which has not yet been investigated, and on optimising process technology. In addition, long-term stability tests are needed to evaluate satisfactory microencapsulation efficiency. Consequently, bacteriophages have the potential to be an effective biocontrol agent in the fight against bacterial tomato diseases and other phytopathogens.

6 Declarations

6.1 Study Limitations

None.

6.2 Funding Source

None.

6.3 Competing Interests

There is no conflict of interest in this study.

6.4 Authors' Contributions

Conceptualization, D.B.E. and C.D.; writing-original draft preparation, D.B.E. and A.E.; writing-review and editing, C.D. and A.E.; All authors contributed sections to the manuscript and approved the final version of the text.

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