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RESEARCH

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Isolation of *Clavibacter michiganensis* subsp. *michiganenesis*-specific bacteriophages from tomato fields in Turkey and their biocontrol potential

Duygu Bekircan Eski[1*] and Cihan Darcan[2]

Abstract

Background *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is causing wilt and canker and significant economic losses in tomato production. Current control methods with conventional chemicals or antibiotics lost their effectiveness due to the development of bacterial resistance to this agent. Bacteriophages, viruses of bacteria, have been attracting increasing attention as an environmentally friendly means of controlling bacterial diseases. In this study, *Cmm* and *Cmm*-specific lytic bacteriophages were isolated from rhizosphere soil and phyllospheres of diseased tomato plants, and the biocontrol potential of bacteriophages was investigated.

Results Eight isolates that showed typical symptoms of *Cmm* infection in biochemical and pathogenicity tests contained the virulence gene *pat*-1 in the genome. Twenty-two native bacteriophages were isolated from 90 samples. As a result of the host range tests performed on the local *Cmm* isolates obtained in the study, temperature and pH tolerance tests were performed with ten phages with a wide host range. Phage24, Phage33 and Phage41, which have higher temperature and pH tolerance than other phages, were used to kill curve assay. Although the combined applications of phages were performed to increase efficacy, it was found that the application of Phage33 alone was more effective.

Conclusion Phage33 may prove to be a suitable agent to be used as part of the microbial control strategy of *Cmm*. In further studies, Phage33 should be formulated to protect it from adverse environmental conditions and tested under field conditions to confirm the results obtained in this study.

Keywords Bacteriophages, *Clavibacter michiganensis* subsp. *michiganensis*, Biocontrol

Background

The tomato, *Solanum lycopersicum* L. (Solanaceae), is one of the most widely grown vegetables in the world, with an annual production of over 180 million tons (FAO 2021). The large proportion of production has been affected by abiotic (temperature, pH) and biotic (microorganisms) factors that damage all parts of the plant (Elnaggar et al. 2018).

One of the most important biotic factors is plant pathogenic microorganisms that cause diseases during plant growth and postharvest storage, leading to economic losses. More than 60 pathogens, including bacteria, fungi, viruses, and nematodes, are known to cause diseases in tomato (Jones et al. 2014). *Clavibacter michiginensis* subsp. *michiganensis* (*Cmm*) is a non-spore-forming, non-motile, gram-positive, and aerobic bacterium that

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causes bacterial canker and wilt disease of tomato (Gleason et al. 1993).

Bacterial canker and wilt disease of tomato was first described in Michigan, USA, in 1909 (Smith 1910). Plant debris, plant tissue, and contaminated seeds allow the bacteria to spread over long distances. Even less than 1% infected seed can cause 60–70% of crop losses in the field (Shaker 2014). The pathogen can remain viable in the soil for 2–3 years. Infected tomato plants showed different symptoms depending on environmental conditions such as temperature and humidity, as well as the virulence of the pathogen and the susceptibility of the variety (de León et al. 2011). Although the disease is seed-borne, wounds and natural openings such as hydathodes and stomata are the main entry points for *Cmm* to penetrate host tissues. Once invaded, the pathogen spreads throughout the plant via the xylem vessels. As the disease progresses, wilting symptoms, stunting, decline in fruit yield, and early plant death occur (Tancos et al. 2013).

Copper-based compounds such as copper hydroxide and copper sulfate are commonly used to control *Cmm* (Kasselaki et al. 2011). However, the excessive use of copper-based chemicals not only leads to the emergence of resistant pathogens, but also prevents seed germination and impairs plant growth through oxidative stress and a reduction in photosynthetic pigments (Raymaekers et al. 2020).

On the other hand, bacteriophages, the viruses of bacterial cells, are highly specific to a single bacterial species, and may even be specific to a single strain of that species. In addition, they are self-replicating, can remain in the environment for a long time, and are safe to use as they are not infectious to non-target organisms such as humans, animals, and plants. Therefore, the use of bacteriophages has gained importance in recent years as a promising tool to control bacterial plant diseases (Svircev et al. 2018). There are important studies on phage-mediated biocontrol of *Ralstonia solanacearum* and *Xanthomonas campestris*, which are other important bacterial pathogens of tomato plants (Elnaggar et al. 2018). However, *Cmm*-specific bacteriophage was first described by Echandi and Sun (1973), and since then no studies have been carried out to isolate novel bacteriophages.

In this context, the aim of the study was to isolate lytic bacteriophages against *Cmm* strains causing bacterial wilt and canker of tomato in Turkey and to determine their potential use as biocontrol agents.

Methods

Samples collection

In the summer of 2021, soils and plant material were collected from different regions of İzmir, Bilecik, and Çanakkale in Western Turkey. Soil samples were collected from 30-cm root depth of diseased plants from the growing areas. For pathogen isolation, samples of the aboveground parts of tomato plants (leaves, fruits, and stems) were collected in Kraft bags from tomato plants showing typical disease symptoms. A total of 90 samples (45 soil samples and 45 samples of diseased plants) were placed in an ice box and transferred to the laboratory to isolate *Cmm* and *Cmm*-specific bacteriophages (Akbaba and Ozaktan 2021).

Clavibacter michiganengis subsp. *michiganensis* isolation

To isolate *Clavibacter michiganengis* subsp. *michiganensis* (*Cmm)* from diseased plant tissue, the plant material was treated with 70% ethanol for surface sterilization and then rinsed with sterile distilled water. All samples were crushed separately in mortars containing 2 ml of sterile water and then a loop full of suspension was inoculated onto yeast extract dextrose calcium carbonate agar (YDCA) medium (Fatmi et al. 2017).

To isolate *Cmm* from soil samples, 20 g was placed in an Erlenmeyer containing 100 ml of sterile phosphate buffer saline (PBS) and incubated at room temperature on an orbital shaker for 3 h. The suspension was then filtered through sterile gauze, and the filtrate was centrifuged at 10,000 rpm for 5 min. After suspension of the pellet in PBS was serially diluted tenfold, 100 µl of each dilution was spread on the YDCA medium. After overnight incubation at 30 °C, presumptive identification of *Cmm* was determined by the presence of yellow-pigmented colonies on YDCA (Schaad et al. 2001). Purified bacterial isolates were then stored in YDC medium supplemented with 50% glycerol at − 80 ∘C.

Morphological and biochemical identification of *Cmm*

Morphological characteristics such as the shape and size of colonies were examined under a stereomicroscope. The cell shape of the bacterial isolates was determined using a light microscope with 1000× magnification. Gram staining of all isolates was performed according to Claus (1992). Catalase, starch hydrolase, growth in 5% NaCl, and gelatin liquefaction assay were performed to determine biochemical properties (Li et al. 2018). The morphological and biochemical characteristics of the isolates were defined according to Bergey's Manual of Systematic Bacteriology (Brenner et al. 2005).

Molecular identification of *Cmm*

Total DNA isolation was performed using the ZYMO ZR Fungal/Bacterial DNA Miniprep Kit according to the manufacturer's protocol. DNA concentration and purity were checked using a Nanodrop spectrophotometer (Shimadzu, Tokyo, Japan) and stored at −20 °C until

use. The presence of the virulence gene *pat-1* (614 bp) located on the plasmid was determined by PCR using the primer pair CMM-5F (5′-GCGAATAAGCCCATA TCAA-3′) and CMM-6R (5′-CGTCAGGAGGTCGCT AATA-3′) (Rivera-Sosa et al. 2021). PCR amplification was performed in a thermal cycler (Thermo Scientific). Reactions were performed in 25 µl: 1.0 µl DNA template, 1.5 µl 2.5 mM $MgCl_2$, 0.5 µl 10 µM dNTPs, 0.5 µl of each 10 µM primer, 0.125 µl 5U/µl *Taq* DNA polymerase, 10 µl 5 × reaction buffer, and 11.87 µl dH_2O. PCR amplification was performed with initial denaturation of template DNA at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. PCR products were electrophoresed on 1.0% agarose gels with ethidium bromide and visualized under UV light.

Pathogenicity assay

Cmm isolates were grown in King's B medium (KB) (20 g/l protease peptone, 1.5 g/l K_2HPO_4, 1.5 g/ml $MgSO_4{\cdot}7H_2O$, 10 ml/l glycerol) at 30 °C for 24 h, and the bacterial suspension was centrifuged at 4500 rpm for 15 min. The pellet was washed three times with PBS and finally resuspended in sterile distilled water to an optical density of 0.1, which corresponded to approximately 10^8 cfu/ml. For the pathogenicity test, tomato plants with 2–3 true leaves were used assay, and three replicates with three plants per pot were performed. One hundred µl of the bacteria were injected into the first true leaf node using a sterile syringe. After inoculation, the pots were kept in a growth chamber at a 85% relative humidity and a photoperiod 16/8-h day/night at 25 °C. Disease symptoms were monitored weekly for three weeks, and the pathogen was recovered from the stem of the plants to confirm *Cmm* infection (Stancu and Mitrea 2020). *Cmm*-DB3 (Acc. No: OP534745) was used as a positive control, and water as a negative control.

Bacteriophage isolation and purification

Ten grams of the collected sample (soil/plant material) was placed in a sterile 250-ml Erlenmayer containing 40 ml Ringer's solution and incubated overnight at 30 °C with shaking at 250 rpm. Samples were centrifuged at 10,000 × *g* for 15 min at 4 °C, and the supernatant was filtered using a sterile 0.22-µm syringe filter. For phage enrichment, 10 ml of the filtrate was incubated with 5 ml of *Cmm* strain DB3 and 10 ml of KB medium in an Erlenmeyer at 30 °C for 48 h. After incubation, the culture was centrifuged at 5000 × g for 20 min at 4 °C, and the supernatant was filtered through a sterile 0.22-µm syringe filter. Phages infecting *Cmm* strain DB3 were determined by spot assay. One hundred µl of an overnight culture of bacteria was added to 4 ml of soft KB agar (0.7% agar), poured onto a KB plate, and allowed to solidify for 5 min. Enriched phage filtrate (10 µl) was spotted onto the bacterial lawn and incubated overnight at 30 °C (Wang et al. 2022).

Bacteriophages were isolated from positive samples using the plaque pick-up method. A single plaque was collected from the double-layer plate using a sterile pipette in 250 µl of SM buffer (100 mM NaCl, 8 mM $MgSO_4.7H_2O$, 1 M Tris–HCl, pH 7.5). Then, 100 µl of the SM buffer containing the phages was serially diluted in SM buffer and double-layer agar (DLA) plaque assay was performed. The procedure was repeated at least three times to obtain a pure single bacteriophage culture (Pekkle Lam et al. 2022).

Determination of bacteriophage titer

Bacteriophage titer was determined by plaque assay using the DLA plaque assay technique (Kropinski and Martha 2009). The phage culture was diluted tenfold in the SM buffer. One hundred µl of each dilution and one hundred µl of host bacteria were mixed with 4 ml of soft agar and poured onto KB medium plates. They were then incubated overnight at 30 °C. After incubation, the phage titer was calculated according to Baer and Kehn-Hall (2014). Experiment was performed with three replicates.

Host range of bacteriophages

The host range of purified bacteriophages was determined against eight *Cmm* strains isolated in this study, as well as against local *Acidovorax citrulli* and *Pantoea agglomerans* strains. Each bacterium was grown overnight at 30 °C in KB broth, and 100 µl of the bacterial culture was added to soft agar (4 ml) cooled to 48 °C and spread on KB agar plates. Ten µl of the purified phage was spotted onto the plate and allowed to dry. Then the plate was incubated overnight at 30 °C to observe plaque formation (Ranjani et al. 2018).

Thermal and pH stability of bacteriophages

Stability of phages (10^8 pfu/ml) at different temperatures (15, 25, 30, 37, and 45 °C) was evaluated after incubation in a water bath for one hour (Lim et al. 2013). Similarly, pH stability of phages (10^8 pfu/ml) at different pH values (5, 6, 7, 8, and 9) was assessed after incubation at 30 °C for one hour. All experiments were performed in triplicate, and phage titer was calculated by DLA plaque assay KB agar plate (Li et al. 2022).

Killing curve assay

To determine the effect of the three bacteriophages and their combinations on bacterial growth, a killing curve assay was performed. A 96-well flat-bottomed plate was

inoculated with 40 µl of a *Cmm*-DB3 culture (10^8 cfu/ml) and 40 µl of fresh KB medium. Then the bacterium was infected with 40 µl of phage at different infection rates (MOI = 1, 10, 100, 1000). The plate was incubated at 30 °C with shaking. The optical density of the culture was measured every hour for 12 h at 600 nm and shaken for 5 s before each measurement (Elisa-Reader, Thermo Scientific) (Álvarez et al. 2019). The experiment was performed with three replicates. For experiments with double and triple phage combinations, each phage was used in equal amounts and the experiments were performed as described above (Rabiey et al. 2020).

Results

Clavibacter michiganengis subsp. *michiganensis* isolation and characterization

A total of 70 bacteria producing yellow pigment on YDC agar were isolated, 42 from soil samples and 28 from plant samples. Fourteen of these isolates were Gram positive. The cell shape of these isolates was determined to be bacilli, which were examined with a light microscope at 1000× magnification. The isolates that were found to be Gram positive were subjected to various biochemical tests, and results are displayed in Table 1. Morphological and biochemical identification revealed that isolates DB11, DB12, DB13, DB18, DB23, DB24, DB35, DB38, and DB42 were possibly *Cmm*. The DNA of these isolates was amplified with *pat-1* virulence gene-specific primer and all isolates, except DB13 showed bands with the predicted size of 614 bp on the agarose gel (Fig. 1).

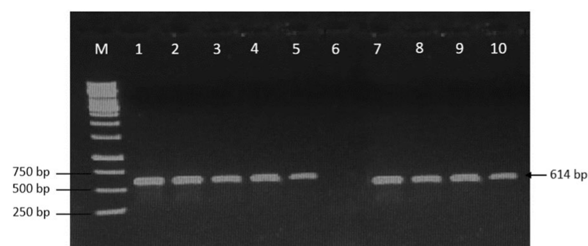

Fig. 1 Agarose gel electrophoresis of *pat-1* virulence gene of putative *Cmm* isolates. M: Marker, 1: DB3 (control), 2: DB11, 3: DB12, 4: DB18, 5: DB23, 6: DB13, 7: DB24, 8: DB35, 9: DB38, 10: DB42

Pathogenicity assay

In the inoculated plants, the first symptoms appeared about 10 days after inoculation. No symptoms were observed in the control group, which was inoculated with water. About 15 days after inoculation, fissures appeared at the inoculation sites and longitudinally along the length of the stem. Small lenticular cankers were also observed all over the stem. After 25 days, some plants were dead and others had completely wilted leaves. The plants with symptoms remained smaller than the control group. Differences were observed in the virulence of the inoculated isolates. Disease symptoms were first observed in plants infected with DB11, DB23, and DB38. However, disease symptoms appeared later in plants infected with DB35 and DB12.

Bacteriophage isolation, purification, and host ranges

A total of 90 samples (45 soil and 45 plant samples) were tested for the presence of lytic bacteriophages against

Table 1 Morphological and biochemical characteristics of putative *Cmm* isolates

Bacterial strains	Gram reaction	Cell shape	Colony pigment	Gelatin liquefaction	Catalase	Growth in 5%NaCl	Starch hydrolyze
DB3*	+	Bacilli	Yellow	+	+	+	–
DB11	+	Bacilli	Yellow	+	+	+	–
DB12	+	Bacilli	Yellow	+	+	+	–
DB13	+	Bacilli	Yellow	+	+	+	–
DB14	+	Bacilli	Orange/Yellow	–	–	–	–
DB15	+	Bacilli	Orange	–	–	–	–
DB18	+	Bacilli	Yellow	+	+	+	–
DB19	+	Bacilli	Yellow	–	–	–	–
DB22	+	Bacilli	Orange/Yellow	–	–	–	–
DB23	+	Bacilli	Yellow	+	+	+	–
DB24	+	Bacilli	Yellow	+	+	+	–
DB33	+	Bacilli	Orange	–	–	–	–
DB35	+	Bacilli	Yellow	+	+	+	–
DB38	+	Bacilli	Yellow	+	+	+	–
DB42	+	Bacilli	Yellow	+	+	+	–

* Control strain

Cmm-DB3. It was found that 22 samples formed clear plaques on the plates. Fourteen of these were from soil samples and eight from plant material. Overall, positive plaques were detected in 24.4% of the samples. The host range of twenty-two phages was examined on eight indigenous *Cmm* strains isolated in this study, one *Pantoae agglomerans*, and one *Acidovorax citrulli* strain. As expected, all bacteriophages tested did not form plaques in *Pantoae agglomerans* and *Acidovorax citrulli* strains. Twelve bacteriophages formed clear plaques on the *Cmm*-DB3 strain but not on eight *Cmm* strains tested (Table 2). Therefore, ten other phages (Phage5, Phage14, Phage24, Phage33, Phage39, Phage41, Phage44, Phage49, Phage61 and Phage82) with a broader host range were selected for temperature and pH stability tests.

Thermal and pH stability test of phages

The temperature and pH stability of ten bacteriophages was evaluated. Phage33 was stable after 1 h incubation at all the temperatures tested, compared to the initial titer of the phage. Although Phage24 showed a reduction of ~ 1.5 log than the initial titer, the temperature tolerance was higher than the other phages (Fig. 2). On the other hand, the titer of the other phages decreased by ~ 3 log compared to the initial titer. The titer of Phage 41 was the same at all pH values tested compared to the initial titer. In particular, this bacteriophage was completely stable at pH 8 and 9. Although the titer of Phage24 and Phage33 was reduced by ~ 1 log than the initial titer, the pH tolerance was higher than the other phages (Fig. 3).

Killing curve assay

Three phages (Phage24, Phage33, and Phage41), which had high temperature and pH tolerance and a broad host range, were used for the killing curve assay. The inhibitory effect of the phages and phage cocktails was observed by inoculating *Cmm*-DB3 (OD_{600} of 0.1) with different MOIs. The growth of *Cmm*-DB3 infected with the Phages24 at MOI of 100 showed minimal inhibition than to another infection rate. Maximum inhibition was observed at the infection rates used; Phage24 reduced bacterial growth by 46.94% at an OD_{600} of MOI 1000 (Fig. 4A). In contrast to Phage24, Phage33 produced maximum inhibition at MOI 10 (57.77%) and 100 (57.50%), and minimum inhibition at MOI 1000 (33.33%) (Fig. 4B). Phage41 showed the highest inhibition than the control bacteria at MOI 1 (48.33%) (Fig. 4C). In addition, bacterial inhibition of phage cocktail D1 (consisting

Table 2 The host range of twenty-two phages forming clear plaques on the *Cmm*-DB3 isolate

Phages	Bacterial strains									
	DB11	DB12	DB18	DB23	DB24	DB35	DB38	DB42	*P. agglomerans*	*A. citrulii*
2	–	–	–	–	–	–	–	–	–	–
5	+	–	–	–	–	+	–	+	–	–
14	+	–	–	+	+	+	+	+	–	–
16	–	–	–	–	–	–	–	–	–	–
21	–	–	–	–	–	–	–	–	–	–
24	+	+	–	+	+	+	+	+	–	–
27	–	–	–	–	–	–	–	–	–	–
33	+	–	–	–	+	+	+	+	–	–
34	–	–	–	–	–	–	–	–	–	–
39	+	–	–	+	+	–	+	+	–	–
41	+	–	–	+	+	+	+	+	–	–
44	–	–	–	–	–	+	–	+	–	–
49	+	+	–	+	+	+	+	+	–	–
43	–	–	–	–	–	–	–	–	–	–
46	–	–	–	–	–	–	–	–	–	–
52	–	–	–	–	–	–	–	–	–	–
57	–	–	–	–	–	–	–	–	–	–
61	+	–	–	+	+	+	+	+	–	–
71	–	–	–	–	–	–	–	–	–	–
80	–	–	–	–	–	–	–	–	–	–
82	+	–	–	–	–	–	+	+	–	–
85	–	–	–	–	–	–	–	–	–	–

(–) no, (+) clear plaque

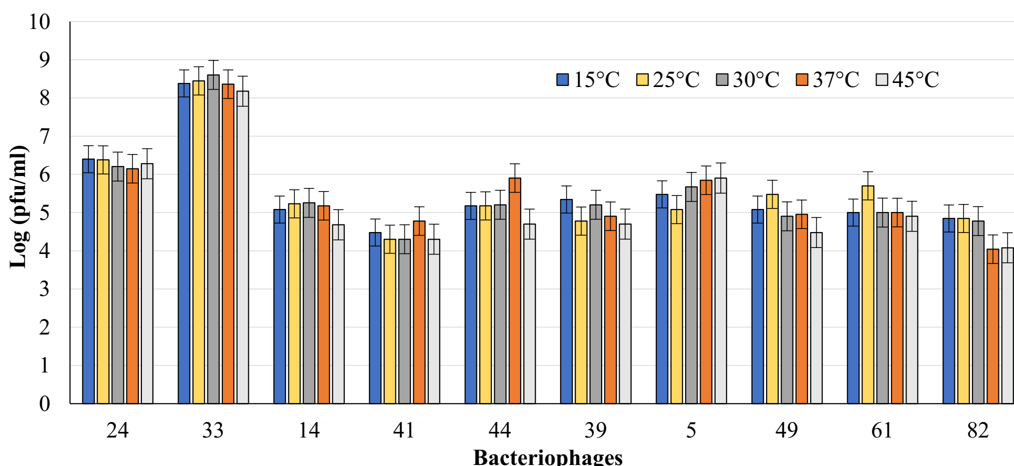

Fig. 2 Survival of *Cmm*-specific bacteriophages exposed to different temperatures for one hour. Bars show means of three reputations. Error bars indicate standard deviation

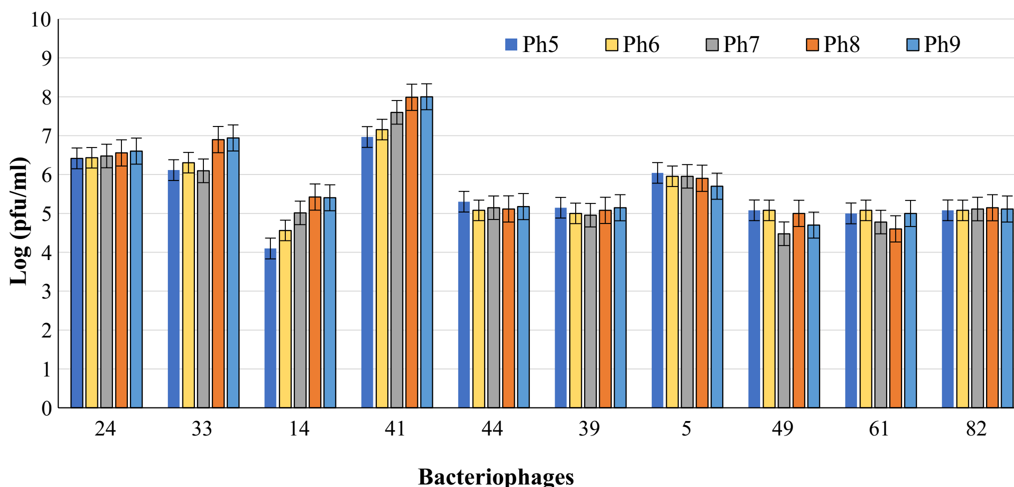

Fig. 3 Survival of *Cmm*-specific bacteriophages exposed to different pH for one hour. Bars show means of three reputations. Error bars indicate standard deviation

of Phage33 and Phage41), D2 (consisting of Phage24 and Phage33), D3 (consisting of Phage24 and Phage41), D4 (consisting of Phage24 Phage33 and Phage41) was evaluated, and it was found that the most effective cocktail was D2 at MOI 1 with 48.33% (Fig. 5A, B, C, D).

Discussion

In recent years, phage-mediated biocontrol has gained attention as an alternative strategy to control bacterial plant diseases. *Clavibacter michiganensis* subsp. *michiganensis* which led to bacterial wilt and canker in tomato plants is one of the most important pathogens in tomato plants. In this study, bacteriophages from rhizosphere soil and phyllospheres of tomato plants were isolated and evaluated their biocontrol potential against *Cmm*. A total of twenty-two native bacteriophages were isolated from 90 samples (45 soil and 45 tomato phyllospheres), 14 from rhizosphere soil and the others from phyllospheres. According to the literature, the most of the isolated bacteriophages were obtained from soil samples near infected plants. Gašić et al. (2011) isolated bacteriophages from soil, leaf, fruit, and root samples from pepper cultivation areas and obtained 25 bacteriophages from soil only. They attributed the inability to isolate bacteriophages from aboveground parts to the fact that bacteriophages are sensitive to UV radiation and temperature. However, James et al. (2020) collected samples (60 soil, 60 leaf, and 20 bark samples) from cherry tree fields

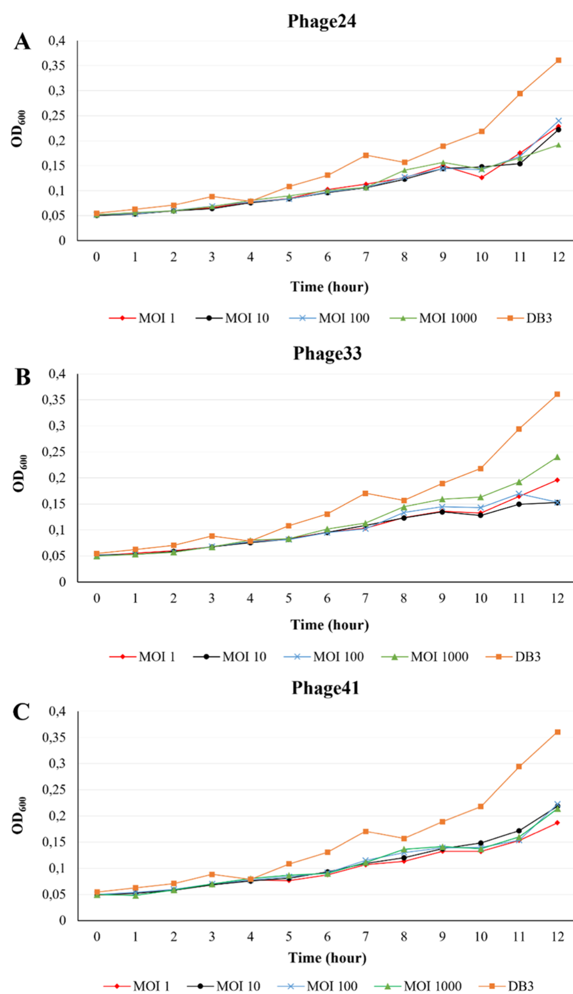

Fig. 4 Growth curves of bacteriophages infected *Cmm*-DB3 at different MOI. **A**. *Cmm* infected with Phage24, **B**. *Cmm* infected with Phage33, **C**. *Cmm* infected with Phage41

and a total of ten bacteriophages were detected from phyllospheres of plants.

On the other hand, among 70 yellow-pigmented bacteria obtained from infected plant material, eight isolates with similar morphological and biochemical characteristics to *Cmm* were detected. Eight isolates that showed typical symptoms of *Cmm* infection in pathogenicity tests and contained the virulence gene *pat-1* in the genome, which was an important virulence factor, confirmed that the isolates were *Cmm*. The presence of the pathogenicity gene *pat-1* in the genomes of the Greek strains was investigated by Malliarakis et al., (2023) using conventional PCR. On the other hand, different molecular approaches have been proposed as an alternative strategy to conventional PCR for the identification of *Cmm*. Chip digital PCR based on the *pat-1* gene sequence of *Cmm* was developed by Morcia et al., (2023). Studies have shown that the *pat-1* gene can be used for molecular identification, albeit with different molecular techniques. In host range experiment conducted with isolates obtained in this study, six of the eight phages isolated from the phyllospheres, and six of the fourteen phages isolated from the rhizosphere had a narrow host range compared to other phages, which is consistent with previous reports that bacteriophages can infect only a few strains of the same species (Gill and Abedon 2003). Similarly, Ramírez et al. (2020) showed that five of the eight phages inhibited the growth of more than half of the 65 strains of *R. solanacearum*. This was also reported by Nakayinga et al. (2021) who showed that 52 of the 140 *Xanthomonas* phages described in the literature have a narrow host range and 88 have a broad host range. Moreover, as expected, our obtained phages did not infect the local isolates of *Acidovorax citrulli* and *Pantoea agglomerans*. Therefore, the temperature and pH stability tests were performed with ten phages (two phages from phyllospheres and eight phages from the rhizosphere soil) that showed a wide host range.

Determining the stability of bacteriophages to be used for biological control is important for use in the field. Temperature is one of the critical factors for bacteriophage survival and persistence, as it plays a fundamental role in adsorption and multiplication. This study has shown that temperature has different effects on different bacteriophages. Only Phage33 isolated from soil remained stable at all applied temperatures compared to the initial titer. Interestingly, although significant decreases in titer were observed for the other phages, the decreases were generally statistically the same at each temperature. There are studies showing that the temperature tolerance of phages is highly variable. Fujiwara et al. (2011) showed that three different bacteriophages of *R. solanacearum* were stable below 28 °C, while one of them, RSL1, showed greater stability at the highest temperatures (37 and 50 °C) than the other phages. Also, Kazantseva et al. (2022) isolated bacteriophages infecting *Pseudomonas syringae* and reported that phage Pf-10 remains almost 100% viable at 30–60 °C, but its viability was completely lost when the temperature reached 70 °C and above. Another important factor affecting phage stability is the acidity of the environment. Previous studies have shown that phages can remain stable under neutral or slightly alkaline conditions, but low and high pH values lead to a significant decrease in phage titers. The bacteriophages Eir4 and Eisa9 of *P. syringae* pv. *tomato* retain their infectivity at pH 7 and pH 9, but phage titers decrease significantly at pH 4 and pH 12 (Korniienko et al. 2022). This is because a bacteriophage that can infect a bacterium attaches to a receptor site on the cell surface and uses the bacteriophage's lysozyme to weaken the bacterial cell wall. However, pH may affect

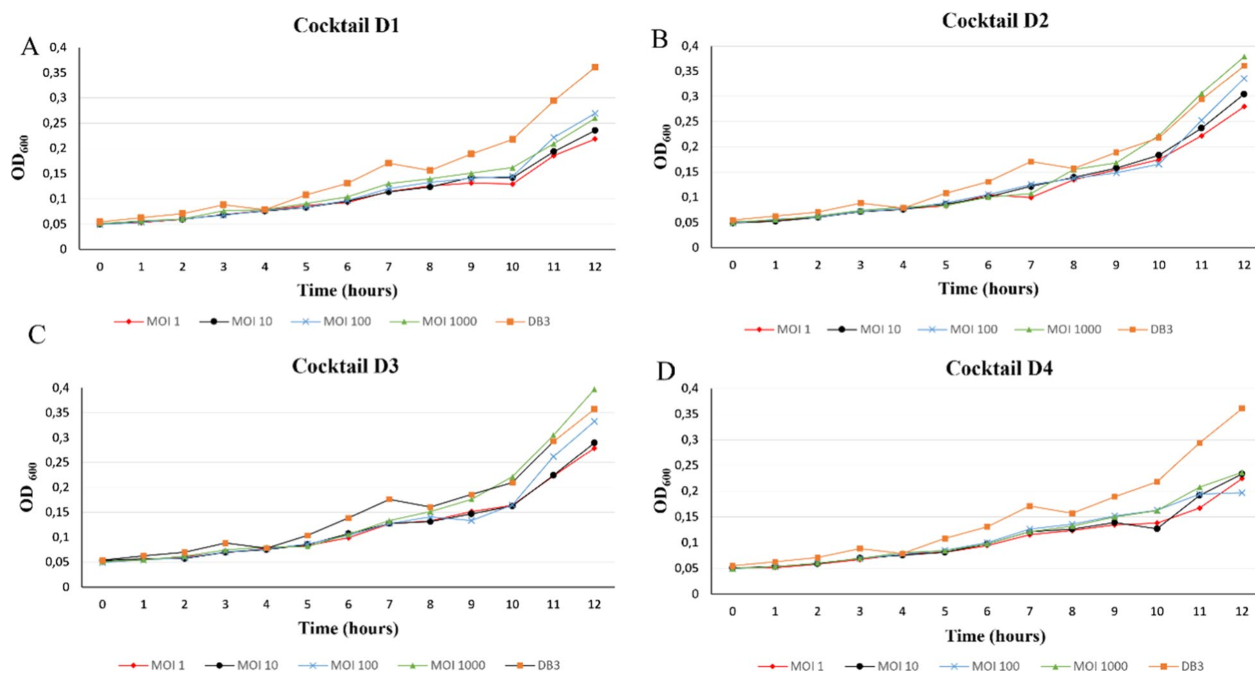

Fig. 5 Growth curves of bacteriophages cocktails infected *Cmm*-DB3 at different MOI. **A**. *Cmm* infected with Cocktail D1 (consisting of Phage24 and Phage33), **B**. *Cmm* infected with Cocktail D2 (consisting of Phage24 and Phage41), **C**. *Cmm* infected with Cocktail D3 (consisting of Phage33 and Phage41), **D**. *Cmm* infected with Cocktail D4 (consisting of Phage24, Phage33, and Phage41)

the lysozyme or other capsid proteins and prevent the phage from attaching to the receptor sites on the host cell (Leverentz et al. 2004). On the other hand, Kizheva et al. (2023) reported that *Xanthomonas euvesicatoria*-specific bacteriophages showed tolerance to low (pH 2) and high pH (pH 12). Of the phages isolated here, only Phage41 was completely stable at pH 8 and pH 9, but phage titer decreases significantly with decreasing pH.

Since bacteriophages with a broad host range and high pH and temperature tolerance have greater potential for controlling *Cmm* under field conditions, killing curve assays were performed by Phage24, Phage33, and Phage41. The bacteriophages were evaluated at MOI values of 1, 10, 100, and 1000 by measuring the OD_{595} of infected *Cmm*-DB3 cultures. All three bacteriophages were found to significantly suppress the growth of *Cmm* than the control. Although the maximum inhibition with Phage33 was observed at MOI 10, the minimum inhibition with phage 33 was observed at MOI 1. To improve the efficacy, the combination of bacteriophages was tested. Although the combination of Phage33 and Phage41 achieved the highest inhibition rate at MOI 1, this indicates that the combination did not show synergistic effect considering that Phage33 alone produced higher inhibition. The same is true for other phage combinations. On the other hand, bacteriophage cocktails have been shown to be more effective than individual strains in previous studies. The bacteriophage cocktail P1, containing six phages, was more effective than single phages against *R. solanacearum* in potato (Wei et al. 2017). This was also demonstrated by Ramírez et al. (2020), who showed that a cocktail of phages M5 and M8 reduced the severity of Moko disease of banana caused by *R. solanacearum* more than a single phage. On the contrary, there are studies showing that phage combinations are less successful than single phage treatments. In agreement with the present study, the combination of nine bacteriophages of *Pseudomonas syringae* pv. *syringae* was less effective than single phage treatment (Akbaba and Ozaktan 2021).

At a time when integrated management is becoming increasingly important for sustainable agriculture, phages are also a good alternative. As bacteriophages are species specific, they have the potential to be used in combination with other biological control methods such as antagonistic bacteria. Abo-Elyousr et al. (2019) previously reported that Rhizobacteria, *Bacillus subtilis, B. amyloliquefaciens, Pseudomonas fluorescens* and *P. aeruginosa* reduced the severity of bacterial canker and wilt disease under greenhouse conditions. Phage33 obtained in this study can be used in combination with these antagonistic bacteria to control *Cmm* infection.

Conclusion

Twenty-two *Cmm*-specific local bacteriophages were isolated in this study, and three phages stood out for their properties such as a broad host range, high pH and temperature tolerance. Among them, Phage33 was found to be the most effective bacteriophage in the killing curve assay. The results suggest that Phage33 may prove suitable as part of an integrated program to control *Cmm*. In further studies, Phage33 should be formulated to protect it from adverse environmental conditions and tested under greenhouse or field conditions to confirm the results obtained in vitro.

Abbreviations

PBS	Phosphate-buffered saline
rpm	Revolutions per minute
cfu	Colony-forming unit
pfu	Plaque-forming unit
OD	Optical density
MOI	Multiplicity of infection
YDCA	Yeast extract dextrose calcium carbonate agar

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Author contributions
All the authors contributed to the conception and design of the study. This study is part of the Ph.D. thesis of DBE under the supervision of CD. DBE conducted the experiments. DBE and CD analyzed the results and wrote the manuscript. The final manuscript has been read and approved by all the authors.

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Declarations

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