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Biocontrol of *Xanthomonas axonopodis* causing bacterial spot by application of formulated phage

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Abstract

Currently phages used as an alternative method for biocontrol plant pathogenic bacteria. However, the efficacy of phage is greatly reduced due to its short longevity on leaf surface of plant. Formulated phages [0.5% corn flour (CF) and 0.5% skim milk (SM)] significantly increased phage longevity on leaves surface were tested in greenhouse and open field trials during the growing season 2014. The leaves of pepper showed halo blight symptoms were tested for the presence of phages specific of *Xanthomonas axonopodis* by spot test. The phages were isolated by single plaque assay from the infected pepper leaves with spots symptoms. The isolated phages produced plaques with 3 to 5 mm diameters translucent spreading halo. The morphological phages particles were isometric head with 70 to 75 nm across with short contractile tails. The formulated isolate phages (CF and SM) were reduced the disease severity to 20.5 and 18.3% under greenhouse and 24.5 and 32.2% under open field conditions respectively, compared with unformulated phages. While, copper mancozeb reduced the disease severity to 45.3 and 39.5% (greenhouse) as well as 60.7 and 35.4% (open field) respectively. The skim milk and corn flour were applied for disease control trials and increased the phage longevity at 20 to 50 hrs and phage population 10^6, 10^5 (skim milk) to 4 x 10^4 and 5 x 10^3 plaque forming unit (PFU) in greenhouse and open field respectively . These formulated phages may be useful as a tool to efficient biocontrol of halo blight disease.

Key wards: biological control, bacteriophages, plant bacteria pathogens; *Xanthomonas axonopodis*

Introduction

Management of plant diseases may be achieved by integration of cultural practices, chemical control, acquired systemic resistance and resistance breeding. Additionally, appearance of antibiotic and cupper resistant bacterial strains in the field hindered the efficacy of chemical control **(Marco and Stall 1983)**. If weather conditions are favorable for disease development, there are no adequate control measures to manage the disease **(Kucharek, 1994).**Several alternative control methods have been investigated in recent years, systemic acquired resistance (SAR) inducers, such as acibenzolar methyl and harpin, have been shown to be active against bacterial disease of tomato **(Louws, *et al.*, 2001)** and their use in control of bacterial spots is the subject of ongoing research **(Obradovic *et al.*, 2002)**. Biological control of the disease has been attempted by using a nonpathogenic, *Ps. putida* B 56 antagonistic *Ps. syringae*_Cit 7 **(Wilson *et al.*, 1997)**. Another approach for biological control is the use of bacterial viruses to control bacterial diseases. Phages have long been proposed as plant disease control agents and have been used in several plant bacterium pathosystem (**Zaccarodelli *et al.*, 1992** and Balogh **and Jones, 2003**). Additionally, phages treated plants were found to be significantly more vigorous than copper treated and untreated ones, and they yielded significantly more extra-large fruits as well. However, **Eman and Afaf (2014)** noted that the phages application were in effective for controlling of bacterial halo blight and speculated that phage endurance was significantly reduced **(Jones and Pernezny ,2003).**

Viruses are very fragile and can't reside long on plant foliage because they are quickly eliminated by harmful environmental factors such as rain and sunlight UV (**McGuire, *et al.* 2001**). Therefore, the need arose to develop for mutations and/or change the application strategy, such as time of application in order to protect phage particles from harmful environmental factors. The accordingly enhanced residual activity of the phages could lead to increase efficacy of phage treatments and to a more convenient application schedule **(Balogh, and Jones, 2003)**.

The objective of this study was to investigate in greenhouse and open field experiments whether the use of selected formulations enhanced the efficacy of phage treatment for controlling bacteria halo blight of pepper plants.

Materials and methods

Plant bacterial pathogen

Three isolates of *Xanthomonas axanopodis* was kindly provided by plant pathology Dept.,Fac. of Agric.,Ain Shams Univ. Cairo, Egypt. The bacteria pathogen was isolated from symptomatic pepper (*Capsicum* spp.) leaves with bacterial spots cultivated in field experimental field Fac. of Agric. Ain- Shams University. Egypt. The bacterial isolate was streaked on nutrient agar medium in glass Petri dishes and incubated at 28 °C for 48 hrs. The bacterial growth were collected and washed with the saline solution (NaCl 0.85 %). The suspensions were shacked by magnetic stirrer for 15min. The concentration of bacteria isolates were counted by the most portable number adjusted at about mean density of 10^8 colony forming unit (CFU). Isolation and identification processes were carried out according to **Fourine (1998) .**The colonies of *Xanthomonas axonopodis* subjected to further characterization to identify the isolates as the standard procedures and confirmed using VETIC kit (Bio Mèrieux, France**)**

Bacteriophages

The infected pepper leaves showing spots symptoms were cut to small pieces and crushed in sterilized water (2:1 w/v) plus sterilized 3 drops of tween 80 per flask. The flasks were shacked on a rotary shaker for 72 hrs at room temperature on 3000 rpm for 20 min. The flasks were inoculated with Xanthomonas axonopodis at log phase culture approximately 5x107 CFU/ml-1 in nutrient glucose broth (2.0 g/L yeast extract and 2.5 g/L glucose).The flask cultures were incubated and shaken continuity for overnight at 28°C in shaking incubator. Bacterial cell and leaves debris were removed by centrifugation at 6000 rpm for 15min. The produced phages suspension was tittered by plaque assay method to make certain there were at least 108 PFU/ml. The phage mixtures consisted of two phage isolates and had an approximate final titer of 1 x 1010 PFU/ml. The phages mixtures were stored in 2 ml Ependorf tube at 4°C in complete darkness.

Phages morphology

Transmission electron microscope (TEM) was used to detect phages typing of *Xanthomonas axonopodis*. The phages were visualized using negative staining method with 1% aqueous uranyl acetate. The grids were air dried and were examined by TEM (JEOL – JEM – 1010 Electron microscope) in (The Regional Center for Mycology Al-Azhar Univ.) according to **Heringa *et al.* (2010).**

Phag formulations

Two formulations were used included:(a) 0.5% pregelatinized corn flour and 0.5% sucrose (PCF) (b) 0.75% skim milk powder and 0.5% sucrose (skim milk),**(Balogh, 2002),** as a formulated phages under greenhouse and field trails conditions.

Phage infectivity: *Xansomonas axonopodis* was suspended in sterile distilled water to a density of 10^7 CFU/ml^{-1} and inoculated on nutrient agar plates. The phage drop (20 µl) of each isolate was over layered on agar. The plates were incubated at 28 °C overnight. Clear confluent lysis and turbid confluent lysis were recorded with two isolets only as positive result, while extremely faint zones were considered negative result **(Eayre, *et al*., 1995**

Biocontrol experiment

Greenhouse experiment

Pepper seeds were cultivated in pots (20 cmQ) containing autoclaved sterilized soil and maintained in greenhouse at 23 to 28°C, fertilized and watered as needed. Three seedlings per pot and ten pots as a replicates for each treatment was done. *Xanthomonas axonopodis* growth was removed from nutrient agar plates 24 hrs post inoculation and suspended in sterile water and adjusted to A_{660} = 0.5 by spectrophotometer which approximately 10^8 CFU/ml^{-1}.Three leaves of each seedling were inoculated with bacterial suspension using a hand-hold plastic sprayer until completely wet. The suspension phage mixtures was adjusted by spectrophotometer which approximately 1 x 10^{10} PFU/ml and used as a biocontrol agent. Control plants were sprayed with tap water, as well as another plant was treated with copper mancozeb (Fungicide). Inoculated plants were sealed within a plastic bag (two hour post inoculation) in order to maintain high relative humidity. The plants were kept in growth chamber at 28°C and 16 hrs photoperiod for 48 hrs (**Fourie, 1998**). The plastic bags were removed and the plants were transferred to the greenhouse where they arranged in a completely randomized block design. The treatments and their constitution are shown in Table (A).

Field experiment:

Also**,** was carried out at spring growing season 2014. Treatments used in the field experiment are shown in Table (A). Three replicates of plots were implemented for each treatment and the plots were arranged in a randomized complete block design. Each plot consisted of 10 pepper plants. The pepper seeds were cultivated according to standard horticultural methods (Ministry of Agriculture). *Xanthomonas axonopodis* isolates suspensions (10^8 CFU/ml^{-1}) mixed with 0.05% tween 80 and misted on pepper leaves in middle of each plot using plastic sprayer. Mixed phages (1 x 10^{10} PFU/ml) treatments were applied twice weekly in the evening.

Copper mancozeb treatment was applied one week from cultivation or sowing. The treatments used in the field experiment are shown as follow:

Table (A) Layout of treatment applied in greenhouse and field experiments with amount based on 1liter spray max

Treatments	*X.axonopodis* (ml)	Skim milk (5%)	Corn flour (5%)	Tween 80	Phages mixture (ml)	Mancozeb (g/l)
unformulated healthy						X
unformulated inoculated	X			X	X	
Formulated skim milk	X	X		X	X	
Formulated corn flour	X		X	X	X	
Mancozeb	X			X		X

Determinations

Disease severity was carried out two times during growing season. Two types of disease severity were performed. Determination of disease severity was estimated according to **Weller (2007)** and calculated the spot areas under the disease progress curve (AUDPC) according to **Shaner and Finney, (1977)**. Also, lesions number in leaves (10 leaves per treatment).

Determination of phage titer was according to **Balogh and Jones, (2003)** during growing season at twice leaves sample were collected from all treatments in the day following the morning application at the afternoon and evening. Each sample consisted of 10 leaves that were located on exposed positions of the plant. The leaves were put in the plastic bags and kept in ice box and weighted and poured 100 ml deionized water in bag. The bags were shaken on shaker for 20 min and 1 ml of rinsing was transferred to 2 ml Ependorf tube and 100 µl chloroform was added. The tube were shaken on vortex for 30 min. The tubes were centrifuged in Ependorf at 6000 rpm for 15 min, then 500 µl of supernatant was put in sterile Ependorf tube. The supernatant was serial diluted for enumeration of the phage titer. *X. axonopodis* cells 24 hrs old culture were scraped from the agar plates and suspended in sterile water. A 100 µl of phage suspensions mixed with 100 µl of bacterial suspension and placed on sterile Petri dish. The nutrient agar medium heated to 45–50°C was added to the mixture and medium was swirled in Petri dish to facilitate mixing of bacterial cells and phage particles. The plate was incubated at 28°C for 48 hrs until the plaques became visible. The plaques were counted at the suitable dilutions. The phage titer was calculated as PFU per gram of leave tissues by the following equation.

Phage titer = plaque number x 1.000/ (weight of sample bag – weight of empty) bag.

Statistical analysis

The results of each sample were transformed using log transformation

[z = log 10 (Y + 1)] and then subjected to ANOVA analysis

Results

Bacterial identification: Three isolates only of *X. axonopodis* isolated from symptomatic pepper leaves spots were identified based on morphological and biochemical tests. Two isolates only revealed negative results with oxidase test, mannitol, sorbitol and inositol as sole carbon sources and positive results with leaven. The isolates were short rods and negative gram staining. The isolates produced brown diffusible pigment on king B medium. Based on morphological and biochemical tests two isolated bacteria were identified as EG1and EG2. The isolated *X. axonopodis* confirmed by VETIC Kit.

Phages propagation

Crude phage suspension prepared from infected leaves of pepper plants and assayed by the over layer technique. Phage isolates were produced different plaque types where regular, irregular circular form clear center and turbidity center with size 3 to 5 mm diameter translucent spreading halo (Fig. A). The phage isolates distinct host specificity when tested with two pathogenic *X. axonopodis*. The specific phage was quantitatively assayed by plaque assay and purified by abtained a single plaque of lysate type. Single plaque isolate was picked up and put 2 ml of *X. axonopodis* broth culture (1 x 10^8 CFU/ml) and macerated then incubated. Specific phage isolates were reacted with 3 *X. axonopodis* isolates

with different type of lysis except one isolate of bacteria did not developed plaques. The produced plaques by specific phages isolates [Clear confluent lysis, turbid confluent with large and small circular with halo and without halo) were mixed for using as biocontrol agents (4.5 x 10^{10} PFU/ml). Electron microscope of phage particles revealed that phages are short, curled, non-contractile tail. The phage particles have an isometric head with different diameter size 70 to 75 nm and short contractile tail (Fig.A).

Biocontrol in greenhouse

Spraying of pepper plants with phage post-inoculation with *X. axonopodis* isolates showed reduction of disease severity and lesion spots either in individual *X.axonopodis* EG or mixture of isolates EG1, EG2 treatments. On the other hand, treating with formulated phages showed significant reduction of disease severity and lesion spots compared with both unformulated phages and copper mancozeb application. However, skim milk formulation achieved the greatest reduction in disease severity to 23.5, 20.3 and 18.3% and corn flour formulation to 27.2, 28.5 and 20.5% for *X. axonopodis* EG isolates EG1, EG2 and their mixture, respectively (Table 1 and Fig.1) while mancozeb, application reduced the disease severity to 30.5, 35.5 and 39.5% and unformulated phages to 52.3, 45.5, 45.3% compared with control since they were 75.5, 72.5, 85.5% respectively (Table 1 and Fig.2). As well as, formulated phages showed reduction in No. of spots (Table1 and Fig.1). The skim milk and corn flour formula without phages did not reduce the disease severity and lesion halo numbers.

Biocontrol in open field

In April, 2014 growing season of pepper plants the formulated phages caused significant reduction in both disease severity and halo lesion blight compared with unformulated phages, mancozeb and untreated control treatments (Table 2 and Fig.2). According to the area under the disease progress curve (AUDPC), the skim milk and corn flour formulated phages were the most effective in disease control being significantly more efficient than both unformulated phages, and copper mancozeb application. The unformulated phage treatment significantly reduced the disease severity compared with untreated control and achieved a level of control similar to copper mancozeb application treatment (Table2 and Fig.2),whereas, for both skim milk and corn flour formulations significantly further increased the efficacy of phage treatment. The skim milk formulated phages was significantly more effective for disease severity reduction with 25.0, 20.2, 19.5% than corn flour formulated phages with 35.2, 30.3, 32.5% for *X. axonopodis* EG 1, 2 and their mixtures, respectively (Table, 2).

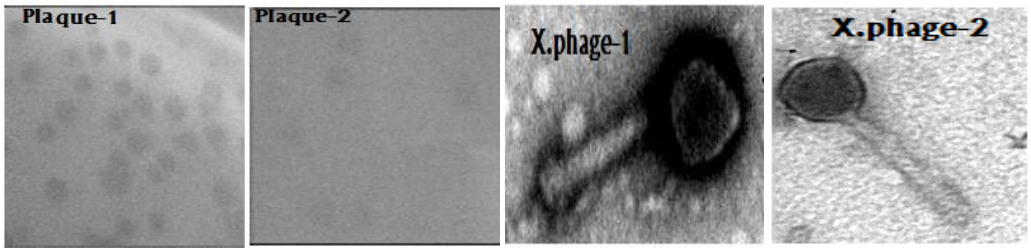


Fig. A: Electrophotogram illustrated negatively stained distinguished morphology of *Xanthomonas* phage particles and showing different two types of plaque morphology.

Table 1. Disease severity and halo blight lesion of infected pepper plants sprayed with formulated phages under greenhouse.

Bacterial isolates	*X. axonopodis* EG 1		*X. axonopodis* EG 2		Mixture of isolate EG1, EG2		
Treatments	DS%	No. of halo	DS%	No. of halo	DS%	No. of halo	AUDPC
Untreated control	75.5	10.5	72.5	10.5	85.5	10.5	52.1
Unformulated phages	52.3	4.2	45.5	4.5	45.3	3.3	48.4
Formulated phages							
Skim milk	23.5	1.2	20.3	1.9	18.3	1.2	23.8
Corn flour	27.2	2.8	28.5	2.1	20.5	1.5	25.5
Copper mancozeb	30.5	2.9	35.5	2.9	39.5	2.9	28.3

DS = Disease severity.
No.of spots = number of spot lesions per leaf.
AUDPC = Area under the disease progress curve.

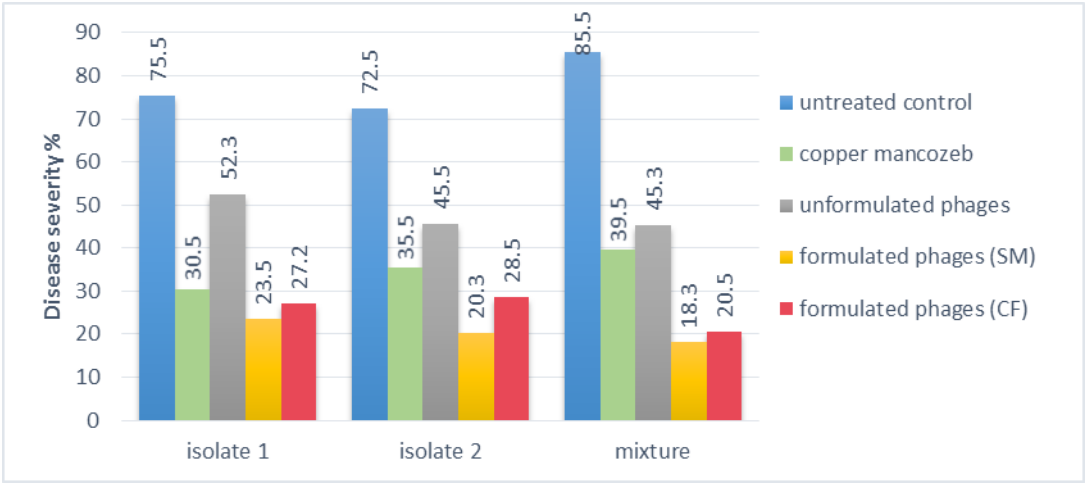


Fig.1: Disease severity percentage of pepper plant infected with *X. axonopodis* EG isolates under greenhouse conditions

Table 2. Disease severity and spot lesions of infected pepper plants sprayed with formulated phages under open field.

Bacterial isolates	*X. axonopodis* isolate EG1		*X. axonopodis* isolate EG2		Mixture of isolate EG1, EG2		
Treatments	DS%	No. of halo	DS%	No. of halo	DS%	No. of halo	AUDPC
Untreated control	80.2	12.5	85.2	15.3	85.5	25.7	62.9
unformulated phages	70.3	6.2	65.5	7.1	66.7	7.5	50.5
Formulated phages							
Skim milk	25.0	3.0	20.2	3.1	19.5	3.2	25.9
Corn flour	35.2	4.5	30.3	4.2	32.2	5.5	45.2
Copper mancozeb	40.5	5.5	37.8	7.2	35.4	9.5	49.2

(AUDPC): Area under the disease progress curve

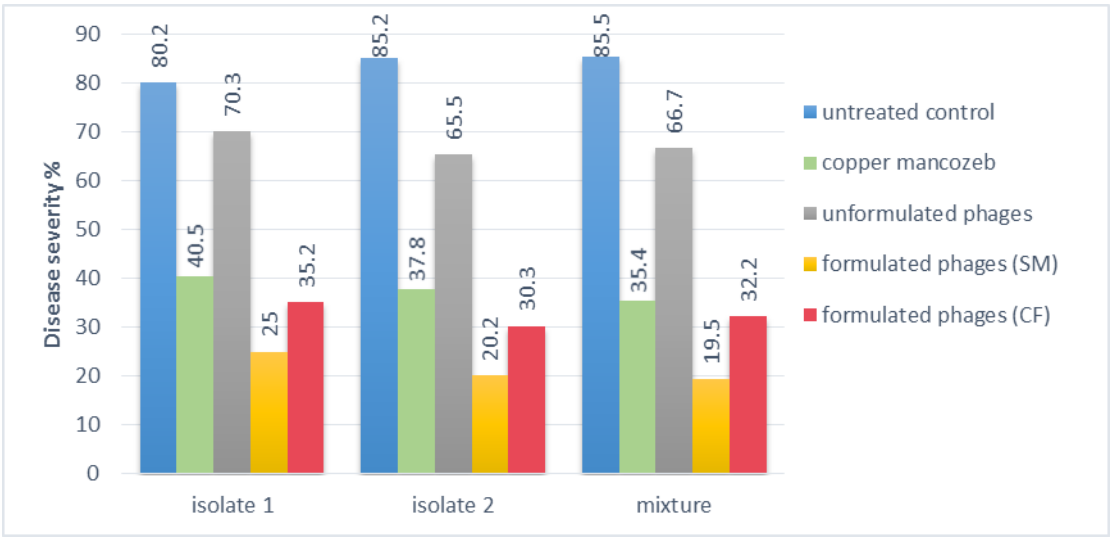
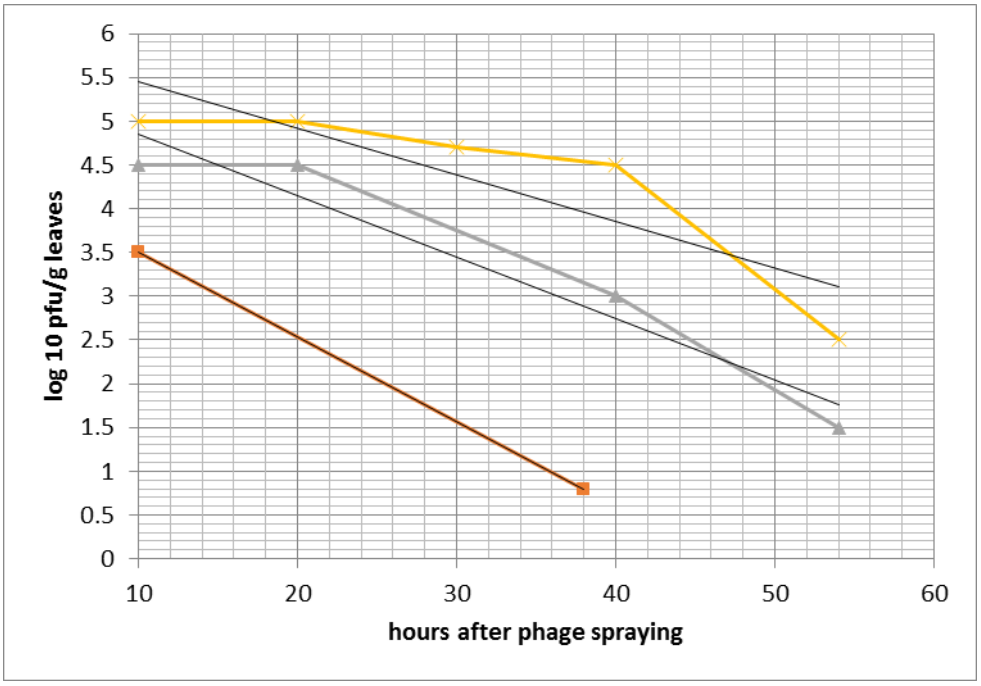


Fig. 2: Disease severity percentage of pepper plant infected with *X. axonopodis* EG isolates under open field conditions.

Longevity of formulated phages in field

The change in phage populations were determined during growth plants in open field. *X. axonopodis* anti-phages was not present in the field, due to the antagonistic effect. The changes in phages reflected as phages populations appeared in a host– free environment. On the other hand, unformulated phage populations were quickly reduced and practically eliminated 36 hrs with in and 48 hrs after spraying (Fig. 3). The skim milk formulated phages decreased the reduction rate of phage populations at 40 to 60 hrs.The corn flour formulated phages decreased the reduction rate of phage populations at 20 to 60 hrs. On the other hand, phage formulations were performed uniformly in greenhouse and open field assessments, showing the reduction rate and contributing to approximately 10^6 PFU/ml after phages spraying compared with phages spraying without protective formulation



Hage without corn flour formulated page skim milk formulated pahge

Fig. 3: Longevity of formulated phages on pepper leaves inoculated with *X. axonopodis* EG under green house and open field.

Discussion

Bacterial halo blight of plants caused by *X. axonopodis* has been a considerable problem in Egypt. The disease symptoms in open field can show considerable variation at the most distractive where temperatures are moderate and abundant inoculum is available **(Kucharek, 1994).**

X. axonopodis EG was isolated from infected pepper plants and distinct halo blight disease in open field. Morphology of colonies were similar to the standard *X. axonopodis* isolates. Morphological and biochemical tests indicated that the isolated bacteria were attributed to Xanthomonadaceae family **(Fourie, 1998 and Sahin and Miller 1996)**.

Bacteriophage specific for *X. axonopodis* were detected in infected pepper leaves and discrete halo blight disease. The crude phages suspension prepared from infected leaves and assayed by the over-layer agar method **(Eman and Afaf, 2014** and **Balogh and Jones 2003**). Single plaque which has the same plaque morphology of each plaque isolate picked up to obtained single phage of each *Ps. syringae* isolate. The phage titer usually assayed by the double layer agar method. The bacteriophages exhibited a regular , irregular , hexagon outline and a short non-contracted or long contracted tail. The isolated phage was characterized with large head and short tail associated with cystoviridae family, morphologically.

One of the greatest challenges in using phages for plant disease control is their extremely short residual activity in the phyllosphere, several studies indicated that phage populations can drop to undetected levels hours after applications **(Iriarte, et al., 2007).** Phages treatment was effective only in the morning

before down. The short residual activity of the control agents hindered the efficacy of

phage treatment when applied during day time. Phages are rapidly degraded in the greenhouse and open field and partially disappear from the pepper foliage about 2 days after application.

The results indicated that the efficacy of phages formulation by skim milk and corn flour could be increased phage protective and proper timing of application. Several protective formulation were identified in earlier studies **(Balogh, 2002and Saccardi, *et al.*1993)** and three of them were selected for disease control trials. These formulations increased the concentration of phage population 2 days after the application.

In this study, protective formulations increased the efficacy of phages treatments for disease control both in the greenhouse and in the open field. Skim milk gave the best results in greenhouse and open field followed by corn flour compared with unformulated phages. Despite the improved efficacy in disease control, none of these treatments achieved a significantly increase in yield. This could have been the result of the highly contagious nature of phage infection. Phages were detected in many untreated plots in the middle of the season in the spring field experiment in Quincy, and in all plots by the end of the growing season in the trail field experiment **(Balogh, 2002).**

Several alternative control methods have been investigated in recent years systemic acquired resistance (SAR) inducers **(Qui, *et al.*, 1997).** Biological control of the disease has been attempted by using a nonpathogenic *Xanthomonas campestris pv vesicatoria* T3 strain to antagonize the TLS strain (**Liu, 1998**) and with other antagonistic bacteria such as *Pseudomonas putida* B56 and *Pseudomonas syringae* Cit 7 **(Swords, *et al.*,1996 and Wilson *et al.*, 1997).** Plant growth promoting rhizobaceria (PGPR1) that induce SAR have been successfully used for controlling bacterial speck of tomato **(Ji et *al.*, 2006).** Another approach for biological control is the use of phages to control bacterial diseases. Phages have long been proposed as a plant disease control agent **(Jones *et al.*, 1998; 2004 and 2007)** and have been used in several plant bacteria pathosystems **(Coons, and** Kotila, 1925 and **Tanaka *et al,* 1990**).

In field trails twice weekly early morning spray applications of mixture of four phages specific to *X. campestris* pv. *vericateria* reduced disease severity of bacterial spot on plants by an average of 17% whereas copper mancozeb application reduced disease severity only 11% (**Bouzar, *et. al.* , 1999 and Flaherty, *et al.*, 2000)**. The phage application in the late morning was ineffective for controlling of bacterial spots and speculated that phage endurance was significantly reduced. Viruses are very fragile and can't reside long on plant foliage because they are quickly eliminated by harmful environmental factors such as rain and sunlight UV **(Mc Guire *et al.*, 2001 and Zaccordelli, *et al.*, 1992**).

Conclusion

In view of the obtained results it could be mentation that, two formulated phages 0.5% corn flour and 0.5% skim milk significantly increased phage longevity on pepper leaves surface and applied to control halo blight disease in open field. So, it could be recommended that these formulated phages may be used as a tool to efficient biocontrol of halo blight disease, to minimize the application of chemical control compounds. Therefore, reduce environmental pollution.

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المقاومة الحيوية للزانثومونس أكسونوبودز المسببة لمرض التبقع بواسطة إستخدام تحميلة الفاج .

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إستخدمت حاليا الفاجات كطريقة بديلة للمقاومة الحيوية للبكتريا الممرضة للنبات. مع أن كفاءة الفاج تنخفض بسرعة نتيجة لقصر عمر الفاج على سطح ورقة النبات . لذلك أستخدمت تركيبتين لتحميل الفاج (كورن فلور 5% و شرش اللبن 5%) تزيد من عمر الفاج على سطح الأوراق حيث أختبرت فى البيوت المحمية والحقل المفتوح . أختبرت أوراق نبات الفلفل والتى أظهرت أعراض الإصابة لوجود فاج بكتيريا الزانثومونس المتخصص بواسطة إختبار البقعة أو الهالة (البلاكات). وبطريقة البلاكات المنفردة أو البقعة قد عزلت الفاجات من أوراق نبات الفلفل المصابة بأعراض التبقع . والفاجات المعزولة تنتج منطقة رائقة شفافة قطرها من 3–5 مم . وقد أظهر الفحص بواسة الميكروسكوب الإلكترونى أن الشكل المورفولوجى لجزيئات الفاج أن الفاج ذو رأس متماثل 70–75 nm مع ذيل قصير ملتصق . وقد وجد أن تحميلة الفاج سواء كانت كورن فلور أو شرش اللبن فإنها تخفض من شدية المرض ل 20,5 (18,3 %) فى البيوت المحمية و 24,5 (32,2%) تحت ظروف الحقل المفتوح وذلك مقارنة مع معاملة الكنترول للفاج الغير محمل ، بينما مبيد المانكوزيب يخفض من شدية المرض بنسبة 45,3 (39,5%) فى البيوت المحمية و 60,7 (35,4%) فى الحقل المفتوح. وقد طبق إستخدام تحميلة الفاج على الكورن فلور وشرش اللبن فى مقاومة المرض فى الحقل و زيادة طول عمر الفاج من 20–50 ساعة وتكاثر الفاج 10^{5}،10^{6} (شرش اللبن) إلى $10X4^{4}$ و $10X5^{3}$ PFU فى البيوت المحمية والحقل المفتوح على الترتيب . وتعتبر تحميلة الفاج مفيدة كوسيلة مؤثرة لمقاومة مرض التبقع.