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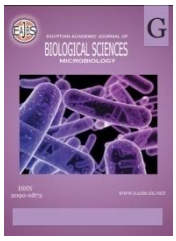

Controlling *Pseudomonas syringae* Causing Halo Blight on Bean Plants Via Biocontrol-Formulated Bacterial Viruses

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ABSTRACT

Bacterial viruses are currently used as a biocontrol agent for bacterial plant disease management. The evaluation of formulated bacteriophages mixed with carriers as a biocontrol agent showed efficacy and reduced disease symptoms (halo blight spots HBS) on the leaves of bean plants caused by *Ps. syringae*. It was isolated on nutrient agar. The single colonies of *Ps. syringae* appeared flat and produced fluorescent diffusible pigment on the king 's B medium as well as phylogenetically based on 16s RNA gene 726 bp by PCR. Two formulated bacteria viruses were applied as a biocontrol agent to control HBS disease. A complete randomized design was adapted in two experiments with three replications under greenhouse and open field conditions. The formulated bacteria viruses (skim milk and corn flour) significantly decreased the severity of the disease under greenhouse between 88.8 and 85.3 % and under open field conditions between 74.9 and 62.0 %, in comparison to pure bacteria viruses and copper oxychloride treatments to 83.3 and 76.3% in the greenhouse in addition to 74.9 and 62.0 % in open field respectively. The use of SM and CF for disease control experiments enhanced phage lifetime by up to 120 hours and phage density by Log 1.5 and 1.2 PFU/mL in the greenhouse and open field, correspondingly. The formulated bacteriophages (CF and SM) compared with bacteria viruses and copper oxychloride in a greenhouse and open fields showed the lowest HBS and AUDPC values. These phages may be effectively used to control halo blight disease.

INTRODUCTION

Integrated plant diseases control program applications might be eliminated bacterial pathogens by bactericides, cultural practices, the plant needed resistance by biotic and abiotic inducers and production resistance plants by traditional breeding. In addition, most bacterial pathogens exhibited resistance against antibiotics and copper in protected greenhouses and the open field (Marco and Stall 1983 and Kucharek, 1994).

Integrated plant disease management is applied by bacterial viruses and traditional common as well as sustainable organic agricultural practices for controlling phytopathogenic bacteria. (Lindsey *et al*., 2020). In recent years, Louws, *et al*., (2001) used systemic acquired resistance (SAR) biotic and abiotic inducers like harpin' and acibenzolar methyl against tomato bacterial disease, and Obradovic *et al*., (2002) used bacteriophages in control of bacterial to control bacterial spots. Wilson *et al*., (1997) used the nonpathogenic bacteriophages and *Ps. putida* B 56 an antagonistic bacterium to *Ps. Syringae* Cit 7 as a biocontrol agent. Long considered as plant disease control agents, phages have been utilized in numerous plant bacterial control initiatives (Saccordi *et al.*, 1993; Zaccarodelli*et al.*, 1992; Eman and Afaf, 2012; Balogh and Jones, 2003). In addition, bacterial viruses treated plants significantly showed disease reduction than copper treated as well as revealed significant yield. The application of bacterial viruses is significantly reduced bacterial halo blight (Eman and Afaf, 2012). On the other hand, the bacteria viruses are inactivated and eradicated from plant leaves because they are exposed to abiotic inhibitors stress such as ultraviolet, sunlight and rain (McGuire, et al. 2001). Therefore, recent new strategies of bacteria viruses application were used to preserve particles of viruses from factors of terrible surroundings. The residual activity of bacterial viruses increased the efficiency of bacterial viral therapies and made administration easier (Balogh, and Jones, 2003).

This study aimed to use the formulated bacteriophages specific to *Ps. Syringae* as a biocontrol agent against halo blight spots (HBS) on bean plants under greenhouse and open field conditions.

MATERIALS AND METHODS

Isolation of Pathogenic Bacteria of HBS Disease:

Leaves of bean (*Phaseolus vulgaris*, L.) naturally infected with halo blight were collected from Qalyb village Qalubia Governorate. The HBS were cut into tiny pieces and smashed in sterilized water (2:1 w/v) with three drops of sterile tween 80 in a flask. The mixture was shaken for 72 hours at room temperature at 3000 rpm for 20 minutes using a rotary shaker. The pathogenic bacterium was isolated as described by Fourine (1998). The nutrient agar medium was inoculated with macerated HBS and incubated for 48 h at 28°C**.** two single colonies were selected and inoculated on an LB agar plate medium. LB broth contained, 1 g lab-lemco powder, 2 g yeast extract, 5 g peptone and 5 g NaCl per l L. The pH was adjusted to 6.8 with 0.01M HCl and 0.01M NaOH. Plates were incubated at 30^0C for 24-48 hrs**.**

Identification of Pathogenic Bacterium:

Isolated bacterium was identified on the basis of the standard taxonomic descriptions of culture characters **(**Sneath 1986**)**.

Detection of Pathogenicity:

The pathogenicity of the isolated bacterium was bio-assayed on detached leaf enrichment according to Verdier *et al*. (2008) and Fawizia *et al*. (2018).

The bioassay was done in sterilized 5 cm Petri dishes. Ten ml of 1.5% agar solution in sterile water was poured into each plate. Young leaves of *Nicotiana tabacum* cv. Samson susceptible hosts were surface-disinfected with 1% NaCl for one minute, washed three times with sterile distilled water and surface-dried in a laminar flow. The lower surface of the leaves was punctured with a needle. Discs were obtained using sterile cork purer and placed on agar Petri dishes. Fifty microliters of macerated HBS tissues were added. As a positive control, a Ps. syringae suspension containing 10^5 cfu/ml is used, while sterile saline serves as a negative control. Petri dishes are sealed with cling film and incubated under constant light for 12 days at 28 ºC and 100% relative humidity. Post-third day from incubation the leaf discs were investigated using binocular microscopy for the formation of incipient whitish pustules. The pathogenic bacterium was isolated on a

nutrient agar medium as described by Verdier *et al.* (2008).

Morphological Study of Pathogenic Bacterial Isolates:

A smear from the pathogenic bacterial isolate was prepared on a glass slide and Gram stained. The prepared smear was microscopically examined. Cell morphology, arrangement and Gram reaction were recorded (Collins *et al.*, 2004)

Polymerase Chain Reaction (PCR):

Using MicroSeq® 500 16SrRNA Bacterial Identification Kit at CliniLab was identified the plant pathogenic bacteria at (Colors Lab, Maadi, Cairo, Egypt) as follow:

DNA Extraction: Five ml of liquid culture of the isolated bacterium (10^8 cfu/ml) was centrifuged at 5000 rpm/m. The pellet was resuspended in 2 mL of PrepMan™reagent in a new microcentrifuge tube and vortexed for 30 sec. and centrifuged for 5 min at 6,000 rpm. The pellet was resuspended in 200 uL of sterile deionized water and heated at 100°C for 20 minutes. then cooled at room temperature for 2min to release the DNA. The tubes were centrifuged at 6000 rpm / 2min. 5µL of supernatant was added to 495µL nuclease-free water to obtain a dilution ratio of 1:100 and saved for PCR.

Amplification by PCR: One pair of primers, Ul and U2, having patterns maintained across all bacteria, was chosen according to Rahmani, *et al.,* (2006) and Al Hag, *et al.,* (2020). The sequence of forward primer is 5' CCAGCAGCCGCGG TAA TACG-3', corresponding to nucleotides 518 to 537 of16S rRNA gene, and that of reverse is 5'-ATCGG(C/ T)TACCTT GTTACGACTTC3', corresponding to nucleotides 1513 to 1491 of the same gene, The PCR mixture in a volume of 50 ul was made by adding 15µl of extracted DNA (approximately 50 ng) l0 X PCR buffer, a 20 pmol concentration of each PCR primer, a 2.5 mM concentration of dNTPs and 2.5 u of Taq DNA polymerase in a total volume of 50 ul was prepared. The negative control contained 15µL nuclease-free water with 15µl of PCR mixture. The PCR program has 10 min denaturation at 94°C for 1 cycle, denaturation at 95ºC for 20 sec., and annealing at 60ºC for 20 sec. extension, 72ºC for 1 min. for 30 cycles and extension 72ºC for 10 min for 1 cycle.

Gel Electrophoreses: 10 ul of PCR product was separated by electrophoresis on a 2% agarose gel to measure the product's size via RF of DNA ladder (100 to 1000bp) and developed by ethidium-bromide staining (0.5µl/ml). The fragments were photographed using a UV lamp in gel documentation. The regulatory control included all PCR ingredients besides template DNA.

Sequencing 16S rRNA Gene*:* After cycle sequencing, excess dye terminators were removed and the purification product was purified using MicroSEQ™ ID purification combo kit, with clean-up cartridges (Includes ExoSAP-IT™ Reagent). Finally, after the sequence was completed, a review of the data was analyzed using MicroSEQ™ ID Analysis Software

Isolation of Bacteriophages: Bacteriophages are specific to *Ps. Syringae* were isolated from a rhizosphere soil sample of bean plants. The liquid enrichment technique of Adams (1966) was used to isolate phages as described by Othman and Shamloul (1997).

Phage *Detection:* The spot test was used for the detection of phages in agar double-layer plats as described by Borrego *et al.* (1987). Plates were prepared by pouring a base layer of 20 ml of nutrient agar medium containing 1.5% agar in petri dishes 10 cm in diameter. The basal layer was allowed to solidify. A mixture of 3 ml nutrient agar melted medium containing 0.7% agar and 300 µl of the indicator bacterium (*Ps. Syringae*) was poured into each plate. The indicator bacterium was a liquid culture of 24 h old (10^8 cfu/ml). The phage lysate was spotted with a sterile micropipette on the upper layer after it had solidified. Plates were incubated at 30-33°C for 36-48 h and then examined for lysis of bacterial lawn at the sites where drops had been applied.

Titration of the Isolated Phages: Titer was assayed using the plaque assay according to Lillehaug (1997). Decimal serial dilutions of

the crude viruses suspension were carried out in SM buffer from 10^{-1} up to 10^{-6}. The base layer of NA medium was poured into the sterilized Petri dishes and allowed to solidify. About 500 µl of virus suspension from each dilution and 300µl of the pathogenic bacteria ($1x10^8$cfu/ml) were mixed well with 3 ml of melted Semi-solid of NA medium (0.7% agar). Then, the mixture was poured over the base layer and allowed to solidify. Plates were then incubated at 25°C for 24h. After incubation, resulting in plaques were counted and the virus titer was calculated as pfu/ml.

Isolation of Lytic Individual Phages: The individual viruses were isolated using a single plaque isolation method (SPI) according to Othman *et al.* (2008). Different plaque types (size, shape and clearness) were picked and transferred to about 500µl SM buffer and stored overnight at 4°C. Then, single plaque isolation was repeated 3 times for each isolate.

Purification of Phages: Purification of bacterial *viruses* was carried out using differential centrifugation method according to Bachrach and Friedmann, (1971). The isolated phages were propagated in their main host within *a put* ratio of 1:10 and then phage suspensions were centrifuged at 3000 rpm/30 min. About 10% chloroform was added to the supernatant to remove the small contaminated bacteria and then centrifuged at 16000 rpm/2hrs/4°C. The supernatant was removed and the pellet was washed in 10 ml SM buffer before being centrifuged for 30 minutes at 3000 rpm. The precipitate was then centrifuged at 16000rpm/2hrs /4°C. The pellet was finally reconstituted in 5 ml SM buffer.

Characterization of the Isolated Phages:

Particle Size and Morphology: Each phage isolate was negatively stained with filtered 0.5% uranyl acetate pH 4 on a Farmavar 200 mesh coated grid (Heringa *et al*., 2010). The grids were air dried, then examined at 50 kv in a transmission electron microscope (TEM) (JEOL – JEM – 1010 Electron microscope) in The Regional Center for Mycology Al-Azhar Univ.

UV stability: The stability of the purified viruses was determined according to Setlow and Boyce (1960). The purified phages were exposed to UV lamp 60 w at distances of 30 cm for 30, 60, and 90 min. The infectivity of UV-irradiated phages was assayed quantitatively using plaque assay.

Stability of the Isolated Phages to Different Temperature Degrees: The Eppendorf tubes each containing l ml of high titer phage suspension ($6x10^{10}$pfu/ml) of each single phage isolate were prepared. Tubes were kept at 10, 20 and 30°C for 60 days. The log pfu/ml of each phage was **assayed** quantitatively using plaque **assay** according to Basdew and Laing (2014).

Formulated Phages: The isolated phages **(**$6x10^8$pfu/ml**)** were mixed with (5%) corn flour and skim milk (w/v) and tween 80 as diffusible material (individually or mixed phages) and stored for two months at 4^0C. The infectivity of formulated **viruses** was assayed quantitatively using plaque assay according to Balogh and Jones (2003)**.**

Phages Infectivity: Four *Pseudomonas* spp. were kindly supplied by Microbiology Lab. , Fac. of Agriculture, Ain Shams Univ. The susceptibility of each isolate to the isolated phages was tested using the spot test. Each bacterial isolate was used as an indicator bacterium in agar double-layer plates (Eayre, *et al*., 1995).

Biocontrol Experiment*:* Liquid culture of *Ps. Syringae* of 24 h. old (10^8cfu/ml) was prepared to be used as inoculum. A mixture of the high titer phage suspensions of the isolated phages (10^{10} pfu/ml) was prepared to be used as a biocontrol agent.

Greenhouse Experiment: A pot experiment was carried out to evaluate the efficiency of Ps. Syringae bacteriophages in controlling halo blight on bean plants. Bean seeds were planted in 20 cm pots filled with sterilized soil. Pots were kept in a greenhouse at temperatures between 23

and 28°C. Each pot contained three seedlings, fertilized and watered as required. Two to three leaflets of every seedling were sprayed thoroughly with the prepared bacterial inoculum using a handheld plastic sprayer. Pots were divided into three groups. The first group comprised untreated plants. The second group contained plants sprayed with a mixture of phage suspension. Whereas, the Third one included plants sprayed with copper oxychloride 2.5 g per 1 liter plus Tween 80 until completely wet as a positive control. The plants used as a negative control were doused with tap water. Two hours after treatments, inoculated plants were enclosed in plastic bags to preserve humidity relatively high. The plants were housed in a growing medium for 48 hours at 28°C and 16 hours of photoperiod (Fourie, 1998). After removing the plastic bags, the plants were carried to the greenhouse and put in a totally random block configuration.

Field Experiment: The experimental field layout was conducted during the 2021/2023 spring planting season. The experimental plots were set in a randomized full design with three replication plots for every therapy under circumstances of spontaneous infection. Every allotment had ten bean plants. The bean seeds were grown using typical horticulture techniques (Ministry of Agriculture). The isolate Ps. Syringae suspension (10^8 CFU/ml^{-1}) combined with 0.05% tween 80 was misted on bean leaves in the middle of each plot using a plastic sprayer. Formulated mixed bacterial viruses (1 x 10^8 PFU/ml) were mixed with 5% of each Corn flour (CF) or Skim milk and 0.05% tween 80 used two times per week in the evening. Copper oxychloride 2.5 g per 1 liter plus Tween 80 treatment was applied once weekly treatment as a positive control

Calculation of Area Under the Disease Progress Curve (AUDPC): The severity of the disease was documented on the lowest leaves at two different crop stages (vegetative and flowering development) using the severity scale according to Saari and Prescott (1975); Eyal *et al.* (1987). Whereas (D1) indicated the disease progress on a long the bean plants and (D2) related to disease severity in the leaf area. The proportion of illness severity was estimated for each score using the given equations (Sharma and Duveiller 2007):

% severity = (D1/9) (D2/9) 100

The phases of development of bean plants were documented. AUDPC was determined for each therapy using the given equation (Shaner and Finney 1977, Mukherjee, *et. al.,* 2009 &Parachivu, *et.al.,* 2013):

$$\text{A.U.D.P.C.} = \sum_{i}^{n-1}\left[\left\{\frac{Yi + Y(i+1)}{2}\right\} x(t(i+1) - ti)\right]$$

In which *Yi* = disease severity on the *I* th date; *ti*= *i*th day; *n* = a number of dates on which HBS was recorded.

Determination of Disease Severity: The severity of the illness was evaluated twice throughout the growing season in accordance to Weller (2007) depending on the quantity of halo blight patches on leaves (10 leaves per treatment) and calculated and AUDPC according to Shaner and Finney (1977).

Determination of *Ps*-viruses Titer on Treated Plants: A sample of 10 leaves that were positioned in the same place were collected in the morning and afternoon of the day from all treatments. The leaves sample were weighed and put in 100 ml of deionized water in plastic bags and saved in an ice box. The bags were shaken with 100 ml of water. One ml of solution was put in an Eppendorf tube (2 ml) and added 100 µl chloroform. The tubes were vortexed for 30 minutes and then spun at 6000 rpm for 15 minutes. 500 µl of

supernatant was put in 1 ml Eppendorf tube and serially diluted. A 100 µl of *Ps*-virus suspensions of each dilution was mixed with 5 ml of a broth culture of isolated *Ps. syringae* logarithmic phase (10^5CFU/ml) and added to semi-solid nutrient agar medium and poured on sold nutrient agar medium on Petri dishes. The prepared Petri dishes were incubated for 48 hours at 28°C until the plaques became visible. The Ps-virus titer was determined in pfu per gramme of leaf tissue as follows equations according to Balogh and Jones, (2003)

$$\text{Ps-virus titer} = \frac{\text{No.of plaque X 1.000}}{\text{weight ofsample bag - weight of empty bag}} \text{ Dilution factor}$$

Statistical Analysis: Log transformation was applied to each sample's findings [z = log 10 (Y + 1)]. Afterward submitted to an ANOVA analysis.

RESULTS

Pathogenic Bacteria: The pathogenic bacteria were isolated from HBS tissues on nutrient agar plates. The isolated bacteria exhibited variability in growth rate on a selective medium. Typical colonies on nutrient agar were circular, moist, smooth, and of entire margin, appeared flat and produced fluorescent diffusible pigment on king B. It appeared as single cells, motile by flagella, non-spore-forming, Gram negative, straight rod (3-6 µm x 0.4-0.7 µm) and Facilitative anaerobic. Based on cultural and morphological characters, three bacterial isolates were identified as *Ps. Syringae* according to Bergey's manual (2009).

Pathogenicity of Isolated *Ps.* Syringae: Inoculation of *N. tabacum cv.* Samson detached immature leaves with *Ps. syringe* resulted in the appearance of spot lesions after 15–20 days

Molecular 16s rDNA Gene: The integrity and quantity of purified DNA of three *Ps. syringae* isolates were 1.7, 1.5, and 1.6 ratios A 260 / A 280 OD, UV spectra. The concentration of DNA was 85, 75 and 92 ug/ml ($5x10^8$ CFU/ml). The isolated DNA for 16s rDNA gene PCR amplification of the three *Ps. syringae* isolates were amplified using the universal PCR method and analyzed by agarose gel electrophoresis. The size of PCR products of the three bacterial isolates was found to be 800 bp calculated by comparing it to the DNA leader (100 bp) as shown in Figure (1).

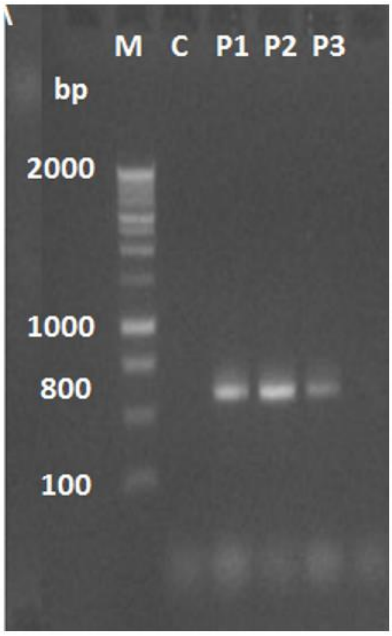

Fig. 1: Agarose gel electrophoresis (1.5%) showing fragments of 16s rDNA gene of *Ps. syringae* isolates (line P1, P2, P3) using universal PCR method and DNA ladder (lane M).

Nucleotide Sequence Analysis: Amplicon (PCR product of 16s rRNA gene) forming of *Ps. syringae* isolate (P1) was allowed for sequencing reaction through cycle sequencing method. The DNA amplicon data exhibited discrete peaks and high values for each base cell. The Primers were readily identifiable in either the forward or backward orientation within every sequencing segment and could be utilized without difficulty to put together the separate sequences as in Figure (2). Each primer's resulting sequence was sufficiently separated from the next to generate a single uninterrupted pattern (Contag).

Ps. Syringae isolate (P1) **(16s rRNA) gene sequence**

```
GATGTCACTAGCCGTTGGATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGG
AGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTG
CCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGT
GCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGT
CCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGC
TAGTCTAACCTTCGGGAGGACGGTTACCACGGTGTGATTCATGACTGGGGTGAAGTC
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Fig. 2: The partial nucleotide sequences of *Ps. syringae* isolate (P1) 16s rDNA gene (726 bp) isolated from bean Leaves infected with halo blight.

The sequence of a partial nucleotide of 726 bp 16s rDNA gene for *Ps. Syringae* separate (P1) from macerated HBS of bean leaves was performed to identify the association with other suggested 16s DNA gene *Ps. syringae* strains registered in GenBank. Phylogenic tree of 16s rDNA gene for *Ps. Syringae* on dependent on nucleotide patterns. The dendrogram displays the percentage similarity of sequence homology among *Ps. Syringae* and *other isolates* in gene bank (Fig.3). Mocrogen 3730X l6-1518-003 Korea sequencing was performed in the forward direction (Fig, 2) *Ps. Syringae* was confirmed with an excellent probability of 97.94 % similarity with recorded other isolates in the gene bank (Table,1).

Table 1: *Ps. Syringae* was the alignment of nucleotide sequences of 16s rDNA gene with recorded other isolates in the gene bank.

Description	Per. Ident	Accession
Ps.syringae partial 16S rRNA gene, type strain ICMP 3023T	97.94%	AJ308316.1
Ps.syringae strain NCPPB 281 16S ribosomal RNA, partial sequence	97.94%	NR_043716.1

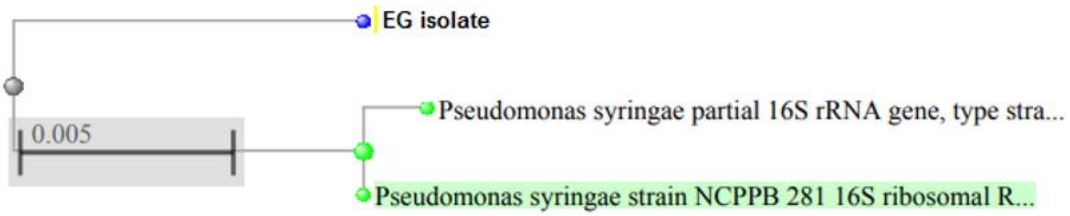

Fig.3: Phylogenic tree of 16s rDNA gene for *Ps. syringae* based on the nucleotide sequences. The dendrogram displays the genetic distance similarity of sequence homology among *Ps. Syringae* and other isolates in the gene bank.

***Ps. Syringae* Bacteriophage:** Bacteriophages specific to *Pseudomonas Syringae* were isolated from rhizosphere soils and macerated HBS tissues by the overlayer technique.

Phages were purified by a single plaque isolation technique. Six *Ps.* phages were isolated and designated *Ps. virus*-1 to *Ps. virus*-6. As shown in Figure (4 A) the isolated phages produced single plaques of different morphologies. The isolated phages formed regular and irregular circular single plaques of 3 to 5 mm in diameter and clear in appearance surrounded with halo. High titer phage suspensions were prepared by liquid enrichment technique. The high titers of the six phage isolates were combined together to be used as a biocontrol agent. The mixture of phage suspensions was quantitatively assayed by plaque assay and found to be 4.5 x 10^{10} PFU/ml.

Size and Morphology of Phage Particles: Bacteriophage isolates were negatively stained and examined by electron microscope. The six phage isolates were found to be of head and tail type (Fig. 4, B). All phages were found to have isometric heads ranging in diameter between 70 to 75 nm, long contractile and short uncontractile tails. Therefore, these six phage types are belonging to *Order Caudovirales* and could be classified under Families *Myoviridae* and *Podoviridae* according to the International Committee on the Viruses Taxonomy (ICTV).

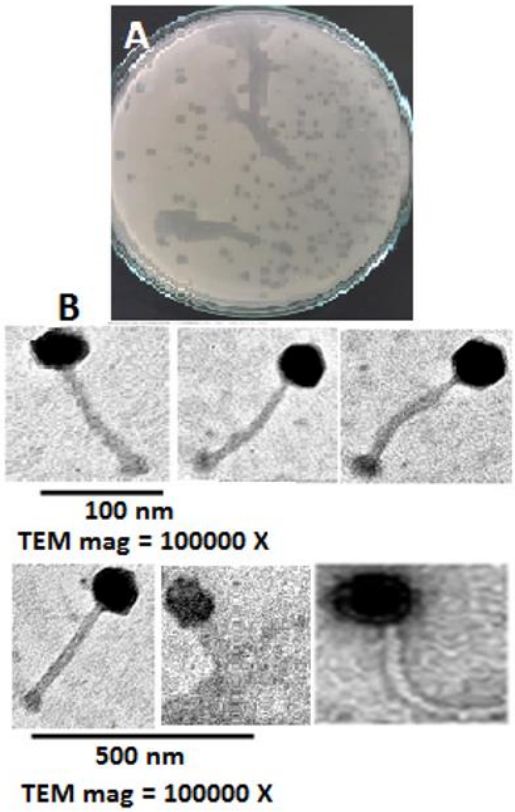

Fig. 4 A: plate containing single plaques of bacteriophages specific to *Ps. syringae*. The differences in morphology of the single plaques are clearly seen. **B**: Electron micrographs of negatively stained phage particles specific to *Ps. Syringae*.

Host Range of The Isolated Phages:

Each of the six phage isolates of *Ps. Syringae* was tested against six different *Pseudomonas* spp. isolated from different sources. As shown in Table (2), *Ps. virus*-1 &*Ps. virus*-3 were infectious to four *Pseudomonas* spp. Whereas isolates *Ps. virus*-2 and *Ps. virus*-4 were infectious to three *Pseudomonas* spp. Moreover, *Ps.virus*-5 and *Ps.virus*-6 infected three and two *Pseudomonas* spp., respectively.

Table 2: The infectivity of six *Ps. Syringae* phage isolates six *Pseudomonas* spp. collected from different sources.

Ps. Isolates	Source	*Ps*-viruses					
		***Ps.*-1**	***Ps.*-2**	***Ps.*-3**	***Ps.*-4**	***Ps.* -5**	***Ps.* -6**
Ps. *Syringae* P1	Current isolate	+	+	+	+	+	-
Ps.syringae P2	Current isolate	+	+	-	+	-	+
Ps.floucense	Sewage water	-	-	-	-	-	+
Ps.syringae	Agric. drainage	-	-	+	-	+	-
Ps.ouregonsa	Sanitation drainage	+	+	+	+	-	-
Ps.putida	Soil Agric. drainage	+	-	+	-	+	-

- = Negative result + = positive result

Stability of Phages at Room Temperature:

The six phage isolates, their mixture and their formulated were kept at room temperature for 60 days. The titer of each phage was estimated after 10, 20 40 and 60 days and expressed as log pfu/ml. As shown in Table (3) non si decrease in titer was recorded for neither the tested phages individually nor their mixture after 10 and 20 days. The titer of all phages and their mixture was slightly reduced after 40 and 60 days. *i.e.* all phage isolates kept their infectivity for 60 days. Phage isolate ps virus-3 was found to be the less stable one among the six phage isolates. Whereas the mixture of the six phages exhibited the highest stability as compared to the individual phages.

Stability of Phages at Different Temperature Degrees:

The six phage isolates, their mixture and their formulated were kept for 60 days at 10, 20, 30 and 60ºC. Data in Table (3) indicated that the titers of all phages were found to be higher and more stable under any temperature at 60 days. The highest titer was recorded for the phages mixture and formulated compared to the individual phages at any tested temperature.

Formulated *Ps*-viruses:

Data presented in Table (3) indicated that the corn flour and skim milk provided high protection to the isolated phages and kept their high infectivity for 60 days at 4ºC. A high titer for each formulated phage was recorded. Moreover, skim milk was found to be more protective as compared to corn flour since, the recorded log. Pfu/ml of skim milk formulated phages was higher than that of corn flour formulated ones.

Stability of Phages to UV:

The isolated phages, their mixture and formulated were exposed to UV radiation for 30, 60 and 90 min. The infectivity of UV irradiated phages was assayed quantitatively using plaque assay and expressed as log pfu/ml. As shown in Table (3) all phage isolates and their mixture exhibited high titers after exposure to UV for 30 min. then a marked reduction in titers was recorded after exposure to UV for 60 and 90 min. The lowest titers were recorded after exposure to UV for 90 min. Whereas, the titer of the formulated phages was the highest even after exposure to UV for 90 min. as compared to the titers of individual and mixed phages.

Table 3: The stability in potential infectivity of the isolated phages under different conditions expressed as log pfu/ml.

VIRUSES		ROOM TEMPERATURE AT DIFFERENT PERIODS (DAYS)				TEMPERATURES (°C)				EXPOSURE TIME (MINS) TO UV		
		10	20	40	60	10	20	30	60	30	60	90
PS VIRUS-1		5.3	5.0	4.1	3.5	5.2	5.1	4.2	4.0	4.5	4.2	4.0
PS VIRUS-2		5.4	4.5	3.8	3.4	4.2	3.8	3.6	3.5	4.3	3.5	3.2
PS VIRUS-3		5.1	4.6	4.1	3.7	5.1	4.8	4.6	4.5	5.1	4.9	4.6
PS VIRUS-4		4.8	4.6	4.2	3.8	4.2	3.9	3.8	3.5	5,2	5.0	4.8
PS VIRUS-5		4.5	4.2	4.0	3.5	4.2	4.0	3.8	3.5	5,5	5.2	4.6
PS VIRUS-6		4.2	4.0	3.8	3.5	4.2	4.1	3.8	3.6	5,2	4.5	4.2
PS- MIXED VIRUSES		7.5	7.2	7.0	6.4	7.1	7.0	6.9	6.8	7.2	7.0	6.8
FORMULATE PHAGES	CF	8.4	8.2	8.0	7.2	8.1	7.8	7.6	7.5	8.2	7.8	7.2
	SM	8.8	8.6	8.2	7.5	8.4	8.2	8.0	7.8	8.6	7.8	7.2

CF= corn flour SM= skim milk

Stability of Formulated Ps-viruses to UV:

The formulated viruses with skim milk and corn flour were exposed to UV radiation at different distances for 30 min. The spot test was used to determine the infectivity of the tested viruses after UV exposure. As shown in Table (4) the formulated phages exhibited higher stability to UV at different distances as compared to unformulated phages. The unformulated viruses lost their infectivity completely after exposure to UV at 120 cm. for 30 min.

Table 4: Stability of unformulated and formulated Ps-viruses after exposure to UV for 30 min at different distances.

Treatments		Distance (cm.) between UV lamp (60 w) and bacterial viruses				
		25	50	75	100	120
Unformulated phages (UFV)		++	++	++	+	-
formulated Ps-virus	Skim milk (FSMV)	+++	+++	++	++	+
	Corn flour (FCFV)	+++	+++	++	++	+

Biocontrol of *Ps. syringae*: Data presented in Table (5) indicated that under greenhouse conditions, spraying of bean plants with unformulated mixed *Ps* viruses (*i.e.*, a mixture of *Ps* virus-1**;** *Ps* virus-2**,** *Ps* virus-3, *Ps* virus-4, *Ps* virus-5 and *Ps* virus-6) post-infection with *Ps. Syringae* reduced the severity of the disease and HBS as compared to the control plants. Moreover, spraying plants with formulated phages resulted in more reduction in the severity of the disease and HBS than in the case of spraying with unformulated phages or copper mancozeb.

Table 5: Disease severity and HBS of infected bean plants sprayed with unformulated and formulated phages under greenhouse.

Treatments		Infectivity index		Disease severity		AUDPC
		No. of Infected plants	% Infection	No.of halo spots / leave	%	
Infected ***Ps. Syringae*** Control		27	90	4.2	92.5	85.12
Ps. viruses		4	13.3	1.3	28.6	23.25.
Formulated ***Ps.*** viruses	Corn flour	3	10	0.9	11.0	15.50
	Skim milk	2	6.7	0.5	11.0	12.20
Copper mancozeb		8	26.7	1.4	30.8	28.30

Number of total plants = 30 for each treatment
DS = Disease severity percentage.
No.of halo = number of HBS per leave.
AUDPC = Area Under the Disease Progress Curve.

Biocontrol in the Open Field: In the bean plants' April 2019 growth season, formed phages induced a considerable decrease in disease intensity and HBS, accompanied by unformulated and formulated phages in comparison to uncontrolled treatments (Table 6 &7). According to AUDPC, SM and CF-formulated phages were much more helpful in preventing illness than unformulated phages and copper oxychloride. The unformulated phage therapy decreases the severity of the illness in comparison to the untreated control and achieves a degree of control comparable to copper oxychloride therapy (Table 6 ,7 & Fig.5). While formulations of both SM and CF considerably boosted the efficiency of phage therapy. SM and CF formulated phages were considerably more efficient to decrease the intensity with 15.0, 10.2 than CF formulated phages with 25.2, 10.7 and 62.34% followed by unformulated phages30.3, 25.5and mancozeb 40.5, 27.8 compared to untreated control treatments 80.2 and 85.2% for Open field and greenhouse, respectively (Table 6 ,7 &Fig.5).

Table 6: Disease severity and halo blight lesions of infected bean plants sprayed with formulated phages under the open field.

Treatments		Open field			Greenhouse		
		DS%	No. of HBS	AUDPC	DS%	No. of HBS	AUDPC
Untreated control		80.2 e	12.5 e	65.2 e	75.2 e	9.3 e	42.9 e
unformulated phages		20.3 b	4.2c	26.3 c	12.5 c	1.2 c	20.5 d
Formulated	Skim milk	15.0 a	2.9a	17.9 a	8.4 a	0.6 a	9.5 a
phages	Corn flour	25.2 c	3.2b	22.4 b	10.7 b	0.9 b	10.2 b
Copper oxychloride		30.5 d	6.5d	32.2 d	17.8 d	2.7 d	25.2 c

Fig. 5: Bean plant disease intensity percentage infected with *Ps. Syringae* isolates under open field conditions.

Table 7: Influence of SM and CF formulated Ps viruses titer as Log PFU/mL on tomato leaves in the field in spring 2019.

Treatments		Greenhouse			Open field		
		April	May	Bacteria virus titer	May	June	Bacteria virus titer
Untreated control		12.5 e	15.3f	2.01a	15.8g	16.1 g	3.07b
Unformulated phages		3.2 c	2.3b	4.36 c	6.2e	7.1 f	4.75c
Formulated phages	Skim milk	2.1 b	1.1a	6.72e	3.1a	3.7 b	6.92e
	Corn flour	2.7 b	2.2b	6.16d	3.4a	4.2 c	6.21d
Copper oxychloride		4.5 d	4.2d	-	5.5d	7.2 f	-

Longevity of Formulated Phages:

The phage titers were measured throughout the greenhouse and open-field on infected growth bean plants with *Ps. syringae*. Due to the antagonistic action, no antagonistic action between phages was found in the greenhouse and field. The alterations in phage numbers represented in phage numbers emerged in an environment devoid of hosts. In contrast, unformulated phage plaques were rapidly decreased and eradicated at 60 hours following formulated phage at up to 100 hours (Table,8 &Fig. 6). The SM-formulated phages lowered phage population rate declined at 120 hours, CF-formulated phages lowered the phage population decline rate by 100 hours. In contrast, phage formulations worked equally in the greenhouse and open field evaluations, demonstrating a decrease rate and providing roughly 1.2 Log PFU /ml following phage spraying relative to phage treatment without preventive formulation.

Table 8: The persistence of formed Ps-viruses on infected bean leaves with Ps. syringae Under greenhouse and field conditions as Log pfu/mL.

Treatments		Time longevity (h)							
		15	30	45	60	75	90	100	120
Unformulated phages	Greenhouse	3.5	3.3	3.2	2.5	1.7	1.2	0.8	0.3
	Field	3.5	3.3	2.5	2.3	1.2	1.0	0.4	0.0
Skim milk - formulated Ps-virus	Greenhouse	3.8	3.6	3.5	3.2	3.0	2.6	2.2	1.2
	Field	3.8	3.4	3.3	3.1	2.5	2.2	1.8	1.0
Corn flour - formulated Ps-virus	Greenhouse	3.6	3.2	3.0	2.8	2.3	1.8	1.5	1.0
	Field	3.8	3.1	3.0	2.2	2.2	1.5	1.2	0.8

Phage inoculum = Log 3.5 pfu / mL

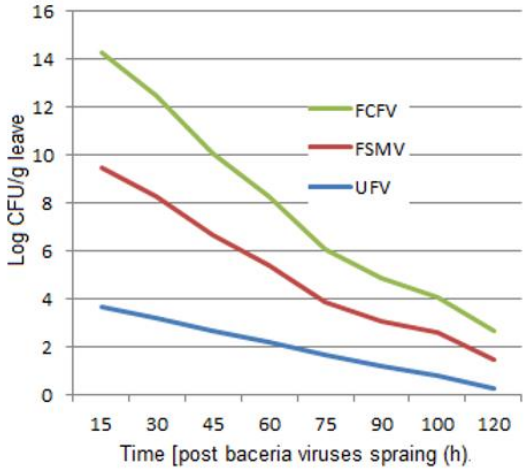

Fig. 6: Chart showing formulated phage longevity on bean leaves injected with *Ps.* syringae in a greenhouse and field. UFV= Unformulated phages, FCMV = Corn flour - formulated Ps-virus , FSMV = Skim milk - formulated Ps-virus.

DISCUSSION

In Egypt, bacterial plant diseases have a significant concern. Antibiotics have been used to manage plant pathogenic bacteria, and antibiotic-resistant bacteria have become a concern in patho systems. Pseudomonas-induced halo bacterial blight of plants. Where temperatures are mild and inoculum is abundant, syringae in the open field may exhibit significant variety at their most distracting (Kucharek, 1994).

Ps.syringae was isolated from bean plants with infected leaves showing halo blight disease in open ground. colonies' shape and culture characters were similar to standard isolates of *Ps. syringae* and attributed to *Pseudomonadaceae* family according to Fourie, (1998) and Bergey's manual, (2009). Isolated *Ps.* syringae was identified by bioassay on detached leaves of *N. tabacum*cv. samson (indicator host) showed spot lesions post 15–20 days inoculation, that is 65–75% to fully expanded Fawzia *et al*., (2018). PCR utilizing 16s rDNA gene was amplified using the universal PCR method and analyzed by electrophoresis of agarose gel. PCR product size with expected size~ 800 bp of two *Ps. syringae* isolates. As a result, in common bacterial pathogens, just one pair of universal primers is employed to amplify the preserved portion of the 16S rRNA gene. Hence, utilizing the universal PCR technique, these bacteria are only observable by a single set of primers; thus, for the identification of pathogens, only one set of primers is required. As compared to the culture approach, the universal PCR has a selectivity of 92,3%. This approach was proven to be sensitive to around 11 gram-negative and 190 gram-positive bacteria, correspondingly (Rahmani, *et al*.,2006). The existence of a single sequence type was shown by Sequence analysis of *Ps. syringae* amplicons and direct sequencing analysis revealed a highly devoted amplified area. That sequence, which represents a portion of the 16s rDNA gene, was later placed in Gen Bank as accession number *Ps. syringae* reference. AJ308316.1 and NR_043716.1. This sequence demonstrated 97.94% similarity. The present library of accessible 16S rRNA gene sequences is comprised of highly conserved gene loci; therefore, it may not include sequence data for a number of prevalent bacteria and emerging new CF species (Karpati & Jonasson, 1996).

Bacterial viruses were specific to *Ps. syringae* (*Ps.-virus*) were detected in halo blight diseased bean leaves and rhizosphere soil by over-layer agar method. Single isolates of *Ps.-virus* were obtained using a single plaque isolation technique (Balogh and Jones 2003 and Eman and Afaf, 2012). The bacterial viruses were found to be of head and tail type. All phages were found to have isometric heads, long contractile and short uncontractile tails. Therefore, these six phage types are belonging to Order *Caudovirales* and could be classified under Families *Myoviridae* and *Podoviridae* according to the International Committee on the Viruses Taxonomy (ICTV) (Eman and Afaf, 2012). One of the most difficult aspects of employing phages for plant disease management is their relatively short residual activity in the phyllosphere. According to many studies, phage numbers may decline to undetectable levels within hours following application (Iriarte, *et al*., 2007). Phages therapy was only efficient in the morning prior to sunset. When given throughout the day, the brief remaining activity of the control agents reduced the effectiveness of the phage therapy. Approximately two days after treatment, phages are quickly destroyed in the greenhouse and open field and partly vanish from the bean foliage due to climatic variables such as UV and temperature.

The findings revealed that the protective efficiency of phage formulations including SM and CF could be enhanced, as could the timing of phage administration for disease management in greenhouses and open fields. Earlier investigations (Balogh, 2002) revealed a number of protective formulations and three of them were chosen for illness prevention studies. These formulations

increased the concentration of the phage population after the application.

SM produced the greatest outcomes in the greenhouse and open field, followed by CF in comparison to phages without formulation. Despite the increased effectiveness in disease management, none of these therapies resulted in a substantial yield gain. This may have occurred due to the very infectious characteristics of phage infection. In the spring field experiment in Quincy, phages were found in many untreated plots in the middle of the planting season, and in all unprotected plots at the conclusion of the growth period (Balogh, 2002).

Phage treatment in the late morning failed to suppress bacterial spots, and it was hypothesized that phage persistence was greatly diminished. Viruses are very delicate and cannot survive on plant leaves for an extended period of time since they are swiftly killed by damaging environmental conditions such as rain and ultraviolet radiation (Mc Guire *et al.,* 2001 and Zaccordelli, *et al.*, 1992). Six *Ps*. virus isolates namely (*Ps. virus*-1 to *Ps. virus*-6) were isolated from macerated HBS tissues and soils produced different plaque types. When tested with *Ps*. syringae, *Ps* virus isolates exhibit unique host specificity. The purified *Ps*. viruses were combined to be used as biocontrol tools (4.5 x 10^{10} PFU/ml).

The long-lasting effect of formulated *Ps*-virus populations was increased at room temperature due to the corn flour and skim milk protective effect for up to 100 days compared to unformulated ones.

Using bacterial viruses or bacteriophages (phages) to control bacterial illnesses is an additional method of biological control. They have been long advocated as plant disease control agents (Moore, 1926) and have been employed in a number of plants-bacterial pathosystems (Jackson1989, Saccardi, *et al*., 1993, Tanaka, *et al*.,1990 and Zaccardelli, *et al*., 1992). In greenhouse and field experiments in 2018 and 2019, Applications of a combination of six phage isolates as a spray specific to *Ps. Syringae* decreased the intensity of bacterial HBS on bean leaves compared to a copper–mancozeb application, the conventional chemical therapy (Flaherty, *et al.*,2000). On the other hand, bacterial viruses-treated bean plants were proven to be much more robust than copper–mancozeb-treated and untreated ones.

Spraying bean plants with *Ps*. virus populations post-infected with *Ps. syringae* reduced illness intensity and HBS. Nevertheless, SM formulation exhibited the largest illness intensity decrease and CF formulation and their mixture, respectively.SM and CF formulations devoid of phages had no effect on disease intensity or lesion halo counts.

Spraying of infected bean plants with *Ps* virus populations post-inoculation in the greenhouse resulted in a disease intensity decrease and lesion holes. In contrast, treatment with formulated phages reduced disease intensity and lesion spots much more than unformulated phages and copper mancozeb.

Virus loads were evaluated during open-field plant development. Owing to the antagonistic impact, there were no anti-*Ps.* syringae virus in the field. The viral populations are associated with an environment devoid of hosts. In contrast, unformulated phage colonies were rapidly decreased and eradicated between 48 and 36 hours after treatment. In contrast, phage formulations behaved equally in greenhouse and open field evaluations, demonstrating a decrease rate and providing roughly 10^6 PFU/ml following phage treatment relative to phage spraying without preventive formulation.

In field trials, viruses are very delicate and cannot survive on plant leaves for an extended period of time due to their rapid elimination by rain and sunlight-UV (McGuire, *et al*.,2001 and, Zaccardelli, *et al*., 1992). In order to save phage particles from hazardous environmental variables, it became necessary to design formulations and/or modify the application strategy, such as the timing of treatment. The consequently increased residual activity of the phages might boost the effectiveness of phage

therapies and make the application schedule greater comfortable. Previously, two formulations developed Skim milk Corn flour that significantly enhanced tomato foliage phage longevity (Balogh, 2002 and Flaherty, *et al*.,2000).

In conclusion, based on our findings, the success of phage therapy in preventing bacterial plant diseases is dependent on the time of administration. However, additional study is required to improve formulations, administration interval, phage dosage, and the composition of phage mixes so that phage therapy might be a viable option for chemical bactericides in the future for the control of infectious plant diseases.

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