



## Bacteriophages as Promising Agents for Biocontrol of *Ralstonia solanacearum* Causing Bacterial Wilt Disease

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**T**O COMBAT the growing antibiotic and chemical resistance in bacterial phytopathogens like *Ralstonia solanacearum*, which causes bacterial wilt disease, bacteriophages are an efficient biocontrol technique and an environmentally benign therapy.

This study reports the isolation and characterization of three phages, designated  $\phi$ RS1,  $\phi$ RS2, and  $\phi$ RS3, for their effectiveness against *R. solanacearum* EMCC 1274 in a bioassay using *Solanum lycopersicum* (tomato) pots. It was discovered that the three isolated bacteriophages were viable and stable over a wide pH range (3.0–10.0) and at temperatures as high as 80°C. The phage  $\phi$ RS1 was measured using a transmission electron microscope to be approximately 291.84nm in size, with an icosahedral head (134.4nm) and tail, while also belonging to the Myoviridae family. The phage  $\phi$ RS2 is almost 232nm in size, with an icosahedral head (116nm) and a tail, and it belongs to the Myoviridae family, while the phage  $\phi$ RS3 is roughly 88.32 nm in size, with an icosahedral head (69.12nm) and a short, non-contractile tail, and it belongs to the Podoviridae family. In comparison to just pathogen-treated controls, plant bioassays demonstrated 91.3, 95.6 and 95.6% reversal of disease symptoms employing phages  $\phi$ RS1,  $\phi$ RS2, and  $\phi$ RS3 respectively. A biocontrol formulation for preventing the bacterial wilt disease caused by *R. solanacearum* appears to be developable using a combination of isolated bacteriophages (100% reversal in disease symptoms).

**Keywords:** Bacterial wilt, Bacteriophage, Biocontrol, DNA extraction, *R. solanacearum*, Restriction enzymes.

### Introduction

According to research conducted worldwide, *Ralstonia solanacearum* is the second most destructive pathogen responsible for bacterial wilt in crops, mostly those of the Solanaceae family (Ramesh et al., 2014). Based only on symptomology, Briton-Jones (1925) probably was the first record of it in Egypt at El-Gemmeiza Farm in El-Gharbeya Governorate. *Ralstonia solanacearum* tries to invade the intercellular tissues of roots, where it multiplies before penetrating xylem vessels and forming exopolysaccharide (EPS), which causes the infected plant to wilt and get worse (Prior et al.,

1998). Due to the organisms' growing tolerance to conventional control methods like copper, pesticides, and antibiotic treatments, controlling the phytopathogen is challenging. A promising biocontrol method involves bacteriophages, viruses that live inside bacterial cells as parasites and can pierce biofilms to kill phytopathogens (Farooq et al., 2022). Bacteriophages are effective in controlling a variety of bacterial phytopathogens, including *Pectobacterium carotovorum*, *Erwinia amylovora*, *Pseudomonas syringae*, *Dickeya solani*, and *Xanthomonas campestris* (Buttimer et al., 2017). Lytic phages are very valuable for the efficient biocontrol of bacterial phytopathogens because of their

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capability to lyse certain host cells, availability in nature, non-toxicity for the environment, and auto-dosing effect (Umrao et al., 2021). *R. solanacearum* bacteriophages were discovered and characterized in numerous investigations. These include lytic phages with great biocontrol, temperate phages with improved pathogenicity, and phages with no apparent biocontrol effect in studies using plant tests (Álvarez et al., 2010). Both the lytic and lysogeny properties of phage PE226 showed pathogenicity toward *R. solanacearum* (Murugaiyan et al., 2011). By reducing exopolysaccharide synthesis, another phage, Rs551, displayed reduced virulence stress of *R. solanacearum* race 3 biovar 2 (Ahmad et al., 2017). The pathogenic *R. solanacearum* strain RUN302 was considerably stunted in growth by the bacteriophage RsoM1USA *in vitro*, however this stunting did not result in a decrease in pathogenicity in tomato plants when compared to an uninfected strain (Addy et al., 2019). There has been reported evidence of Egypt-specific *in vitro* phage lytic activity against *R. solanacearum* (Elhalag et al., 2018).

The main goal of the investigation was to increase the effectiveness of biological treatments against *R. solanacearum* by examining the synergistic effects of applying multiple phages to an Egyptian infected tomato plant. This was done in an effort to come up with innovative, effective approaches for treating diseases that affect the Solanaceae family in Egypt.

## **Materials and Methods**

### *Bacteria and culture conditions*

The Microbial Resource Center (MIRCEN), Faculty of Agriculture, Ain Shams University in Egypt, provided the *R. solanacearum* EMCC 1274 strain. The other studied bacteria were isolated and identified as *R. solanacearum*, *Pectobacterium carotovorum* and *Pectobacterium atrosepticum*. The bacteria were cultivated in a casamino acid-peptone glucose (CPG) medium (Oxoid, UK) with 0.1% casamino acids, 1% peptone, and 0.5% glucose at 28°C while being shaken at 200-300rpm (Fujiwara et al., 2011).

### *Bacteriophage sampling and isolation*

In Gharbia, Qalubeia, and Menufia, Egypt, soil samples were taken from mixed rhizospheres of several varieties of solanaceous crops, and bacteriophages were isolated from these samples.

After being infected with the *R. solanacearum* EMCC 1274 strain at a concentration of  $4 \times 10^8$  CFU/ml, the soil (5g) was suspended in a CPG medium (50mL). The shaker incubator (VIS-180, Taisite Lab Sciences Inc., USA) was used to incubate the inoculated flasks for 24h at 150rpm per minute and 28°C. Centrifugation was performed on the resultant cell suspension for 10min. at 3000rpm. Through a membrane filter with a 0.22- $\mu$ m pore size, the supernatant was filtered. Utilizing the double-layer agar (DLA) overlay method, the plaque assay was carried out (Umrao et al., 2021).

### *Single plaque isolation*

Based on the appearance of the plaques, several bacteriophages specific to the *R. solanacearum* EMCC 1274 strain were obtained (Abo-Senna et al., 2018). Single-described plaques were placed, picked up, and put into flasks with 10mL of the liquid host culture ( $4 \times 10^8$  CFU/mL) using a sterile bacterial inoculation needle. The flasks were then incubated at 28°C for 24h while being shaken at 150rpm in a shaker incubator. Then, the liquid cultures were centrifuged at 6000rpm for 15min after incubation to get rid of the cell debris. To remove any leftover bacteria, chloroform was added to the supernatant (1: 10 v/v), which was then vigorously shaken for 3-5min. The cleared suspension was then taken for testing using the plaque assay procedure. The properties of the produced plaques have been compared to those of the original plaques to ensure that they are similar. Three repetitions of the single plaque separation were necessary to attain an elevated level of plaque purity.

### *Bacteriophage propagation*

From single-plaque isolates, phages were multiplied, as described in Didamony et al. (2015). A single plaque was chosen and added to an *R. solanacearum* EMCC 1274 log phase culture (24h culture). The phage-host mixture was incubated at 28°C for 24h before being centrifuged in a chilled centrifuge (INO-BRCMicro, INOVIA TECHNOLOGY, Turkey) for 10min at 6000rpm. The phage particles were then precipitated in the presence of 0.5M NaCl and 5% polyethylene glycol 6000 after the filtrate had been passed through a membrane filter with a 0.22- $\mu$ m pore size. Moreover, SM buffer, including 100mM NaCl, 10mM MgSO<sub>4</sub>, and 0.01% gelatin, was used to dissolve the pellet after it had been centrifuged at 6000rpm for

30min at 4°C. Phages that had been purified were stored at 4°C until use.

#### *Transmission electron microscopy (TEM)*

Phages were examined according to the instructions provided by Luftig (1967). A drop of the phage suspension was put on 200 mesh copper grids coated with carbon coat, and any excess was wiped away using filter paper. Then, a 2% uranyl acetate solution was applied to the grids, and any extra was eliminated. A transmission electron microscope (JEOL JEM-2100 model) was used to examine samples on October 6 at Nano Tech Egypt in Cairo, Egypt.

#### *Phages host range*

The host range of phages  $\phi$  Rs1,  $\phi$  Rs2 and  $\phi$  Rs3 was assessed by performing both the spot test and plaque assay against different twenty-five isolates (twelve different *R. solanacearum* isolates, eight *P. carotovorum* isolates and five *P. atrosepticum* isolates) isolated from different infected plant and soil samples.

#### *Phages stability at different temperatures*

The stability of the phages at various temperatures was tested in vitro using the techniques outlined by Jamal et al. (2015) with some modifications. Ten minutes were spent incubating 1.5mL Eppendorf tubes with a diluted suspension of the bacteriophage at a temperature ranging from 30°C to 90°C.

#### *Phages stability at different pH*

Following a 1-hour incubation period at 28°C and stability tests on each of the three isolated phages at pH values from 3 to 11, the double-layer agar plate approach was used to count the phage particles that survived instantly.

#### *Phages stability to gamma and UV radiation*

The ability of bacteriophages to survive at various gamma radiation doses was assessed by exposing each phage suspension to various doses of gamma radiation (0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4kGy) employing a cobalt-60 (60 Co Gamma Chamber, MC20, Russia) source at a dosage rate of 372.58 Gy/60sec. The spot-test method should then be used to confirm survival qualitatively (Mahmoud et al., 2021). Additionally, the stability of the isolated phages to UV irradiation was qualitatively evaluated after being exposed to UV wavelength (254nm) for (10, 20, 30, 40, 50, 60min) at a constant distance (50cm) from the

UV lamp (Hussein et al., 2018).

#### *Extraction and restriction endonuclease analysis of phages DNA*

Using the Phage DNA Isolation Kit (NORGEN, BIOTEK CORP, Cat. 46800), genomic DNA was purified in accordance with the manufacturer's instructions. Also used as a DNA ladder was Quick-Load® Purple 2-log DNA Ladder (0.1- 10.0kb) - New England Biolabs, Inc.: N0550S. Additionally, in accordance with the manufacturer's procedure, a mixture of HindIII (10U/L) and EcoRI (10U/L) from Thermo Fisher Scientific was utilized for DNA fragmentation. Finally, data interpretation was carried out using Totallab (Ver.1.1) software. MultiSUB Maxi, Maxi Horizontal Electrophoresis System Cleaver Scientific was used for fragmentation.

#### *Plant bioassay*

A plant bioassay investigation employing the procedure of soaking the soil in a set-up of a greenhouse with pots (16cm in diameter) holding 1500g of sterilized soil per pot was conducted to evaluate the effectiveness of bacteriophage biocontrol (Addy et al., 2012). For the experiment, tomato plants Cheyenne E448 (measuring 20-25cm in length) were employed in triplicate. A sterile needle scratched the base of the plant's stem, and plants were divided into six groups and inoculated with 20mL of sterilized distilled water as a negative control, 20mL of the tested bacterial strain with different isolated phages as follows:

(Group 1): sterilized distilled water as negative control.

(Group 2): *R. solanacearum* EMCC 1274 ( $6 \times 10^7$  CFU/ g) as positive control.

(Group 3): *R. solanacearum* EMCC 1274 and phage  $\phi$ Rs1 (1.0 MOI).

(Group 4): *R. solanacearum* EMCC 1274 and phage  $\phi$ Rs2 (1.0 MOI).

(Group 5): *R. solanacearum* EMCC 1274 and phage  $\phi$ Rs3 (1.0 MOI).

(Group 6): *R. solanacearum* strain EMCC 1274 and mix of all isolated phages.

Pots were kept under natural light and temperature conditions and were irrigated

regularly every three days with equal amounts (250mL) of sterilized distilled water. Twice in one week, tomato plant disease symptoms using a wilting grade scale according to Kempe (1983). Grade 1 plants showed 25% wilted leaves, grade 2 plants showed 26-50% wilted leaves, grade 3 plants showed 51-75% wilted leaves, grade 4 plants showed 76% or more wilted leaves and stem collapses, and grade 5 plants showed mortality. The percentage of disease symptoms appearance was estimated according to the following equation:

#### Statistical analysis

Data for phage stability to different temperature degrees, pH and UV radiation were analyzed by One-Way ANOVA using PASW STATISTICS 18 software. The software included the Duncan Significant Difference test employed to compare means. If  $P < 0.05$ , differences were regarded as statistically significant.

## Results

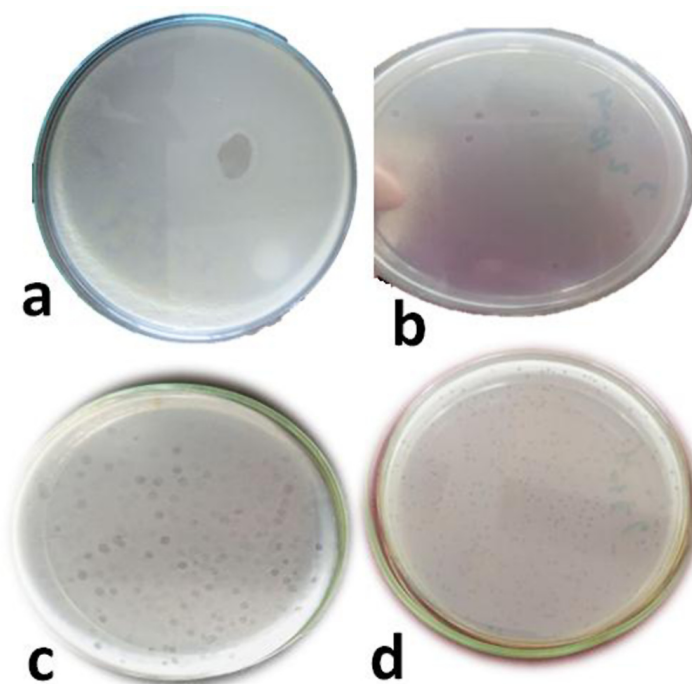
#### Isolation and characterization of bacteriophages

Lytic bacteriophages ( $\phi$ RS1,  $\phi$ RS2, and  $\phi$ RS3) that could infect the bacterial wilt-causing

*R. solanacearum* strain EMCC 1274 were found in mixed rhizospheres of various solanaceous crop cultivars from the Egyptian regions of Gharbia, Qalubeia, and Menufia. The diameter of the plaque varied from 1.0 to 3.0mm.  $\phi$ RS1 was a clear circular plaque measuring 2 mm in diameter;  $\phi$ RS2 was a clear circular plaque measuring 3mm, while  $\phi$ RS3 was a clear circular plaque measuring 1mm (Fig. 1) (Table 1). The lytic phage's pathogenic activity was visible in the shape of round transparent plaques that developed on the host's lawn.

#### Transmission electron microscopy

The phage  $\phi$ RS1 was found to be approximately 291.84nm in size and to have an icosahedral head (134.4nm) and tail, indicating that it belonged to the Myoviridae family. The phage  $\phi$ RS2 was found to be approximately 232nm in size and to have an icosahedral head (116nm) and tail, indicating that it belonged to the Myoviridae family. Furthermore, the phage RS3 was estimated to be 88.32nm in size, with an icosahedral head (69.12nm) and a short, non-contractile tail. It was classified as a member of the Podoviridae family (Fig. 2) (Table 2).



**Fig. 1. Isolation and characterization of phages  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 against host *R. solanacearum* strain EMCC 1274 [a. spot test showing the bacterial lysis by virulent bacteriophages, b.  $\phi$ RS1 clear circular plaques measuring 2mm in diameter and c.  $\phi$ RS2 clear circular plaques measuring 3mm in diameter, d.  $\phi$ RS3 clear circular plaques measuring 1mm in diameter on double layer agar plates]**

**TABLE 1** Plaque morphology of the isolated *R. solanacearum* phages  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3

Plaque character	Phage		
	$\phi$ RS <sub>1</sub>	$\phi$ RS2	$\phi$ RS3
Shape	Circle	Circle	Circle
Diameter in mm	2	3	1
Transparency	Clear	Clear	Clear
Presence of halo	Absent	Absent	Absent

**Fig. 2.** Electron micrograph of phage particles under TEM [The virions were negatively stained with uranyl acetate. A.  $\phi$ RS1, B.  $\phi$ RS2 and C.  $\phi$ RS3 displaying a typical Podovirus. Myoviridae family bacteriophages:  $\phi$ RS1 and  $\phi$ RS2. The scale represents 100nm]**TABLE 2** Morphological characters of the isolated phages as illustrated by transmission electron microscopic examination

Phage	Head diameter (nm)	Tail length	Tail width	Base plates	Phage diameter	Order	Family
$\phi$ RS1	134.4	157.44	26.88	26.88	291.84	Caudovirales	Myoviridae
$\phi$ RS2	116	116	27.84	9.28	232	Caudovirales	Myoviridae
$\phi$ RS3	69.12	19.2	19.2	-	88.32	Caudovirales	Podoviridae

The Myoviridae family was characterized by the morphological traits of the phages  $\phi$ RS1 and  $\phi$ RS2 (large heads and long tails).  $\phi$ RS3 displaying typical polygonal head and short tail of the Podoviridae.

#### Phage host range

Table 3 lists the different isolates examined for susceptibility to  $\phi$ RS1,  $\phi$ RS2, and  $\phi$ RS3 infection. The findings of the spot test and plaque assay revealed that all these strains were resistant to the phages, except *R. solanacearum* (Ral1), which was sensitive to each of the three isolated phages and *R. solanacearum* (Ral8), which was exclusively susceptible to  $\phi$ RS2 phage.

#### Phages stability at different temperatures

Data in Fig. 3 illustrates how sensitive the phages  $\phi$ RS1,  $\phi$ RS2, and  $\phi$ RS3 are to different temperatures. The three phages' activity was steady up to a temperature of 80°C but decreased after being exposed to temperatures of 90°C for 10min. Interestingly,  $\phi$ RS1 and  $\phi$ RS2 phages survived at 50°C, while  $\phi$ RS3 survived at 60°C with no significant loss in phage particle number. The loss of the number of virus particles was about 30.00% at 60°C, 75% at 70°C and 90% at 80°C for both  $\phi$ RS1 and  $\phi$ RS2 in laboratory conditions. For  $\phi$ RS3, the loss of the number of virus particles was

56.25% at 70°C and 75% at 80°C. A total loss of the number of the three virus particles occurred at 90°C.

#### Phages stability at different pH

The sensitivity of phages  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 to various pH is represented in Fig. 4.  $\phi$ RS2 and  $\phi$ RS3 retained their lytic capability against *R. solanacearum* strain EMCC 1274 for up to 1h incubation at pH from 4 to 10, and showed maximum stability at pH 5, 6, and 7. The activity of  $\phi$ RS1 remained stable for up to 1h incubation at pH from 3 to 10, and showed maximum stability at pH 6, 7, and 8.

#### Phages stability to gamma and UV radiation

After receiving a dose of gamma radiation of more than 3 kilo grey (kGy),  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 lost their capacity to lyse *R. solanacearum* strain EMCC 1274 (Table 4). In contrast, after being exposed to ultraviolet radiation for 60min at a distance of 50cm,  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 still had the ability to lyse *R. solanacearum* as shown in Fig. 5.

**TABLE 3.** Host specificity of the isolated *R. solanacearum* phages  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 on different phytopathogenic bacterial isolates as determined by spot testing with serial dilutions of phage suspensions

Bacterial isolates	Isolation source	Isolation place	Phages			
			$\phi$ RS1	$\phi$ RS2	$\phi$ RS3	
<i>Ralstonia solanacearum</i>	Ral1	Kafr shebin , Qalubeia	+	+	+	
	Ral2	Diseased potato tuber	Kafr taha , Qalubeia	-	-	-
	Ral3		Noub taha, Qalubeia	-	-	-
	Ral4		Shebin elqanater, Qalubeia	-	-	-
	Ral5		Kafr taha ,Qalubeia	-	-	-
	Ral6		Menufia	-	-	-
	Ral7		Fayoum	-	-	-
	Ral8	Soil sample	Behaira	-	+	-
	Ral9		Kafr shebin , Qalubeia	-	-	-
	Ral10		Kafr El Ziat, Gharbia	-	-	-
	Ral11		Potato brown rot project, Doki, Giza	-	-	-
	Ral12	Diseased potato tuber	Faculty of agriculture, Zagazig university	-	-	-
EMCC 1274	<b>Reference bacterial strain from MIRCEN</b>		+H	+H	+H	
<i>Pectobacterium carotovorum</i>	Pc1	Diseased onion	Zagazig	-	-	-
	Pc 2		Zagazig	-	-	-
	Pc3	Diseased potato tuber	Kafr taha , Qalubeia	-	-	-
	Pc4		Fayoum	-	-	-
	Pc5		Behaira	-	-	-
	Pc6	Soil sample	Kafr shebin , Qalubeia	-	-	-
	Pc7		Kafr El ziat, Gharbia	-	-	-
	Pc8		Potato brown rot project, Doki, Giza	-	-	-
DSM 30170	<b>Reference bacterial strain from MIRCEN</b>		-	-	-	
<i>Pectobacterium atrosepticum</i>	Pa1		Behaira,	-	-	-
	Pa2	Soil sample	Kafr Taha, Qalubeia	-	-	-
	Pa3		Fayoum	-	-	-
	Pa4		Potato brown rot project, Doki, Giza	-	-	-
	CMM 322	<b>Reference bacterial strain from MIRCEN</b>		-	-	-

(+) Indicates that the strain is susceptible to the phage and produces plaques, with titers in parenthesis, while (-) indicates that no plaques were observed. (+H) Indicates that the strain is the host of the phage.

MIRCEN: Microbiological Resources Centre

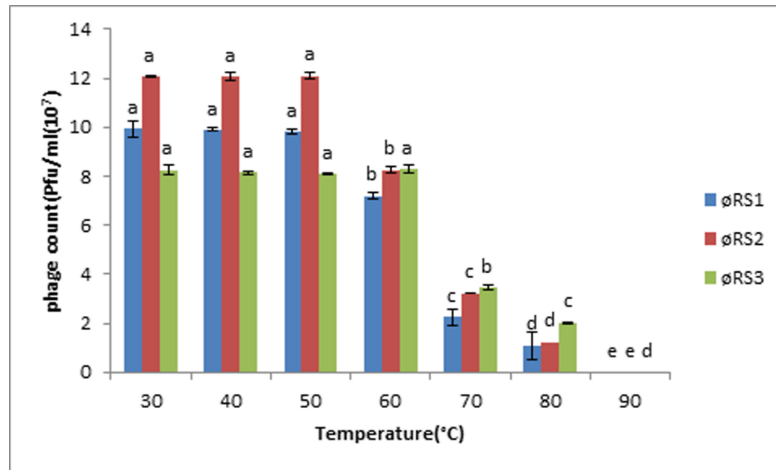


Fig. 3. Bacteriolytic stability of phages  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 against host *R. solanacearum* strain EMCC1274 at different temperatures [Note: The bacteriolytic stability was expressed by Plaque forming unit (PFU) per ml. All values represent means of three run determinations  $\pm$  SE. Error bars represent  $\pm$  the standard error]

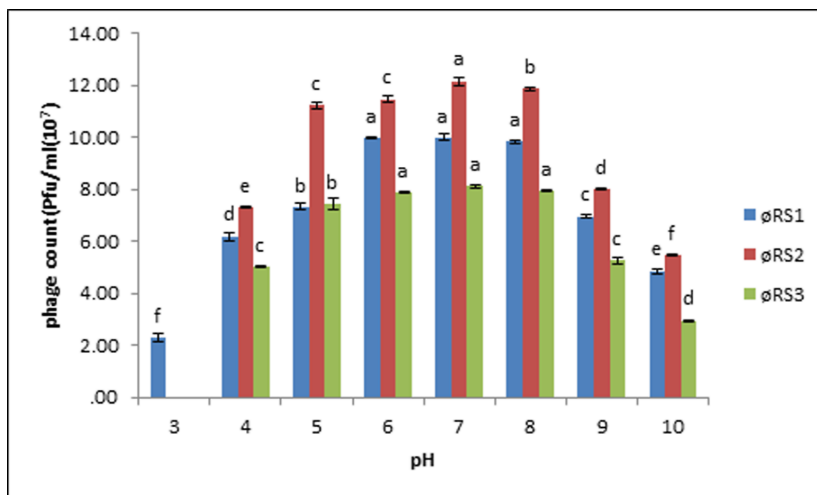


Fig. 4. Bacteriolytic stability of phages  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 against host *R. solanacearum* strain EMCC1274 at different pH values [Note: The bacteriolytic stability was expressed by Plaque forming unit (PFU) per ml. All values represent means of three run determinations  $\pm$  SE. Error bars represent  $\pm$  the standard error]

TABLE 4. Stability of the isolated *R. solanacearum* phages  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 to gamma radiation

Gamma radiation dose (kGy)	Phage lytic activity		
	$\phi$ RS1	$\phi$ RS2	$\phi$ RS3
0.5	+	+	+
1	+	+	+
1.5	+	+	+
2	+	+	+
2.5	+	+	+
3	+	+	+
3.5	-	-	-
4	-	-	-

(+) indicates bacterial lysis. (-) indicates no lysis.  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 lost their lytic capability against *R. solanacearum* strain EMCC 1274 after exposure to gamma radiation of dose more than 3 kilo gray (kGy).

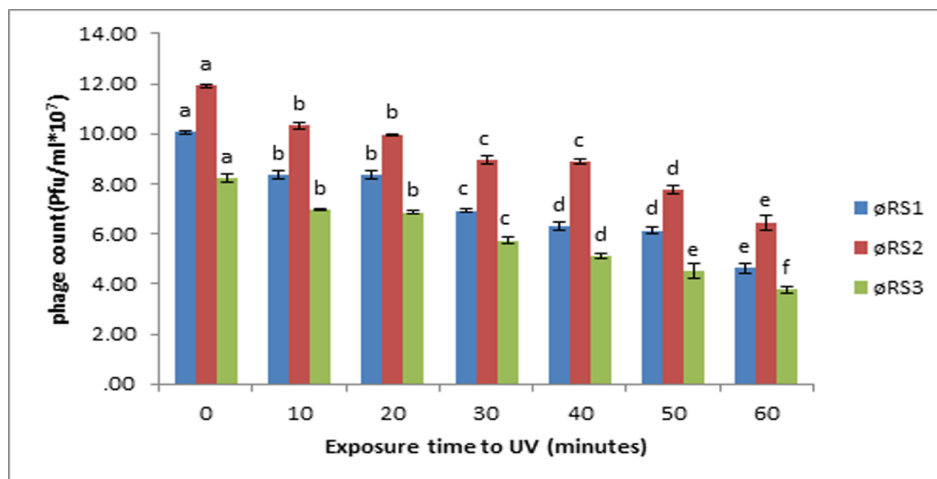


Fig. 5. Effect of UV radiation on the bacteriolytic stability of phages  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 against host *R. solanacearum* strain EMCC1274 [Note: The bacteriolytic stability was expressed by Plaques forming unit (PFU) per mL. All values represent means of three run determinations  $\pm$  SE. Error bars represent  $\pm$  the standard error]

#### Restriction endonuclease analysis of phages DNA

From each phage, genomic DNA was extracted and digested using a combination of HindIII and EcoRI restriction enzymes, which only cleaved dsDNA. All phages contained dsDNA, as evidenced by the effective digestion of the DNA (Fig. 6a). The genomic DNA of  $\phi$ RS1, digested in fourteen restriction bands,  $\phi$ RS2 digested in ten restriction bands and  $\phi$ RS3 digested in thirteen restriction bands of varied sizes. According to the total of the restriction fragment sizes for  $\phi$ RS1,  $\phi$ RS2, and  $\phi$ RS3, the bacteriophage genome was roughly 21, 15, and 14kbp long (Fig. 6b).

#### Plant bioassay

The tomato plants with the pathogen inoculation were found to be stunted (20cm in height with 22 leaves), and there were signs of yellowing to browning of the leaves. The control plants, which had not been inoculated, were discovered to be healthy and had 46 leaves and a height of 52cm. Because four out of the forty-six wilted leaves were found in tomato plants infected with *R. solanacearum*, phage RS1 demonstrated 91.3% control of the disease's symptoms. In tomato plants infected with *R. solanacearum*, phage RS2 and RS3 showed 95.6% control of the bacterial wilt symptoms; only two of the forty-six leaves were found to be wilted. The three phages  $\phi$ RS1,  $\phi$ RS2, and  $\phi$ RS3 together showed 100% of the bacterial wilt symptoms in tomato plants infected with *R. solanacearum*, as no wilted leaves were discovered and the plant looked to be in good health like the control as shown in Fig. 7.

#### Discussion

The discovery of new biocontrol options is relevant to the bioprospecting of native bacteriophages (Scortichini, 2022). *R. solanacearum* is a soil-borne pathogen that affects a variety of crops, including tomatoes and potatoes. As a result, there is a high probability that the rhizosphere soil of these crops contains bacteriophages that are active against this bacterial species (Nion & Toyota, 2015; Kones et al., 2020).

The choice of environmental reservoirs as the best sources for antibacterial phages was confirmed in this study by the isolation of three lytic bacteriophages ( $\phi$ RS1,  $\phi$ RS2, and  $\phi$ RS3) through enrichment of *R. solanacearum* that contaminated different soil samples from various origins in Egypt. Two other lytic phages targeting *R. solanacearum* were only recently reported, both from soil (Elhalag et al., 2018; Addy et al., 2019).

The Myoviridae family was characterized by the morphological traits of the phages  $\phi$ RS1 and  $\phi$ RS2, while the morphological trait of  $\phi$ RS3 was unique to the Podoviridae. With varying degrees of success, the use of podoviruses as biological control agents to combat the bacterial wilt by *R. solanacearum* was documented (Elhalag et al., 2018; Álvarez et al., 2019; Biosca et al., 2019; Zaki et al., 2022a). To combat the bacterial wilt induced by *R. solanacearum* in Solanaceae crops, Myoviruses were utilized as biocontrol agents with varying degrees of success (Fujiwara et al., 2011; Umrao et al., 2021).



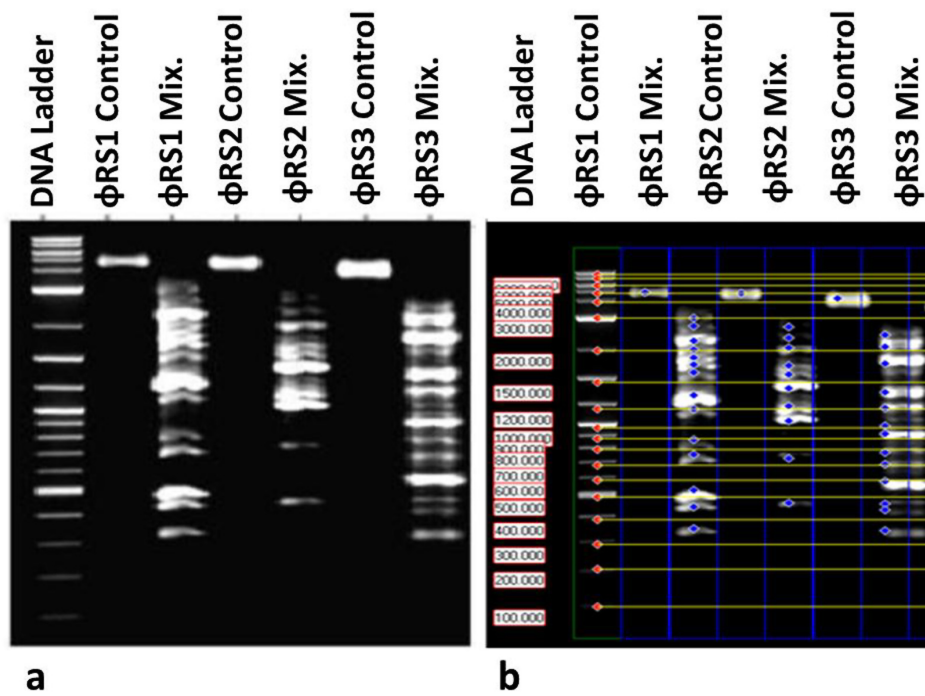


Fig. 6 a) Fragmentation pattern of phages  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 genomic DNA after digestion with a mixture of Hind III and EcoRI restriction enzymes, b) Fragments length calculation

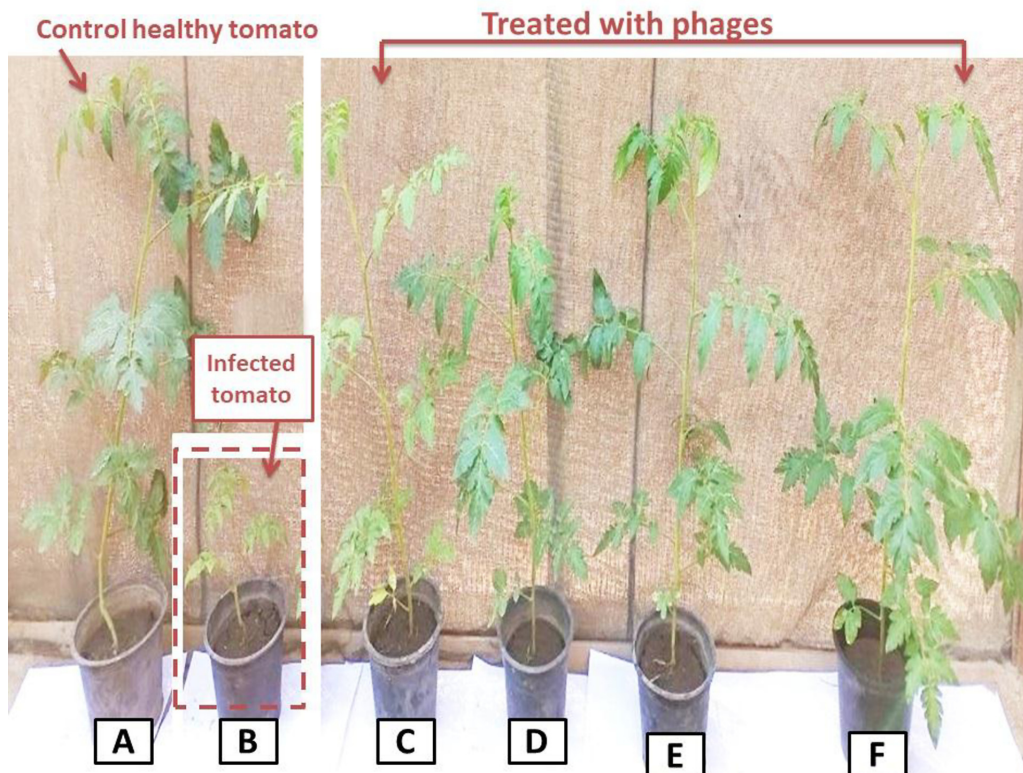


Fig. 7. Plant bioassay (pot test) [A. untreated plant, B. treated with *R. solanacearum* strain EMCC 1274, C. treated with *R. solanacearum* strain EMCC 1274 and  $\phi$ RS1, D. treated with *R. solanacearum* strain EMCC 1274 and  $\phi$ RS2, E. treated with *R. solanacearum* strain EMCC 1274 and  $\phi$ RS3 and F. treated with *R. solanacearum* strain EMCC 1274 and a mixture of  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3]

A phage should exhibit reasonable specificity, as one method of assessing phage effectiveness (Weber-Dąbrowska et al., 2016; Abedon et al., 2017). In contrast to non-target pathogenic or environmental bacteria, which were not affected, the three bacteriophages specifically infected a collection of plant pathogens *R. solanacearum* (former phylotype II) strains, known to be a genetically homogeneous group (Safni et al., 2014; Hasanién et al., 2022). Similarly, bacteriophages M13 and M18 isolated from the rhizosphere of banana plants displayed some degree of specificity to bacterial strains isolated from the same agroecological zone and banana plants. These strains were all of the sequevar 6 and phylotype II varieties (Ramirez et al., 2020). This prevented an impact on other bacteria from the environment, which is necessary for a safe biocontrol agent (Buttimer et al., 2017). The bacteriophage specificity to different strains of *R. solanacearum* was investigated in studies from Japan and Spain (Biosca et al., 2019; Zaki et al., 2022b). This specificity might be explained by the fact that the host range of bacteriophages is restricted to particular bacterial populations with whom they have co-evolved. Several phages interact specifically with certain receptors on the cell surface of the host bacteria, but they are unable to do so with receptors with other structural configurations (Piracha et al., 2014).

The three phages also demonstrated efficient lytic activity on populations of *R. solanacearum* across a variety of environmental conditions, including temperature, pH and UV irradiation. Temperature has a significant impact on how long bacteriophages can survive because it can degrade viral capsids and damage viral DNA or RNA (Olson et al., 2004). Various phage strains react differently to heat and pH. Our findings demonstrated that the activity of the three isolated phages was constant up to a temperature range of 80°C but lost when exposed to temperatures of 90°C for 10min. Between pH values of 4 and 10, all tested phages' activity remained steady against *R. solanacearum* strain EMCC 1274, showing the greatest stability at pH 6 and 7. In contrast, the activity of  $\phi$ RS1 only remained stable at highly acidic conditions (pH3).

The results are in harmony with those reported by Elhalag et al. (2018), who isolated and identified the phage RsPod1EGY that infected *R. solanacearum* from Egyptian soil by exhibiting a strong tolerance for a wide range of temperatures,

from 28 to 60°C. Still, it eventually went inactive at 70°C. The phage showed good pH stability at pH values varying from 5 to 9, while pH 7.0 displayed the most stability. The *Ralstonia* Phage RsoMIUSA isolated in the United States by Addy et al. (2019) was stable from 4 to 50°C, as evidenced by its titer staying constant at around  $10^8$  PFU/mL. However, at 60°C, a significant decrease in phage titer was seen. After 1h of incubation at 70°, 80°, and 90°C, no phage particles were found.

Phage  $\phi$ sp1 was discovered to be viable and stable at temperatures up to 55°C when it was identified by Umrao et al. (2021) against the *R. solanacearum* that causes wilt. At a variety of pH levels, bacteriophage was discovered to be stable and effective (3.0–9.0). Virion protein denaturation in acidic settings can be used to explain the phage's inactivity at lower pH values (Hazem, 2002).

$\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 lost their lytic capability against *R. solanacearum* strain EMCC 1274 after exposure to gamma radiation of dose more than 3 kilo gray (kGy). About other bacteriophages that infect *R. solanacearum*, no information is currently known. According to Sommer et al. (2001), 17kGy was approximately the dose needed to reduce bacteriophages by 2 logs. Microorganisms exposed to  $\gamma$ -rays can face actual damage from the rays and indirect effects as the production of OH-radicals, H atoms, and solvated electron radicals (Sommer et al., 2001). According to de Roda Husman et al. (2004), dissolved oxygen, alkalinity, and organic matter acting as radical scavengers are some of the factors that have a significant impact on these indirect effects. Virus inactivation is more dependent on these indirect effects than bacterial inactivation is, according to Thompson & Blatchley III (2000).

A significant barrier to the widespread use of phages to prevent bacterial infections of crops appears to be UV-induced phage mortality (Iriarte et al., 2007; Jones et al., 2007). In addition to mutagenic and harmful DNA damage, UV is known to change certain peptide bonds (Regan et al., 1992; Suttle & Chen, 1992). Since, UV is mutagenic, it may result in mutations that increase UV resistance. This may be a possible explanation for why  $\phi$ RS1,  $\phi$ RS2 and  $\phi$ RS3 retained their lytic capability after exposure to ultraviolet radiation for 60min at a distance of 50cm. Inversely, phage  $\phi$ sp1 isolated by Umrao et al. (2021) against wilt-causing *R. solanacearum* was drastically affected

by UV C irradiation (254nm) as no plaques were observed in UV-treated DLA plates after exposure for 5, 10, and 15min.

The DNA of the three isolated phages in our study was successfully digested into multiple bands when treated with a mixture of Eco RI and Hind III restriction enzymes indicating that all phages contain dsDNA. According to the total of the restriction fragment sizes for  $\phi$ RS1,  $\phi$ RS2, and  $\phi$ RS3, the bacteriophage genome was roughly 21, 15, and 14kbp long. These results are in agreement with those reported by Addy et al. (2019), who isolated the *Ralstonia* phage RsoM1USA in the United States genome consisting of 39,309 bp. The nucleic acid of phage RsPod1EGY, which was isolated by Elhalag et al. (2018), was double-stranded DNA, as evidenced by the distinct band patterns that were produced when it was digested by EcoRI, ClaI, NotI, AccI, SpeI, and HindIII. The total length of the restriction fragments pointed to a bacteriophage genome of 41kbp.

Plant bioassays (pot assay) showed about 100% reversal of disease symptoms using a cocktail of  $\phi$ Rs1,  $\phi$ Rs2 and  $\phi$ Rs3 phages compared to only pathogen-treated controls. It was noted that other phages that infected *R. solanacearum* might have biocontrol potential. In tests with 12 tomato plants treated with  $10^{12}$  PFU/pot under greenhouse conditions, soilborne podovirus RsPod1EGY

appeared to be efficient in suppressing wilting symptoms for 10 days (Elhalag et al., 2018). On the other hand, the tomato plant virulence of *R. solanacearum* was not decreased by soilborne myovirus RsoM1USA (Addy et al., 2019; Hasaniien et al., 2021). This phage shared a tight relationship with myovirus  $\phi$ RSA1, which failed to prevent bacterial wilt effectively.

### Conclusion

The authors conclude in Fig. 8 that, the two isolated Myoviridae lytic phages designated  $\phi$ RS1 and  $\phi$ RS2 and the podovirus  $\phi$ RS3, isolated from geographically distant origins in Egypt, can be used as an efficient biological control agent against *R. solanacearum*. The three phages were found to be highly host specific and their activity remained stable in a wide range of environmental conditions such as temperature, pH, and ultraviolet irradiation. High reductions in bacterial wilt incidence were observed for all of them and their combinations in vivo under greenhouse conditions. It will be beneficial to minimize infections in cold storage if *R. solanacearum* can lessen its ability to cause pathogenic wilting in tomato seedlings. The authors concluded in Fig. 8 that the bacteriophages are an efficient biocontrol technique and an environmentally benign therapy for controlling the diseases caused by *R. solanacearum*.

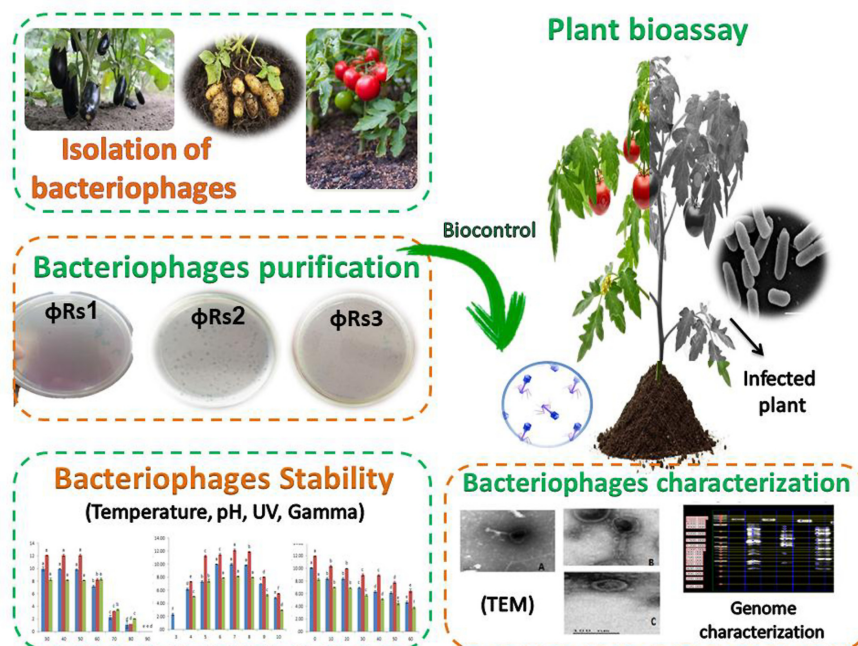


Fig. 8. Graphical representation of the whole isolation, identification, characterization and application of the control of the wilt disease caused by *Ralstonia solanacearum* using bacteriophages

**Abbreviations:** EMCC: Egypt Microbial Culture Collection; pH: Logarithm of the hydrogen ion concentration; °C: The degree Celsius; nm: nanometer; UV: Ultraviolet; CPG: Casamino acid-Peptone-Glucose; rpm: Rotation per minute; CFU: Colony-forming unit; Gy: Gray; kGy: Kilo Gray; bp: Base pair; kb: kilo base pair; PFU: Plaque-forming unit; MOI: Multiplicity of infection; DLA: Double-layer agar; mm: Millimeter; dsDNA: Double Stranded DNA.

**Competing interests:** The authors declare that they have no competing interests.

**Authors' contributions:** YAH conducted experimental methodology, participated in data analysis and representation, and participated in manuscript revising and editing. All authors read and approved the article. MHA performed the experiments and wrote the manuscript and participated in manuscript revising and editing. NAY conceived and designed research, provided the used chemicals, provided practical guidance, and participated in manuscript revising and editing. AA conceived and designed the research, conducted experimental methodology, participated in data analysis and representation. GED suggested the research point, investigated the article, conceived and designed the research, conducted experimental methodology.

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**Declarations**

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## البكتريوفاجات كعوامل مبشرة في المقاومة الحيوية للرالستونيا الباذنجانية المسببة لمرض الذبول البكتيري

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تعد البكتريوفاجات (الفاجات) تقنية فعالة لمقاومة الامراض النباتية التي تسببها البكتيريا مثل بكتيريا *Ralstonia solanacearu* التي تسبب مرض الذبول لإفراد العائلة الباذنجانية. تشير هذه الدراسة إلى عزل وتوصيف ثلاث فاجات، تم تسميتها ب  $\phi$ RS1 و  $\phi$ RS2 و  $\phi$ RS3، واختبار فعاليتها ضد *R. solanacearu* EMCC 1274 في اختبار حيوي باستخدام شتلات الطماطم .

تم اكتشاف أن الفاجات الثلاثة المعزولة كانت قابلة للحياة ومستقرة على نطاق واسع من الأس الهيدروجيني (3.0-10.0) وفي درجات حرارة تصل إلى 80 درجة مئوية. بعد فحص الفاجات الثلاثة المعزولة باستخدام الميكروسكوب الإلكتروني النافذ ودراسة الخصائص الجينومية لها وجد ان  $\phi$ RS1 و  $\phi$ RS2 تنتمي إلى عائلة ال Myoviridae بينما  $\phi$ RS3 ينتمي الي عائلة ال Podoviridae.

بعد اختبار مدي قدرتها على القضاء على *R. solanacearu* EMCC 1274 وجد ان استخدام خليط من الفاجات الثلاثة  $\phi$ RS1 و  $\phi$ RS2 و  $\phi$ RS3 قادر علي القضاء على *R. solanacearu* EMCC 1274 داخل نبات الطماطم وعدم ظهور اعراض الذبول البكتيري بنسبة %100.