

Isolation of Bacteriophages and Determination of their Efficiency in Controlling *Ralstonia solanacearum* Causing Bacterial Wilt of Tomato

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ABSTRACT: Bacterial wilt caused by *Ralstonia solanacearum* is a devastating disease of many economically-important crops. Management of *R. solanacearum* is difficult using a single method hence incorporation of a biological component in an integrated programme would be a promising approach. In the present study biocontrol potential of bacteriophages in controlling bacterial wilt of tomato (variety Thilina) was investigated. Bacteriophages were isolated from soil collected from vegetable fields or locations rich with organic matter and a mixture of six different phages were used in the study. Their effectiveness in controlling bacterial wilt caused by two isolates of *R. solanacearum* (isolate 6 and AB3) was investigated under planthouse conditions. Bacteriophage mixtures at a concentration of 2.86×10^6 pfu/ml were applied to the rhizosphere as a soil drench by several methods. The phage isolates had different lytic patterns on host *R. solanacearum* isolates and varied in their plaque morphology. Percentage wilt incidence by isolate 6 was reduced by 10%, due to application of the phage mixture immediately before the inoculation of the pathogen or when applied three times as a soil drench. Wilt incidence by isolate AB3 was reduced by 20% due to the application of the phage mixture by the two methods. Survival of the bacteriophage in soil treated with phages ranged from 0.2×10^3 – 3.5×10^4 pfu/g of soil, after 15 days of the last application of phages.

Keywords: Biological control, Lytic pattern, Percentage wilt incidence

INTRODUCTION

Production of tomato (*Solanum lycopersicum*) worldwide is affected by biotic and abiotic constraints. Among the biotic constraints, bacterial wilt caused by *Ralstonia solanacearum* (E.F.Smith), formerly named as *Pseudomonas solanacearum* has been considered to be the most damaging factor. A large body of information confirms that the bacterial wilt caused by *R. solanacearum* is the most ubiquitous and damaging disease of crops grown in tropical and subtropical countries (Kelman, 1985). The bacterium is well adapted to soil and survives in the rhizosphere. It possesses a diverse range of virulence factors, enters the roots from artificial or natural openings and colonizes vascular tissues resulting in internal and external symptoms leading into complete wilting of the plant. *R. solanacearum* is considered as a species complex comprising of heterogeneous group of related strains. *R. solanacearum* has been subdivided into “races” based on host range and five such races have been classified (Hayward, 1991). Race 1 has the highest number of host species including solanaceous crops (Elphinstone, 2005).

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In general, control of bacterial diseases is a challenge due to various factors of the pathogen including its genetic variation, ability to overcome plant genetic resistance, development of resistant bacterial strains, ability to reach high populations in a relatively short period of time under conditions favourable for disease development and lack of effective bactericides (Iriarte *et al.*, 2012). Hence, control of bacterial wilt caused by *R. solanacearum* is also a difficult task.

Conventional control measures of bacterial diseases involve a range of cultural practices, aiming to reduce inoculum density of the pathogen, spreading rate of the disease and time interval that the host and pathogen interacts with each other. Other methods to control bacterial pathogens include application of chemicals such as insecticides (for the control of insect vectors), antibiotics (e.g. tetracycline and streptomycin) and copper fungicides. However, presence of many plant pathogenic bacteria resistant to copper and antibiotics is a common problem (Ronald, 2011). Bacteriophages (phages) offer an alternative to conventional management strategies for controlling bacterial plant diseases (Balogh *et al.*, 2008; Addy *et al.*, 2012). Phages are viruses that specifically infect bacteria, yet have no direct negative effects on animals or plants. Infection of a bacterium by a virulent phage typically results in rapid viral replication, followed by the lysis of the bacterium and the release of numerous progeny phages. These phages can then proceed to infect neighbouring bacteria. Therefore, the numbers of phage will expand when target pathogens are encountered and the therapy will essentially be amplified in response to the bacterial infection. Potential of isolating bacteriophages from different environmental sources (e.g. river water, soil, phyllosphere etc.) and efficiency of them in controlling plant pathogenic bacteria, namely cabbage rot caused by *Xanthomonas campestris* pv. *campestris* (Mallmann and Hemstreet, 1924), blackleg disease of potato caused by *Pectobacterium carotovorum* subsp. *atropeticum* (Kotila and Coons, 1925) and bacterial spot disease on tomato and pepper caused by *Xanthomonas campestris* pv. *vesicatoria* (Flaherty *et al.*, 2000) have been reported.

The present study was conducted with the objectives of isolating potential bacteriophages as biological control agents of *R. solanacearum* and determining their efficiency in controlling bacterial wilt of tomato under *in vitro* and *in vivo* conditions.

METHODOLOGY

The laboratory and planthouse experiments were conducted at the Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya, Peradeniya.

Isolation of *R. solanacearum*

Tomato plants of varieties Thilina, T-245, KWR and Marglobe showing typical wilt-symptoms were collected from selected fields in mid country wet zone (Gampola, Gampolawela), mid country intermediate zone (Kolabissa, Gampolawela, Kottagasmula, Naula, and Thalathu Oya) and low country dry zone (Kithulhitiyawa, Kalundewa) of Sri Lanka. Bacterial ooze collected from surface-sterilized stems of the wilted tomato plants were plated on Nutrient Agar plates. A total of 20 bacterial isolates were purified and those isolates were subjected to biochemical and molecular methods for confirmation as *R. solanacearum* isolates. To determine Gram reaction both Gram staining and 3% KOH test were done. Isolates were grown on selective media namely, sucrose peptone agar (SPA),

semi selective medium, South Africa (SMSA) and Kelman's 2,3,5-triphenyl tetrazolium chloride (TZC). As molecular methods, PCR based identification was done for the confirmation of the isolates using BP4-R/BP4-L and PS-1S-R/PS-1S-F primers which are specific to *R. solanacearum* (Lee and Wang, 2000; Lee *et al.*, 2001). The primer sequences for BP4-R and BP4-L were (5'-GACGACATCATTTCCACCGGGCG-3') and (5'-GGGTGAGATCGATTGTCTCCTTG-3'), respectively and the expected PCR product size was 1100 bp. PS-1SR and PS-1S-F primers which are specific to *R. solanacearum* race 1 were used for the identification of *R. solanacearum* isolates. Base pair sequences of the primers PS-1S-R and PS-1S-F were 5'-CGCAACGCTGGATGAACCC-3' and 5'-CAGACGATGCGAAGCCTGAC-3' respectively and the expected size of the PCR product was 1070 bp (Lee *et al.*, 2001).

Isolation of bacteriophages

Bacteriophages were isolated from soil samples collected from fields at different locations e.g. Thalatu-Oya (agroecological zone IM3c), Pallekele (agroecological zone IM3a) and Peradeniya (agroecological zone WM2b) representing soils managed under different crop management practices (Table 1). Bacteriophages were isolated according to the method described by Crosse and Hingorani (1958). CPG medium was used as the basal medium for growing the lawn of host bacteria and isolating bacteriophages. Isolates of bacteriophage were separately-identified based on plaque morphology. Accordingly, six bacteriophage isolates were identified and a mixture of the six isolates was used in the biocontrol assays of the present study. Identified bacteriophage isolates were stored in SM buffer (NaCl 5.8g, MgSO₄ 7H₂O 2.0 g, 1M Tris HCl (pH7.4) 50 ml and 2% gelatin 5 ml per liter) at 4 °C under dark conditions until future use.

Table 1. Description of phages used in the experiments.

Identification code given for each bacteriophage	Location of Sampling Area	Cropping systems practiced in the fields from which the bacteriophages were collected
C	Peradeniya (WM2b)	Organic matter rich soil uncultivated leaf litter area
F	Pallekele (IM3a)	Organically-cultivated Tomato (<i>Solanum lycopersicum</i>) field
J	Iriyagama (WM2b)	Home garden having vegetables
N	Pallekele (IM3a)	Organically-cultivated Okra (<i>Abelmoscus esculentus L.</i>) field
O	Pallekele (IM3a)	Banana (<i>Musa sp.</i>) grown area
P	Pallekele (IM3a)	Organically-cultivated Brinjal (<i>Solanum melongina</i>) field

Determining the broad spectrum ability of phages

Phage typing was done *in vitro* to determine the lysing ability of each bacteriophage isolate listed in Table 1 against the 19 isolates of *R. solanacearum*. The *R. solanacearum* isolates

were streaked separately as a thick smear on well-dried double layer agar plates. Once the streaks have dried completely, a 10 µl phage suspension (using a micropipette) was spot inoculated close to one end of the streak. The phage inocula were allowed to dry completely, incubated at 37 °C for 1-2 days and clear, lysed areas around the spot inoculations were observed.

Establishment of tomato plants

Tomato nurseries were prepared in Polyurethane (Rigiform) blocks. A commercial compost mixture (purchased from Hadabima sales centre at Gannoruwa) was sterilized by autoclaving and used as the potting medium. Seeds of tomato (variety Thilina) were treated with Thiram 80% WP (Tetramethylthiuram disulfide) at a rate of 5 g/kg to prevent seed-borne fungal pathogens. Tomato seeds were sown and watering was done as needed. Potting medium was treated with Thiram solution (Thiram 80% WP diluted in water to apply in a rate of 70 g/ 50 l/ 10 m²) one week after sowing the seeds. Seedlings were transplanted at three leaf-stage (*i.e.* 4-5 weeks after sowing) in black polyethylene bags (16 cm in height and 16 cm in diameter). Potting medium was a mixture of commercial compost, top soil and coir dust at a ratio of 2:2:1. The bags were filled with the potting medium treated with Thiram (Thiram 80% WP diluted in water to apply in a rate of 70 g/ 50 l/ 10 m²) one day prior to transplanting. The basal fertilizer application was done at a rate of 65 kg/ha Urea, 325 kg/ha TSP and 65 kg/ha MOP as recommended by the Department of Agriculture, Sri Lanka.

Inoculation with *R. solanacearum*

Two weeks after transplanting, tomato plants were inoculated with *R. solanacearum* according to the method described by Abdhullah and Rahman (1998) as follows. Tomato plants were kept without watering for 24 hours and roots of each plant were cut 1 cm away from the base of the stem diagonally. Out of the 19 isolates of *R. solanacearum*, two isolates, namely AB3 and 6 were used for inoculation after confirming their virulence by *in vitro* (TZC test) and *in vivo* (inoculating to tomato plants) tests. Approximately 10 ml of an aqueous cell suspension of each *R. solanacearum* isolate was poured around the base of the stem. The OD value of the above bacterial suspension was adjusted spectrophotometrically to reach OD of 0.26 at 600_{nm} (which is approximately 1x10⁸ cfu/ml).

Mass preparation of bacteriophages

Bacteriophages were mass prepared under *in vitro* conditions as follows: *R. solanacearum* isolate AB3 was used as the host bacterium to propagate the phages. A volume of 250 ml of CPG broth medium was inoculated with isolate AB3 and incubated for overnight at 28 °C in a shaking water bath. Aliquots of 150 µl were taken in to microfuge tubes from the six isolates of bacteriophages. Chloroform was added to each tube to have a final concentration of 5% (v/v). The microfuge tubes were vortexed and centrifuged at 8000 g for 20 min at room temperature (Gill *et al.*, 2003). The supernatants were collected and mixed with the overnight bacterial broth culture. The broth culture was shaken and incubated overnight in a shaking water bath at 28 °C.

The resulted in clear solution containing progeny of bacteriophages was centrifuged with 5% v/v chloroform at 8000 g under room temperature. Supernatants of the six bacteriophage isolates were collected and added into 1000 ml of sterile tap water to have a bacteriophage titer of 2.86 x 10⁶ pfu/ml. The titre of the final mixture of the bacteriophages was calculated

by plating serially diluted phage mixture on a lawn of host bacteria using double layer agar plates.

Determination of the efficiency of phage mixture in controlling bacterial wilt

Isolates 6 and AB3 of *R. solanacearum* were used for inoculation of separate sets of tomato plants. Bacteriophages were mass propagated using isolate AB3 as the host bacterial cells. The treatment structure used for one set of plants (inoculated with one isolate of *R. solanacearum*) was as follows:

P3times - The phage mixture was applied thrice to the soil (twice before the inoculation and once after the inoculation) with a 2 day interval between each application of bacteriophages

RS+ P3times - The phage mixture was applied thrice to the soil (twice before the inoculation and once after the inoculation) with a 2 day interval between each application of bacteriophages and inoculated with *R. solanacearum* (isolate 6)

P14d(BI)- The phage mixture was applied on the 14th day after transplanting RS+ P14d(BI)- The phage mixture was applied on the 14th day after transplanting then inoculated with *R. solanacearum* (isolate 6) in the same day

RS- Inoculated only with *R. solanacearum* (isolate 6)

Negative control- Neither inoculated with *R. solanacearum* (isolate 6) nor treated with the phage mixture

Same set of treatment structure was duplicated for *R. solanacearum* isolate AB3. Each treatment was replicated ten times according to a Completely Randomized Design. Percentage wilt incidence (number of plants showing wilt symptoms out of total number of plants per given treatment) was recorded. Significance of the treatments was determined by ANOVA and mean separation was done by LSD. The above experiments were conducted twice, independently.

Enumeration of the bacteriophages in soils treated with the phage mixture

After the completion of the above two experiments, plants were uprooted (at 30 days after transplanting) and soils were tested for the presence of bacteriophages. Three pots were selected randomly from each treatment and soils in the three pots were mixed to get a composite sample. From the composite sample, 2.5 g of soil was taken and added into flasks separately. A volume of 50 ml of sterilized water was added in to each flask and gently agitate for 10 minutes. Chloroform was added to each beaker to reach a final concentration of 5% v/v. The contents in the flasks were shaken gently. An aliquot of 1 ml of slurry was taken from each flask into sterile microfuge tube and added 5% chloroform (v/v). The contents in the microfuge tubes were vortexed and centrifuged at 8,000 g for 20 minutes at 4 °C. The supernatants containing the extracted bacteriophages were separately collected in to sterilized microfuge tubes under aseptic conditions. The bacteriophage lysates were serially diluted and phage titer was calculated by plaque assay technique.

RESULTS AND DISCUSSION

Confirmation of *R. solanacearum*

All the 20 isolates were gram negative. Results given by all the *R. solanacearum* isolates when grown on semi-selective media and *in vivo* pathogenicity are summarized in Table 2.

Table 2. Summary of the results of biochemical and *in vivo* pathogenicity test

Test	Bacterial Isolate																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	AB1	AB2	AB3
Growth on SPA	+	+	+	+	+	+	+			+	+	+	+	+	+	+				
Growth on TZC	+	+	+	+	+	+	+			+	+	+	+			+		+	+	+
Growth on SMSA	+	+	+	+	+	+	+			+	+	+	+			+				
Pathogenicity test				+	+	+				+		+						+	+	+

Bacterial colonies developed on SMSA medium were irregular, white and fluidal after an incubation period of 48-72 hours. Round to irregular, creamy white and fluidal colonies were produced on SPA medium. The colony morphology on the above two media was similar to the positive results should be produced by *R. solanacearum*. Appearing of whitish colour colonies with pink centres on TZC medium was an indication that those colonies are virulent *R. solanacearum*. Isolates 1, 3, 9 and 17 did not give positive results to any of the pathogenicity tests (Table 2). There could be gram negative endophytes or saprophytes those could get co-isolated during the isolation procedure, which are not *R. solanacearum* but several other *Pseudomonas* spp. and *Enterobacter* spp (Ramesh *et al.*, 2009).

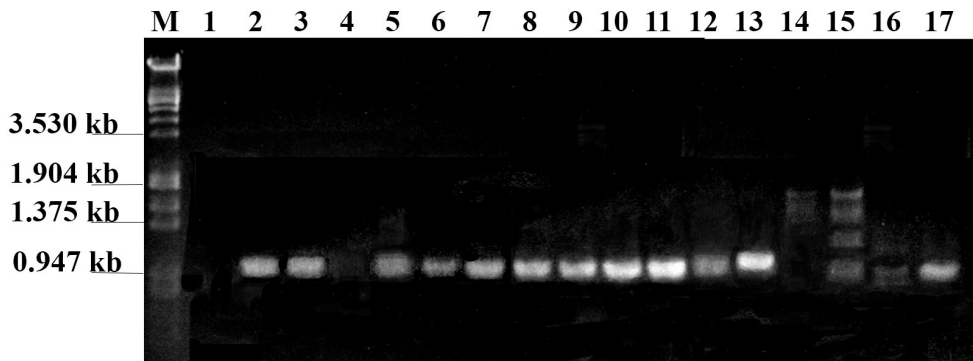


Fig. 1. PCR products given by different isolates of bacteria obtained from infected plant stems when amplified by PS-IS-R and PS-IS-F primers

Fig. 1 shows the PCR products produced by isolates 1-17 when amplified by PS-IS-R and PS-IS-F primers. Isolates 1 and 14 did not result in the PCR product of the expected size which was 1070 bp. However, the rest of the isolates were positive for the expected size of the PCR product both PS-IS-R/PS-IS-F and BP-4R/BP-4L (data not shown) indicating they belonged to *R. solanacearum* race 1. PCR amplification was done using the above two primer pairs for isolates AB1, AB2 and AB3 and they also produced the expected sizes of

PCR products (data not shown). It confirmed that those three isolates also belonged to race 1 of *R. solanacearum*.

A polyphasic approach based on biochemical tests, semi-selective media and molecular methods was used to identify and select virulent *R. solanacearum* isolates. A number of saprophytic soil borne bacteria could grow on SMSA agar and outcompete the culture medium when *R. solanacearum* population is lesser in the sample than the saprophytic population (Pradhanang *et al.*, 2000). Moreover, the isolates (with different origins) identified by PCR methods as *R. solanacearum* and as virulent isolates by TZC medium may not develop wilt symptoms on the variety of host plant used for inoculation due to variations in pathogenicity (Chandrashekhara *et al.*, 2012). Therefore, based on all tests, isolates 4, 5, 6, 10, 11, 12, AB1, AB2, and AB3 were identified as virulent *R. solanacearum* isolates.

Determination of broad spectrum ability of phages

The bacteriophage isolates showed a variation in plaque morphology on host bacterium isolate 6 (Fig. 2).

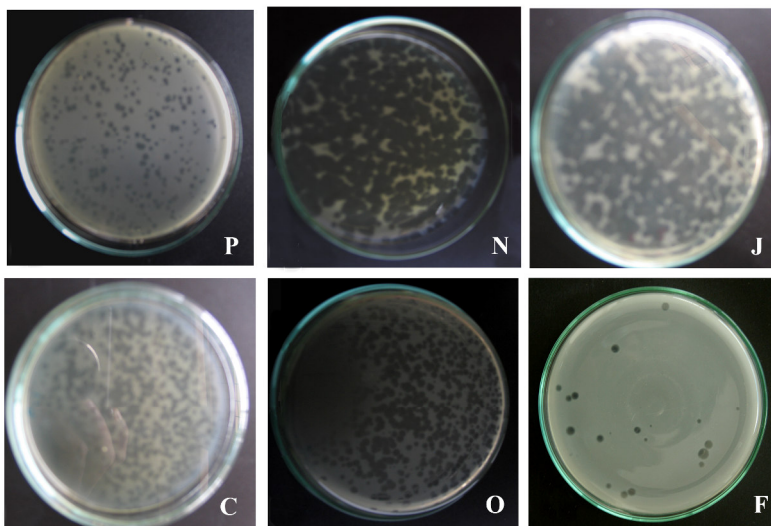


Fig. 2. Variation of plaque morphology produced by six selected phages on doubled layer agar plates, when *R. solanacearum* isolate 6 (i.e. P, N, J, C, O and F) was used as the host bacterium

Table 3 summarizes the phage typing results of the six phage isolates tested on 19 isolates of *R. solanacearum*.

Table 3. Broad spectrum ability of lysis of isolated bacteriophages against *R.solanacearum* isolates

Bacteriophage code	<i>R. solanacearum</i> Isolate																		
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	AB 1	AB 2	AB 3
C	√	√							√				√		√				
F		√	√	√	√	√	√					√		√	√		√	√	√
J	√	√	√	√	√	√	√	√				√	√	√	√		√	√	√
N	√	√	√	√	√	√	√					√	√	√		√			√
O	√	√	√	√	√	√	√					√		√		√			√
P	√	√	√	√	√	√	√	√				√	√	√					√

A heterogenous lytic pattern was shown by the six bacteriophage isolates against the 19 isolates of *R. solanacearum*. However, isolates 11 and 12 of *R. solanacearum* were not lysed by any of the bacteriophage isolates. All the phage isolates were effective against *R. solanacearum* isolate 3. Nine isolates of *R. solanacearum* were lysed by five phage isolates out of the six. *R. solanacearum* isolate 10 was lysed by one isolate of phage mixture.

According to Hayward (2000), *R. solanacearum* has an unusually wide host range covering over 200 plant species belonging to more than 50 botanical families, and shows great phenotypic and genotypic diversity between strains. Therefore, determining the host range of each phage isolate should be a prerequisite before deciding on the member isolates to be used in the phage mixture. Determining the host range of each phage enables to design of a phage mixture capable of infecting all known pathogenic strains involved in the disease. Although the host range of most phages is usually narrow, bacteria isolated from the plant environment should be tested for lysis by the putative phages to be used as biological control agent, to ensure a minimal impact of the phages on the wider microbial community and potential commensal strains (Frampton *et al.*, 2012). Bouzar *et al.* (1999) used 26 bacteriophages to type approximately 100 *Xanthomonas euvesicatoria* strains isolated from various countries in the Caribbean region including Central America, and identified at least 26 different phage lysis patterns. Our results on phage typing also showed varying lysis patterns between the used bacteriophage and *R. solanacearum* isolates, indicating the specific nature of each phage and bacterial combination.

Efficiency in controlling bacterial wilt by phage mixture under *in vivo* conditions

Percentage wilt incidence did not differ significantly among the treatments until 7th day in the plants inoculated with isolate 6, however, the wilt incidence differed significantly among treatments thereafter ($p < 0.0001$). Plants inoculated with isolate AB3, showed a significant difference of % wilt incidence among the treatments from 5th day after inoculation.

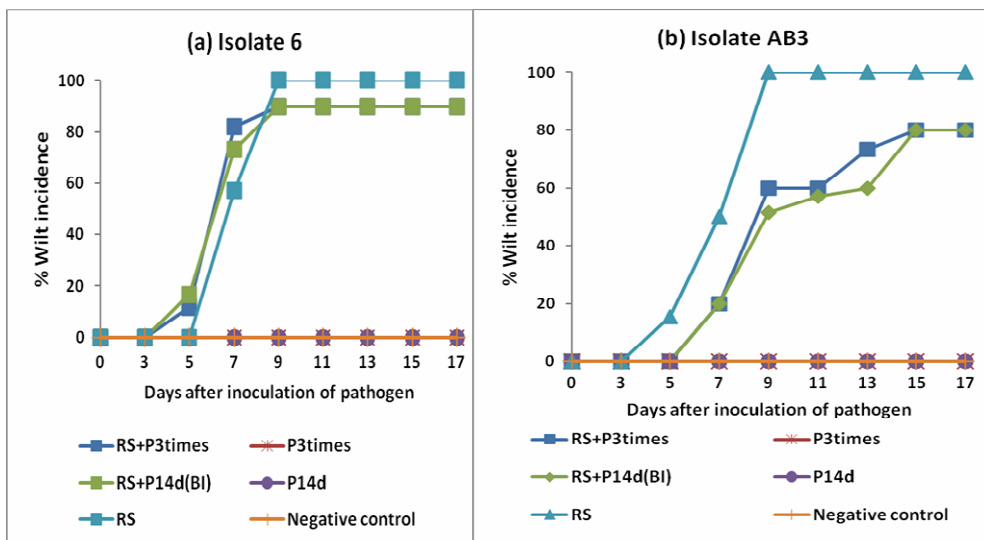


Fig. 3. Percentage wilt incidence shown by tomato (var. Thilina) when inoculated with two isolates of *R. solanacearum* and treated with different methods of phage treatments

As expected the plants treated with the negative control and only with the phage mixture (e.g. P3 times and P14d (BI) did not develop wilt disease (Fig. 3). Only the plants inoculated with isolate AB3 and applied with the phage mixture by two methods [RS+P3times and RS+P14d (BI)] significantly reduced ($p < 0.05$) the % wilt incidence. This indicated the efficiency of reducing wilt development by the two application methods of phage mixture, when the plants are infected with isolate AB3. However, the two application methods had no significant difference on the reduction of wilt incidence when plants are inoculated with either of the isolates. Even though a 10% reduction of wilt incidence was observed in plants inoculated with isolate 6, by the two application methods, a significant reduction was not achieved due to phage treatments. However, a 20% reduction of wilt incidence was achieved due to treatments of phage mixtures in plants inoculated with isolate AB3, which was a significant reduction.

The two isolates showed a difference in wilt symptom development. Initial wilt development was shown five days after inoculation with isolate 6, whereas symptom development occurred 3 days after the inoculation with isolate AB3, indicating the varying degree of virulence of the two *R. solanacearum* isolates. It confirmed the necessity of an effective phage mixture for control of bacterial wilt under natural infections. Adriaenssens *et al.* (2012) have reported success of using a mixture of phages against *Dickeya solani* and *Pectobacterium atrosepticum* to control soft rot caused by both species.

According to three consecutive field trials done by Balogh and Jones (2003) nonformulated phage had reported 9-20% reduction in bacterial spot on tomato in comparison to the non-treated plants. It is reported that the plants treated with casecrete-formulated phage had reduced 12-43% of the tomato bacterial spot disease (Frampton *et al.*, 2012). Accordingly, the disease reduction achieved by the present study is in a reasonably acceptable level.

Iriarte *et al.* (2012) has applied a phage mixture at various time points prior and following the application of *R. solanacearum* to the soil. According to Iriarte *et al.* (2012) the most effective wilt control had been achieved when a phage mixture was applied immediately after inoculation of the pathogen. The same study reported that there was no effect on disease control when a single phage was applied immediately after inoculation of the pathogen. Findings of the present study agree with the observations of Iriarte *et al.* (2012). According to Fig. 3 of the present study, the lowest percentage wilt development (highest disease reduction) was shown when the phage mixture was applied immediately before inoculation of the pathogen. According to Iriarte *et al.* (2012) phage mixtures as well as the single phage treatments were less effective in controlling bacterial wilt of tomato when applied 3 days before or after inoculation of the plants. Success of the phage treatment has shown variable efficiencies with the application time and no. of times. For example, when controlling above-ground bacterial pathogens, Civerolo and Keil (1969) achieved a significant reduction of peach bacterial spot only if the phage treatment was applied one hour or one day before inoculation with the pathogen. Bergamin Filho and Kimati (1981) reported that the highest disease reduction was achieved when the phages were applied at the day of inoculation for the two pathosystems; black rot of cabbage caused by *Xanthomonas campestris* pv. *campestris* and bacterial spot of pepper caused by *X. campestris* pv. *vesicatoria*.

Phage survival and their persistence at the required site of action are affected by conditions such as pH, temperature, desiccation, rain, and UV. The most damaging factor appears to be UV irradiation in sunlight (Iriarte *et al.*, 2007). In the present study, density of bacteriophages in phage treated soils (approximately 15 days after the last treatment of bacteriophages) ranged from 0.2×10^3 - 3.5×10^4 cfu/g of soil. Soils that were not treated with bacteriophages did not show any presence of bacteriophages. In general phages persisted in the rhizosphere and roots of treated plants, although they did decline between ten and a hundred-fold over a 14 day period (Iriarte *et al.*, 2012). Hence, increasing the retention efficiency of bacteriophages in soil, use of formulated phage mixtures and use of phage treatment in combination with other agronomic and/or chemical methods will increase the disease reduction. Marco and Stall (1983) have evaluated phage treatment in combination with copper-mancozeb for the control of *Xanthomonas* leaf blight of onion and found that combination resulted in enhanced disease control. Considering the potential of bacteriophages in controlling bacterial wilt, future studies should be focused on increasing the efficiency of wilt reduction through improving the application methods or by combining integrated methods.

CONCLUSION

The present study identified 19 isolates of *R. solanacearum* from wilt-infected tomato plants. Application of a mixture of six phage isolates at a concentration of 2.86×10^6 pfu/ml as a soil treatment three times at a two day interval (starting from 14 days after transplanting) and treating the soil immediately before inoculation of the pathogen reduce the wilt incidence at a range of 10-20%.

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