Effect of phage infection on pathogenic activity of *Ralstonia solanacearum* in tomato
(Kesan serangan faj terhadap aktiviti patogenik *Ralstonia solanacearum* pada tomato)

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Keywords: phage, pathogenic activity, *Ralstonia solanacearum*, tomato

**Abstract**

Phages are obligate intracellular parasites that multiply inside the bacteria and making use of host biosynthetic machinery. They are highly host specific and only attack a particular group species of bacteria. Phage will multiply once they attack the bacteria cells, and releasing several hundred new phages. As a result, the bacteria are destroyed and leaving a clear area or plaque on the agar.

Recently, the usage of certain phages against bacterial infection has regained scientific interest due to the resistance of bacteria to antibiotic has become a serious treat to human. Studies showed that upon infection by phages, the host *Ralstonia solanacearum* cells showed several abnormal behaviours, such as a reduction of culture turbidity, decrease in coloration of the colonies, increase in sensitivity against certain antibiotics and a serious decrease in growth rate, approximately 40–60% from the normal. This study also indicated that phage infection may affect the pathogenicity of the host cells, resulting in the increase of the survival rate of tomato plants.

**Introduction**

*Ralstonia solanacearum* (Yabuuchi et al. 1995) is one of the most devastating plant diseases and predominantly in the tropical, sub-tropical and temperate regions. It is a soil-borne gram negative bacterium. The infection takes place through the roots and exhibits strong tissue-specific tropism within the host, specifically invading and extensively multiplying in the xylem vessels. It further spreads throughout the plant and multiplies to a high population density. It is easily spread through the contaminated soil and irrigation water. It can survive for many years in association with alternate host (Yamada et al. 2007). In the susceptible host plants, this pathogen disrupts water transport, alters physiology and induces a severe wilting symptom.

In Malaysia, more than 35 families of plants are affected by this disease, and the major economic host includes potato, tomato, eggplant, chili, ginger and groundnut (Hamidah and Lum 1992; Hayward 2000). This disease was first reported in Peninsular Malaysia in 1910 on potato and tomato (Abdullah 1992; Masyitah 2004). The large scale cultivation of tomatoes in Malaysia, especially in lowlands has been limited by the widespread incidence of this disease.

The initial symptom in tomato plants was started by wilting of the upper leaves during the hottest part of the day, followed...
by recovery during the evening and early hours of the morning. The wilted leaves maintain their green colour and do not fall off as disease progresses. However, the complete wilting may occur under condition favourable to the disease and the vascular tissues of the stem will show a brown discoloration (Momol et al. 2004).

In the cropping field, the plant, soil or water need to be treated with sodium hypochlorite once identified as being infected by *R. solanacearum*. Normally the crop debris need to be removed at the end of the season and the planting sites rotated every year to avoid contamination by the disease. Many researches were carried out to develop methods for controlling bacterial wilt, including searching for resistant varieties of tomato variety MT1 (Ho 1988), cultural practices, composting (Masyitah 2004), and using biological and chemical control. However, none of them found to be effective against bacterial wilt disease.

Thus, the usage of certain phages against bacterial infection has gained scientific interest due to the fact that phages are highly host specific, only attack a particular group species of bacteria, and does not develop resistance as pathogens do to antibiotics.

The objective of this study was to investigate the effect of phages on pathogenicity of *R. solanacearum* cells with the aim for developing biocontrol agent against bacterial wilt disease in tomato.

### Materials and methods

#### Isolation and purification of phages

Sewage and soil samples were collected from Indah Water Konsortium Sdn. Bhd., Puchong, Cameron Highlands, Port Dickson and Serdang. Three different types of *Escherichia coli* strains [TG1 (supE, hsdA5, thiA(lac-pro AB), F’[traD36, proAB+, lacP, LacZ, DαM15]), ER2738 (F’ pro A+B+ lac 9 Δ (lacZ) m15 zsfr::Tn10 (TerR)/ffhu Aα gln vD (lac-proAB) thi-1 A (hsds-mcrB) 5) and BL21 (F-ompThsdSB (rB-mB-)galdcm)] were used as a host for the amplification of the phages.

Each of the *E. coli* culture (5 ml) was added into the fresh Luria Bertani (LB) broth [tryptone (1%), yeast extract (0.5%), NaCl (1%); pH 7.5, 100 ml] containing tetracycline (5 mg/ml, Sigma). The mixture was incubated with shaking until OD₆₀₀ about 0.5. Sample (50 ml) was added into *E. coli* culture separately, which was prepared previously and incubated overnight at 37 °C at 250 rpm.

The phage mixture was centrifuged at 10,000 rpm for 5 min and the supernatant was transferred to the new conical flask. The supernatant was filtered with membrane filter (0.45 μm, Whatman) to remove the unnecessary particles. The phage particles in the supernatant were precipitated by adding polyethylene glycol (20% PEG 8000) and NaCl (2.5 M). The suspension was kept at 4 °C for 1 h to overnight and centrifuged at 13,000 rpm for 30 min at 4 °C. Finally, phage pellet was resuspended in TBS (50 mM Tris; 150 mM NaCl, pH 7.5).

#### Virulence of strains

The virulence of *R. solanacearum* (isolated from infected tomato plants in Serdang) was checked by streaking the bacteria suspension on tetrazolium chloride agar (TZCA) [peptone (10 g/ litre), casein hydrolysate (1 g/ litre), glucose (5 g/ litre), 2,3,5-triphenyl tetrazolium chloride (0.05 g/ litre), agar (15 g/ litre)] and incubated overnight at 28–30 °C. This technique is commonly used to differentiate wild colony types (white with pink centres) from low virulence mutants or avirulent mutants (deep red colony) that could occur on subculturing (Kelman 1954).

#### Turbidity study

A total of 12 potential phages isolated from sewage and soil samples were tested against *R. solanacearum* (Tan et al. 2009). Two ml overnight culture of *R. solanacearum* was
transferred into fresh nutrient broth (NB; 20 ml, BD, USA) and incubated at room temperature on the shaker of 250 rpm for 6 h. The isolated phages (1 x 10^{10} pfu/ml, 100 µl) were then added into the conical flask and incubated further on shaker at 250 rpm overnight.

The turbidity of the culture was measured at the absorbance of 600 nm by using spectrophotometer (Model Helios, UK) (Yamada et al. 2007). The uninfected R. solanacearum was used as positive control and each treatment was repeated three times.

**Colony colour observation**
The broth culture of infected and uninfected R. solanacearum was streaked on the nutrient agar (NA, BD, USA) and incubated overnight at 30 °C. The changes of colony colour was observed and compared. Each treatment was repeated three times.

**Growth rate study**
Two ml overnight culture of R. solanacearum was transferred into fresh nutrient broth (NB, 20 ml) and incubated at room temperature on the shaker at 250 rpm for 6 h. The isolated phages (1 x 10^{10} pfu/ml, 100 µl) were then added into the conical flask and incubated further on shaker at 250 rpm overnight. The Ralstonia cells were then harvested and streaked on NA plate, and incubated overnight at 30 °C.

A single cell of each infected R. solanacearum was inoculated into nutrient broth (NB, 20 ml) and incubated on the shaker at 250 rpm for 1 h. A 10-fold serial dilution of broth culture was prepared and plated for the first hour of inoculation. The cultures were further incubated for another hour and the same procedures were followed.

The plating was carried out for each hour of incubation time in order to measure the growth rate of cells based on colony forming unit (cfu/ml) (Tanaka et al. 1990). The uninfected R. solanacearum was used as positive control and each treatment was repeated three times.

**Sensitivity study to antibiotic**
The infected R. solanacaerum were used for sensitivity study against the following antibiotics: ampicillin (5–200 µg/ml); kanamycin (30 µg/ml, Oxiod, UK); tetracycline (30 µg/ml, Oxiod, UK); carbenicillin (100 µg/ml, Oxiod, UK); streptomycin (10 µg/ml, Oxiod, UK); penicillin G (10 µg/ml, Oxiod, UK) and gentamicin (10 µg/ml, Oxiod, UK).

The infected R. solanacearum was spread on the NA plate with sterile cotton bud, and the different concentration of antibiotic was applied on the NA, incubated at 30 °C overnight. The formation of clear zone was measured and compared with the uninfected R. solanacearum (Freifelder 1987). Each test was conducted in three replicates.

**Pathogenicity study on tomato plant**
The infected R. solanacearum were grown in NB medium for 1–2 days at 30 °C. After centrifugation, cells were resuspended in distilled water (20 ml) at a density of 1 x 10^8 cells/ml. The absorbance of culture was measured at 600 nm before inoculated into the soil planted with tomato plants when the plants are 6 weeks old with 6–10 leaves. Plants were cultivated at open condition at 27 °C (16 h light/8 h dark) for 4 weeks, and the observation was made everyday from day 1 until day 30.

The symptoms of wilting were graded from 1 to 5 [Grade 1: only a slight change, Grade 2 and 3: a few leaves showed wilting symptom, Grade 4: leaves with most of wilting symptom and Grade 5: plant almost dead] as described by Winstead and Kelman (1952). As a control, distilled water and uninfected R. solanacearum were inoculated in the same method and each test was repeated three times.
Results and discussion

Turbidity and colour changes

A total of 12 phage isolates (P36, P45, P47, P71, P72, P630, P631, P482, P483, P459, P535 and P536) were individually used to infect *Ralstonia solanacearum*. The turbidity of *R. solanacearum* in broth culture was decreased to 30–80% after being infected by the phages as compared to uninfected *R. solanacearum* (Figure 1). In addition, the colony colour of the bacteria was also slightly reduced (Plate 1).

This phenomenon may occur due to the infection of the phages through specific receptor on the host cell. They injected and released their DNA/RNA into the cells. The transcription of phage DNA took place and spontaneously incorporated into bacterial DNA. As a result, the bacteria lost its ability to replicate their own DNA, and phages have taken over the mechanisms to produce their coat protein for the progeny.

Once the DNA was packaged into phage, and assembly of phage particles completed, the lyses process occur whereby the bacterial cells burst and released all the new phages into the surrounding medium. Normally 50–100 phage particles are produced per cell, and the number depending on the particular phage species (David 1986). In addition, the ‘string like particles’ of death cells were also detected in the broth culture after the *R. solanacearum* was infected by phages (Plate 2).

![Figure 1. Turbidity of Ralstonia solanacearum following the infection by the various phages in broth culture](image)

![Plate 1. Some examples for colony colour study of Ralstonia solanacearum after infected by phages. The bacteria colony infected by phage P36 (b); phage P72 (d) and phage P631 (f). The uninfected R. solanacearum (a, c and e) was served as positive control](image)
The formation of “string like particles” of death cells of *Ralstonia solanacearum* in broth culture after been infected by phages

**Figure 2. Growth rate of *Ralstonia solanacearum* as affected by phages**

The growth rate and antibiotic study

Upon infection by the phages, the host *R. solanacearum* cells showed a reducing growth rate as compared to the normal growth (Figure 2). The growth rate reduced by 40-60%. This phenomenon may be due to the shut down of several mechanisms inside the bacterium host (David 1986). The same observation was also reported by Yamada et al. (2007), whereby the growth rate of *R. solanacearum* strain C319 in tobacco plants was decreased approximately 60% after infected by φRSS1 phage.

The sensitivity of phage infected *R. solanacearum* to various antibiotics

is shown in Table 1. The phage-infected cells were more sensitive to kanamycin as shown by the development of which formed the wider inhibition zone (Plate 3). This study indicated that phage can cause destruction of principal barriers of the outer membrane in Gram negative bacteria called efflux system MexAB-OprM (Xian-Zhi et al. 2000). Thus, the sensitivity of Gram negative bacteria will increase and become susceptible to the antibiotic (Hagens et al.)
Plate 4. Effect of phages infection on the pathogenic activity of *Ralstonia solanacearum*. Tomato plants (6 weeks old) were inoculated with phages-infected cells (a) and non-infected cells into the rhizosphere of the plants (b). After 20 days of post infection, the plants which inoculated with phage-infected cells still maintain healthy, while the plants inoculated with non-infected cells were completely died.

Table 1. Sensitivity of phage-infected *Ralstonia solanacearum* against certain antibiotic on agar plate as indicated by the size of the inhibition zone (mm) after 24 hour of incubation

<table>
<thead>
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<th>P36</th>
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<th>P71</th>
<th>P72</th>
<th>P630</th>
<th>P631</th>
<th>P482</th>
<th>P483</th>
<th>P459</th>
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Amp = Ampicillin; K = Kanamycin; Tet = Tetracycline; Carb = Carbenicillin; Strep = Streptomycin; Pen. G = Penicillin G; Genta = Gentamicin
As a result, low doses of antibiotics were able to inhibit growth or kill the pathogens (Hagens et al. 2006).

**Pathogenicity as affected by phage infection on tomato plant**

The pathogenicity of *R. solanacearum* was also affected by the phages. From the observation, tomato plants which were inoculated with non-infected *R. solanacearum* showed wilting symptom after 20 days of post inoculation and the plants died completely after 21 days (*Plate 4*). However, the other plants which were inoculated with phage-infected cells still remain healthy up to 30 days after inoculation.

However, the period taken by the plant to show the wilting symptom in this study was slightly longer compared to Yamada et al. (2007). The differences could be due to the inoculation method, whereby the method applied by Yamada et al. (2007) was direct injection into the stem while here, the phage-infected cells were inoculated into the rhizosphere of the plants.

The wilting symptom observed in this study was slightly different from normal wilting. Normally the young leaves will show wilting symptom once infected by *R. solanacearum*, and followed by the whole plant. However, in this case, the wilting only occurred on the leaves and lower parts, and the stem remain healthy.

**Conclusion**

This study showed that phages isolated from sewage and soil samples are potential as biocontrol agent against *R. solanacearum*. Great care is necessary during development, production and application of phage treatment to avoid unnecessary risks. In addition, constant monitoring for the emergence of resistant bacterial strains is essential. Phage- based disease control management is a dynamic process which needs continuous adjustment in preparation of the phage in order to be effective for pathogenic bacteria in agriculture crops.

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**References**


**Abstrak**

Faj merupakan parasit yang dapat menggandakan bilangan dengan menggunakan sistem biosintetik di dalam perumah. Faj sangat spesifik dan hanya menyerang bakteria dalam kumpulan spesies yang tertentu sahaja. Apabila faj menyerang bakteria, ia akan menggandakan bilangan dan menghasilkan beberapa ratus faj baru, dan membentuk zon jernih di sekitar bakteria yang musnah. Kebelakangan ini, penggunaan faj dalam menangani jangkitan bakteria telah mencetuskan minat para saintis disebabkan masalah peningkatan daya tahan oleh sesetengah bakteria terhadap antibiotik merupakan ancaman kepada manusia. Dalam kajian ini, sel *Ralstonia solanacearum* menunjukkan perubahan sifat-sifat tidak normal seperti pengurangan kekeruhan kultur di dalam cecair, pengurangan warna koloni, peningkatan kadar ketidaktahanan terhadap sesetengah antibiotik dan pengurangan kadar pertumbuhan sebanyak 40–60%. Daya ketahanan pokok tomato juga bertambah setelah diinokulat dengan *R. solanacearum* yang dijangkiti oleh faj.

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