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CHARACTERIZATION OF BACTERIAL SOFT ROT STRAINS AND THEIR SPECIFIC PHAGES ISOLATED FROM SOIL AT TAIF

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ABSTRACT

In this study some bacteriophage(s) specific to some plant pathogenic bacteria (the causal agent of soft rot diseases), were isolated and characterized from soil of Taif, KSA. Soil samples were randomly collected from some rhizosphere soils cultivated with various plant species including potato. Ten bacterial isolates were obtained from soil and used as hosts for enrichment and isolation of the virulent bacteriophages. The spot test and turbidity tests were used to detect the presence of the phage of interest in the suspension. The area of clear zones were represented by threes levels, *i.e.*, weak lysis (+) (three isolates), moderate lysis (++) (one isolate) and high lysis (+++) (two isolates). The phage(s) was propagated and partially purified for determining the morphology of viral particles via electron microscopy. Sperm shape virus-like particles with long tail and icosahedral head were shown in the electron micrographs of partially purified phages specific to the two selected bacterial isolates (# 08 and # 10). These two bacterial isolates were then biologically and molecularly identified. The nucleotide sequences of 16S rRNA gene of the two bacterial isolates was determined and final sequences of 942 and 940 nts for the 16S rRNA gene of two soft rot bacteria (# 08 and # 10), respectively were recorded. Data of the phylogentic trees show that the two bacterial isolates (# 08 and # 10) could be strains of *Pseudomonas stutzeri* (LC053456.1) and *Bacillus pumilus* (LC053854.1).

Keywords: Bacteriophage, soft rot, spot test and turbidity tests, electron microscopy, 16S rRNA gene.

INTRODUCTION

Soft rot disease is major cause of several economically important losses of growing plants, harvesting crops and also affects succulent leaves of vegetables. Bacterial soft rots are most commonly caused by species of Gram-negative bacteria, such as *Erwinia*, *Pectobacterium* and *Pseudomonas* (Elphinestone 1987; Simth *et al*., 1994 and Agrios, 2005).

The bacteriophage is a virus that infects and replicates within a bacterium (Othman 1997; Othman *et al*., 2008 and Sadik *et al*., 2014). Bacteriophages are composed of proteins that encapsulate a DNA or RNA genome and replicate within the bacterium following the injection of their genome into its cytoplasm. Bacteriophages are among the most common and diverse entities in the biosphere (McGarth and Sinderen 2007).

Phages are widely distributed in locations populated by bacterial hosts, such as soil bacteriophages (phages) that have been proposed as potential biological control agents against plant pathogenic bacteria. They have been evaluated for different pathogens e.g. *Erwinia amylovora*, *Xanthomona spruni*, *Pseudomonas tolaasii*, *Streptomyces scabies* and *Ralstonia solanacearum* (Eayre *et al*., 1995 and Jones *et al*., 2008). Phages were also experimentally tested to control *Pectobacterium* spp. (Czajkowski *et al*., 2013) and *Dickeya* spp. (Czajkowski *et al*., 2013) in potato and other crops with relative success but only limited attempts have been made to characterize these lytic bacteriophages.

In Saudi Arabia, Sadik *et al*. (2014) isolated and characterized some bacteriophages from some rhizosphere soil samples collected from Taif region. The presence of bacterial viruses using plaque-forming assay (spot test technique) was confirmed in the tested soil samples. In Europe, most direct losses to potato production caused by *Pectobacterium* and *Dickeya* (Adriaenssens *et al*., 2012). Phages have a unique potential which may lead to new types of applications and allow phage therapy to occupy specific niches in the pesticide market.

Therefore, this study was designed to isolate, purify and identify on molecular basis of a plant pathogenic bacterium from soft rot-diseased plant and soil from Taif region. The presence of bacteriophage(s) specific to the identified bacterial strain(s) and their electron microscopy was also studied.

MATERIALS AND METHODS

Source of samples and their physical and microbiological analyses: Some soil samples were randomly collected from some rhizosphere

soils cultivated with various plant species including potato from Taif area. The mixture of soil samples was prepared as described by Mohamed (1998). Physical properties, *i.e.*, parent material, presence of salt or alkali, calcium carbonate content, pH, level of elements (cations), electrical conductivity (EC) and texture of the soil were determined in the Central Laboratory, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. A number of potato tubers exhibited soft rot were gathered from the vegetable market in Al-Haweiah suburb, Taif, KSA. The total bacterial count was determined using the plate count method as described by Jacobs and Gerstein (1960). The most common bacteria that found in the soil as well as the soft rot-diseased potato tubers were isolated and maintained on slants of nutrient agar medium maintained at 4-5°C until used (Mohamed 1998).

Phage isolation, detection and propagation: The method of Stenholm *et al*. (2008) was carried out for preparation of phage suspension from collected soil samples. The plaque (bacterial-free area) was used as indicator for the presence of the phage of interest in the phage suspension. In case of turbidity test, a volume of 1.00 mL of the enriched phage solution was added to 9.00 mL of nutrient broth medium inoculated with the culture of the bacterial isolates (# 8) or (# 10). Tubes of nutrient broth medium were inoculated with the culture of the bacterial strains used as control. On incubation at 37°C for 48-72 h, the turbidity was visualized by light compared to the control one (Which contain no phage) (Dr. Othman B.A., personal communication). The method of Carey-Smith *et al*., (2006) and modified by Othamn (1997) was applied for virus propagation.

Phage purification and its electron microscopy: Dextran sulfate-polyethylene glycol two phase liquid system was applied as described by Othman (1997). The phage suspension was used negatively stained as described by Nugent and Cole (1977). Photographs were taken with a Model Beckman 1010 transmission electron microscope at the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Molecular characterization of bacterial isolates: Two copies of each of the selected *Pseudomonas* and *Bacillus* isolates were sent to Macrogen Inc in Korea for molecular identification by determining the nucleotide sequences of 16S rRNA gene. Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (App-lied Biosystems, Foster City, CA). Nucleotide sequences were determined on both strands of PCR amplification products at the Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). NCBI blast tool was used for blast search result. The DNA sequences were analyzed using BLAS TN 2.2.23 + software (http://www.ncbi.nlm. nih. gov/blast) against the strains and/or isolates collected from the database for genotyping.

Phage stability: A serial two fold dilutions (½, ¼, 1/8, 1/16 and 1/32) of enriched phage solutions were prepared using sterilized water (d.H_2O). The phage was detected by spot test technique as above mentioned. A number of five fresh tubes containing 1.00 mL of the enriched phage solution for each were prepared. All tubes were stored at 4°C in a refrigerator. The phage was detected after 0, 2, 4, 6, 8, 10, 12, 14 and 16 weeks from storage at 4°C by spot test technique.

RESULTS AND DISCUSSION

We are herein focusing on isolation and identification of some bacterial soft rots causal agents and their phages. *Pectobacterium carotovorum* or *Erwinia carotovora*, *Dickeya dadantii* or *Erwinia chrysanthemi*, and certain species of *Pseudomonas*, *Bacillus* and *Clostridium* were most commonly recorded as soft rots causal agents in different crops (Adriaenssens *et al*., 2012; Czajkowski *et al*., 2013 and Lunt 2013).

In this study, the physical properties of collected soil samples were: sandy loam or loamy sand textures, alkaline, and EC values ranged from 1.30 to 1.70 ds/m. At the level of microbiological analysis, the bacterial total count was ranged from $2.5X10^3$ to $4X10^4$-cfu/mL and a number of 10 bacterial isolates were selected, purified and separately used as hosts for bacteriophage(s) isolation.

Bacteriophages were often considered alternative agent for controlling bacterial infection and contamination (Yang *et al*., 2010), and soil phages were suggested as potential biological control agents against plant pathogenic bacteria (Eayre *et al*., 1995; Calendar and Abedon 2005; Jones *et al*., 2008; Adriaenssens *et al*., 2012 and Czajkowski *et al*., 2013). Therefore, the presence of bacteriophages in soil specific to some plant pathogenic bacteria isolated from soil and/or soft rot-diseased plant was studied. Spot test technique was successfully used for detection of the presences of bacteriophages in phage suspensions prepared from soil. This was based on phage ability to form plaques on the 10 indicator bacterial hosts isolated

from soil and soft rot-diseased plant of Taif (Figure-1). These results are in harmony with that reported by Othman (1997); Othman *et al*. (2008); Lee *et al*. (2011); Delfan *et al*. (2012); Sadik *et al*. (2014) and Bhunchoth *et al*. (2015). Turbidity test was confirmed the specificity of the phages to the two bacterial isolates (# 08 and # 10) as clear lysis tube was observed compared to bacterial culture un-inoculated with phage suspension as a control (Figure-2). The plaques on nutrient agar medium showed three levels of lysis, *i.e.*, weak (+), moderate lysis (++) and high lysis (+++). Our results are in agreement with that found by Eman and Afaf (2014) who showed different phages specific to *Pseudomonas syringae* pv. phaseolicola from infected bean leaves growing in four localities and were isolated. By using enrichment technique and produced plaques with 3 to 5 mm diameter and a distinct translucent spreading halo.

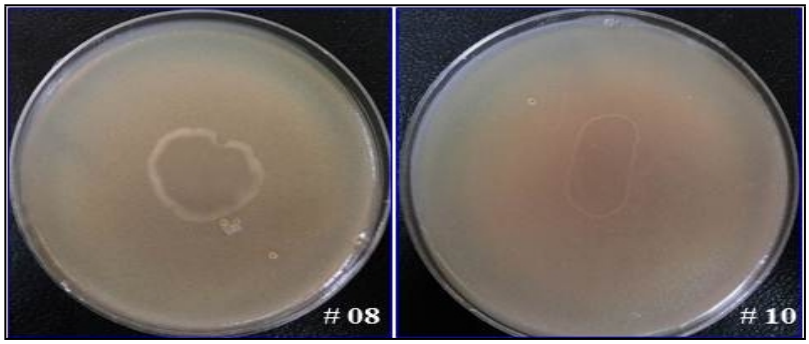

Figure -1: Spot test technique shows the presence of phage specific to soft rot bacteria (# 08 and # 10) isolated from soft rot-diseased tuber potato and soil.

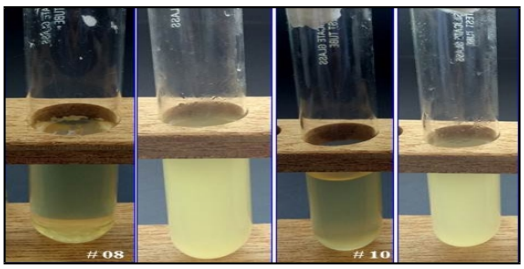

Figure-2: Turbidity test used for detection of phage specific to soft rot bacteria (# 08 and # 10) isolated from soft rot-diseased tuber potato and soil.

Electron microscopy of phage suspensions of the two selected bacterial isolates (# 08 and #10) showed the presence of sperm shape virus-like particles with long tail and icosahedral head were shown in (Figure-3). Similar results were reported by some investigators Keel *et al*. (2002) and showed the Phi GP100 phage with an icosahedral head, and a stubby tail. Analysis of morphology of phage specific to *Pseudomonas tolaasii* with an electron microscope revealed that phi Pto-bp6g contains an icosahedral head and a long tail, which was classified as the family of Siphoviridae (Thi *et al*., 2012). Transmi ssion electron microscopy examination showed that the purified phage specific to *Bacillus subtilis* was a tailed, isometric shape and the diameter was 59 nm (Marie 2013).

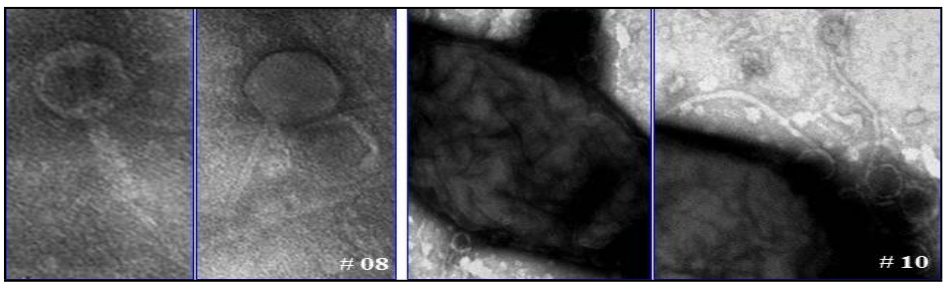

Figure-3: Electron micrograph of phage specific to soft rot bacteria (# 08 and # 10) isolated from soft rot-diseased tuber potato and soil.

The selected bacterial isolates (# 08 and # 10) were microbiologically identified as short rods (Gram negative bacterium) and bacilli (Gram positive bacterium) respectively (Fig

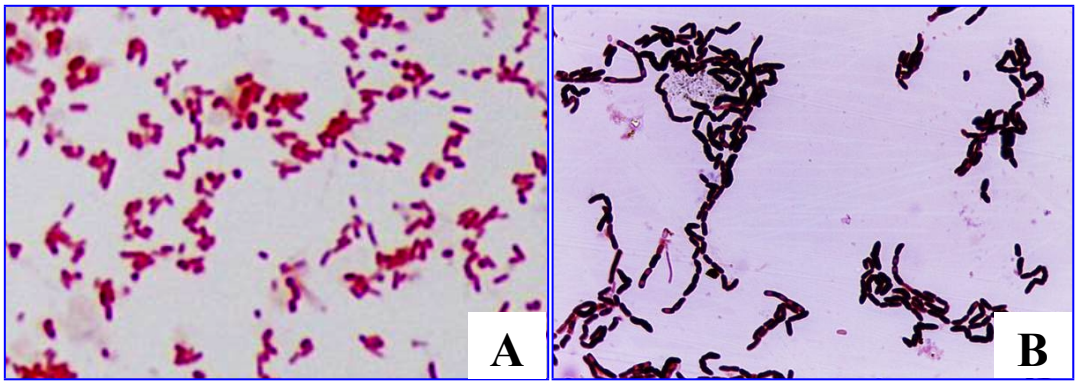

Figure-4: A: Gram negative soft rot bacterium (# 08) isolated from soft rot-diseased tuber potato of Taif region. B: Gram positive soft rot bacterium (# 10) isolated from soil.

In this study, the nucleotide sequences of 16S rRNA gene of the two bacterial isolates (# 08 and # 10) was determined and final sequences of 942 and 940 nts for the 16S rRNA gene of two soft rot bacteria ((# 08 and # 10), respectively, were recorded. The 16S rRNA gene was used as the standard for classification and identification of microbes because it is present in most microbes and shows proper changes (Bottger 1989; Palys *et al*., 1997; Kol-bert and Persing 1999 and Harmsen and Karch 2004). On sequences producing significant alignments between the query sequences of the two isolates and those related strains recorded in GenBank, the two bacterial isolates ((# 08 and # 10) could be strains of *Pseudomonas stutzeri* (LC053456.1) and *Bacillus pumilus* (LC053854.1), respectively (Fig

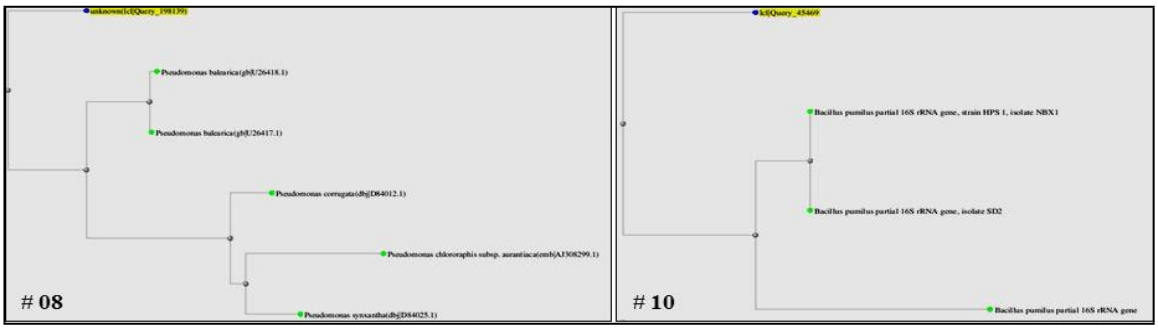

Figure-5: Phylogenetic tree shows the taxonomy of partial nucleotide sequence of 16S rRNA gene of soft rot bacteria (# 08 and # 10) isolated from soft rot-diseased tuber potato and soil, respectively, of Taif region and some bacterial soft rot strains recorded in GenBank.

Using both of dilution end point (DEP) and longevity at 4°C the phage stability was studied. Results proved the presence of phage specific to both of *Pseudomonas stutzeri* (isolate # 08) and *Bacillus pumilus* (isolate # 10) strains in the two fold dilutions, *i.e.*, ½, ¼, 1/8, 1/16 and 1/32 of their enriched phage solutions (Figure-6). *In vitro* determination of thermal inactivation point (TIP), DEP and longevity in vitro (LIV) of phage specific to *Bacillus subtilis* were found to be 68 degrees C/10 min, 10.4 and 96 hrs, respectively (Marie 2013). The phages were detected 0, 2, 4, 6, 8, 10, 12, 14 and 16 weeks post storage at 4°C, this was obvious from the clear zones of spot tests for each period (Figure-6). Zain and Al-Othman (2013) showed that the activity of phage-resistant *Bacillus* strains was not affected when stored in refrigerator or freezer up to 35 and 30 days, respectively. However, there was no activity when phages were stored in refrigerator or freezer for 90 and 150 days. On the other hand, phage activity was not affected by temperature up to 50°C; however, the activity was lost at 60°C.

Dilutions	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**
Spot test	+	+	+	+	+

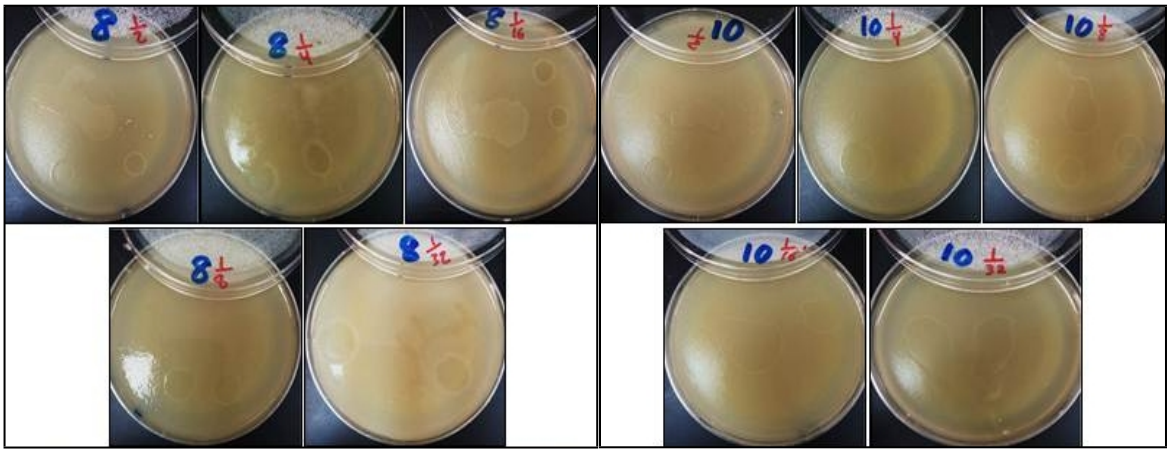

Figure-6: Dilution end point of phage suspension specific to soft rot bacteria (# 08 and #10) isolated from soft rot-diseased tuber potato and soil

CONCLUSION

Bacteriophage(s) specific to plant pathogenic bacteria (the causal agent of soft rot diseases) were isolated from soil and morphologically characterized. Sperm shape virus-like particles with long tail and icosahedral head were shown in the electron micrographs of partially purified phages specific to the two selected bacterial strains. Based on the nucleotide sequences of 16S rRNA gene the two isolates were identified as strains of *Pseudomonas stutzeri* and *Bacillus pumilus* and submitted in GenBank with accession numbers of LC053456.1 and LC053854.1, respectively.

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