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Biocontrol of Halo Blight of Bean Caused by *Pseudomonas phaseolicola*

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ABSTRACT

Different phages parasiting *Pseudomonas syringae* pv. *phaseolicola* were isolated from infected bean leaves growing in four localities using enrichment technique. The isolated phages produced plaques with 3 to 5 mm, diameter and a distinct translucent spreading halo. Presumptive phage particles associated with *P. syringae* pv. *phaseolicola* were observed by Transmission Electron Microscope (TEM). Bioagent and garlic extract were inhibited *P. syringae* pv. *phaseolicola in vitro*. Halo blight disease severity was determined under greenhouse condition using the effective phage isolates. The effect of phage isolates (namely Ph1, Ph2 and ph1+2) were reduced disease severity by 58.57-61.14 and 70.8%, respectively compared with *P. fluorescence* treatment (35.82%); *P. putidae* treatment (22.17%) and Garlic extract (30.54). These phages may be useful as a tool to efficient detection and control of halo blight pathogen. Mixed phages (1+2) was more effective than single phage treatments on all disease characteristics. Although all phages that used as single or mixed were more effective to reduce disease incidence when compared with control. These results indicated that these phages may offer a new possibility for the identification and diagnosis of *P. syringae* pv. *Phaseolicola* strains from bean plant.

Key words: *Pseudomonas syringae* pv. *phaseolicola*, bean, halo blight, TEM

INTRODUCTION

Bacterial halo blight of beans (*phaseolus valgarius* L.), caused by *P. syringae* pv. *phaseolicola* (Saettler, 1991) has been a considerable problem in Egypt, especially in beans field, since the importation of infected seed in the early 1960s. The pathogen attacks both foliage and pods. The symptoms of the disease in the field can show considerable variation at The most destructive where temperatures are moderate and abundant inoculum is available.

Biological control by antagonistic microorganisms is a well developed concept, mainly for soil-borne pathogens (Kloepper, 1993). A strain of *P. putida* isolated from pepper fruits was able to inhibit *P. syringae* pv. *Phaseolicola in vitro* studies (Liao, 1989). The potential for garlic extract to inhibit growth of *P. syringae* pv. *phaseolicola* (Burk.), as an input in the management of the halo blight disease (Satya *et al*., 2005).

Bacteriophages have been used in identification of bacteria, strain characterization, epidemiological studies and even in attempts which have been made in biological control studies (Fahy and Persley, 1983).

This study was aimed to use safety alternative control to halo blight of beans under Egyptian conditions.

MATERIALS AND METHODS

Isolation and identification of bacterial isolates: Five bacterial isolates from infected bean leaves (*phaseolus valgarius*) with halo blight were isolated from Qaha and FAC. of Agric. Ain Shams Univ., Qalubia Government. Isolation and identification processes were carried out as Fourie (1998) described.

Isolation and purification of bacteriophage: The leaves of bean with halo blight symptoms were cut to small pieces and crushed in Sterilized Distilled Water (SDW) plus sterilized tween 80 (2-3 drops flask^{-1}) and shaked on a rotary shaker for 72 h at 28°C at 3000 rpm for 20 min. The enrichment culture technique was used to isolation of phage according to Eayre *et al.* (1995).

After shaking, the cultures were centrifuged at 10000 rpm for 10 min. A double layer method was routinely used for plaque isolate, where 3 mL of phage supernatant was mixed with 3 mL of 48 h old broth culture of *Pseudomonas syringae* pv. *Phaseolicola* and 0.7% soft agar. These mixtures were overlaid on the basal medium (peptone-sucrose-agar) according to Myung *et al.* (2002).

The phages presumptively responsible for the plaques were purified by three cycles of single plaque isolation to obtain a single purified plaque lysate type. Purified phage was stored in 2 mL plastic vials at 4°C in complete darkness.

Bacteriophages plaque assay: The pure culture of *P. syringae* pv. *Phaseolicola* was prepared on nutrient agar plates. After 24 h, 10 μL of filtrated bacteriophage isolate was added to culture plate and incubated at 28°C for 24 h (Tanaka *et al.*, 1990).

Phage host specificity: Bacteriophage isolates of *P. syringae* pv. *Phaseolicola* were tested against 10 bacterial isolates belong to *P. fluorescence, P. putidae* and *Xanthomona vesicatoria* isolates representing three genera and five species were obtained from the stock culture collection maintained in Department of Plant Pathology, Faculty of Agriculture, Ain Shams University.

Tested bacteria were suspended in SDW to a density of about 1×10^7 colony forming unit (cfu)/mL. Aliquot (0.5 mL) of mixture with 2 mL of melted yeast extract peptone agar (0.8 agar) and then overlaid on plates of yeast extract-peptone agar as previously described 20 μL drops of phage suspension were placed on the agar over layers and the plates were incubated at 28°C overnight. Clear confluent lysis, turbid confluent lysis or individual plaques were recorded as positive reactions, while extremely faint zones were considered negative (Eayre *et al.*, 1995).

TEM examination: Transmission Electron Microscope (TEM) was used to determine phages typing of *P. syringae* pv. *Phaseolicola*. This study was carried out at the electron microscopy laboratory of the microbiology and cell science department, faculty of science, Al Zaher University, Cairo, Egypt.

The phages were visualized using negative staining method with 1% aqueous uranyl acetate. Where, a drop of the phage suspension was added to a 300-mesh copper grid and the droplet was partially wicked off using a triangle-shaped piece of filter paper. The remaining thin layer of liquid was left on the grid.

The grids were air dried and examined by transmission electron microscope (Sharma *et al.*, 2008).

Bacteriophage infectivity: The tested bacteria were suspended in Sterile Distilled Water (SDW) to a density of 10^7 cfu mL^{-1} and then overlaid on plates of yeast extract-peptone agar as previously described. The 20 µL drops of each phage isolate suspensions were placed on the agar over layers and the plates were incubated at 28°C overnight. Clear confluent lysis, turbid confluent lysis or individual plaques were recorded as positive reactions, while extremely faint zones were considered negative (Eayre *et al.*, 1995).

***In vitro* assay**

Garlic extract: Fresh garlic cloves (*Allium sativum*) about 50 g were washed under SDW and blotted with paper towels. Samples were then sliced into small pieces and blended using a twister blender (Hamilton Beach, 16-speed Turbo Twister blender) for 10 min at room temperature. The extract was obtained by centrifuging samples (Sorvall RC5B centrifuge, Newton, CT) at 8000×g for 45 min. The supernatant was collected and sterilized using a Nalgene filter (diameter 0.42 mm, Fisher Scientific). The extract was then kept at 4°C until use within 24 h. As final concentration, after preliminary tests according to Balestra *et al.* (2009). Four concentrations of garlic extract were tested against pathogenic bacteria. Nutrient glucose agar medium was inoculated by pathogenic bacteria (1×10^8 cfu mL^{-1}) at rate 0.1 mL 100 mL^{-1} medium. Saturated filter paper disks (5 µL) of each concentrate (2.5, 5, 10, 15%) of garlic extract was placed on the surface of inoculated plates. Disks saturated with sterilized water only were used as a control. Four disks/plate and 3 plates for each treatment were done then the plates were incubated at 28°C for 72 h. Effect of each concentrate of garlic extract was measured in form of inhibition zone surrounding the disks according to EL-Mahmood (2009).

Bioagents: Two isolates of *P. fluorescence* and *P. putidae* were tested against pathogenic bacteria. Nutrient agar medium was inoculated by pathogenic bacteria (1×10^8 cfu mL^{-1}) at rate 0.1 mL 100 mL^{-1} medium. A loopfull of bioagents (24 h old cultures) was inoculated at the center of each plate. Plates without inoculation by bioageants were used as a control. Four replicates for each bioagent isolates were used then the plates were incubated at 28°C for 72 h. Diameter of inhibition zone was measured according to Boudyach *et al.* (2001).

***In vivo* assay**

Plant growth and inoculation: Germinated seeds of beans were planted in 10 cm diameter plastic pots in sterile soil and maintained in a greenhouse (Fourie, 1998). Two seeds were planted per pot and 10 pots for each treatment.

P. syringae pv. *phaseolicola* isolates were grown on King's B agar medium for 24 h at 28°C. Inoculum was prepared by suspending culture in sterile distilled water and adjusting it by spectrophotometer to contain approximately 10^8 cfu mL^{-1}. Seedlings, 7-10 days old, with fully expanded primary leaves were used for inoculation. The bacterial suspension was sprayed onto the abaxial surface of the leaves using atomizer until completely wet. Control plants were inoculated with sterile distilled water. Inoculated plants were kept in a humidity chamber for 48 h (Fourie, 1998; Taylor *et al.*, 1996; Fett and Dunn, 1989).

Phage and bioagent treatments: High titer suspensions of phage were prepared by methods described by Tanaka *et al.* (1990). The indicator virulent isolate of *P. syringae* pv. *Phaseolicola* mixed with the phage was grown in sucrose pepton broth on a rotary shaker (125 rpm) at 28°C for 72 h. The infected *P. syringae* pv. *Phaseolicola* culture was centrifuged at 10.000 rpm for 15 min to remove the bacterial cells and debris then the supernatant was filtrated via 0.22 μm membrane. The filtrate was diluted ten-fold dilutions with sterilized water (10^7 plaque forming unites (pfu).

Two isolates of bacteriophage were selected to determine disease severity of halo blight of beans. A liquid culture of phages was amended with 3% corn flour+5% sucrose. Bean seedlings were sprayed with phage isolates either individual or mixed before inoculation with *P. syringae phaseolicola* (Balogh *et al.*, 2003).

Bioagent treatment: Two previous bioagents *P. fluorescence* and *P. putidae* isolates were grown on Yeast extract Peptone Dextrose Agar (YPDA) medium for 48 h at 28°C. The bacterial cells were suspended in sterile distilled water and centrifuged at 3000 rpm for 30 min. The pellet was re-suspended in distilled water and adjusted to the denisity of 10^8 cfu mL^{-1} tested Bioagents were used as soil drench. The seedlings were treated with bioagents (20 mL $seedling^{-1}$) one weak before and after inoculation. water was used as control treatment. Treated plants were placed in humidity chamber for 48 h before and after inoculation (Abd El-Ghafar and Mosa, 2001).

Garlic extract treatment: Extract of garlic cloves was sprayed at 20%, which inhibited *P. syringae phaseolicola in vitro*. Spraying was applied two days before inoculation by pathogenic bacterium (EL-Mahmood, 2009).

Plants were rated for infection 10 days after inoculation on a 1-5 scale, with 1 being highly resistant and 5 being highly susceptible (Fourie, 1998).

RESULTS

The morphological and biochemical tests confirmed that two isolates from the isolated bacteria were identified as *P. syringae phaseolicola*. These isolates tested positive for levan, negative for the oxidase test and no utilization for mannitol, sorbitol and inositol as sole carbon sources. The two isolates were produced a brown diffusible pigment on King's B medium.

The two phages specific for *P. syringae phaseolicola* were isolated and identified which showed complete lyses of bacterial cells.

Bacteriophage stained by 2% uranyl acetate in pH 4-4.5 was observed by transmission electron microscopy. The first phage isolate was characterized a cubic nucleocapsid, about 74 nm across with short tail in its structure (Fig. 1a).

The second phage isolate was characterized by a hexagonal nucleocapsid, about 72 nm across, without tail (Fig. 1b).

The two phages produced different types of plaques. The first type produced a plaques circular, clear center (3 mm in diameter) with a distinct translucent spreading halo.

The second type produced a plaques circular, clear center (5 mm in diameter) with a distinct translucent spreading halo. One plaque of each phage isolates was picked up and used for preparing purified phage lysate. The purified phage lysate was propagated to obtain large scale production specific for *P. syringae* pv. *Phaseolicola*.

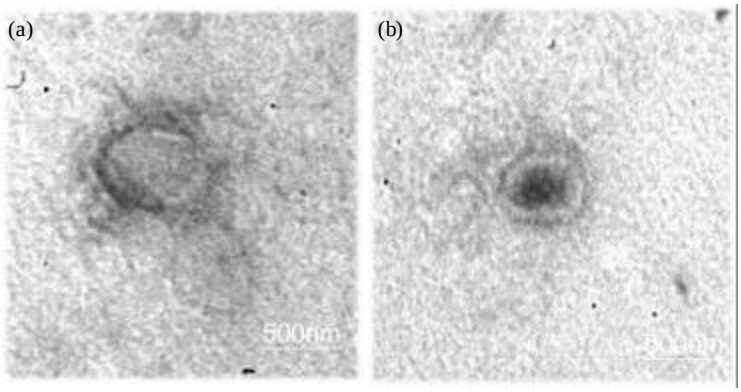

Fig. 1: Electron micrograph of *P. syringae* pv. *Phaseolicola* bacteriophage

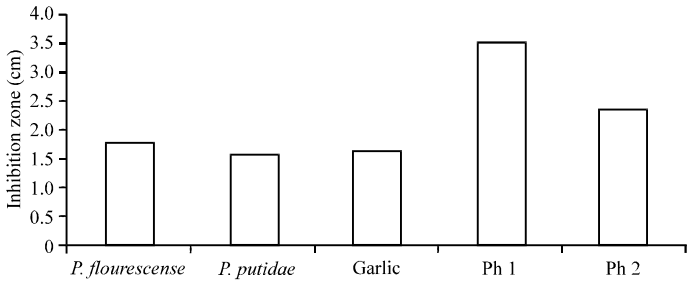

Fig. 2: Histogram showing the inhibition zone of *P. syringae* pv. *Phaseolicola* treated with different bacterial bioagent and bacteriophage

The bacteriophage of *P. syringae* pv. *Phaseolicola* showed distinct host specificity when tested with 10 isolates of different phytopathogenic bacteria. Only one isolate of *P. syringae* pv. *Phaseolicola* out of five isolates from been was lysed when tested with the phages, while other tested bacterial isolates did not develop plaques.

Extract of garlic cloves (*Allium sativum*); *p. fluorescence; p. putidae*; phage 1 (ph1) and phage 1 (ph2) were tested for its effect on growth of two isolates of *P. syringae* pv. *phaseolicola*. Figure 2 showed that, all of these treatments had inhibitory effect on growth of the pathogenic bacteria compared with the control.

Spraying of bean plants with phage before inoculation with bacterial pathogen showed significant reduction of disease severity either in individual or mixing treatment. Treating with phage 1 , phage 2 and phage 1+2 before inoculation with *P. syringae* isolate (1) Reduced disease index to be 35.5, 33.3 and 25%, respectively compared with control (DI = 85.7%). While disease index with garlic extract was, *P. fluorescence* and *P. putidae* treatment was 45.2, 55 and 66.7%, respectively. On the other hand, spraing with phage 1, phage 2 and phage 1+2 before inoculation with *P. syringae* isolate (2) Reduced disease index to be 42.1, 45.3 and 33.3%, respectively compared with control (DI = 73.2%). While disease index with garlic extract was, *P. fluorescence* and *P. putidae* treatment was 47.5, 52.7 and 57.7%, respectively (Table 1, Fig. 3).

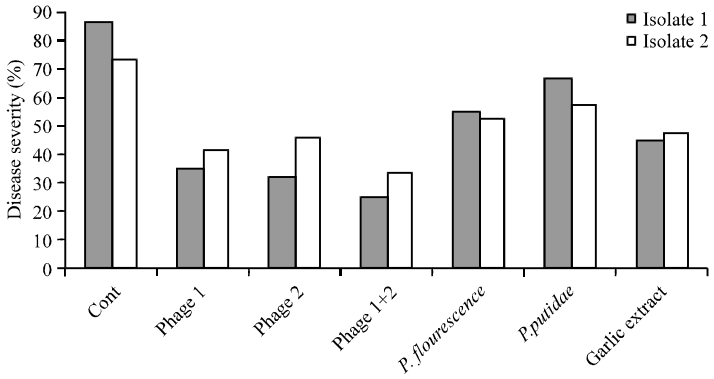

Fig. 3: Effect of phage treatments on disease severity of been halo blight compared with different bioagents

Table 1: Disease severity of halo blight of beans after spraying by different treatments before bacterial inoculation

	Isolate 1		Isolate 2	
	DI	Mean No. of spots leaf^{-1}	DI	Mean No. of spots leaf^{-1}
Cont	85.7	5.47	73.2	4.9
Phage 1	35.5	1.50	42.1	2.1
Phage 2	33.3	1.10	45.3	2.5
Phage 1+2	25.0	0.50	33.3	1.1
P. flourescence	55.0	2.78	52.7	2.3
P. putidae	66.7	3.40	57.7	3.3
Garlic extract	45.2	2.00	47.5	2.5

DI: Disease index

DISCUSSION

In this study *P. syringae* pv. *phaseolicola* was isolated from infected bean plants. Biochemical tests indicated that isolated bacteria was attributed to *Pseudomonadales* family. Morphology of colony was similar to the standard isolates of *P. syringae* pv. *phaseolicola*. Lee and Yen were studied the effect of bacteriophage on *P. syringae* pv. *Phaseolicola* in oil enhanced recovery. This bacteriophage exhibited a regular hexagonal outline, about 50 μm across and a short, wedge-shaped tail attached to a corner corresponded to the morphology and size of bacteriophage head and non-contracted tail.

In this study, the isolated bacteriophage was characterized, about 74 nm across with short tail. the phage was associated with Cystoviridae family, morphologically. In previous studies, two types of bacteriophages from Podoviridae and Myoviridae families were reported for *P. syringae* pv. *Phaseolicola* but not from Cystoviridae family. Based on international committee on taxonomy of viruses (ICTV) only one bacteriophage (named Phage f6) was reported to act against *Pseudomonas syringae*, but there was no report about *P. syringae* pv. *Phaseolicola*.

Our experiments demonstrated that the glycine buffer is a suitable medium to preserve bacteriophages in refrigerator without any effect on the population. we suggest that phages isolated from natural habitat of plants against infectious agents might be employed to control plant diseases in the same area. Experiments to determine the effectiveness of phage against potato infections should be performed in order to develop a phage control treatment for the disease.

One of the greatest challenges in using bacteriophages for plant disease control is their extremely short residual activity in the phyllosphere. Several studies indicated that phage populations can drop to undetected levels hours after applications (Balogh, 2002; Iriarte *et al*., 2007).

On the other hand, *Pseudomonads* possess many traits that make them well suited as biocontrol and growth-promoting agents. These include the ability to: (1) Grow rapidly in vitro and to be mass produced (2) Rapidly utilize seed and root exudates (3) Colonize and multiply in the interior of the plant (4) Produce a wide spectrum of bioactive metabolites (i.e., antibiotics, siderophores, volatiles and growth-promoting substances) (5) Compete aggressively with other microorganisms and (6) Adapt to environmental stresses (Weller *et al*., 2002).

P. fluorescens and *P. putida* usually have antagonistic effect against *Fusarium* and *Ralstonia* (Chang and Yen, 1985).

The major weakness of *Pseudomonades* as biocontrol agents is their inability to produce resting spores (as do many *Bacillus* spp.), which complicates formulation of the bacteria for commercial use (Weller, 2007).

Previous studies reported that plant extracts and essential oils show antimicrobial activity against a wide range of pathogens. Especially garlic extracts contain a potent fungicide (Wilson *et al*., 1997).

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