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Disease control of *Ralstonia solanacaerum* in tomato and *Xanthomonas campestris* in pitaya using bacteriophage

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Research Paper

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Bacterial outbreaks are generally problematic to be controlled due to lack of effective bactericides and resistance development from the pathogen itself. Bacteriophages have recently been evaluated for controlling a number of phytobacteria. The efficacy test of phage incorporated with All Cosmos Industries (ACI) fertilizer for controlling tomato bacterial wilt and pitaya (dragon fruit) soft rot disease were determined. In tomato study, after two seasons of application, results indicated that 80 % of plants showed no wilting symptom was occurred. The vegetative growth of plants increased for both seasons. The survival rate of phage-cocktail in ACI fertilizer was determined and they can be detected after two months of incorporated process (7-Log PFU/ml). In pitaya study, application of phages managed to control disease spreading which caused the plants to be sustained. Treatment T1 had the highest shoot number (25.8), fruit number (0.4) and shoot length increment (12 cm) after one month of application. After four months, results showed that T1 had the highest shoot number per plant (19 cm) and shoot length increment (3.4 cm) compared to all treatments. However, there was no significant on reproductive parameters been observed. T1 performed better than Treatment T3 by 1½ and 0.7 folds in shoot number and shoot length increment, respectively. It was noted that T2 also showed some positive impacts on vegetative growth.

Key words: Bacterial wilt disease, *Ralstonia solanacearum*, Bacterial soft rot disease, *Xanthomonas campestris*

INTRODUCTION

Plant pathogens are responsible for the major economic loses in agriculture (Agrios, 2004). The failure of bacterial disease management in agriculture usually caused by several factors, such as lack of effective bacteriacides against the diseases; high pathogen variability; rapid population build-up under optimal conditions; mutation rates resulting resistance developing and high mutation rates also resulting in bacteria overcoming (Balogh *et al*., 2010). Copper products and certain antibiotics have been used for more than a century to overcome the disease problems in agriculture. However, continuous copper usage poses environmental hazards due to build-up to toxic levels in the soils (Koller, 1999) and led to emergence of resistant strains and loss control in several patho-systems (Cooksey, 1990; Thayer and Stall, 1961; Manulis *et al*., 1998; Louws *et al*., 2001).

Bacteriophages were first found in association with plant pathogenic bacteria when Mallman and Hemstreet in 1924 demonstrated that the filtrate of decomposed cabbage inhibited growth of the “cabbage-rot organism” *Xanthomonas campestris pv. campestris* (Balogh *et al*., 2008). Phage therapy has been found to be effective for the control of a number of phytobacteria (*Xanthomonas sp* (Balogh *et al*., 2008; Civerolo and Kiel, 1969; Saccardi *et al*., 1993; Flaherty *et al*., 2000;McNeil *et al*., 2001); Pseudomonas sp (Munsch and Olivier, 1995); *Erwinia sp* (Ravensdale *et al*., 2007; Svircev et al., 2005; Schnabel *et al*., 2010); *Ralstonia* (Tanaka *et al*., 1990; Tan *et al*., 2010) and Streptomyces (McKenna *et al*., 2001) since 1990.

Ralstonia solanacearum is one of the most devastating tomato diseases in lowland Malaysia. 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 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). Another disease which is common is bacterial soft rot disease, *Xanthomonas campestris* which cause problem in Pitaya (Hylocereussp). Due to environmental concern, phage therapy was exploited as alternatives to chemical antibacterials and would allow for targeting a broader range of plant diseases. Thus, the objective of this study is to use incorporated bacteriophage with fertilizer for controlling bacterial wilt disease in tomato and bacterial soft rot in pitaya, and examine the efficacy of this product in the field.

MATERIALS AND METHODS

Isolation and purification of phages

Sewage and soil samples were collected from Indah Water Konsortium Sdn. Bhd., Puchong (latitude: 2.9937°; longitude: 101.6000°; temperature: 26 ℃; humidity: 89%), Cameron Highland (latitude: 4.5000°; longitude: 101.5000°; temperature: 18 ℃; humidity: 84%), Port Dickson (latitude: 2.5364°; longitude: 101.8000°; temperature: 30 ℃; humidity: 70%) and Serdang (latitude: 3.0231°; longitude: 101.7000; temperature: 26 ℃; humidity: 89%). Three different types of *Escherichia coli* strains [TG1 (supE, hsdΔ5, thiΔ(lac-pro AB), F'[traD36, proABΔ, lacIq, LacZ, ΔM15]), ER2738 (FΔ pro A+B+ lac1qΔ (lacz) m15 zzf::Tn10 (TetR)/fhuAzgln vΔ (lac-proAB) thi-1 Δ (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 ℃ 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 ℃ for 1 h to overnight and centrifuged at 13,000 rpm for 30 min at 4 ℃. Finally, phage pellet was resuspended in TBS (50 mMTris; 150 mMNaCl, pH 7.5).

Biomass production of phages

All the chemicals used in this study were of analytical grade and were purchased from BDH Chemicals Ltd., Amresco, Genaxis GIBCO BRL, Merck, Promega, Fermentas Inc., Amersham Pharmacia and Sigma Chemicals. The phages were propagated in LB broth (5 Liter), precipitated with PEG/NaCl, and finally purified through cesium chloride density gradient centrifugation according to (Smith and Scott,1993). The incorporated phages with ACI fertilizer were incubation from two weeks to eight months. The titer of phage was determined using plaque assay method (Smith and Scott, 1993; Tan *et al*., 2009).

Efficiency study of incorporated-phages with ACI fertilizer on tomato

This experiment was carried out using peat soil, in the pot, under control environment (temperature: 33 ℃, humidity: 83%). The four weeks old plants were challenged with pathogen (*R. solanacearum*, 10-Log CFU/ml). Three treatments were applied on the plants, as follow: Treatment 1 (T1): ACI fertilizer + phages cocktail (6-9 Log PFU/ml); Treatment 2 (T2): ACI fertilizer; Treatment 3 (T3): Commercial fertilizer with standard formulation of NPK. Each of the plot size is 5 plants/ treatment/ replicate. Fertilizer application was done in accordance with the schedule/ fertilization program from All Cosmos Bio-tech Holding Corporation. Data collection was based on soil analysis, vegetative growth, soils and root microbial population before and after applying the phage-fertilizer, disease scoring (at stage before phages-fertilizer application, 1 month and 3 months phage-fertilizer after application).

Efficiency study of incorporated-phages with ACI fertilizer on dragon fruit

The pitaya study was carried out on the Bris soil, Siri Baging, open field planting at Station MARDI Sg Baging, Pahang (latitude: 4.0793°; longitude: 103.3807°; temperature: 27 ℃; humidity: 89%). Five treatments were applied on the plants, as follow:

Table 1. Survival rate of phages in ACI fertilizer.

Incorporated phages/ Log PFU/ml	Day-1	1 week	2 weeks	1 month	2 months	8 months
P36	7	7	7	7	7	2
P45	7	7	7	7	7	2
P47	7	7	7	7	7	1
P72	7	7	6	7	7	1
P630	6	6	6	6	6	0
P631	8	8	8	8	8	2
P482	7	7	7	7	7	1
P483	6	6	6	6	6	0
P459	6	6	6	6	6	0
P536	6	6	6	6	6	0

Treatment 1 (T1): ACI solid fertilizer + phage cocktail (6-9 Log PFU/ml), application every 2 months (700 g/pole) and ACI pure organic + phage (6-9 Log PFU/ml), application every 3 months (3 kg/pole); Treatment 2 (T2): ACI solid fertilizer alone, application every 2 months (700 g/pole) and ACI pure organic, application every 3 months (3 kg/pole); Treatment 3 (T3): Standard NPK, application every 2 months (700 g/pole) and organic manure (fecal from sheep), application every 3 months (3 kg/pole); Treatment 4 (T4): ACI liquid fertilizer + phage cocktail (6-9 Log PFU/ml), application every 2 months by spraying on the infected areas and into the plants (800 ml/pole) and ACI pure organic + phage cocktail (6-9 Log PFU/ml), application every 3 months (3 kg/pole); Treatment 5 (T5): ACI liquid fertilizer, application every 2 months by spraying on the infected areas and into the plants (800 ml/pole) and application of ACI pure organic for every 3 months (3 kg/pole). Each of the plot size is 3 plants/ treatment/ replicate. Data collection was based on soil analysis, no flowering on the branch, fruit number and size per plant, shoot number, soil and root microbial population before and after applying the phage-fertilizer, disease scoring (at stage before phages-fertilizer application, 1 month and 3 months phage-fertilizer after application). Assays were performed in triplicate and standard deviation from the arithmetic mean of the data.

RESULTS AND DISCUSSION

The survival rate of phage in ACI fertilizer was determined from day-1, 1 week, 2 weeks, 1 month, 2 months and 8 months of incubation. Phages can be detected after two months of incubation (7-Log PFU/ml). However, it was dropped after 8 months (0-2 Log PFU/ml) (Table 1). Even though phages are virus, and they can become dormant without host. However, when they are incorporated into certain chemical compounds, the viability may drop due to uncertainty. The study showed that phages need some protective formulation to increase their longevity under green house and field conditions (Balogh *et al*., 2010). Three identified potential carriers mainly for phages suspension consist of milk, sugar and flour for alleviate the effect of light irradiation and provide rain-fastness.

From the tomato study, plants treated with T1 have the highest average height and weight [4 weeks: 36.84 cm; 8 weeks: 75.68 cm; 12 weeks: 96.2 cm (height) and 462.16g (fresh weight)], as compared to T3 [4 weeks: 24.88 cm; 8 weeks: 67.32 cm; 12 weeks: 68.32 cm (height) and 226.88 g (fresh weight)] (Figure 1 and Figure 3). For disease scoring, treatment T2 is higher than T1 (Figure 5). The average size of stem is about 7.7 cm at 4 weeks; 8.34 cm at 8 weeks and 9.72 cm at 12 weeks in T1; while in T3, it was only 6.46 cm at 4 weeks; 7.56 cm at 8 weeks and 6.92 cm at 12 weeks. The result was not significant different between the treatments (Figure 2). However, T1 gave the highest fruit weight score as 90.8 g/plant (Figure 4). The incorporated phages with ACI fertilizer can control 80% of wilting disease and enhancing of vegetative growth due to the nutrient update from the plants.

The reason why we need to incorporated phage with fertilizer because the agriculture production is carried out in an open environment. By using phages alone, they can only deployed into the system where there is little or no control over the environment factors, such as temperature, light irradiation, moisture levels and pH. Additional, in agriculture settings, phages alone have to provide long-lasting protection of susceptible plant tissues (weeks or months), which are distributed over large areas (hectares), which is not practical. By incorporated phages with fertilizer, the application can be expensed to the broader areas and much more available phages expose to phytobacteria.

There are many factors which can affect the efficiency of phage application in controlling diseases. The application of phages usually prepared in the mixtures (so-called cocktails) in order to manage arising of bacterial strains in diversity population and build-up of bacterial strains that display resistance to specific phages. Studies also showed that phages have been found to survive considerably longer, when applied close to sunset rather than in the morning or early afternoon. Evening applications thus have provided better control of tomato bacterial wilt than day time applications.

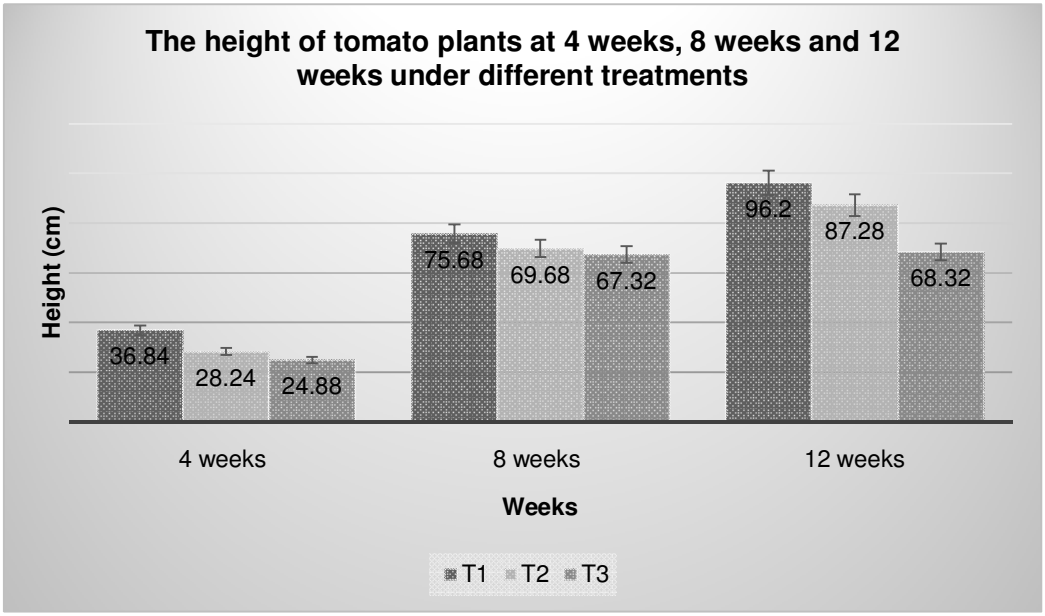

Figure 1. The height of tomato plants at 4 weeks, 8 weeks and 12 weeks under different treatments (T1, T2 and T3). Assays were performed in triplicate and the error bars represent the standard deviation from the arithmetic mean.

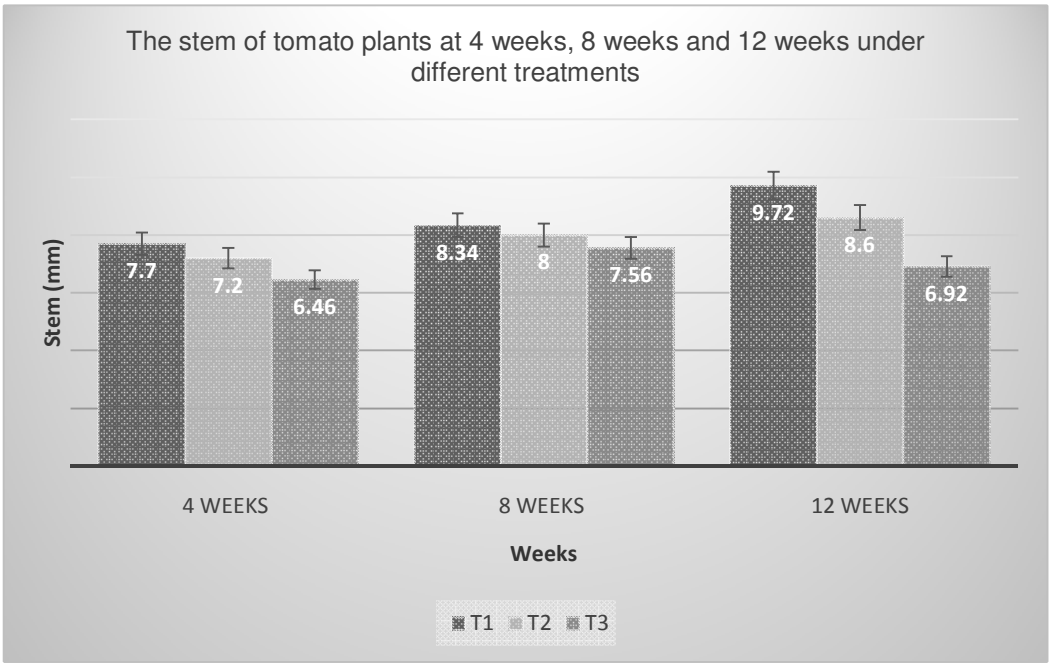

Figure 2. The stem diameter of tomato plants at 4 weeks, 8 weeks and 12 weeks under different treatments (T1, T2 and T3). Assays were performed in triplicate and the error bars represent the standard deviation from the arithmetic mean.

In several studies, phage treatment was most effective if applied shortly before or concurrently with bacterial inoculants. It may lost efficacy if applied following inoculation as reported by Civerolo, 1971. However, there is no consensus on application frequency. In several field studies, phage treatments were effectively when applied

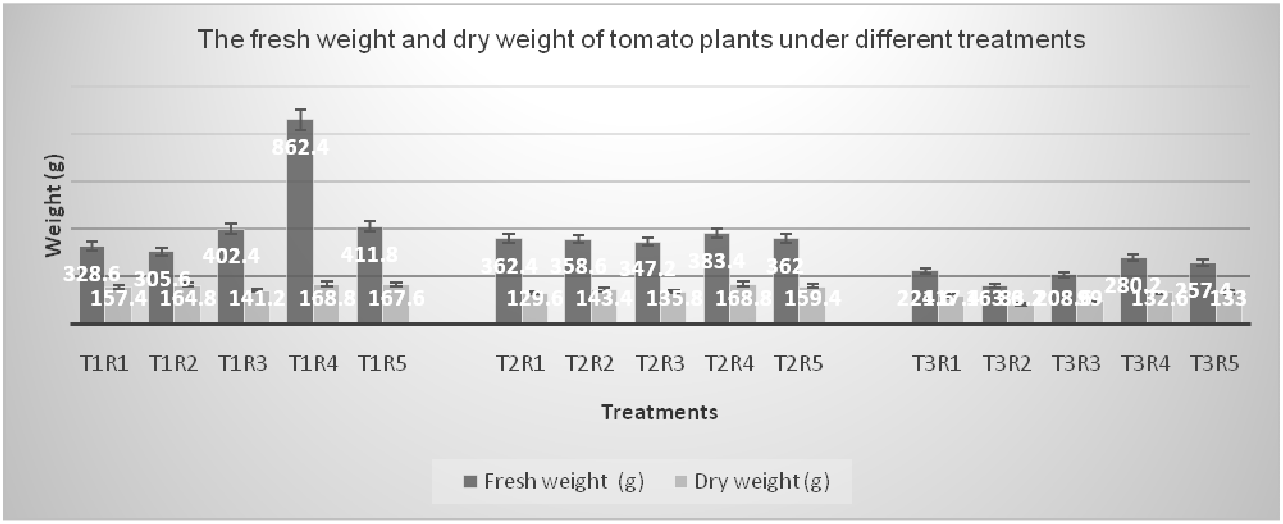

Figure 3. The fresh weight and dry weight of tomato plants under different treatments (T1, T2 and T3). Assays were performed in triplicate and the error bars represent the standard deviation from the arithmetic mean.

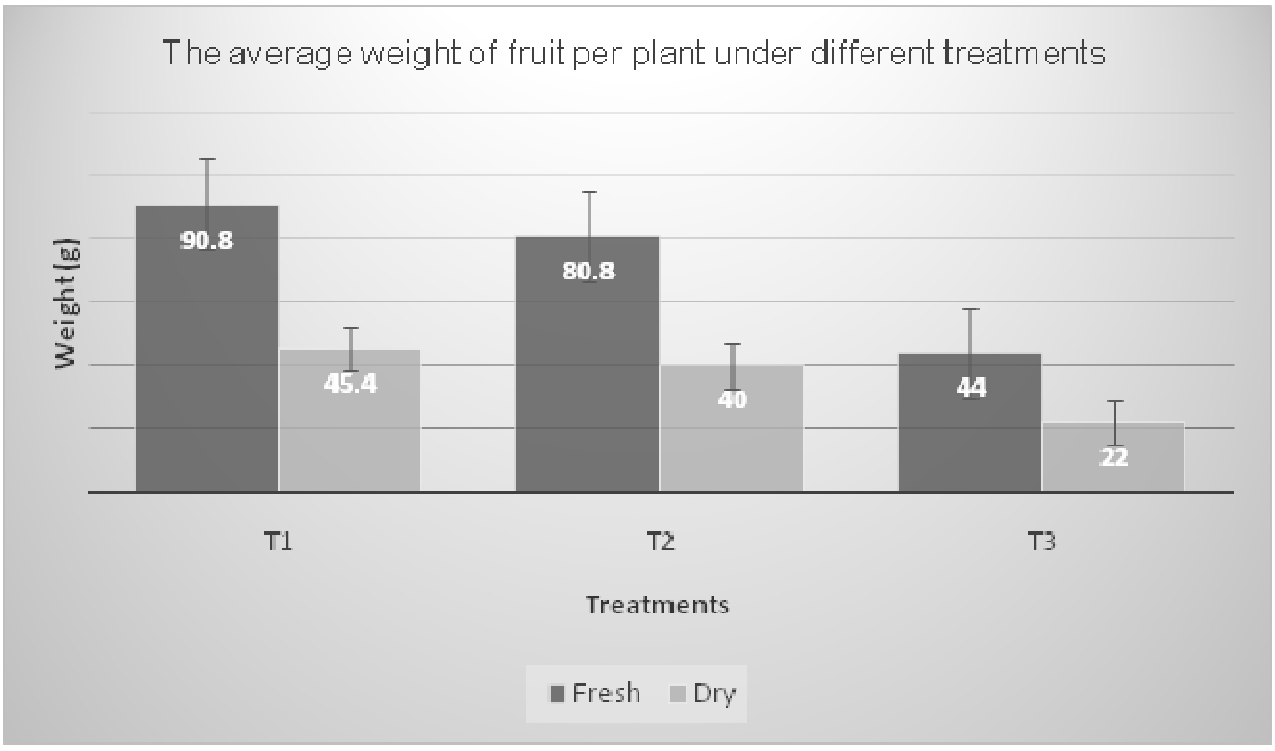

Figure 4. The average weight of tomato fruit per plant under different treatments (T1, T2 and T3).

twice weekly, based on studied by Obradovic *et al*., 2004; Balogh *et al*., 2008; Borah *et al*., 2000 and Balogh *et al*., 2003). However, it is not clear about the phage concentration is required for efficient disease control, but it is likely depends on the patho-system and phages. Some reports showed that phage manage to control tomato bacterial spot when applied at 6-8 Log PFU/ml; but not less than 4Log PFU/ml (Balogh *et al*., 2002). In this study, the effective phage cocktail concentration was between 6-9 Log PFU/ml.

The same results obtained in pitaya showed that plants treated with T1 had the highest shoot number (25.8), fruit

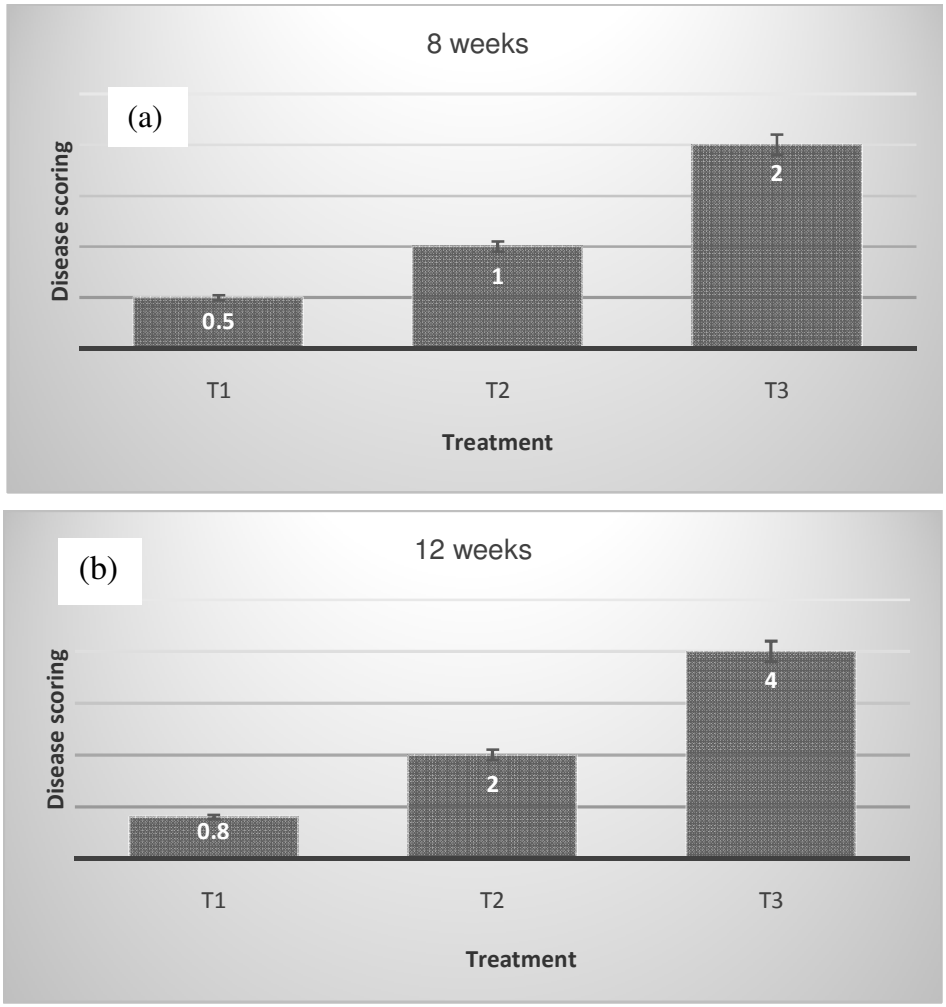

Figure 5. Disease scoring of tomato plants at (a) 8 weeks (b) 12 weeks under different treatments (T1, T2 and T3). There is no wilting symptom at the initial stage of growing. Scoring 1= very little (sparse); 2= low; 3= medium; 4= medium to high; 5= high. Assays were performed in triplicate and the error bars represent the standard deviation from the arithmetic mean.

number (0.4) and shoot length increment (12 cm) after one month of application (Figure 6). After four months, results showed that T1 had the highest shoot number per plant (19) and shoot length increment (3.4 cm) compared to all treatments which is according with the data presented by Tan *et al*., 2010. However, there was no significant on reproductive parameters been observed. T1 performed better than Treatment T3 by 1½ and 0.7 folds in shoot number and shoot length increment, respectively.

It was noted that T2 also showed the positive impact on vegetative growth (Figure 6 and Figure 7). For disease scoring, T4 treatment managed to control bacteria disease spreading and only caused 0.3% from the infection. This may be due to the application by spraying directly onto the infected areas, so the disease can be controlled (Figure 8).

CONCLUSION

Incorporated bacteriophage in fertilizers have potential to occupy a specific niches in the bio-pesticide market. Bacteriophages are environmental friendly compared to the other chemical-pesticide. By using incorporated technology with All Cosmos Bio-tech Holding Corporation, the process of fertility and disease prevention can be carried out at the same time. The efficacy test of bacteriophage incorporated with fertilizer helped 80% of wilting symptom in tomato and positive impact of vegetative growth of pitaya.

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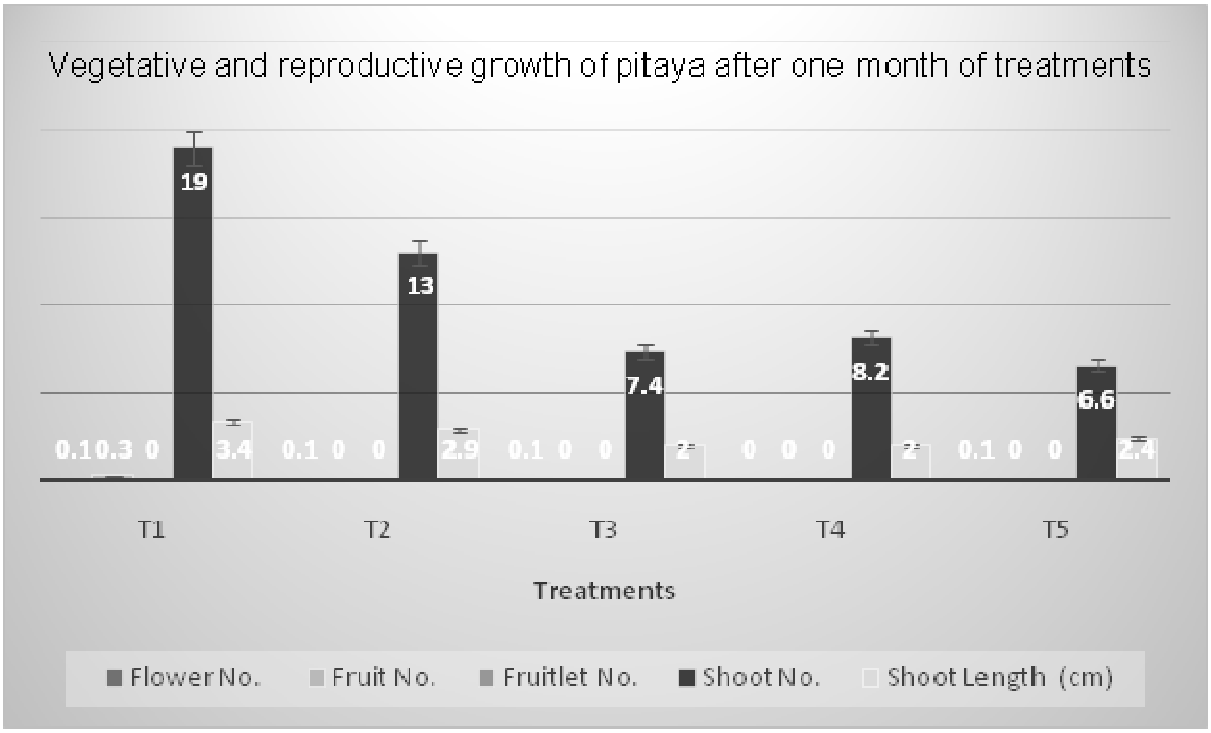

Figure 6. Vegetative and reproductive growth of pitaya after one month of treatments (T1, T2, T3, T4 and T5). Assays were performed in triplicate and the error bars represent the standard deviation from the arithmetic mean.

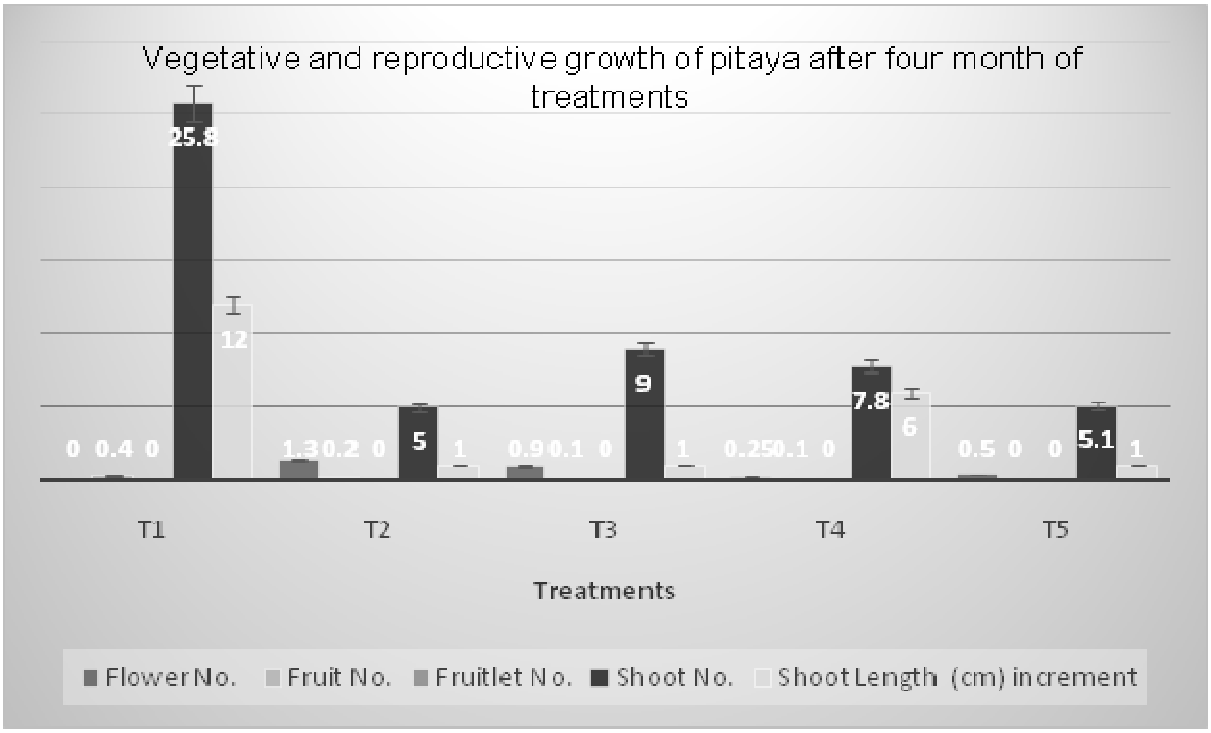

Figure 7. Vegetative and reproductive growth of pitaya after four month of treatments (T1, T2, T3, T4 and T5). Assays were performed in triplicate and the error bars represent the standard deviation from the arithmetic mean.

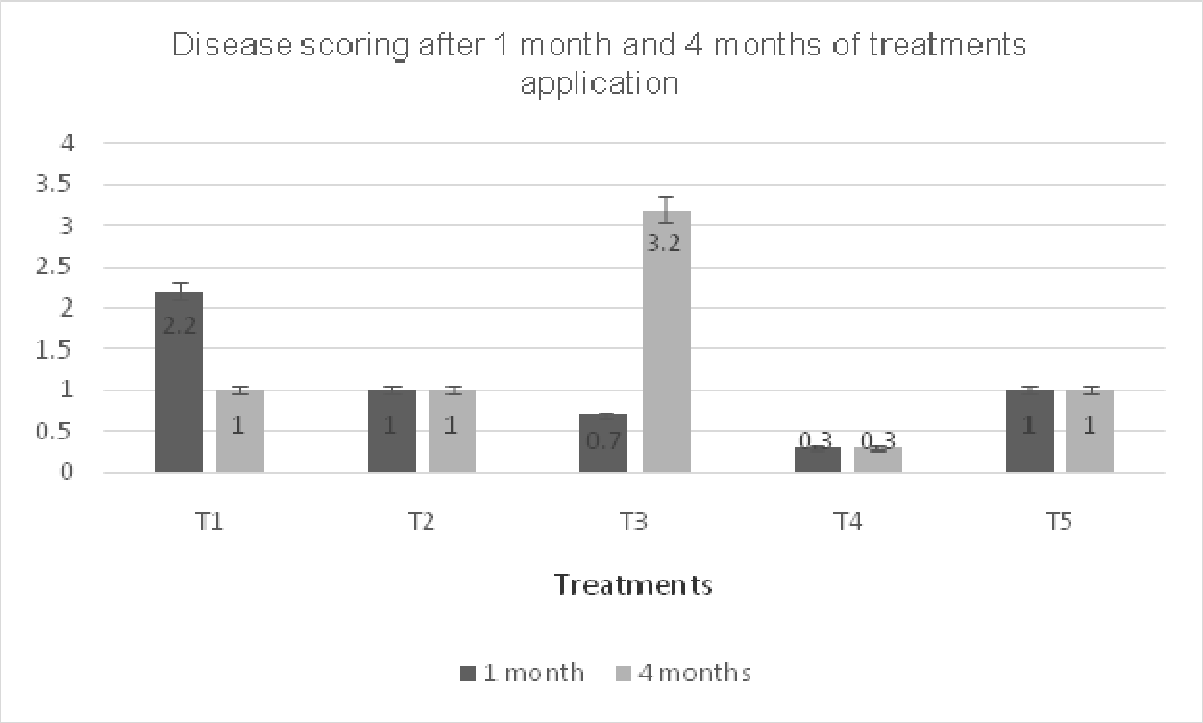

Figure 8. Disease scoring of pitaya after one month and four months of treatments (T1, T2, T3, T4 and T5). Scoring 1= very little (sparse); 2= low; 3= medium; 4= medium to high; 5= high. Assays were performed in triplicate and the error bars represent the standard deviation from the arithmetic mean.

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