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Research Article
Chitosan and Plant Growth Promoting Rhizobacteria Application to Control *Squash mosaic virus* on Cucumber Plants

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Abstract

Background and Objective: *Squash mosaic virus* (SqMV) is a seed-borne virus infecting Cucurbitaceae, including cucumber plants. The SqMV cause systemic infections in plants and it is very difficult to find the effective control strategies to reduce its spread. Chitosan and Plant Growth Promoting Rhizobacteria (PGPR) has been reported to suppress plant diseases. The objective of this study was to determine the potential of chitosan and PGPR to reduce disease intensity caused by SqMV and enhance plant growth on cucumbers. **Methodology:** Cucumber cultivars was first screened by blotter test and dot immunobinding assay (DIBA) for virus detection. Application of chitosan and PGPR was given as seed treatment before planting seeds and as foliar spray or soil drench during plant growth. Data was subjected to statistical analysis using two-way ANOVA and means were separated by DMRT $p \leq 0.05$. **Results:** Virus detection from seeds of several cucumber cultivars indicated high incidence of SqMV infection, i.e., 72.22-100%. Application of chitosan and PGPR caused delay incubation period, reduce disease severity as well as titer of virus especially on generative phase (4-7 weeks after planting). In addition, chitosan and PGPR application increased plant growth especially plant height. **Conclusion:** Application of chitosan and PGPR as individual or combination treatment effectively enhanced plant growth and suppressed disease severity. Therefore, application of chitosan and PGPR should be recommended to control disease caused by SqMV on cucumber.

Key words: Disease severity, plant growth, seed-borne virus, titer of virus

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Squash mosaic virus (SqMV) is a seed-borne virus in cucurbit plants and may spread during transplanting and at harvest after primary infections by infected seeds[1]. This virus can also be transmitted mechanically or by insect vectors[2]. Infection of SqMV cause yellow-green mosaic with dark green vein-banding on cucumber leaves. The leaves become rigid, rugose and narrowing of leaf size and the plant becomes stunted. Cucumbers were infected by SqMV showed shrinkage of fruit base and malformed fruit. Fruit was infected and may cause changes in taste and nutrition content, seed germination, fruit weight and number of fruit production[3].

Many attempts to control the disease have not been successful to reduce its spread. No resistance or tolerance response was found in commercial varieties of cucumber, therefore alternative methods to effectively control this virus should be searched. Application of antiviral compounds or agents that induce systemic resistance of plants has been reported for virus infection. Use of chitosan and Plant Growth Promoting Rhizobacteria (PGPR) showed the potential for inducing plant resistance[4,5].

Chitosan is a natural biopolymer derived from deacetylation of chitin and has properties of environmentally friendly and easily degradable. Chitosan has been reported to induce antiviral resistance of plants but the degree of induced resistance was mostly determined by plant species. The antiviral activity was determined by structure of the chitosan molecule and its molecular weight[4]. Chitosan was effective on agriculture such as acting as the carbon source for microbes in the soil, accelerating transformation process of organic matter into inorganic matter and assisting the root system of plants to absorb more nutrients from the soil[6]. Application of chitosan at concentration of 0.9% effectively suppressed the infection of *Bean common mosaic virus* and reduced population of its vector *Aphis craccivora* on yard long bean[7].

Plant Growth Promoting Rhizobacteria (PGPR) are bacteria that actively colonizing rhizosphere and can enhance plant growth and increasing plant nutrient via several mechanisms such as siderophore production, antibiotics and phosphate solubilization[5]. Application of *Pseudomonas* sp., in combination with chitosan reduced the severity of disease caused by *Tomato leaf curl virus* (ToLCV) up to 90.33% in field conditions[8]. In contrast to other diseases, a few studies have been reported for the control efficiency of chitosan and PGPR for viral diseases[9,10]. Therefore, it is important to conduct a research to determine the effectiveness of chitosan and PGPR to suppress SqMV infection on cucumber.

MATERIALS AND METHODS

Time and place of research: Field experiment and virus diagnosis was conducted at Situ Gede village, Darmaga, Bogor, West Java and Laboratory of Plant Virology, Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University, respectively. This research was conducted from February to November, 2016.

Screening of cucumber seeds: Five cultivars of cucumber, i.e. 'Amanda', 'Calista F1', 'Japan File', 'Yupiter' and 'Vario F1' was tested for seed-borne virus using blotter test followed by virus detection using dot blot immunobinding assay with antibody against SqMV as described below. Cucumber seeds were planted according to ISTA protocol, i.e., 400 seeds for each cultivar.

Detection of SqMV by dot immunobinding assay (DIBA) method: The DIBA was conducted following method described by Asniwita *et al.*[11] with slight modifications. Analytical grade of chemicals was used for solution preparation in this assay. Nitrocellulose membrane were dotted with 2 µL of the sap extract prepared by grinding leaf samples in Tris Buffer Saline (TBS) pH 7.5 containing tris-HCl 0.02 M and NaCl 0.5 M with ratio of 1:10 (w:v). The membranes were dried for 15 min, followed by incubation in blocking solution (2% skim milk and 2% triton X-100 in TBS) for 1 h followed by washing five times in dH_2O. The membranes were then incubated overnight at 4°C in a 1:100 dilution of primary antibody (2% skim milk in TBS). The membranes were washed in 0.05% Tween in TBS (TBST) (five washes, 5 min per wash). The membranes were incubated for 3 h in a 1:100 dilution of secondary antibody. The membranes were washed again in TBST as described before. Finally, the membranes were incubated 30 min in substrate solution containing NBT/BCIP tablet (Sigma Fast) in Alkaline Phosphate (AP) buffer pH 9.3 (Tris-HCl 0.1 M, NaCl 0.1 M, $MgCl_2$ 5 mM and aquadest). The reaction was stopped by soaking the membranes in dH_2O. Determination of virus titer was based on color intensity of DIBA reaction with a score range: O For negative reaction (-), 1: For weak reaction (+), 2: For moderate reaction (++), 3: Strong reaction (+++) and 4: Very strong reaction (++++).

Preparation of chitosan and PGPR solutions: Chitosan and PGPR solution containing *Bacillus polymixa* and *Pseudomonas fluorescens* was obtained from CV. WISH Indonesia (Bogor, West Java). Chitosan was dissolved in acetic acid (1.5%) to make stock chitosan solution (5%) and then

diluted to obtain 0.9% application solution. The PGPR solution was dissolved in aquadest for making 1% PGPR solution for application.

Planting of cucumbers in the field: Two cultivars of cucumber were chosen based on the result of seed screening. Cucumber seeds were planted on polybag with size of 35×35 cm containing growing, madia i.e., soil and manure with ratio of 2:1 (w/w). Fertilization was applied following method described by Susila[12].

Application of chitosan and PGPR: Cucumber seeds were soaked for 2 min in a commercial grade of sodium hypochlorite solution (2%) and rinsed five times with sterile distilled water. The treated seeds were transferred to the chitosan solution (0.9%) for 60 min or in a PGPR solution (1%) for 30 min. As for treatment combination of chitosan and PGPR, seeds were first treated with chitosan before PGPR. Application at 2, 4 and 6 Weeks After Planting (WAP) was given by foliar spray for chitosan and soil drench for PGPR in a dose of 100 mL per plant.

Experimental design: The experiments were arranged in split plot design with 4 treatments, i.e., chitosan, PGPR, combination chitosan and PGPR and control with 3 replications for each treatment. Observation was consisted of virus incubation period, type of symptoms, titer of virus, disease incidence and severity, Area Under Disease Progress Curve (AUDPC), number and weight of fruit. Titer of virus was confirmed by taking leaf samples for detection using DIBA method. Disease and symptoms severity were recorded according to the scale described by Ayo-John *et al.*[13], i.e. 1: For no symptom, 2: For mild symptom (10% of leaves), 3: For moderate symptom (10-30%), 4: For severe symptom (30-50%) and 5: For leaf distortion and death (over 75%). The AUDPC was calculated by using the formula suggested by Simko and Piepho[14]:

$$AUDPC = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) (t_{i+1} - t_i) \quad (1)$$

where, y is intensity of disease at each observation, t is time of each observation and n is number of observation.

Statistical analysis: All data on incubation period, titer of virus, disease incidence and severity, AUDPC value, number and weight of fruit were subjected to the two-way analysis of variance (ANOVA) with SPSS program version 16.0 and means were separated by Duncan's Multiple Range Tests (DMRT) $p \leq 0.05$[15].

RESULTS

Screening of cucumber seeds: Seed germination of five cucumber cultivars was very good, indicated all seeds has a good viability (Table 1). However, SqMV infection was also detected in high incidence, ranged from 72.22-100% in all cultivars. Most seedlings infected by SqMV showed obvious symptoms of green mosaic, malformation of leaves and chlorosis of leaves. The average score of virus titer on five cultivars ranged from 1.91-3.35 with the lowest virus titer found on cv. Japan file and the highest on cv. Calista F1 (Table 1). The two cultivars were then selected for further field experiment.

Effects of chitosan and PGPR application on virus infection: Disease incidence was high (83.33-100%) and was not affected by cultivars, treatments, or interactions between cultivars and treatments. Disease symptom was developed faster on cv. Japan file than on cv. Calista F1. However, interaction between cultivars and treatments did not cause differences for incubation period. This result indicated that symptom development was influenced only by plant cultivars or treatments. All treatments on cv. Japan file tends to cause fast symptom development, in contrary all treatments on cv. Calista F1 tends to cause slow symptom development. Application of chitosan and PGPR effectively delayed symptom development (Table 2).

Variability of symptoms was observed on the plants in the field, i.e., green mosaic, yellow mosaic, yellow-green mosaic, vein banding, vein clearing, cupping and rugose. The most

Table 1: Seed germination, disease incidence, score of titter virus and type of SqMV symptoms on cucumber seedlings

Cucumber cultivars	Seed germination (%)	Disease incidence (%)	Score of virus titer	Type of symptoms
Amanda	99.50	100.00	2.79	MG, MF, RG, CL
Calista F1	85.75	100.00	3.35	MG, MF, CL
Japan file	94.67	72.22	1.91	MG, MF, CP, CL
Vario F1	98.00	100.00	2.59	MG, MF, CP, RG, CL
Yupiter	98.50	100.00	2.72	MG, MF, CP, RG, CL

MG: Green mosaic, MF: Malformation of leaves, CP: Cupping, RG: Rugose, CL: Chlorosis of leaves, SqMV: *Squash mosaic virus*

Table 2: Effect of chitosan and PGPR on incubation period and disease incidence on cucumber plants

Parameters	Incubation period (DAP)*	Disease incidence (%)*
Cultivars		
Calista F1	13.56±3.02[b]	92.50±14.22[a]
Japan file	11.52±1.02[a]	87.50±9.65[a]
Treatments		
Control	10.12±0.48[a]	98.33±4.08[a]
Chitosan	14.52±3.00[c]	81.67±13.29[a]
PGPR	12.43±1.35[b]	90.00±12.65[a]
Combination	13.08±2.05[bc]	90.00±12.65[a]
Interaction(Cultivars×treatments)		
Calista F1×control	10.03±0.68[a]	100.00±0.00[a]
Calista F1×chitosan	16.63±2.91[a]	80.00±20.00[a]
Calista F1×PGPR	13.13±1.43[a]	90.00±17.32[a]
Calista F1×combination	14.43±2.25[a]	100.00±0.00[a]
Japan file×control	10.20±0.30[a]	96.67±5.77[a]
Japan file×chitosan	12.40±0.79[a]	83.33±5.77[a]
Japan file×PGPR	11.73±1.01[a]	90.00±10.00[a]
Japan file×combination	11.73±0.15[a]	80.00±10.00[a]

PGPR: Plant growth promoting rhizobacteria, DAP: Days after planting, *Different letters within one column indicate significant difference between treatment ($p<0.05$) on DMRT analysis. Values consisted of Mean±SD

Table 3: Effect of chitosan and PGPR on virus titer based on virus detection using DIBA

	Score of virus titer		
Parameters	2 WAP (Vegetative phase)*	4 WAP (Flowering phase)*	7 WAP (Fruiting phase)*
Cultivars			
Calista F1	1.31±0.41[a]	1.22±0.34[b]	1.12±0.69[a]
Japan file	1.12±0.32[a]	1.01±0.25[a]	1.10±0.58[a]
Treatments			
Control	1.65±0.46[b]	1.47±0.30[b]	2.03±0.46[b]
Chitosan	1.12±0.19[a]	0.93±0.21[a]	0.77±0.22[a]
PGPR	1.10±0.21[a]	1.07±0.22[a]	0.93±0.31[a]
Combination	0.98±0.17[a]	0.98±0.22[a]	0.70±0.15[a]
Interaction (Cultivars×treatments)			
Calista F1×control	1.73±0.66[a]	1.67±0.25[a]	2.13±0.63[a]
Calista F1×chitosan	1.17±0.21[a]	0.93±0.30[a]	0.67±0.21[a]
Calista F1×PGPR	1.20±0.26[a]	1.13±0.21[a]	0.87±0.21a
Calista F1×combination	1.13±0.06[a]	1.13±0.06[a]	0.80±0.17[a]
Japan file×control	1.57±0.25[a]	1.27±0.21[a]	1.93±0.30[a]
Japan file×chitosan	1.07±0.21[a]	0.93±0.11[a]	0.87±0.21[a]
Japan file×PGPR	1.00±1.00[a]	1.00±0.26[a]	1.00±0.43a
Japan file×combination	0.83±0.06[a]	0.83±0.23[a]	0.60±0.00[a]

PGPR: Plant growth promoting rhizobacteria, WAP: Weeks after planting, DIBA: Dot immunobinding assay, *Different letters within one column indicate significant difference between treatment ($p<0.05$) on DMRT analysis. Values consisted of Mean±SD

common symptoms were green mosaic, yellow mosaic and vein banding. The SqMV-infected plants developed leaves with distorted margins, vein clearing and mild to severe mosaic and infected fruit have mottling on the skin and malformed[2].

In general, virus titer on infected plants tends to decrease by time (Table 3). Virus titer was higher in the vegetative phase (2 WAP) than those in the flowering phase (4 WAP) and increased again in the fruiting phase. This condition indicated relationship between multiplication of the virus and plant growth. Virus multiplication tends to be induced by active growth of the plants. Interaction between cultivars and treatments did not cause differences for virus titer, although the lowest virus titer was observed on cv. Japan file treated with combination of chitosan and PGPR. Virus titer was influenced by treatments or cultivars. Treatment using chitosan, PGPR, or their combination significantly $p\leq0.05$ decreased virus titer. Virus titer on cv. Calista F1 was higher than those on cv. Japan File.

Further observation revealed that disease severity has a positive correlation with the titer of virus. Disease severity of all treatments was decreased at similar trend with those of virus titer, whereas those in control treatment constantly increased by time of observation (Fig. 1). However, the lowest disease severity was observed on plants not showing the lowest titer of virus. This result indicated that disease severity based on symptoms may not correspond with titer of SqMV. The symptoms observed in plants is probably caused by other

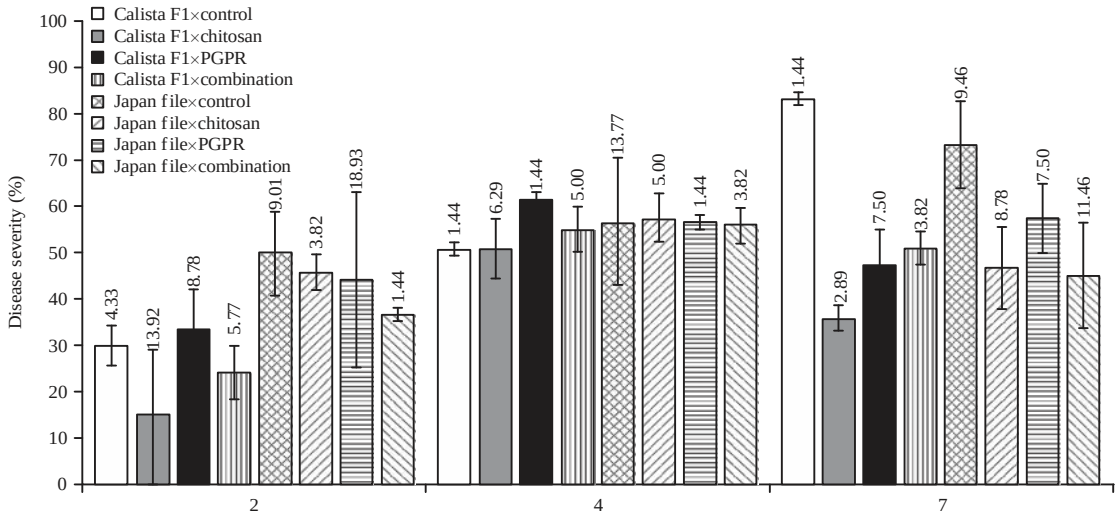

Fig. 1: Disease severity of SqMV on each treatment in the field
Bars represent standard deviation

Table 4: Value of Area Under Disease Progress Curve (AUDPC) in the field

Parameters	AUDPC*
Cultivars	
Calista F1	256.25±30.09[a]
Japan file	271.98±27.90[a]
Treatments	
Control	286.25±32.09[b]
Chitosan	240.00±33.91[a]
PGPR	276.04±9.03[b]
Combination	254.17±12.81[ab]
Interaction (Cultivars×treatments)	
Calista F1×control	279.17±8.78[a]
Calista F1×chitosan	218.75±33.56[a]
Calista F1×PGPR	274.58±12.83[a]
Calista F1×combination	252.50±13.75[a]
Japan file×control	293.33±48.45[a]
Japan file×chitosan	261.25±19.84[a]
Japan file×PGPR	277.50±5.73[a]
Japan file×combination	255.83±14.60[a]

PGPR: Plant growth promoting rhizobacteria, *Different letters within one column indicated significant difference between treatment ($p<0.05$) on DMRT analysis. Values consisted of Mean±SD

viruses. Most common viral diseases infected cucumber was caused by *Cucumber mosaic virus* (CMV), *Papaya ringspot virus* (PRSV), *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV)[16]. The higher value of AUDPC was observed on cv. Japan file with control treatment, whereas the lowest value of AUDPC was on cv. Calista F1 with chitosan treatment (Table 4). This result showed that chitosan treatment was effectively suppressed SqMV infection.

Effects of chitosan and PGPR application on crop yield: Analysis on yield components showed that number of fruits and fruits weight was not influenced by cultivars, treatments, or interactions between cultivars and treatments (Table 5). Application of chitosan, PGPR and combination of them effectively improved plant height. Interaction between cultivars and treatments did not cause differences on plant height. This result indicated that symptom development was influenced only by plant cultivars or treatments (Fig. 2).

DISCUSSION

The SqMV was detected from seed in high incidence, i.e., 72.22-100% on several cucumber cultivars. Infected seeds will become primary virus inoculum in the field and may effect disease spread. Application of chitosan and PGPR significantly $p\leq0.05$ delayed incubation period and reduced disease severity as well as titer virus of SqMV especially on generative phase. This result indicated their potency to inhibit disease spread in the field. The effectiveness of chitosan and PGPR application for viral disease control has been reported previously[17,18]. Chitosan treatment caused inhibition of tomato yellow leaf curl disease symptoms[9]; application of PGPR in combination with chitin significantly $p\leq0.05$ reduced ToLCV and TLCV infection from 80.33, 93.33 and25%, respectively on tomato under field conditions[8,10]. It was suggested that the application of PGPR along with chitosan may induce antiviral mechanism and elicitation of biochemical defense responses. In addition to promoting bacterial growth and stimulating the activation of chitinase enzymes, chitin has also been shown to have other beneficial effects on rhizobacteria. Application of chitosan, PGPR and treatment combination of them also effectively improved plant height of cucumber plants. Similar results were reported for application of chitosan and PGPR alone as well as in combination on tomato, maize and scots pine[9,19-21].

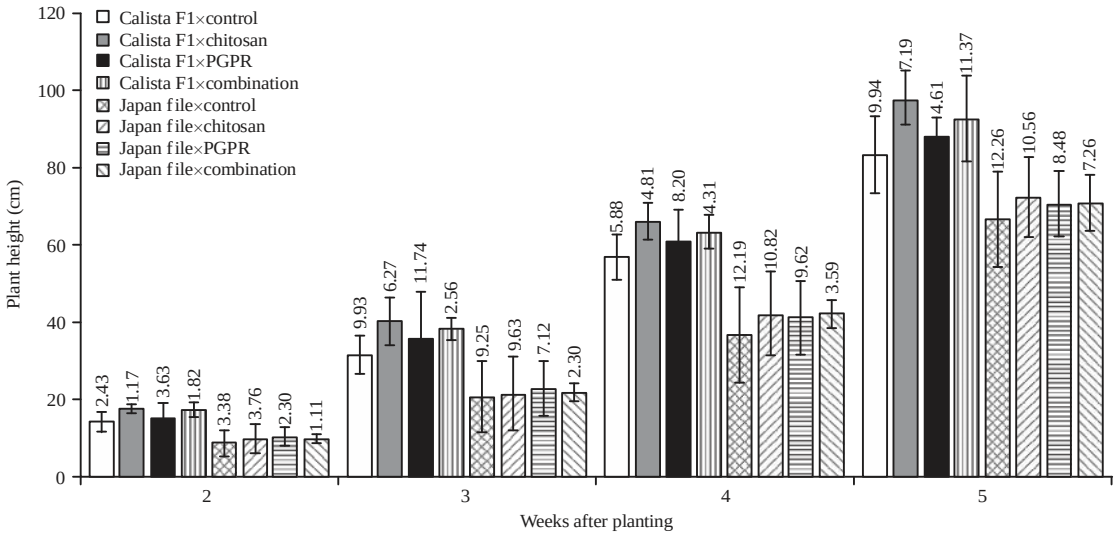

Fig. 2: Effect of chitosan and PGPR on plant height on cucumber plants
Bars represent standard deviation

Table 5: Effect of chitosan and PGPR on number and weight of fruit on cucumber plants

Parameters	No. of fruits per plants*	Fruits weight per plants*
Cultivars		
Calista F1	0.17±0.06[a]	13.33±6.80[a]
Japan file	0.18±0.05[a]	13.78±4.37[a]
Treatments		
Control	0.18±0.08[a]	12.81±6.92[a]
Chitosan	0.20±0.00[a]	15.42±2.40[a]
PGPR	0.15±0.05[a]	9.86±4.32[a]
Combination	0.15±0.05[a]	16.14±6.54[a]
Interaction (Cultivars×treatments)		
Calista F1×control	0.20±0.10[a]	14.26±9.66[a]
Calista F1×chitosan	0.20±0.00[a]	15.29±2.91[a]
Calista F1×PGPR	0.13±0.06[a]	7.36±1.21[a]
Calista F1×combination	0.13±0.06[a]	16.42±8.79[a]
Japan file×control	0.17±0.06[a]	11.37±4.50[a]
Japan file×chitosan	0.20±0.00[a]	15.55±2.43[a]
Japan file×PGPR	0.17±0.06[a]	12.35±5.15[a]
Japan file×combination	0.17±0.06[a]	15.85±5.44[a]

PGPR: Plant growth promoting rhizobacteria, *Different letters within one column indicate significant difference between treatment ($p<0.05$) on DMRT analysis. Values consisted of Mean±SD

The current research indicated that chitosan and PGPR treatment shown inhibitory activity against virus infection through antiviral or virus inhibitors mechanism. This condition was called induced local acquired systemic resistance, reflected in the synthesis of salicylic acid, phytoalexins and PR proteins (chitinase and β-1, 3-glucanase), the lignification of cell walls and callose synthesis[17]. Chitosan may also inhibit viral infection by inactivating virus replication, multiplication and cell-to-cell movement. In addition, nanoparticles of chitosan can bind to nucleic acids during virus penetration and cause damage to virus. Chitosan can also disable the synthesis of mRNAs encoded by genes for metabolic and infection from virus or viroid[4,22]. The PGPR as biocontrol agents can Induce Systemic Resistance (ISR) involving jasmonate and ethylene signaling within the plant and these hormones stimulate the host plants defense responses against a variety of plant pathogens. The PGPR also promote plant growth by facilitating resource acquisition, modulating plant hormone levels and decreasing the inhibitory effects of various pathogens as biocontrol agents[23].

The knowledge regarding the potency of chitosan and PGPR for enhancing plant growth and inhibit SqMV infection will help the development of viral disease management in the field, especially for those whom concerned on environment-friendly control of plant diseases. Further research is required to determine the antiviral mechanism of chitosan and PGPR and its most effective application.

CONCLUSION

The study indicated that application of chitosan, PGPR and combination of them effectively improved plant height. Chitosan and PGPR application also delayed symptom development and reduced disease severity as well as titer of virus, especially on generative phase. Therefore, application of chitosan and PGPR should be recommended to enhance plant growth and induce systemic resistance against SqMV infection.

SIGNIFICANCE STATEMENTS

This study discovers the potency of chitosan and Plant Growth Promoting Rhizobacteria (PGPR) to control a seed borne disease of cucumber caused by *Squash mosaic virus*. Application of chitosan and PGPR improved plant growth, delayed disease development and decreased disease severity. This study will help the researcher to uncover the critical areas of plant virus infection inhibition that many researcher were tried to explore. Thus, a new recommendation for plant viral disease management using chitosan and PGPR could be employed.

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