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Research Article

The Effect of Chitosan Application against Plant Growth and Intensity of Stunting Disease on Black Pepper (*Piper nigrum* L.) Seedlings[ж]

Pengaruh Aplikasi Kitosan terhadap Pertumbuhan dan Intensitas Penyakit Kerdil pada Bibit Lada (Piper nigrum *L.*)

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ABSTRACT

Black pepper (*Piper nigrum* L.) is an important estate crops in Indonesia. Some pathogens that have been known to infect black pepper plants include fungi, nematodes and viruses. The stunting disease on black pepper plants was caused by *Cucumber mosaic virus* (CMV). Molecular detection using RT-PCR method showed that the samples were positively infected by CMV which were amplified by specific primers CMV 111 with bands of 111 bp in size. This virus can be carried by vegetative propagation material of plants. Many control strategies against this virus have been investigated, especially inducing plant resistance with chitosan. Chitosan is a natural biopolymer that play an important role in reducing disease incidence and severity and stimulate plant growth. The aim of this study was to figure out the inhibiting ability of chitosan solution against infection of stunting virus on black pepper seedlings through spraying applications. Chitosan treatments were prepared in concentrations of 0.5%, 0.75%, and 1%. The result showed that application of chitosan at all concentrations affected the decrease of disease incidence and intensity and improved plant growth with insignificant different amongst all treatments but significantly different with control. The highest decrease in incidence was found at 0.75% of chitosan concentration (26.37), while the highest decrease of intensity was expressed at 1% of chitosan (37.62). Application of chitosan also significantly affected to all parameters of plant growth either plant height or leaf diameter. Application of 1% of chitosan increased the percentage of plant growth rather than other treatments, with the increase of plant height 58.12 % and leaf diameter 54.74 %.

Keywords: black pepper, chitosan, stunting disease

INTISARI

*Lada (*Piper nigrum *L.) merupakan salah satu tanaman perkebunan penting di Indonesia. Beberapa patogen telah diketahui menginfeksi tanaman lada di antaranya jamur, nematoda, dan virus. Penyakit kerdil pada tanaman lada disebabkan oleh* Cucumber mosaic virus *(CMV). Deteksi molekuler menggunakan metode RT-PCR menunjukan bahwa sampel positif terinfeksi CMV yang diamplifikasi menggunakan primer spesifik CMV 111 dengan ukuran pita band target 111 bp. Virus ini dapat terbawa bahan perbanyakan tanaman secara vegetatif. Banyak strategi pengendalian virus yang telah diuji, diantaranya induksi ketahanan tanaman dengan kitosan. Kitosan adalah biopolimer alami yang berperan dalam menurunkan insidensi dan intensitas penyakit dan menstimulasi pertumbuhan tanaman. Tujuan dari penelitian ini adalah untuk mengetahui kemampuan penghambatan dari larutan kitosan terhadap infeksi dari virus kerdil pada bibit lada dengan aplikasi penyemprotan. Konsentrasi kitosan yang digunakan adalah 0,5%; 0,75%; dan 1%. Hasil penelitian menunjukan bahwa apliksi kitosan pada semua konsentrasi berpengaruh dalam menurunkan insidensi dan intensitas penyakit dan meningkatkan pertumbuhan tanaman dengan tidak berbeda nyata di antara perlakuan tetapi berbeda nyata dengan kontrol. Penurunan nilai insidensi tertinggi yakni pada aplikasi kitosan 0,75% (26,37), sedangkan penurunan nilai intensitas tertinggi yakni pada aplikasi kitosan 1% (37,62). Aplikasi kitosan juga berpengaruh signifikan terhadap semua parameter pertumbuhan tanaman baik tinggi tanaman maupun diameter daun. Pada aplikasi kitosan 1% meningkatkan persentase tinggi tanaman lebih baik dibandingkan dengan perlakuan lainnya,yakni tinggi tanaman sebesar 58,12 % dan diameter daun sebesar 54,74 %.*

Kata kunci: lada, kitosan, penyakit kerdil

[ж]The article has been presented at International Conference on Biodiversity on 20th–21st May 2017

INTRODUCTION

Black pepper (*Piper nigrum* L.) is one of important estate crops in Indonesia. Stunting disease on black pepper plants is one of the inhibiting factors on the growth of black pepper plants. This disease was reported as the result of infection from pathogenic virus such as *Cucumber mosaic virus* (CMV) and *Piper yellow mottle virus* (PYMoV) (de Silva *et al*., 2002). Multiple infections of these viruses would generally cause more severe symptoms and inhibit plant growth. In addition, the recognition of visual symptoms in the field was very difficult due to their infection occurred at the same time (Miftakhurohmah *et al*., 2016). CMV is a single-stranded RNA virus with a broad host range and causes various symptoms. The common symptom on pepper is characterized by shrink, curly, brittle, hardened and chlorotic spotting leaves. In other cases, the leaves become abnormal, narrow and shortened of segment length, and the plant was stunting (Bhat *et al*., 2004). de Silva *et al*. (2002) reported that CMV could be transmitted through vegetative propagation materials (cutting and grafting), insect vector (*Aphis gosypii* Glover) and mechanically from black pepper to other host plants.

The prevalence of virus on plant could not be only observed based on the symptom (Bhat & Siljo, 2014; Miftakhurohmah & Balfas, 2014). Therefore, rapid detection method can determine the presence of this virus infection. Reverse Transcription Polymerase Chain Reaction (RT-PCR) is one of high sensitive molecular method in detecting RNA virus. The accurate information regarding the virus infection on plant can assist in determining the management strategies. The control measures for inhibiting the spread of this virus are the selection of healthy seeds, control of insect vector and sanitation (Miftakhurohmah & Balfas, 2014). Meanwhile, the development of the insect vector can be inhibited by synthetic pesticides as one of the common applied strategies. However, negative impacts of pesticide application limited the implementation of this technique.

Black pepper plants were generally propagated with vegetative material from cuttings. This method was applied due to effective and easy in obtaining the generation with similar characteristics to parenting plants (Suparman *et al*., 1992). Cuttings from infected plants can widely spread the virus. This is caused by the systemic infection of virus, so the selection of cuttings from infected plants will produce new infected plants. The use of healthy seedlings was one of principle technique on black pepper cultivation in order to enhance plant growth and production (Bhat *et al*., 2016). Therefore, it is required the investigation to suppress viral infection on cuttings and increase plant growth, primarily the use of natural and eco-friendly products such as chitosan.

Chitosan is a natural product derived from shells extract of crustaceans (Crustacea) such as shrimp and crab. Chitosan has been widely used to control plant pathogenic fungi, bacteria, viruses, pests and insect vectors. Chitosan has been reported to control some plant pathogenic viruses such as *Potato virus X*, *Tobacco mosaic virus*, *Tobacco necrosis virus*, *Alfalfa mosaic virus*, *Bean stunting virus* and *Cucumber mosaic virus* (Pospieszny *et al*., 1991). Faoro (2013) reported that chitosan could reduce the infection of *Bean common mosaic virus* and suppress the vector population. This is also confirmed by Damayanti *et al*. (2013) validated that the use of chitosan on seed treatment or leaf spraying could extend the incubation time *Bean common mosaic virus* (BCMV), express the variations of milder symptoms and significantly produce lower titer of BCMV compared to control. Remembering to the benefit of chitosan and systemic spread of the virus in plant cutting, it is important to apply chitosan on pepper cuttings which were infected by CMV.

MATERIALS AND METHODS

The research was conducted at Virology Laboratory and Greenhouse of Department of Crop Protection, Faculty of Agriculture, Universitas Gadjah Mada and at pepper plantations in Putat Village, Patuk, Gunungkidul, Province of Daerah Istimewa Yogyakarta (D.I.Y), in November 2016 until April 2017. The materials were CMV-infected pepper cutting, 1% of acetic acid solution, chitosan powder, Total RNA Minikit (Plant) (Geneaid), ReverAid First Strand cDNA synthesis kit and PureTaq Ready To Go PCR beads. The primers were forward primer (CMV-F 5'-TGTGGGTGACAGTCCGTAAA-3') and the reverse primer (CMV-R 5'-ACGCGGCATACT GATAAACC-3') with expected amplicon of 111 bp. The specificity of the primers was checked using the BLAST, and the secondary structure, intra- and

inter-primer complementarities were checked using Oligo Calc. The primers were synthesized at Integrated DNA Technologies (Coralville, Iowa, USA) (Bhat & Siljo, 2014).

Total RNA Extraction

RNA extraction was performed using Total RNA Minikit (Plant) following provided method by manufacturer (Geneaid). The tested samples were plants with mosaic and stunting symptoms which were collected from pepper plantations.

Reverse Transcription PCR

The synthesis of cDNA in this study was conducted by using ReverAid First Strand cDNA synthesis kit (Thermo Scientific, Waltham, MA USA). The RT-PCR run templates were samples of extracted RNA. The master mix was prepared according to the procedure shown in the cDNA synthesis instructions. The RT-PCR conditions were denaturation at 65°C for 5 min, annealing at 42°C for 60 min, extension at 70°C for 5 min and final extension at 4°C for 5 min.

PCR (Polymerase Chain Reaction)

The obtained cDNA was used as template in PCR analysis using PureTaq Ready To Go PCR beads. The procedure was based on the product instructions by adding 20 µl of free water (dH20), 1 µl of 5 pmol CMV111 F and CMV111 R primers and 3 µl of cDNA template, to make the total volume of 25 µl. PCR was run under conditions of pre-denaturation at 94°C for 3 min, denaturation at 94°C for 1 min, annealing at 55°C for 15 s, extension at 72°C for 1 min and final extension at 72°C for 5 min.

The PCR products were electrophoresed within a 0.8% agarose gel which was dissolved in 0.5× Tris-Borate EDTA (TBE) at 50 Volt for 45 min. The agarose gel was then immersed in a solution of ethidium bromide (EtBr) for 15 min, visualized on UV transluminator and documented with a digital camera.

Preparation of Chitosan Solution

Chitosan was collected from Laboratory of Microbiology, Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada. Chitosan powder was dissolved perfectly in a solvent (1% acetic acid). Those chitosan solutions were prepared by dissolving 5, 7.5, and 10 gram of powdered chitosan in 1000 ml of acetic acid solvent for getting concentrations of 0.5 %, 0.75%, and 1%, respectively.

Preparation of Pepper Cutting

Black pepper cuttings were collected from pepper growing areas in Gunung kidul, Yogyakarta. These cuttings were positively infected by CMV (confirmed with molecular detection RT PCR) showing mosaic, uneven surface, curling, shrink, malformations, narrowed leaves and shoestring-like shoot leaf symptoms. The fresh selected cuttings originated from climbing shoot with 4 segments. Cuttings were transferred to 5 kg in volume-polybags in green house by adding soil and compost with a ratio 2:1. Plants were then maintained until ready to use for treatment about 2 months old.

Application of Chitosan

Chitosan was applied on viral-infected seedling at the beginning of nursery (1st weeks) and repeated every week (for 9 times application) during 10 weeks observation, according to predetermined concentrations. It was sprayed using 500 ml of hand sprayer in the morning as much of ± 20 ml at the upper and lower surfaces of the leaves.

The Observed Parameters

The Development of Stunting Disease Incidence and Intensity

Observations were conducted weekly to monitor the development disease incidence and intensity in the greenhouse at the plant vegetative stage as long as 10 weeks. Weekly disease incidence was calculated by this following formula:

$$DI\ (\%) = \frac{Total\ number\ of\ infected\ leaves}{Total\ number\ of\ leaves\ observed} \times 100\ \%$$

The observation of disease intensity was demonstrated by measuring the score of disease severity on each tested plants, i.e. 0 for healthy leaves or no symptoms of disease; 1 for mild mosaic leaf and percentage of symptomatic leaves 5−10%; 2 for leaf mosaic clear and percentage of symptomatic leaves 11−25%; 3 for leaf mosaic clear, shoestring and the percentage of symptomatic leaves 26−50%; 4 for leaf mosaic clear, shoestring and the percentage of symptomatic leaves 50−75%; and 5 for leaf mosaic clear, shoestring, stunting and leaf percentage of symptomatic > 75%. The observed scores were then converted into the disease intensity with the following formula:

$$DI\ (\%) = \frac{\sum(n \times v)}{(Z \times N)} \times 100\%$$

With : DI = disease intensity, n = number of infected leaves with certain grade, v = disease score; Z = maximum disease score, N = total number of leaves observed

Observation Effect of Chitosan Application on Growth of Black Pepper Seedling

The plant height was weekly observed by measuring the seedlings from above of soil surface to growing point. The measurement was started on 1 to 10 weeks old plants after transferring. Meanwhile, total leaf diameter was measured at the last observation (10th week) by measuring the diameter of 5 young leaves for each treatment and then was averaged.

Experimental Design and Data Analysis

Experiments were arranged in completely randomized design (CRD), consisting of 3 treatments and 1 control and each treatment was repeated for 5 times, i.e. A = control (untreatment); B = 0.5% of chitosan, C = 0.75% of chitosan and D = 1% of chitosan. Data was analyzed by variance analysis and significant different data was proceeded with DMRT at level of α 5% using SAS version 9.13 program. The percentages of plant height and leaf diameter were compared to control using formula;

$$PI\ (\%) = \frac{Treatment\ plant\ scoring - original\ scoring}{original\ scoring} \times 100\ \%$$

with PI = percentage increase; Original scoring = untreatment plant scoring.

RESULTS AND DISCUSSION

Amplification of CMV RNA Using RT PCR Method

The infection of CMV in black pepper plants generated typical symptoms such as mosaic and stunting. The use of infected plant samples in the field successfully detected the presence of viral. The RT-PCR method could amplify DNA band product from symptomatic leaves with single band of 111 bp in size under annealing temperature of 55°C for 15 s and 0.8 % of agarose gel (Figure 1).

Stunting Disease Progression

The observations on disease incidence and intensity showed that all concentration treatments affected decrease of disease incidence and intensity. Variation of the symptoms on the black pepper

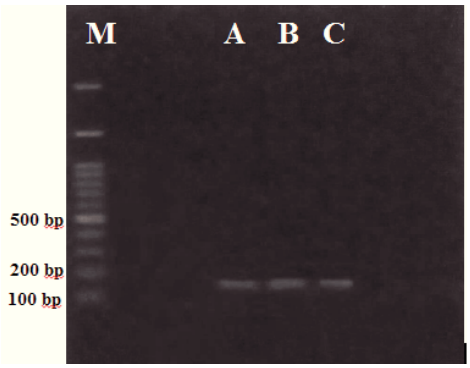

Figure 1. Visualization of PCR products of mosaic leaves and stunting samples; the DNA bands were amplified by CMV 111 specific primer (111 bp); lanes A–C = infected pepper samples; lane M = 100 bp DNA ladder

leaves appeared mild mosaic, chlorotic, curly, uneven surface, and wrinkled leaves, as well as shoestring-like leaf shoot and stunting plants (Figure 2). Treatments of B (Chitosan 0.5%), C (Chitosan 0.75%) and D (Chitosan 1%) showed that the scores were not significantly different between treatments in weekly observation, but they were significantly different with treatment A (control) at 3rd week observation.

The advanced of DMRT on incidence scoring of stunting disease revealed that the average scoring of A (control) at 10th week was 84.83%, while the scores of decrease in disease incidence were 40.045%, 26.037% and 34.107% for treatments B, C, and D, respectively (Figure 3). Further DMRT on intensity scores of stunting disease documented the percentages of decrease in disease intensity, i.e. 97.86%, 40.05%, 39.15% and 37.63%, for treatments of A, B, C, and D, respectively (Figure 4).

The application of chitosan showed that all concentrations of chitosan significantly affected the decrease of stunting disease and symptoms in black pepper seedling compared to control. Percentage of incidence and intensity among the treatments were not significantly different, but it was significantly different to control. Under DMRT analysis, score of incidence showed that treatment of 0.75% chitosan was more able to reduce the number of infected leaves rather than other treatments and control, while the lower score of decrease in disease intensity was reached by concentration of 1% chitosan.

Figure 2. Stunting symptoms on Black Pepper leaves; healthy leaves (a), mild mosaic and uneven surface leaf (b), mosaic and curly leaf (c), mosaic and shrink leaf (d), malformation leaf (e), mosaic leaf buds and narrowed leaf (f), mosaic and shoestring-like leaf shoot (g)

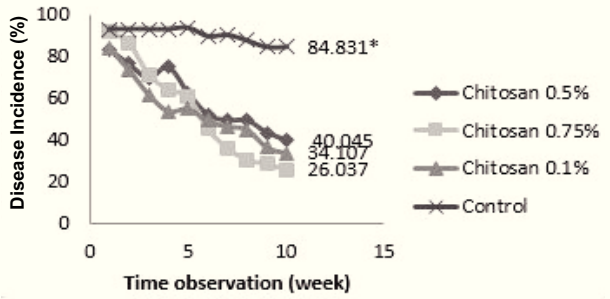

Figure 3. Effect of chitosan application on the incidence of stunting disease on black pepper seedling (*: significant difference between the chitosan treatments and control with a DMRT further test level α 0.05).

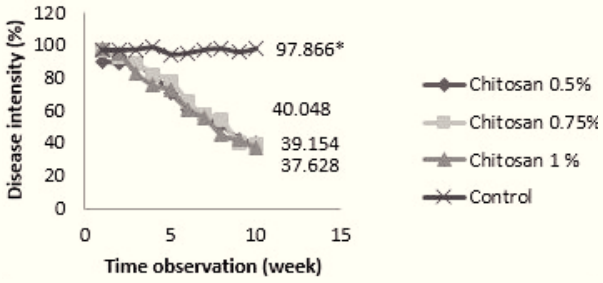

Figure 4. Effect of chitosan application on the intensity of the stunting disease on black pepper seedling (*: significant difference between the chitosan treatments and control in a DMRT further test level α 0.05)

The Effect of Chitosan Application on the Growth of Black Pepper Seedling

The Application of chitosan on black pepper seedling showed a significant effect on the growth and development of plants (Table 1). Observation of plant height and leaf diameter showed that application of chitosan was able to increase the percentage of plant height and leaf diameter compared to control. The percentage of increase in plant height were obtained i.e. 20.40%, 28.45 %, 38.44% and 58.12%, for treatments A, B, C, and D, respectively (Figure 5).

The decline intensity of pepper stunting mosaic disease was also indicated by narrowing the leaf size which was observed in leaf diameter. Application of chitosan showed significant effect on leaf diameter rather than the control. The increasing percentages in the leaf diameter were 29.92%, 30.65% and 54.74% for treatments B, C, and D, respectively. Data explained that the plant growth were higher under treatment of 1% concentration than other treatments and control (Figure 6).

Disscussion

CMV is one of the virus which infecting black pepper plants with typical symptoms mosaic and stunting. However, the types of symptoms caused by virus infection are very diverse and systemic. Further infection will produce more severe leaves symptoms such as mottle, vein banding, shrink, surface uneven, curly and shoestring leaves, while flower or short fruits cannot produce seeds. High variation in symptoms is very important for method of detection and characterization of this virus. Virus detection is generally performed by molecular methods such as Polymerase Chain Reaction (PCR) and Reverse Transcriptation Polymerase Chain Reaction (RT-PCR). Both methods will give rapid and high sensitivity results. The availability of

Table 1. The effect of chitosan application on the growth of black pepper seedling

Parameter	Treatment		Percentage of increase (%)
Plant height	Chitosan 0.05 %	26.230a	28.45
	Chitosan 0.75 %	28.270a	38.44
	Chitosan 1 %	32.290a	58.12
	Control (Untreatment plant)	20.420b	
Leaf diameter	Chitosan 0.05 %	3.5600a	29.92
	Chitosan 0.75 %	3.5800a	30.65
	Chitosan 1 %	4.2400a	54.74
	Control (Untreatment plant)	2.7400b	

Remark: The data followed by the same letter in the same column did not significantly different according to the Duncan test at the 95% confidence level.

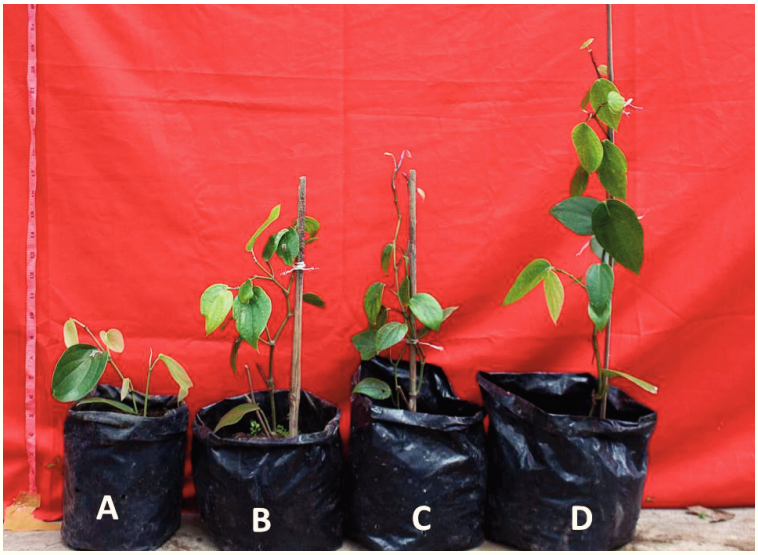

Figure 5. Effect of chitosan application on plant height; untreated plant (A), treated plants: B (0.5% of chitosan), C (0.75% of chitosan), and D (1% of chitosan)

Figure 6. The observation on leaf diameter of black pepper: (1) mosaic and stunted leaf (untreatment plant); (2) mosaic, chlorotic, narrow leaf and uneven leaf surface (treatment of chitosan 0.5%); (3) mosaic and narrow leaf (treatment of chitosan 0.75%); (4) mild mosaic leaf (treatment of chitosan 1%)

molecular detection with RT-PCR will help to detect the virus quickly and accurately as a first step in determining a management strategy for target virus. The pepper plant showing the presence of mosaic and stunting symptoms from Yogyakarta were identified infected by CMV. This was successfully indicated by the amplification of bands at 111 bp in size using a specific primer CMV 111. This research was not applied negative and positive control, due to the unavailability of samples as a comparison (Figure 1) and the research was finished. The detection of CMV in black pepper was also reported by Bath and Siljo (2014) using the same primer. The similar infection in pepper plants has also been reported by Siju *et al.* (2007) in India. In Indonesia, stunting disease has been reported since 1987 (Firdausil *et al*., 1992), while serological detection with ELISA method on pepper seedlings in Sukabumi and Purbalingga has demonstrated the infection of CMV and PYMoV on all tested varieties (Mariana & Miftakhurohmah, 2016). Meanwhile the reports about the presence of CMV infection in Yogyakarta have been known before in banana (Sumardiyono *et al*., 1996) and cucurbit plants (Daryono & Natsuaki, 2009). All these revealed that the infection of CMV has been widely distributed in Indonesia. However, the possibility of PYMoV infection on pepper in this research was not confirmed.

CMV has a broad host range with diverse symptoms. Such insights suggested that the control of selected pathogen should emphasize the general defense response. The use of chitosan has been known to boost the plant resistance against various types of plant pathogens and increase plant growth. Therefore, this research used chitosan as one alternative in controlling CMV on black pepper.The results of this research showed that the application of chitosan 0.5%, 0.75%, and 1% significantly influenced the inhibition of virus on black pepper plants. It was due to the ability of chitosan in inhibiting virus growth directly or indirectly. Direct inhibition is conducted by blocking the spread of the virus systemically throughout the plant and indirectly by increasing the hypersensitive response of the host to infection through inducing plant resistance. Induction mechanism of plant resistance with chitosan applayed through the formation of enzymes, protein and structure inhibitor such as callus, micro-oxidativeburst, micro hypersensitive response, the production of nitric oxide and activity of the enzyme phenylalanine ammonia-lyase (PAL) (Iriti *et al*., 2006; Zhao *et al*., 2007). Damayanti *et al*. (2013) confirmed that the treatment of chitosan could increase plant resistance under biotic stress (pathogen infection) and increases tolerance of abiotic factors. They also explained that long bean plants treated with chitosan showed a longer time of virus incubation and the variation of symptom was lower than the untreated control. This was confirmed by the presence of peroxidase activity and the lower titers of BCMV. The chitosan mechanism in inhibiting the virus movement was initiated by inducing the inhibitor enzymes, for example is peroxidase. Peroxidase enzymes could enhance inducing the plant resistance through physical barriers such as lignification. Lignification of cell wall was capable of inhibiting penetration of insect vector and preventing the virus movement (Goodman *et al*., 1986).

In this research, DMRT also showed that treatment of chitosan at all concentrations affected plant growth parameters such as plant height and leaf diameter. Scoring indicated that 1% chitosan treatment had a greater impact on all plant growth parameters, i. e., plant height and leaf diameter than other treatments and control (Table 1). In addition, the chitosan-treated plant demonstrated better growth than untreated ones. This was supported by research of Noiket *et al*. (2014) which stated that chitosan was a natural compound that could improve plant growth, induce plant resistance against diseases and reduce damage from plant pathogens. The results of this experiment proved that the application of chitosan at 10−20 ppm revealed lower scores of disease severity than the applications at 60 ppm. However, the concentration of 60 ppm was able to increase the height of tomato Sridathip 3 from Thailand compared to other treatments. The investigation of Mishra *et al*. (2014) also supported that the co-application of chitosan and beneficial microbes such as *Pseudomonas* sp. could reduce the severity of disease and stimulate the growth of tomato plants compared to control. Chitosan is known as good inducer to stimulate the resilience of the plant. Suptijah *et al*. (2010) stated that the combination of dipping and spraying treatments of 25 ppm chitosan gave the best effect on each parameter of plant growth such as height, number of branches and leaves, length and width of leaf as well as wet and dry weight of plants.

The ability of chitosan in inducing the growth of plants was regulated by the signal boosters to produce plant growth hormones such as gibberellins and also to enhance the signaling pathways to auxin biosynthesis (Uthairatanakij *et al*., 2007). Wanichpongpan *et al*. (2001) reported that chitosan treatment on Gerbera plants could increase the average number of buds, length of stem, number, width and length of leaf and number of flower per clump. The use of single chitosan without any chemical fertilizer was able to increase the population of microbes and fasten the transformation process of nutrient from organic to be inorganic compounds, so that they were easily absorbed by plant roots (Boonlertnirun *et al*., 2008). Meanwhile, Borkowski *et al*. (2007) *cit*. Suptijah *et al*. (2010) reported that the spraying of 25 ppm of chitosan on tomato plant could increase its harvested yield.

Based on all above explanations, it is known that chitosan is a natural product that can induce the decrease of disease in infected plants, although the plants remain infected by virus with lower intensity. It is recognized that the decrease in disease incidence and intensity could indirectly reach up to 100% in which there is still virus accumulation in plant tissue which displays as light mosaic symptom. Therefore, further investigation regarding the use of chitosan in reducing the CMV infection on black pepper is required, particularly pre-application of chitosan prior to infection and increasing the concentration of chitosan application. Some reports also suggest that stunting disease in black pepper was known has two viruses infection, those viruses were PYMoV and CMV (de Silva *et al*., 2002; Miftakhurohmah *et al*., 2016). The use of infected cuttings from the field in this study is likely carry multiple infections. Therefore it is also suspected that chitosan can also inhibit the development of PYMoV infection. However, accurate research is needed to ensure this inhibitory ability.

In conclusion, application of chitosan in all concentrations (0.5%, 0.75%, and 1%) were significantly affected the reduction of disease incidence and intensity and improved the growth of black pepper seedling compared to control. The highest decrease percentage in incidence was found at treatment 0.75%, while the highest decrease percentage of intensity was recorded at treatment of 1%. The application of 1% chitosan in treatment increased the percentage of plant growth parameters, such as plant height and leaf diameter rather than other treatments.

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