AGRIVITA Journal of Agricultural Science. 2021. 43(1): 56–68

AGRIVITA
Journal of Agricultural Science
www.agrivita.ub.ac.id

Control of Banana Wilt Disease Caused by *Fusarium oxysporum* Schlecht f.sp. *cubense* (E. F. Smith) Using Crab Shell Powder and Chitosan

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ARTICLE INFO

Keywords:
Antifungal
Biological control
Chitinoliytic bacteria
Induced resistance
Soil amendment

Article History:
Received: August 5, 2020
Accepted: November 3, 2020

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ABSTRACT

This research was conducted to evaluate the effectiveness of crab shell powder and chitosan to control Fusarium wilt disease on bananas and to analyze the involved control mechanisms. The effectiveness of crab shell powder and chitosan to *F. oxysporum* f.sp. *cubense* was examined in a laboratory (in vitro) and greenhouse (in planta). In vitro evaluation showed that chitosan has an antifungal effect while crab shell powder did not. Application of crab shell powder and chitosan suppressed the disease in green house test. The concentrations of crab shell powder and chitosan that most effective to control *Fusarium* wilt incidences were 0.25% and 0.10% with an efficacy rate of 66.7% and 83.3%, respectively. The highest disease severity reduction was showed by crab shell powder 0.25% and chitosan 0.50% with an efficacy rate of 56.8% and 59.4%, respectively. Suppression of the disease might be due to the fungicidal effect of chitosan and the increase of the total population of bacteria and chitinolytic bacteria in the rhizosphere when banana seedling roots were treated with crab shell powder or chitosan. Experiment results using the split roots technique exhibited the role of crab shell powder and chitosan potentially to induce the resistance of banana to *Fusarium* wilt.

INTRODUCTION

Banana is the most popular fruit and consumed by millions of people around the world. Even, in some countries, e.g. Central America, banana is also a major crop for economic turnaround. In the banana-producing countries, this fruit is a potential commodity to be developed both for export as well as domestic food resources. However, this sustainability faces the obstacle due to the presence of serious disease threats, one of which is *Fusarium* wilt. *Fusarium* wilt which is also known as Panama disease is widely regarded as one of the most destructive plant diseases and spreads all over the banana plantation in the world (Moore, Pegg, Bentley, & Smith, 2001; Ploetz & Pegg, 1997; Stover, 1962). This disease is caused by the soil-borne fungus *Fusarium oxysporum* Schlecht f. sp. *cubense* (E. F. Smith) Snyd and Hans (*Foc*) which can survive up to 30 years when the host is absent by formation thick-walled resting spore namely chlamydospore (Stover, 1962). Since the pathogen's high ability to survive in soil, once *Foc* is established in the area, the threat to banana production by this disease will continue to occur in the long term (Dita, Barquero, Heck, Mizubuti, & Staver, 2018). Many research has been conducted to find the best strategy to overcome this disease problem, but only a few control measures exist and applicable for managing this disease in the field (Ploetz & Pegg, 2000).

Since *Foc* is a soil-borne pathogen, many aspects that are determined the soil ecosystem's health are very critical for the management of the disease. Soil ecosystem, especially rhizosphere, has an important role for plants as an essential site for microbial activities to help plants in nutrients uptake and as a battlefield between pathogens and their antagonistic microorganisms (Berendsen,

ISSN: 0126-0537 Accredited First Grade by Ministry of Research, Technology and Higher Education of The Republic of Indonesia, Decree No: 30/E/KPT/2018

Cite this as: Widodo, Harti, H., & Wiyono, S. (2021). Control of banana wilt disease caused by *Fusarium oxysporum* Schlecht f.sp. *cubense* (E. F. Smith) using crab shell powder and chitosan. *AGRIVITA Journal of Agricultural Science*, *43*(1), 56–68. https://doi.org/10.17503/agrivita.v1i1.2780

Pieterse, & Bakker, 2012; Raaijmakers, Paulitz, Steinberg, Alabouvette, & Moënne-Loccoz, 2009). At the beginning of the development of biological control of plant diseases, the studies were mostly conducted with the approach of isolation and selection of potential microbes and then applied by inundation to the field against the target pathogen as studied in suppressive soils. The existence of *Fusarium* diseases suppressive soils as a natural phenomenon have been well known for several decades in which biological control is effectively working (Alabouvette, 1999). Since most soil microbes cannot be cultured, there are many possibilities that potential microbes as biological agents for controlling plant diseases have not yet been explored (Amann, Ludwig, & Schleifer, 1995; Kent & Triplett, 2002). Recently, this limited information has been opened by the development in metagenomics that can provide the more complete configuration of microbes in a certain ecosystem (Bakker, Berendsen, Doornbos, Wintermans, & Pieterse, 2013; Berg, Grube, Schloter, & Smalla, 2014; Siegel-Hertz et al., 2018).

Implementation of biological control of plant diseases can be approached, either by introducing selected biological control agents or by manipulating the environment to be more conducive to indigenous antagonists to suppress the plant pathogens (Cook & Baker, 1983). Some agricultural practices shifted biotic and abiotic soil properties will determine whether the host will be vulnerable or not to soil-borne plant pathogens. Agricultural practices to promote the benefits of soil microbes activities, including soil solarization, crop rotation, residue management, soil tillage, and organic amendments is an important approach in suppressing soil-borne plant pathogens (Raaijmakers, Paulitz, Steinberg, Alabouvette, & Moënne-Loccoz, 2009). One of the methods to manipulate those growth environments is by applications of biopolymer substances such as chitin and chitosan for promoting indigenous soil antagonists (Hallmann, Rodríguez-Kábana, & Kloepper, 1999; Kielak, Cretoiu, Semenov, Sørensen, & van Elsas, 2013; Kloepper et al., 2004). Besides a role in stimulating beneficial soil microorganisms, chitosan is also known as an antifungal substance (Bell, Hubbard, Liu, Michael Davis, & Subbarao, 1998; El Hassni, El Hadrami, Daayf, Barka, & El Hadrami, 2004) and a potential elicitor to induce the plant immune systems (Reddy, Arul, Angers, & Couture, 1999). Chitin is produced from the waste products of processed marine food, e.g. crab shell and shrimp. When chitin is further processed by deacetylation will produce a modified natural carbohydrate polymer called chitosan (Xu, Gallert, & Winter, 2008).

The use of chitin and chitosan to manage some plant diseases has been studied by some researchers on pre-harvest (Abd-El-Kareem, El-Mougy, El-Gamal, & Fotouh, 2006; Cretoiu, Korthals, Visser, & van Elsas, 2013) and post-harvest (Jitareerat, Paumchai, Kanlayanarat, & Sangchote, 2007). There is limited information regarding the use of crab shell powder as a chitin resource and its derivative, chitosan, as a control measure for the *Fusarium* wilt disease on bananas. Considering those potential uses of crab shell powder and chitosan in the control of plant diseases, this study was conducted. The aim of this work was to evaluate the effectiveness of crab shell powder and chitosan to control *Fusarium* wilt disease of banana and to analyze the possible mechanisms involving in the disease suppression.

MATERIALS AND METHODS

Inoculum Preparation of *Fusarium oxysporum* f.sp. cubense (Foc)

The *Fusarium oxysporum* f.sp. *cubense* (*Foc*) IPB 057 used in these trials was the collection from the Laboratory of Plant Mycology, Department of Plant Protection-Bogor Agricultural University from March 2009 to December 2010. The rejuvenated Foc isolate was propagated in Potato Dextrose Broth (PDB) medium, and then shaken for as long as 7 days. The propagated Foc was then filtered using three layers of filter paper. The remaining pellets were then crushed using a find blender (Ultra-Turrax) and then suspended into 200 ml of sterilized water, mixed with 1 kg of soil which has been autoclaved (121^0C, 121 lb, 30 minutes) for two successive days, and incubated for 4 weeks to produce the chlamydospores. Soils contained with chlamydospores (± 10^7 chlamydospores per g of media) were kept within the temperature of ± 17^0C and used as the source of inoculum (Widodo, 2000).

Inhibition of Mycelial Growth and Conidia Germination of Foc by Crab Shell Powder And Chitosan

Crab shell powder (CSP) used as the source of chitin was obtained by crushing the dried crab shells. The chitosan used in this experiment was

obtained from the Department of Marine Science and Technology (Bogor Agricultural University) Indonesia, which was dissolved in acetic acid with a concentration of 6%. The mycelial growth inhibition test was performed with the food poisoning method by mixing the crab shell powder or chitosan with various concentrations in Potato Dextrose Agar (PDA) medium. PDA containing crab shell powder at final concentrations of 0.25, 0.50, 1.00, and 2.00% (w/v) and chitosan at final concentrations of 0.01, 0.05, 0.10, and 0.50% (w/v) were used for growth inhibition tests. PDA and PDA containing benomyl at final concentration of 0.15% (w/v) were used as additional controls. The media mixed with crab shell powder or chitosan of each concentration then shaken using vortex and then poured into Petri dishes (diameter 9 mm) as much as 10 ml. One plug (0.8 cm in diameter) of Foc colony grown on PDA was then put in the center of solidified media containing each concentration, and then incubated at room temperature for 7 days. The relative inhibition rate of mycelium growth was calculated using the formula as follow:

Relative Inhibition Rate = [(DC – DT) / DC] x 100%, ..1)

where: DC and DT are *Foc* colony diameter growth on untreated and treated media, respectively.

The conidia germination inhibition test was conducted using a modified procedure of Widodo (2000). Conidial suspensions of *Foc* were prepared by scraping the colony of 7 days old fungus culture on PDA after added with 10 ml sterilized aquadest per petri dish. To separate the conidia from mycelium, the suspensions were filtered using a-4 layer cheese clothes and then added with sterilized aquadest to be 100 ml final volume. This suspension was diluted to a final conidial concentration of $3x10^2$ conidia/ml and added with L-asparagin and glucose at a final concentration of 0.50% for each substance. Crab shell powder and chitosan with the desired final concentration were amended separately onto each conidial suspension. The final concentration of CSP were 0, 0.25, 0.50, 1.00, and 2.00% (w/v), while for chitosan were 0.0, 0.01, 0.05, 0.10, and 0.50% (w/v). Fungicide (a.i. benomyl) with final concentration of 0.15% (w/v) was used as check treatments. Two drops of each treated suspension were dripped on sterilized object glass and placed on moistened tissue papers. Conidial germination was observed under a light microscope after incubating the suspension at room temperature in the dark for 16 hours. The relative inhibition rate of conidial germination was determined using the formula described above for colony diameter measurements. The experiments were performed using a completely randomized design consisting of 10 treatments and five replications.

Effect of Crab Shell Powder And Chitosan on Banana *Fusarium* Wilt in Potted Banana

Natural sandy loam soil collected from the surrounding area of banana plantations mixed with cow manure (5:1 w/v) were filled into plastic bags (30 cm-diameter) and used as banana-growing medium. The growing medium then were infested with *Foc* chlamydospores in the concentration of 10^3 cfu/g soil (fresh weight). Dry CSP with concentration rates of 0.25, 0.50, 1.00, and 2.00% or chitosan (0.01, 0.05, 0.10, and 0.50%) was mixed in growing medium based on weight/volume. As the check and comparative treatments were 0.0% of CSP or chitosan, and benomyl 0.15%, respectively. Banana var. Raja Bulu seedlings from tissue culture at the age of 1 month after acclimatization were taken out from the medium and thoroughly cleaned using tap water. The seedlings were then soaked within each suspension of CSP and chitosan for 2 hours. The suspension was made by mixing the CSP or chitosan with distilled water to adjust the concentrations used for the treatments and added with Tween 80 0.2% as a dispersing agent. During the experiment, plants were fertilized with NPK (15:15:15) as much as 1g/plastic bag every month until the end of the observation period.

The efficacy of treatments was determined based on the incidence and severity of Fusarium wilt. To assess the efficacy of treatments in the suppression of Fusarium wilt disease incidence, the number of infected plants as indicated with leaf yellowing and/or wilting among total plants in each experimental unit was observed weekly until 16 weeks after treatment. Individual plants were rated for disease severity at 4 months after treatment based on corm discoloration index using a modified method of INIBAP Technical Guidelines (Carlier, De Waele, & Escalant, 2003) with a scale of 1 to 5, where 1 = no discoloration, 2 = only a few points discoloration in vascular tissue, 3 = discoloration of up to one-third of vascular tissue, 4 = discoloration between one-third to two-thirds of vascular tissue, 5 = discoloration greater than two-third of vascular tissue and/or total discoloration of vascular tissue.

Modification of the method was on the index numbering for proper calculation of disease severity due to the treatments. Plants were uprooted and washed with running tap water to separate soil particles and corm. Individual plant corm was then cut into 6 pieces in equal size and rated the discoloration using INIBAP method with scale numbering modification as mentioned above. The experiment was designed in a simple randomized complete block design with three replications where each treatment unit consisted of 4 plants. Efficacy of the treatment was calculated using the formula as follow:

Efficacy = [(DI without treatment – DI treatment) / DI without treatment] x 100%, ...2)

where: DI is disease incidence or disease index.

Banana Induced Resistance due to Crab Shell Powder and Chitosan

Induced resistance mechanisms due to CSP and chitosan treatments were determined using the split roots techniques (Widodo, 2000). The split root technique was done to determine the possibility of induction resistance mechanism by CSP and chitosan in the suppression of Fusarium wilt disease on bananas. One month after acclimatization of the banana seedlings were taken out from the medium and cleaned with tap water. The roots were then divided into two equal parts to be planted with the dual pot system which contained two 400 ml plastic glasses. One plastic glass was filled with sterilized soils amended with the CSP or chitosan, while the other was filled with sterilized soil and infested with 1000 cfu/g *Foc* chlamydospores. Parts of the roots were then cut by approximately 2 cm below the hump and then planted into plastic glasses which were added by CSP or chitosan treatments. Both of the plastic glasses were then placed within a bigger plastic pot (± 30 cm) covering with sterilized sand media to prevent contamination between the two plastic glasses. Disease incidence and severity were determined with the same method as above mentioned. The experiment was designed in a simple randomized complete block design with three replications where each treatment unit consisted of 4 plants.

Effect of Crab Shell Powder and Chitosan on Rhizosphere Microbial Populations

Rhizosphere microbial populations were determined at the end of the experiments. A composite sample of root pieces with soil particles remaining (10 g) was added to 100 ml sterilized water. The flasks were shaken on an orbital shaker at 150 rpm for 30 minutes. A serial dilution was prepared and 0.1 ml aliquots of dilutions 10^{-3}, 10^{-4}, 10^{-6}, and 10^{-7} were then plated on three media using micro pipette. The media were: 10% strength tryptic soy agar (10% TSA) (Difco Laboratories, Detroit, MI) to support the growth of a total bacteria; potato dextrose agar (PDA) (Oxoid LTD, England) added with 2 drops of 20% lactic acid/petri dish before pouring the melted medium for fungal populations and chitin agar containing 0.2% colloidal chitin to estimates the total chitinolytic microorganisms. The clearing zone surrounded the isolated microorganisms on chitin media were counted as chitinolytic.

Data Analysis

The data gained from the experiments, e.g. mycelial growth, conidia germination, and disease incidence were subjected to analysis of variance (ANOVA) and to Tukey Test on 95% confidence level for multiple comparisons among means. Meanwhile, to see the effect of treatment on the disease severity (disease index) was analyzed using Chi-Square test. The disease incidence development based on leaf yellowing of above ground symptom was also described using graphic.

RESULTS AND DISCUSSION

Growth Inhibition of *Fusarium Oxysporum* F.sp. cubense (Foc) by Crab Shell Powder and Chitosan

The effects of CSP and chitosan on the inhibition of *Foc* colony growth were varied (Table 1). Crab shell powder did not significantly reduce the growth of *Foc.* A aigher concentration of CSP treatment showed lower efficacy to inhibit *Foc* colony growth. On the other hand, all treatments of chitosan concentration had a significantly inhibitory effect against *Foc* growth. A higher concentration of chitosan showed a higher relative inhibition rate on *Foc* radial growth, and was more than 90% inhibited at 0.5% concentration which similar result to those with benomyl treatment (Table 1). The above treatments were also showed a similar effect on the germination rate of *Foc* conidia. CSP treatments also did not show significantly different results on the inhibition of conidia germination. Meanwhile, all chitosan treatments significantly inhibited the *Foc* conidial germination, where concentrations of

0.1 and 0.5% showed a similar inhibitory effect on conidial germination as benomyl (Table 1).

The *in vitro* test in this study indicated that CSP did not have a fungicidal effect as shown on the colony growth and conidia germination which were not significantly different comparing with the untreated (Table 1). The crab shell's main contents are chitin (15-30%), protein (19.5%), $CaCO_3$ (40-50%), and various minerals. e.g. P, Fe, Na, K, Cu, Mn, and Zn (Bilgin & Fidanbaş, 2011; Kurita, 2006; Tharanathan & Kittur, 2003). Since protein and various minerals within CSP are able as a nutrient source for fungal growth, the addition of these materials on PDA in *in vitro* test did not show any inhibition to *Foc*. As a fungicidal effect of chitosan, our experiment indicated that this substance within a concentration of 0.50% inhibits the radial growth and conidial germination of *Foc* more than 90% and 80%, respectively (Table 1). It is obvious, that chitosan in our present study has a direct inhibitory effect against the Fusarium wilt pathogen. The fungicidal effect of chitosan in the concentration of 0.1% has been reported to inhibit the mycellial growth and conidial germination of *Fusarium oxysporum* f.sp. *albedinis* by 75% and 100%, respectively (El Hassni, El Hadrami, Daayf, Barka, & El Hadrami, 2004). Other researchers reported that chitosan also has stimulatory effects on plant enzyme activities, e.g. chitinase (Abd-El-Kareem, El-Mougy, El-Gamal, & Fotouh, 2006; Deng, Zhou, & Zeng, 2015; Jitareerat, Paumchai, Kanlayanarat, & Sangchote, 2007) and β-1,3-glucanase (Jitareerat, Paumchai, Kanlayanarat, & Sangchote, 2007) that are able to inhibit the mycellial growth and conidial germination of pathogenic fungi. As other researcher's results and this study indicated that chitosan will be having as an alternative control measure other than synthetical fungicides to *Foc*. Since chitosan was made from a natural product, hopefully, it will be more environmental friendly comparing with synthetic fungicides.

Effects of Treatments on Disease Development

Due to the banana crop damage caused by Fusarium wilt disease and the difficulties of chemical control measures, alternative control of the disease are urgently needed. In this study, the efficacy of CSP as a chitin resource and chitosan in the suppression of banana Fusarium wilt and its possible mechanisms was tested under greenhouse conditions.

Table 1. The effects of crab shell powder and chitosan treatments on the colony growth and conidial germination of *Fusarium oxysporum* f.sp. *cubense*

Treatments	Colony diameter (cm)[1)]	Relative Inhibition rate (%)	Conidia germination (%)[1)]	Relative inhibition rate (%)
Without treatments	9.0±0.0^{a}	0.0	64.2±4.5^{a}	0.0
Crab shell powder 0.25%	8.9±0.2^{a}	1.1	58.9±27.0^{a}	8.2
Crab shell powder 0.50%	8.6±0.8^{a}	4.0	55.1±4.8^{ab}	14.2
Crab shell powder 1.00%	8.8±0.1^{a}	2.2	55.1±17.1^{ab}	14.1
Crab shell powder 2.00%	8.8±0.4^{a}	2.2	63.9±11.0^{a}	0.4
Chitosan 0.01%	6.7±0.9^{b}	25.1	33.4±6.7^{bc}	48.0
Chitosan 0.05%	4.8±0.5^{c}	47.1	23.2±5.7^{cd}	63.8
Chitosan 0.1%	3.7±0.5^{d}	59.1	16.7±1.0^{cd}	74.0
Chitosan 0.5%	0.8±0.0^{e}	91.1	9.2±3.1^{d}	85.6
Benomyl 0.15%	0.8±0.0^{e}	91.1	12.0±3.8^{cd}	81.2
	**		**	

Remarks :[1)] data followed by the same letter at each column are not significantly different ($p < 0.05$) according to Tukey Test; **) significant at $p < 0.01$ with the same test.

The effect of the treatments on banana Fusarium wilt in this study can be determined with the development of disease incidence based on above ground symptom and severity as showed in the corm discoloration index at the end of the experiment. Crab shell powder with a concentration of 0.25% and 0.50%, and chitosan with a concentration of 0.05%, 0.1%, and 0.5% were significantly suppressed the disease incidence development of banana Fusarium wilt comparing with untreated until the 16 weeks after treatment. These treatments even were better than benomyl fungicide which is now widely recommended as Fusarium diseases control measure (Fig. 1; Table 2). Except for CSP 2.0% and benomyl 0.15%, other treatments can delay the appearance of above-ground symptoms from 1 to 2 weeks compared with untreated (Fig. 1). The efficacy of crab shell powder was reduced as the concentration was raised up and contrast with chitosan. Chitosan treatment with a concentration of 0.1% and 0.5% was the most effective in reducing disease incidence with 83.3% efficacy comparing with untreated, but not significantly different between those two treatments (Table 2). Among the CSP treatments, a concentration of 0.25% was the most effective in suppressing the disease incidence with an efficacy value of 66.7% at 4 months after treatment. This study showed that chitosan treatments generally are more effective compared to crab shell treatments. The effective and efficient efficacy of chitosan treatment for reducing the disease incidence occurred when applied at a concentration of 0.50% (Table 2). Based on the Chi-square test, the treatments either direct application or using a split root system were significantly affect the disease index (Table 3). Generally, the CSP up to 1.0% and all applied concentrations of chitosan treatments reduced the disease index comparing without treatment. Among all the treatment's indirect application, the highest reduction was obtained with chitosan treatment at 0.50% in which reduced disease severity up to 59.4% and followed by CSP 0.5% with the efficacy of 51.3%. Although benomyl 0.15% also reduced the disease index, the efficacy was only 26.9% and not better than CSP and chitosan treatment (Table 3).

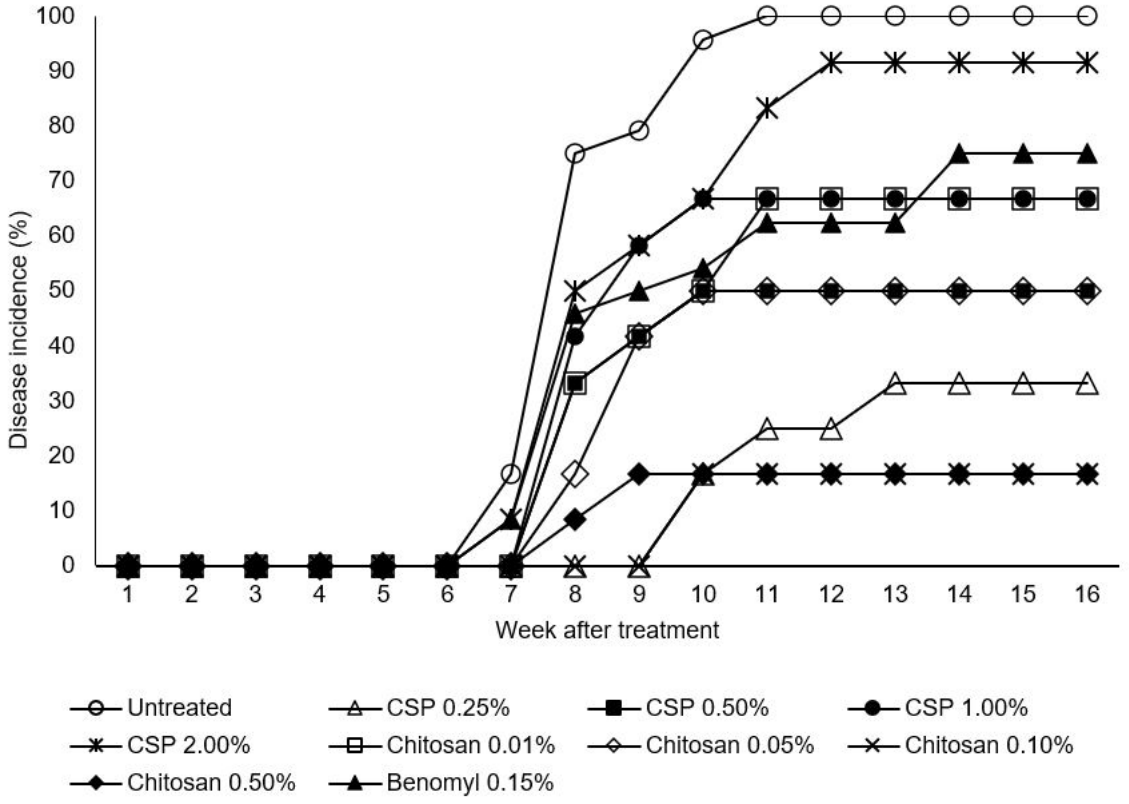

Fig. 1. Banana Fusarium wilt disease incidence development affected by crab shell powder and chitosan

Table 2. The effects of crab shell powder and chitosan on the disease incidence at 4 months after treatment for inducing resistance test

Treatment	Direct Treatment		Split Root System	
	Disease Incidence (%)	Efficacy (%)	Disease incidence (%)*	Efficacy (%)
Without treatments	100.0±0.0^{a}	0.0	95.8±7.2^{a}	0.0
Crab shell powder 0.25%	33.3±14.4^{cd}	66.7	33.3±14.4^{b}	65.2
Crab shell powder 0.50%	50.0±0.0^{bcd}	50.0	8.3±14.3^{bc}	91.3
Crab shell powder 1.00%	66.7±14.4^{abc}	33.3	8.3±14.3^{bc}	91.3
Crab shell powder 2.00%	91.7±14.4^{ab}	8.3	16.7±14.4^{bc}	82.6
Chitosan 0.01%	66.7±28.9^{abc}	33.3	25.0±0.0^{bc}	73.9
Chitosan 0.05%	50.0±25.0^{bcd}	50.0	0.0±0.0^{c}	100.0
Chitosan 0.10%	16.7±14.4^{d}	83.3	0.0±0.0^{c}	100.0
Chitosan 0.50%	16.7±14.4^{d}	83.3	0.0±0.0^{c}	100.0
Benomyl 0.15%	75.0±12.5^{abc}	25.0	70.8±7.2^{a}	41.6

Remarks: * data were transformed to arcsin prior statistical analyzing

Table 3. Effect of banana seedling treatment with crab shell powder and chitosan on Fusarium wilt disease index for inducing resistance test

Treatment	Direct treatment		Split root system	
	Disease Index*	Efficacy (%)	Disease Index*	Efficacy (%)
Without treatments	3.1±1.2	0.00	2.7±0.5	0.00
Crab shell powder 0.25%	1.3±0.5	56.8	1.0±0.0	62.5
Crab shell powder 0.50%	1.5±0.5	51.3	1.0±0.0	62.5
Crab shell powder 1.00%	2.1±1.2	32.5	1.0±0.0	62.5
Crab shell powder 2.00%	3.2 ±1.5	-2.9	1.1±0.3	59.5
Chitosan 0.01%	2.1 ±0.8	32.2	1.0±0.0	62.5
Chitosan 0.05%	1.7±0.9	45.2	1.0±0.0	62.5
Chitosan 0.10%	1.7±0.6	45.2	1.0±0.0	62.5
Chitosan 0.50%	1.2±0.4	59.4	1.0±0.0	62.5
Benomyl 0.15%	2.2±1.0	26.9	1.9±1.2	28.1
df	36		36	
X^2	66.692		102.316	
χ^2crit ($\alpha = 0.05$)	50.998		50.998	
χ^2crit ($\alpha = 0.01$)	58.619		58.619	
p-value	0.0013		0,000000028	

Remarks:* average of 12 plants for each treatment

Table 4. Total populations of banana root surface microbes treated with crab shells powder and chitosan

Treatments	Populations (log cfu/g sample)		
	Total Bacteria	Fungi	Chitinolytic bacteria
Without treatment	7.9	4.4	5.3
Crab shell powder 0.25%	9.4	3.9	6.3
Crab shell powder 0.50%	9.4	4.1	6.6
Crab shell powder 1.00%	9.3	4.2	6.9
Crab shell powder 2.00%	9.1	4.3	6.0
Chitosan 0.01%	7.9	4.6	5.7
Chitosan 0.05%	9.0	4.5	6.3
Chitosan 0.10%	9.4	4.3	6.5
Chitosan 0.50%	9.3	3.9	6.6
Benomyl 0.15%	8.1	3.9	5.8

Although in vitro testing CSP did not suppress the growth of Foc, but when this material was applied in planta using root dipping method within concentration up to 1.00% showed the suppression effect on the disease development. Our results indicated that the mechanism of suppression of Fusarium wilt disease on banana due to the application of CSP or chitosan can occur through two mechanisms, both direct and indirect. The mechanisms of the direct effect of CSP on the suppression of banana Fusarium wilt did not analyze further in this study. It showed that chitin containing in CSP has no fungicidal effect directly as showed in vitro experiment (Table 1), and the further process in nature of this material producing substances inhibited Foc and/or triggering other factors might have a role for suppression of the disease development. Other researchers reported that the involvement of chitin decomposition which releases volatile, e.g. ammonia, which is well known as antimicrobe volatile substance, has a role in suppression of some soil-borne plant pathogens (Hora & Baker, 1972; Sneh & Henis, 1971). Abd-El-Kareem, El-Mougy, El-Gamal, & Fotouh (2006) showed that application of chitin + chitosan at 6 g/kg soil was effectively controlled the root rot diseases on tomato caused by *Rhizoctonia solani*, *Fusarium solani* and *Sclerotium rolfsii*. Meanwhile, chitosan treatment showed the suppression effect both on the growth of *Foc in vitro* as well as to the development of the symptoms of the disease it caused.

Amendment of organic materials containing chitin also stimulates the soil enzyme activities such as dehydrogenase and chitinase (Wongkaew & Homkratoke, 2009) which are essential for maintaining soil fertility as well as soil health (Das & Varma, 2010). The one exception result in our study showed that treatments with CSP at the concentration of 2.00% were not much different compared to the untreated (Table 2). This condition was might result from the changes in soil chemical properties such as the soil pH. The average soil pH treated with 2.00% of CSP was 5.6, whereas on the soil pH of untreated was 6.8 (data not shown). This lower soil pH will make the pathogen was more infective compared to the higher soil pH (Stover, 1962).

This research also suspects that the disease suppression by using CSP was an indirect mechanism due to the increasing of beneficial bacteria populations, especially chitinolytic bacteria, that might be potential as antagonists (Table 4). Generally, the CSP and chitosan treatments increased the number of total bacteria and chitinolytic bacteria and reduced the total fungal population in the banana rhizosphere. The 0.01% chitosan treatment contained a lower count of bacteria population and a higher fungi population compared to other treatments. The chitosan treatment on higher concentration (0.50%) generally reduced the fungi population and increased the number of chitinolytic bacteria (Table 4). Although chitinolytic bacteria isolates obtained in our study were not tested further, presumably those bacteria are capable to hydrolyze the chitinous hyphae of the pathogen (*Foc*) and reduced the populations. When chitin contents materials were applied to the soil, it is possible to stimulate the growth of the useful microorganisms by the production of chitinase by the microorganisms to degrade chitin (Kielak, Cretoiu, Semenov, Sørensen, & van Elsas, 2013), and that may enhance the soil suppressiveness toward soil-borne plant pathogens (Cretoiu, Korthals, Visser, & van Elsas, 2013; Hallmann, Rodríguez-Kábana, & Kloepper, 1999). Bell, Hubbard, Liu, Michael Davis, & Subbarao (1998) demonstrated

that chitin application by drenching on the planting holes and chitosan treatment by seed soaking increased the population of chitinolytic microbes and actinomycetes in the soil. One fungus genus, *Mortierella,* and three bacterial genera, including *Bacillus, Lactococcus,* and *Pseudomonas* were reported to be predominant in the disease-free soils caused by *Foc* (Zhou et al., 2019). Genera of soil microorganisms with antifungal properties in soil microbiomes associated with Verticillium wilt-suppressive broccoli were also reported more abundant in chitin amended soils (Inderbitzin et al., 2018). Based on molecular identification, the population of some genera of rhizospheric microbes potentially as biological control agents, such as *Cellvibrio, Pedobacter, Dyadobacter, Streptomyces, Lecanicillium,* and *Mortierella,* was ten-fold increased in chitin mixed potting soil (Debode et al., 2016). Remediation of Fusarium disease of long-term watermelon monoculture field soil using bio-organic material was also reported in China. This treatment significantly reduced the population pathogenic *F. oxysporum* and enhanced total beneficial bacteria in treated soils (Liu et al., 2018). The complex role of rhizhosphere microbal communities in plant health is well known, especially with regard to abundance and diversity (Fu et al., 2019). Samples of banana with asymptomatic Fusarium wilt showed higher bacterial and fungal diversity and abundance than the infected samples. Several of those dicovered microbes communities were known to play key roles in plant growth and health (Kaushal, Swennen, & Mahuku, 2020). Abundance of some beneficial bacterial of different phyla isolated from healthy banana soils has been demonstrated to correspond with lower Fusarium disease incidence in banana plantation (Efendi, Pambudi, & Pancoro, 2019). From an ecological point of view, biological control agents are often influenced by environmental factors, including the availability of nutrients and other microbes in the ecosystem. The combination of *Bacillus velezensis* HNO_3 with worm compost or cow dung has been shown to increase the suppression of Fusarium wilt in banana plants due to the high accumulation of several elements, such as Mg, P, Zn and Mn (Wu, Shan, Li, Li, & Wu, 2020). The other indirect mechanism may also be involved in suppressing this disease due to the addition of some mineral elements contained in CSP, including Ca, P, Zn, and K as reviewed by Orr & Nelson (2018). The combination of Fe, which is also contained in CSP, and Boron (B) was able to suppress Fusarium wilt in bananas due to the decrease of the phytotoxic synthesis produced by the pathogen (Dong, Wang, Ling, Shen, & Guo, 2016).

The split roots technique was done to determine the possibility of induced resistance mechanisms by CSP and chitosan in the suppression of Fusarium wilt disease on bananas. Our study showed that the CSP and chitosan treatments using the split roots technique significantly suppressed the disease incidence and severity (index) comparing to those without treatments and benomyl, with an efficacy of more than 60% (Table 2 and Table 3). The 0.50%, and 1.00% CSP treatments suppressed the disease incidence to more than 90%, while chitosan treatments with the concentration of 0.05, 0.01 and 0.50% completely reduced the disease incidence and did not show any discoloration on the rhizome. The effective for inducing resistance against *Foc* was chitosan at a concentration between 0.05% and 0.5%, followed by CSP up to 0.10%. Treatments with chitosan at the concentration of 0.05% to 0.5% caused a complete reduction in both disease incidence and disease severity in a split-root system experiment. Research on the potential of chitosan in inducing plant resistance to disease has been widely reported, but the use of chitin as a raw material for making chitosan, e.g. CSP, is still rare. Pre- or post-harvest treatment with oligochitosan has been demonstrated can induce plant defense mechanisms against plant pathogens by stimulating defense-related enzymes, and enhancing activities of plant volatiles (Deng, Zhou, & Zeng, 2015; Yin, Zhao, & Du, 2010; Zhang & Chen, 2009). Inducing plant resistance with chitosan treatments has also been reported by Amini (2009), Benhamou, Lafontaine, & Nicole (1994), and Paz-Lago et al. (2000) against Fusarium wilt diseases, and Farouk, Ghoneem, & Ali (2008) for downy mildew. Chitosan showed two main mechanisms in the suppression of plant diseases, e.g. directly due to fungicidal effect (Abd-El-Kareem, El-Mougy, El-Gamal, & Fotouh, 2006; Amini, 2009; Khiareddine et al., 2015) and indirectly by inducing host resistance (Benhamou, Lafontaine, & Nicole, 1994; Farouk, Ghoneem, & Ali, 2008; Khiareddine et al., 2015; Paz-Lago et al., 2000). The report of Paz-Lago et al. (2000) showed that chitosan has a role as an elicitor in inducing the synthesis of some enzymes created to host defense against *F. oxysporum* f.sp. *lycopersici* on tomato, such as β 1,3 glucanase and peroxydase.

This elicitor also increased the thickness of leaf blade, palisade tissue, spongy parenchyma, midrib region, and dimension of vascular bundles that resulted cucumber more resistant to downy mildew pathogen (Farouk, Ghoneem, & Ali, 2008). Chitosan was also reported induces plant defenses in tomato root exudates against Fusarium wilt and root-knot nematode *Meloidogyne javanica* by triggering reactive oxygen species that mediated to alter chemical components of the cells and lipids (Suarez-Fernandez et al., 2020). On the other hand, reduction of Fusarium wilt disease incidence and severity based on the split-root method in our present study indicates that chitin containing materials e.g. CSP might also be potential as an elicitor in plant defense mechanisms.

This study also showed that CSP in a concentration of 0.25% up to 1.0% and all chitosan treatments were able in delaying of banana Fusarium wilt symptom when applied into seedlings. The symptoms delay could reach between 26 to 28 days comparing with the untreated. Based on the results of our study we reported here, it showed that the nursery treatment with CSP or chitosan has prospects in controlling Fusarium wilt of bananas. It is also interesting to note in the present study that the control effect achieved by seedling roots dipping using chitin (CSP). This method is expected to be more efficient in the use of materials compared to soil amendment and might be applied as seedlings treatment prior to transplanting. However, re-treatment should be performed during the growing season for practical purposes in the field scale to maintain the induced resistant mechanisms and delaying the appearance of the symptoms. Further researches are required to study the CSP and chitosan in terms of methods and application frequencies, especially in field conditions. It is also required to study the various combinations of CSP and chitosan in suppressing the incidence and severity of Fusarium wilt disease on bananas. Until now, Fusarium wilt disease is still one of the main obstacles in banana production worldwide. Due to the nature of the causative pathogens which are able to survive for a long time in the soil and have elastic genetic characters, the effectiveness of controlling this disease by using resistant varieties and fungicides are not able to perform in a long period. This study showed that the mechanism in suppressing this disease by this treatment was inducing plant resistance and increasing the natural antagonistic microbial population, and it is hoped that the effect will be long lasting and more sustainable. With the ease of finding materials and how to apply them as well as their effectiveness in reducing the disease severity and incidence, we hope that the results of this study can be an inexpensive and applicable control measure for the Fusarium wilt diseases on banana, either in big or smallholder banana plantation.

CONCLUSION AND SUGGESTION

The suppression of Fusarium wilt due to the application of CSP through an indirect mechanism, while chitosan treatment occurred through either direct mechanism as a fungicide and/or indirectly as happened in the CSP treatment. Crab shell powder and chitosan treatment were able to induce banana plant resistance against Fusarium wilt disease. The mechanisms of induction of banana plant resistance to Fusarium wilt due to the treatment of both materials should be further investigated.

ACKNOWLEDGEMENT

This research was funded by Research Center for Tropical Horticulture, Bogor Agricultural University through the National Strategic Research (RUSNAS) program. The authors would like to express kindly thanks to above institution for supporting this research.

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