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Original Article

Antagonism and compatibility of biofertilizer bacteria toward *Fusarium oxysporum* F. sp. *Cubense*

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Abstract

Fusarium wilt caused by *Fusarium oxysporum* F. sp. *cubense* (FOC) is an important disease on banana. Fusarium wilt was hard to control because the pathogen can survive in many kind of soils type although there is no host. Therefore, overcoming the disease is urgently needed to develop such as biological control. Biofertilizer bacteria, such as *Azotobacter*, *Azospirillum*, *Streptomyces* and *Bacillus* were begun to use as antagonist agent to the pathogen. This research aimed to study the mechanism of antagonism of the biofertilizer bacteria toward FOC. There were 4 isolates examined *in vitro* to test the production of chitinase, pectinase, and antagonism. The research showed that all isolates of biofertilizer bacteria were able to produce chitinase and pectinase except *Azospirillum*. *Streptomyces* and *Bacillus* were able to inhibit the growth of FOC colony at 80.45 and 87.71% respectively. Combination of *Azotobacter* to *Streptomyces* is compatible as well as *Azospirillum* to *Bacillus*. *Azotobacter* to *Azospirillum* is incompatible as well as *Streptomyces* to *Bacillus*.

Keywords: Banana, Azotobacter, Azospirillum, Streptomyces, Bacillus

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Introduction

Fusarium wilt is an important disease in banana. The disease is caused by *Fusarium oxysporum* F.sp. *cubense* (FOC) (Ploetz, 1994). Intensively cultivated banana plants tend to be susceptible to fusarium wilt disease (Moore et al., 2002). FOC is disseminated by soil, suckers, waters, and cultivation tools (Buddenhagen, 2009). Ploetz (2015) explained that FOC pathogen spreads faster on monocultural bananas grown intensively by using inorganic fertilization. The absorption of inorganic fertilizers by plants directly interferes with soil microbial activity because there was no association between soil and microbes (Meyer and Leveau, 2012). This resulted in the impairment of plant defence causing plant susceptibility toward disease (Rebib et al., 2012). Biofertilizer can provide biological control toward soil pathogens and also able to provide nutrients needed by the plant (Agrios, 2005).

Groups of biofertilizer bacteria such as *Azotobacter*, *Azospirillum*, *Streptomyces* and *Bacillus* have potential as biocontrol agents. Biofertilizer bacteria have been reported to be able to suppress plant disease progression (Rao, 1982; Cahyani et al., 2014). On the other hand, the population of biofertilizer bacteria tends to be unstable when applied in the field due to the competition between bacteria to obtain nutrients

and their adaption to the new location. Regarding the role played by biofertilizer bacteria in plant growth, an interaction between the biocontrol bacteria, the pathogen and the plant is believed to be involved, provided that the environmental conditions are appropriate (Minerdi et al., 2011; Sukmawati and Myarsyah, 2017). The aim of this research was to understand the antagonism mechanism and compatibility of biofertilizer bacteria toward FOC *in vitro*. The ability of biofertilizer bacteria in suppressing pathogen is the first step to obtain potential biocontrol agents.

Material and Methods

Chitinase and pectinase activity test

The chitinase and pectinase activity of biofertilizer bacteria were be held from January to September 2017 at Microbiology Laboratory, PT. Indo Acidatama Tbk., Indonesia. Suspension of biofertilizer bacteria such as *Azotobacter* (10^7cfu mL^{-1}), *Azospirillium* (10^7cfu mL^{-1}), *Streptomyces* (10^5cfu mL^{-1}), and *Bacillus* (10^8 cfu mL^{-1}) were prepared by culturing them in King's B liquid medium incubated for 24 h. Each isolate was placed in the center of a Petri dish containing chitin and pectin agar as treatment and without chitin and pectin (from citrus) as a control (Salvador et al., 2005). Chitinase and pectinase activity tests were qualitatively observed by the diameter of bacterial colony growth in agar medium.

Antagonism test *in vitro*

FOC isolates were obtained from the collection belong to Plant Pests and Diseases Laboratory of Universitas Sebelas Maret (UNS), Indonesia. The inoculums of bacteria were prepared by growing the single colony bacteria in King's B incubated for 24 h with density of 10^7-10^8 cfu mL^{-1}. The inhibitory test of the biofertilizer bacteria toward FOC was performed using dual culture method according to inhibition zone (Kim et al., 2008). A piece FOC colony (Φ 5 mm) was cultured on a PDA with a distance of 3 cm from the edge of the Petri dish. The filter paper (Φ 5 mm) was immersed in the isolate suspension of biofertilizer bacteria with density of 10^8 cfu mL^{-1} and then cultured side by side with the FOC with density of 10^6 spores mL^{-1} and a distance of 3 cm from the edge of the Petri dish in the same culture medium. The antagonism test *in vitro* used a random completely design (RCD) with 5 replication. The growth inhibition of FOC by biofertilizer bacteria was calculated by following formula (Ghildial and Pandey 2008):

$$Growth\ inhibition = \frac{r1 - r2}{r2} x\ 100\%$$

Where r1 = FOC radius away from biofertilizer bacteria, r2 = FOC radius approaching biofertilizer bacteria. The data were analyzed with *F* test and DMRT at level of 5%.

Bacterial toxic filtrate test

A total suspension of 3 mL of biofertilizer bacteria isolates were inserted in the test tube and centrifuged by 5000 rpm for 25 minutes. The supernatant from the isolate suspension was taken then autoclaved for an hour then exposed to UV light for 2 h. Each supernatant was placed on the PDA side by side and incubated for 1-2 weeks. Zone of inhibition was observed for a week. Data analysis was performed as antagonism test (Malinda et al. 2015).

Volatile toxic compound test

The volatile toxic compound test was prepared by preparing as much as 0.1 mL suspension of the biofertilizer bacteria flattened on the Petri dish containing the NA culture medium in a lid of Petri dish and at the same time the FOC colony (Φ 5 mm) were cultured on the other lid of the Petri dish containing the PDA culture medium. The both lids were covered each other with FOC culture upside down and silted using plastic isolator. The growth diameter of FOC colony was observed every 2 days for 8 days (Ting et al. 2011).

Antagonism compatibility test

For compatibility tests between isolates of *Azotobacter*, *Azospirillum*, *Streptomyces* and *Bacillus*, each isolate were suspended in sterile water. Compatibility test (ie.A vs B) was performed by immersing a filter paper (Φ 5 mm) into a bacterial suspense A and put on a nutrient agar (NA) medium that had been dispersed as much as 0.1 mL of bacterial suspension B. Reversed test (B v's A) was also made with the same method. The incompatibility was indicated by the appearance of inhibition zone (Hadiwiyono and Widono, 2012).

Results and Discussion

Chitinase and pectinase activity

The results showed that some bacteria were able to produce chitinases and pectinases except *Azospirillum* (Table 1). It indicated that *Azospirillum* is unable to degrade chitin and pectin. Qualitative chitinase activity determined the presence of clear zones on agar medium.

Table 1. Chitinase and pectinase activity of biofertilizer bacteria

Biofertilizer bacteria	Chitinase		Pectinase	
	Activity*	Colony (ø: cm)	Activity*	Colony (ø: cm)
Azotobacter	(+)	2.60 ± 0.30	(+)	1.62 ± 0.32
Azospirillum	(-)	0.00 ± 0.00	(-)	0.00 ± 0.00
Streptomyces	(+)	1.90 ± 0.63	(+)	1.27 ± 0.44
Bacillus	(+)	1.68 ± 0.31	(+)	1.50 ± 0.46

Description: *) Chitinase and pectinase activity at 24 h after incubation: (+) positive if degrade chitin / pectin and (-) negative if not degrade chitin / pectin.

Azotobacter, *Streptomyces* and *Bacillus* could use the chitin and pectin as carbon source. The pathogenic fungi have cell walls containing chitin compounds (Huan et al., 2005). FOC chitin degradation is naturally occurring by certain types of bacteria and actinomycetes (Fakamizo et al., 1996; Bressan and Fontes, 2010). Aaisha and Barate (2016) described that pectinase is usually found in almost all organisms including yeast, antagonistic bacteria, fungi and pathogens. Testing the ability of biofertilizer bacteria in degrading chitin and pectin in the FOC was supported by antagonism, filtrate and volatile compounds.

Antagonism

Some bacteria were able to inhibit FOC colony (Fig. 1). *Bacillus* and *Streptomyces* are the most effective in inhibiting the growth of pathogen. In the PDA medium the growth of FOC conidia able to cover the suspension of *Azospirillum* reaches the entire culture medium.

Results showed that *Streptomyces* and *Bacillus* were antagonistic to wilt pathogen. Hadiwiyono and Widono (2012) explained *Bacillus* is a potential biocontrol agent of fusarium wilt caused by FOC. *Azotobacter* and *Azospirillum* were not able to suppress the growth of pathogen. FOC is a weak parasite when it is in the soil, but in culture media where nutritional needs are fulfilled its conidial growth is much faster (Garcia et al., 2013). The expected bacteria to be biocontrol agents should have a faster rate of colony growth compared to pathogen growth (Kumar et al., 2010). The use of several biofertilizer bacteria with various action modes can improve the effectiveness of biological control (Grosh et al., 2011).

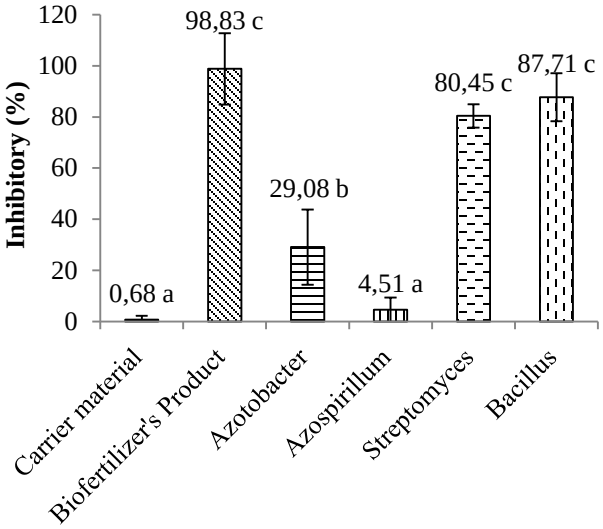

Fig 1. Inhibitory of biofertilizer bacteria toward FOC colonies in vitro.

Description: The average followed by the same letter is not significantly different based on DMRT at 5%

Toxic filtrate and volatile compound

The present results are consistent with antagonism tests that toxic filtrate of *Streptomyces* and *Bacillus* capable to inhibit the growth of FOC colonies (Table 2). *Azospirillum* was unable to suppress FOC growth either directly through antagonism or indirectly through filtrate compounds. The mechanism that plays a role in filtrate test is antibiosis whose function resembles the antifungal compound through the activity of chitinase and pectinase. These antibiotic compounds are thought to interfere with the growth of pathogenic fungal spores. FOC pathogen without biofertilizer bacteria was able to grow optimally with a diameter of 4.77 cm in the volatile compound test.

The formation of clear zone on agar medium was used to select biofertilizer bacteria with the mechanism of inhibition by antibiosis. The mechanism of bacterial suppression of pathogenic growth may have occurred

indirectly through antibiosis (Vessey, 2003; Hadiwiyono and Widono, 2013). Arrebola et al. (2010) explained the growth of mycogenic fungal mycelium may be inhibited by bacterial antibiotic activity. The ability of *Streptomyces* in synthesis of antibiotics is often used in degrading fungi cell walls. *Streptomyces* have various active compounds of several types of antibiotics, antiviral compounds, several enzymes, growth promoters and biofertilizers (Dharmaraj and Dhevendaran, 2008).

Table 2. Bacterial filtrate and volatile toxic compound of biofertilizer

Treatment	Toxic	
	Filtrate (Inhibitory: %)	Volatile (FOC ø: cm)
No Biofertilizer	0.00± 0.00 a	4.77 ± 0.16 a
Azotobacter	18.97± 3.97 b	3.59 ± 0.54 b
Azospirillum	0.69 ± 1.54 a	3.54 ± 0,41 b
Streptomyces	71.82± 11.06 c	2.77 ± 0.14 c
Bacillus	66.76± 8.91 c	2.34 ± 0.80 c

Description: The average followed by the same letter is not significantly different based on DMRT at 5%.

Bacillus have been reported to produce more than 66 types of antibiotics that are toxic to the pathogen (Mochizuki et al., 2005). Hadiwiyono and Widono (2017) explained *Bacillus* produced volatile compounds that are presumably onvolved in the antagonism mechanism showed by decreasing growth of pathogen on the agar medium. *Azotobacter* and *Azospirillum* have not been able to inhibit the growth of FOC colonies.

FOC in PDA that was cupped over by culture of biofertilizer bacteria on NA shows the diameter of varied pathogen colonies. Interestingly, the release of active molecules, including volatiles, and physical contact in antagonism among the biofertilizer bacteria seem important for suppressing pathogen. Rebib et al. (2012) described a kind of volatile alkaloid by biofertilizer activity, especially the *Bacillus* that can inhibit the growth of FOC colonies.

Antagonism compatibility

The pair of *Azotobacter* and *Azospirillum*, *Azosprillum* and *Streptomyces*, *Bacillus* and *Streptomyces* were incompatible (Table 3). *Azotobacter* and *Azospirillum* are consistently incompatible each other. It was presumably due to nutritional competition in NA culture medium. Suryana and Cahyono (2008) reported that if two or more species microbes were placed in agar and they do not obstruct each other, then microbes were compatible. *Bacillus* versus *Streptomyces* was incompatible as indicated by a clear zone of inhibition.

Table 3. Biofertilizer bacteria compatibility

Biofertilizer bacteria	Character	Zone of inhibit (cm)
A against B		
Azotobacter* and *Azospirillum	Incompatible	2.81 ± 0.21
Azotobacter* and *Streptomyces	Compatible	0.00 ± 0.00
Azotobacter* and *Bacillus	Compatible	0.00 ± 0.00
Azospirillum* and *Streptomyces	Incompatible	2.36 ± 0.39
Azospirillum* and *Bacillus	Compatible	0.00 ± 0.00
Streptomyces* and *Bacillus	Incompatible	2.12 ± 0.21
B against A		
Azospirillum and *Azotobacter*	Incompatible	2.26 ± 0.29
Streptomyces* and *Azotobacter	Compatible	0.00 ± 0.00
Bacillus* and *Azotobacter	Compatible	0.00 ± 0.00
Streptomyces* and *Azospirillum	Compatible	0.00 ± 0.00
Bacillus* and *Azospirillum	Compatible	0.00 ± 0.00
Bacillus* and *Streptomyces	Incompatible	0.82 ± 0.18

Description: A against B: Bacteria A wasdyed filter paper by bacterial suspension, while bacteria B was spread on culture medium grown simultaneously.

Azospirillum and *Streptomyces* are not compatible with the inhibition diameter of 2,36 cm. Pryor et al. (2006) explained that the method of detection of inhibition zones in bacteria shows a competitive mechanism for growing space. *Streptomyces* and *Bacillus* were incompatible characterized by both colonies that form a clear zone on NA culture medium. The clear zone caused by both bacteria is also marked when its position is reversed. Han et al. (2005) argue that the diameter of the clear zone formed indicates the magnitude of microbial inhibitory capacity. *Streptomyces* antibiotics tend to have stronger effects on the growth of *Bacillus* colonies. Hasani et al. (2014) explained that

antibiotics produced by *Streptomyces* have different mechanisms of action by damaging the cell wall, disrupt the function of cell membranes, and disrupt protein synthesis and nucleic acid. *Streptomyces* and *Azospirillum* are compatible. Suspension of *Streptomyces* on the filter paper consistently produces an inhibitory compound against the colonization of *Azospirillum* dispersed in culture medium.

Conclusion

Mixed consorsium formulation of biofertilizer bacteria was the most effective in inhibiting the growth of FOC. Biofertilizer bacteria are able to inhibit FOC through the mechanism of competition, antibiosis, enzyme production of chitinase activity and pectinase. *Streptomyces* and *Bacillus* have the potential to biocontrol fusarium wilt disease *in vitro* but are incompatible when grown on the same medium.

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Contribution of Authors

Widyantoro A: Conceived Idea, Data Collection, Manuscript Writing.
Hadiwiyono: Designed Research Methodology, Variable Assesment Method, Data Interpretation, Manuscript Final Reading and Approval.
Subagiya: Statistical Analysis, Data Interpretation Analysis.

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