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Effectivity and compatibility of *Azotobacter* and *Bacillus* for biological control agents of fusarium wilt on banana seedlings

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Abstract. Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *cubense* (Foc), is important banana. This disease infect in any growing and generation stages resulted in banana harvesting failure. For that, effective method to control its spreading need to be developed. Rhizospheric and endophytic bacteria belongs to biological control agent (BCA) which could be effective to control Foc disease. These BCAs are able to produce enzymes and toxics which are negatively impact to pathogen growth, in the other side these compounds are able to promote plant growth. Based on that, this research was aimed to know the effectivity and compatibility of using *Azotobacter* A01 isolate, *Bacillus* B01 isolate (rhizospheric) and *Bacillus* B16 isolate (endophytic) as BCAs for banana wilt. In this study, banana seedlings were produced using tissue culture method, acclimated for 2 months then infested by BCA at 10^8 cfu g^{-1} on day 7 before Foc inoculation at 10^6 conidia g^{-1} of medium. The wilt disease and seedlings growth assessment were conducted weekly for 56 days period. As the result, all BCAs isolate in single or combination, were effective to control fusarium wilt by decrease its intensity. Moreover, isolate combination shows more compatible and effective as BCA in control the disease.

1. Introduction

Banana is the largest commodity in Indonesia by contribute 34.65% of total fruit production [1]. In 2011 – 2015, banana consumption in Indonesia grew 1.32% per year [2], meanwhile in 2015 banana production was reached approximately 7.3 million tons then decreased 4% by 2016. This production decrease was occurred in various provinces in Indonesia [3]. In 2006-2009 bananas in 15 provinces in Indonesia showed fusarium wilt symptoms with the highest incidence in South Sumatra (60.38%), West Kalimantan (42.19%) and Papua (21.62%) [4]. Fusarium wilt is an important banana disease caused by *Fusarium oxysporum* f.sp. *cubense* (Foc). The total damage to banana cultivation in the world due to fusarium wilt is estimated at 100.000 ha and cost around US $ 2 billion [5]. Infected bananas usually cannot bear fruit and the leaves turn yellow [6].

The application of fungicides in banana cultivation can enhance plant resistance in controlling fusarium wilt [7]. However, in long-term, its application will affect to the number and soil microorganism activity. The use of fungicides in addition to lethal pathogens is also able to kill soil microorganism which favorable to plants by suppressing root disease and accelerate plants health by enzymes [8].

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Application of biological control agents (BCA) is one of efforts in order to improve or develop banana cultivation. Endophytic and rhizospheric bacteria are the potential bacterium to be developed for BCA purpose. Endophytic bacteria grow in plant tissues and harmless to the host. These bacteria also play a role in fulfill plant nutrition and diseases control [9], meanwhile rhizospheric bacteria is a bacterium that live in the plant root and play a role to control soil borne diseases [10]. *Azotobacter* sp. belongs to the plant growth promoting rhizobacteria group, which have role to provide N nutrient and some can provide P nutrient for plant growing [11], as well as produce growth hormones [12]. *Bacillus* spp. can produce antifungal compounds that inhibit Foc growth [13] by suppressing the disease for about 19-30% in vitro tests [14]. Therefore the application of these BCAs, both in combination or single, were expected to suppress fusarium wilt that lead to increase plant growth.

2. Materials and methods

The materials are *Azotobacter* A01 rhizosphere from reeds, *Bacillus* B01 rhizosphere from sugarcane and *Bacillus* B16 from banana endophytes as BCAs. The BCA was sub-cultured on Nutrient Agar (NA) medium and incubated for 48 hours. The field experiment was consisted of 7 treatments, arranged by Completely Randomized Design with three replicates. In one treatment unit was consisted of 3 banana seedlings which produced by tissue culture acclimated for 2 months. The banana seedlings were planted in polybags filled with sterilized media consisting soil and compost (3:1, w:w). The inoculation of BCA isolates (10^8 CFU mL^{-1}) was carried out a week before Foc inoculation (10^6 konidia of g^{-1} planting media), leakaged of 25 holes $polybag^{-1}$. Inoculation of Foc was prepared by sub-cultured Foc on Potato Dextrose Agar (PDA) and incubated for a week. Foc konidia were harvested by adding 10 mL sterile water and transfered to Erlenmeyer glass as suspension stock. Observations were made to asses effect on plant growth and suppression of fusarium wilt. Disease intensity (IP) was ditermined by following formula.

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

where n= number of seedling with a certain score; v= score of wilting (0, 1, 2, 3, 4, 5 and 6); N= higher score; Z= all seedling. Score determination was based on the level of wilting using the following groups: 0= no wilting, 1= one of leaf-wilting, 2= two of leaf-wilting, 3= three of leaf-wilting, and 4= four of leaf-wilting, 5= five of leaf-wilting and 6 = six or all of leaf-wilting. The Area Under the Disease Progress Curve (AUDPC) was ditermined by the following formula.

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

where $AUDPC$= area under the disease progress curve; Y_i= disease intensity at first day observation; t_i= Time of the first observation; n= Observation during terminal disease.

Infection rate is determined by:

$$r = \frac{2.3}{t}\left(log\frac{1}{1-Xt} - log\frac{1}{1-Xo}\right)$$

with r= rate of infection (unit per day); t= observation interval (7 days or weekly); X_0= proportion of initial disease observation; and X_t = proportion of disease observations at t. The effectiveness of disease control was ditermined by a following formula.

$$EDC = \frac{e1-e2}{e1} \text{ x } 100\%$$

where EDC= effectiveness of disease control, $e1$= disease intensity on the seedling with no control, and $e2$= disease intensity of BCA treatment. BCA isolates showed biocontrol activities against pathogens were able to increase plant growth.

The compatibility test was performed by preparing 0.1 mL of bacteria suspensin 10^8cfu mL^{-1}droped in Petri's dish and then poured NA, followed with shaking to homogenized the bacterial suspension

with the medium before being solid. The next step is placing filter paper which has been immersed in BCA suspension 10^8cfu mL^{-1} in culture medium then incubated for a week to observe inhibitory zone formation. Antagonism test of BCA to Foc was done with dual culture technique. BCA isolates were streaked on PDA on Petri's dish Ø 9 cm by 3 cm from the point where the Foc was placed and incubated for 7 days. The percentage of inhibition was measured by a following formula.

$$Inhibition\ Percentage = \frac{r1 - r2}{r2} x\ 100\%$$

where r1= colony finger of Foc growing away from BCA, r2= the colony finger approaching BCA. Filtrate preparation was carried out by preparing 3 mL of BCA cell suspesion in a test tube then centrifuged in 5000 rpm for 25 minutes. The supernatant was taken from the suspension and then autoclaved for an hour. This process was performed for two times and then exposed under ultra violet (UV). The capability of BCA to produce toxic compound was studied by approaching the effect of BCA filtrate culture in inhibiting the pathogen growth. The test was carried out using multiple culture method. Volatile testing was done by a pair of cover dish. BCA was cultured on the battom of dish using NA and pathogen using PDA in the second cover dish paired by upside down position of the first dish. The set of dish was sealed by isolator plastic to make sure there were no volatile compounds leaking or exchanged by the air from outside dish. The growth of Foc colony diameter was observed until day 8. Data were analyzed using *F* test followed with and Duncan Multiple Range Test at 5% level.

3. Results and discussion

The results showed that *Azotobacter* A01, *Bacillus* B01 and *Bacillus* B16 were able to suppress Foc infection in both single and/or combination (Table 1). The combination of BCAs could inhibit Foc better than single isolate. This shows that the inoculation of *Bacillus* B01 and *Bacillus* B16 is effective to controle fusarium wilt. *Bacillus* is able to inhibit pathogens through antibiotic compounds, secondary metabolites and produced siderophores. Siderophore plays a role in suppressing the intensity of disease due to Fe^{3+} availability which is limited for pathogen, then the development is inhibited [15]. Foc inhibition is also caused by bacterial activity in secreting chitinase enzymes which play a role in breaking down chitin substrate on Foc cell walls [16]. *Bacillus* sp. able to induce systemic resistance through increased phenolic compounds, phytoalexin, peroxide and salicylic acid which play a role in suppressing disease progression. Colonization of BCA in plant root play a role in inducing resistance of plant through thickening of cell wall which result in inhibition of pathogen penetration into plant tissue [17].

Table 1. The effects of BCA on banana seedlings inoculated with Foc on disease intensity, infection rate, area under the disease progress curve (AUDPC) and effectiveness of disease control (EDC)

Biological control agent (BCA)	Disease intensity (%)	Infection rate (unit day^{-1})	AUDPC (%)	EDC (%)
Without BCA	57.4±6.42 C	0.11±0.04 b	1464.8±78.6	-
Azotobacter A01	48.2±3.21 Bc	0.08±1.00 a	1490.7±258.9	16.1±5.6 a
Bacillus B01	42.6±8.49 Ab	0.07±0.15 a	1387.0±216.7	25.8±14.8 ab
Bacillus B16	44.4±9.62 Ab	0.07±0.05 a	1374.1±345.9	22.6±16.7 ab
A01B01	40.7±3.21 Ab	0.06±0.00 a	1354.9±106.9	29.0±5.6 ab
A01B16	38.9±5.56 Ab	0.06±0.04 a	1315.7±107.1	32.3±9.7 ab
B01B16	35.2±3.21 A	0.05±0.09 a	1225.0±121.4	38.7±5.6 b

Explanation: The values in the same column followed by the same letter are not significantly different in Duncan's multiple-range test (P<0.05), A01B01= *Azotobacter* A01 and *Bacillus* B01, A01B16= *Azotobacter* A01 and *Bacillus* B16, B01B16 = *Bacillus* B01 and *Bacillus* B16.

The combination of *Bacillus* B01 and *Bacillus* B16 were able to suppress fusarium wilt by slowing down infections rate and suppressing disease intensity. The combination of BCA causing the isolation of Foc from the environment, then Foc is difficult to infect the plants [18]. Moreover, BCA is able to

suppress the progress of fusarium wilt [19]. The infection rate of the disease is related to the development of disease which is strongly influenced by environmental factors. Foc can grow optimally at 25°C, while it will be inhibited at temperatures below 15°C and above 30°C [20]. The average temperature in March-May was 29.8%. These environmental conditions support well the development of fusarium wilt because Foc is able to grow in optimal. The area under the disease-progress curve (AUDPC) is one of indicators of the reatment effectiveness in suppressing pathogens development. The combination of *Bacillus* B01 and *Bacillus* B16 showed the lowest AUDPC. *Bacillus* B01 and *Bacillus* B16 which were inoculated simultaneously able to work optimally in suppressing the development of fusarium wilt. This was supported by the compatibility test between BCA isolates.

The results of compatibility tests between BCA showed that the properties were compatible with one another because they did not form a clear zone inhibition. There is no inhibition zone formation showed by each bacterial isolate was compatible [21]. Compatibility between BCA is suspected because each bacterial isolate tested has the ability to compete for nutrients and different living spaces. The compatibility mechanism between BCA is influenced by the capability of microbes to provide nutrients, sensitivity to certain organic materials and the provision of secondary metabolites [22]. Bacterial compatibility is a prerequisite for the successful use of combined bacteria on agricultural land [23]. This is consistent with the results of combination of *Bacillus* B01 and *Bacillus* B16 showed the best inhibitory ability on the progress of fusarium wilt in banana seedlings. Combination of *Bacillus* B01 and *Bacillus* B16 are able to collaborate in colonizing plant roots so as to increase the ability to inhibit Foc. The mechanism of inhibition of pathogens by BCA can be determined by secondary metabolites produced [24]. *Bacillus* B01 and *Bacillus* B16 were able to produce antibiotic compounds that inhibited Foc growth in vitro with inhibition at range 50-60% (Table 2).

Table 2. Inhibitory capability of BCA against Foc in vitro

Biological control agent (BCA)	Growth Inhibition of Foc		
	Dual culture (%)	Culture Filtrate (%)	Volatile compound (ø:cm)
Without BCA	0.00±0.00 a	0.00±0.00 a	5.28±0.20 c
Azotobacter A01	22.26±12.40 b	19.18±15.01 ab	4.38±1.41 b
Bacillus B01	60.60±12.48 c	67.79±10.33 b	3.12±0.93 b
Bacillus B16	53.70±11.88 c	47.13±25.43 bc	4.28±0.64 a

Explanation: the values followed by the same letter are not significantly different based on Duncan's multiple range test (P<0,05).

In vitro test results showed *Azotobacter* A01, *Bacillus* B01 and *Bacillus* B16 were able to inhibit the growth of Foc colonies with the highest inhibitory activity was in *Bacillus* B01. Inhibition occurs because of the presence of antifungal compounds which are produced by the presence of clear zones. Antifungal compounds produced by bacteria cause swelling and shortening of the hyphae so that growth is inhibited [25]. *Azotobacter* and *Bacillus* have antifungal activity that can inhibit the growth of F. oxysporum [26]. *Bacillus* produces antifungal, siderophore, hydrogen cyanide, ammonia and salicylic acids which play a role in controlling pathogens [27]. Volatile compounds produced by *Azotobacter* and *Bacillus* in suppressing the growth of plant pathogens in the form of ammonia [28], and hydrogen cyanide [29]. *Bacillus* endophytes are able to produce volatile and non-volatile compounds, chitinase and pectinase enzymes and growth regulating hormones, especially IAA [30, 31]. This is supported by the testing of biological control agents on the growth of banana seedlings (Table 3).

Azotobacter A01, *Bacillus* B01 and *Bacillu*s B16 have a role in inducing plant resistance and are able to increase plant growth through BCA's phytohormones. Combination of *Bacillus* B01 with *Bacillus* B16 showed the best ability to suppress Foc activity and to increase plant growth. *Bacillus* was able to produce greater P available compared to Azotobacter, each of which was 66837 mg L^{-1} and 23813 mg L^{-1} [11]. Inoculation of *Azotobacter* and *Bacillus* is able to increase plant growth through the ability of bacteria to synthesize IAA, fix nitrogen, provide P nutrients and other elements [32]. *Bacillus* is able to stimulate growth and increase the resistance of banana seedlings from tissue

culture to fusarium wilt. The weight gain of the root is thought to be Foc suppression by biological control agents that have the ability to produce growth hormone, thus it can stimulate root growth [33]. Endophytic *Bacillus* isolates were able to release extracellular compounds that could play a role in increasing growth of banana micro-plantlets [34, 35]. Moreover, *Bacillus* endophytes applied to banana seedlings can increase weight of fresh and dry plants [36].

Table 3. The capability of biological control agents to grow banana seedlings inoculated by Foc

Biological control agent (BCA)	Plant Height (cm)	Number of leaves (piece)	Weight of Fresh Seedling (g)	Weight of Root (g)
Without BCA	42.67±03.93 A	6.44±0.33 a	59.37±4.93 a	18.94±1.74
Azotobacter A01	43.78±10.72 A	6.56±0.77 ab	70.28±2.83 bc	18.23±1.15
Bacillus B01	48.67±05.34 ab	6.78±0.78 ab	72.58±7.28 bc	19.95±6.95
Bacillus B16	53.22±09.62 bcc	6.89±0.80 ab	77.90±5.15 bc	24.57±6.78
A01B01	51.39±03.53 abc	6.78±0.63 ab	73.48±6.05 bc	22.67±1.40
A01B16	58.56±07.73 bcd	7.33±1.15 ab	82.01±5.59 c	21.97±3.17
B01B16	66.72±05.62 B	7.44±0.58 b	95.94±1.83 d	24.54±2.44

Explanation: The values in the same column followed by the same letter are not significantly different based on Duncan's multiple range test ($P<0.05$), A01B01= *Azotobacter* A01 and *Bacillus* B16, A01B16= *Azotobacter* A01 and *Bacillus* B16, B01B16 = *Bacillus* B01 and *Bacillus* B16.

4. Conclusions

Azotobacter A01, *Bacillus* B01 and *Bacillus* B16 are compatible with one to another as BCA of fusarium wilt. *Bacillus* B01 showed effective control Foc in in vitro test. In both, single and combination of BCA could decrease fusarium wilt intensity and promote banana growth including plant height, number of leaves, wet weight of seedling and roots. The inoculation of a combination of *Bacillus* B01 with *Bacillus* B16, however, were more effective to control fusarium wilt and promote banana growth than single inoculation.

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