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In vitro evaluation of the effect of combined indigenous antagonistic bacteria against *Fusarium oxysporum*

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ABSTRACT

This study aimed to evaluate the potential of combined indigenous antagonistic bacteria against Fusarium oxysporum f.sp. *cepae by in vitro. These bacteria were originated from coffee plant litter in UB Forest and already known their ability as a single biocontrol against F. oxysporum. The research was conducted at the Plant Disease Laboratory, Department of Plant Pests and Diseases, Brawijaya University. The methods consisted of isolate preparation, pathogenicity test and in vitro antagonistic test using a modified multiple culture method. The study was conducted with eight treatments and four replications. Based on this study, there were four best treatments in inhibiting the mycelia growth of F. oxysporum more than 50% compared to the control. The highest inhibitory was Bacillus mycoides and Alcaligenes faecalis which were able to inhibit F. oxysporum up to 67,46%. This study proves the potential of a new combination of indigenous antagonistic bacteria to inhibit fusarium wilt disease.*

Keywords:
Alcaligenes faecalis; Bacillus mycoides; Consortium bacteria; Coffee litter bacteria; UB Forest

1. Introduction

Fusarium oxysporum (*Foc*) is a species of pathogenic fungi causing fusarium wilt disease on shallot plant (*Allium ascalonicum* L.). Fusarium wilt disease is one of the most important diseases caused by soil borne pathogens in Indonesia. Symptoms of fusarium wilt are seen in the early and late stages of growth, such as twisted leaves, pale green leaves, smaller and less healthy tubers (Suryadi et al., 2020). Recently, plant disease control caused by soil-borne pathogens such us soil disinfection, using fumigants on storage process, using chloropicrin, hot water, solarization, or resistant cultivars are very popular (Arie, 2019). Researchers also discovered the effectiveness of chitosan which has an antifungal effect to control the incidence of fusarium wilt (Widodo et al., 2021). Farmers usually control this disease using pesticides and generally use pesticides beyond the recommended dosage. There are many negative effects of excessive use of pecticides (Dinata et al., 2023). Farmers are not aware of the damaging potential of pesticide toxicity. Many farmers do not have information about the types of pesticides and their long-term effects on human health. The uncontrolled

use of pesticides has resulted in the decline of non-target species, affecting animal and plant biodiversity, affecting aquatic and terrestrial ecosystems and food webs (Mahmood et al., 2016).

Based on these problems, an appropriate and environmentally friendly alternative to fusarium wilt control is needed. Biological agents from beneficial microbes have the potential to control plant diseases (Vyas et al., 2017). The potential of biological agents is currently being researched to find beneficial microbes in controlling fusarium wilt disease. Utilization of beneficial bacteria in the form of a single isolate shows good potential to be used as biological agents. Researchers found the potential of indigenous antagonistic bacteria from coffee litter bacteria in UB Forest which act as a single biological control (Dinata, 2018). However, it was still not effective in controlling fusarium wilt disease. Consortium has more properties than individual microbes, because the synergy of the microorganisms fills them (Aguilar-Paredes et al., 2020). The use of a combined bacteria in controlling plant disease is considered to have better results than using a single isolate, because it consists of several bacteria that synergize and provide compounds or metabolites that are beneficial to one another. Several studies have shown success using bacterial combination in controlling plant diseases. Research on the microbial consortium between *Bacillus* sp., *Pseudomonas sp*., and *Trichoderma* sp. able to inhibit the growth of *S. rolfsii* in vitro (Silaban et al., 2015). (Wartono et al., 2021) in their research, reported that the use of *B. subtilis* and *T. harzianum* either alone or combination could increase rice plant resistance to blast disease between 15.64% - 21.59%.

Over the years, various studies have described several techniques for reducing fusarium wilt in shallots. However, research on biological control of fusarium wilt on shallots with a combination of indigenous bacteria is still very limited. In connection with this, the current research is focused on the biological control approach of fusarium wilt caused by *Foc* with inoculation from a combination of indigenous bacteria. New biological control that synergizes several indigenous bacteria that have been isolated from UB Forest coffee litter. The purpose of this study was to to obtain the best combination of antagonistic bacteria in vitro for controlling fusarium wilt disease in shallots.

2. Materials and Methods

2.1 Preparation of bacteria and inoculum of *Fusarium oxysporum* f.sp. *cepae*

The research was conducted at the Plant Disease Laboratory, Department of Pests and Plant Diseases, Faculty of Agriculture, Brawijaya University from January – April 2020. Stock cultures were prepared from all of the antagonist bacterial isolates, grown on Natrium Agar (NA) medium to verify their purity. The test used five isolates of coffee litter bacteria, identified *Bacillus mycoides, Alcaligenes faecalis, Pseudomonas* sp., *Erwinia* sp., and *Clostridium* sp. The bacteria and pathogens *Fusarium oxysporum* f.sp. *cepae* (*Foc*) used in this study are a collection from the Laboratory of Plant Diseases, Department of Plant Pest and Disease, Brawijaya University. The *Foc* was cultured on Potato Dextrose Agar (PDA) and incubated at room temperature.

2.2 Pathogenicity test

Pathogenicity test was carried out to prove that the fungal isolates used still had high virulence and were able to cause disease in their host plants. Pathogenicity test started with purification of *Foc* isolate on ECG media (Sugar Potato Extract) then shaken with

an orbital shaker for 3x24 hours. *Foc* suspension as much as 50 ml was poured on the roots of the shallot plant. Furthermore, observations were made for 7 days until symptoms of twisting and wilting disease appeared. After the symptoms of the disease appeared, the part of the onion bulb that was infected with *Foc* was isolated again to ensure that the onion plant was actually attacked by the *Foc* pathogen.

2.3 Research design

Based on the synergy test from (Dinata, Aini, & Abadi, 2021), eight treatments were selected for the combination antagonist test. The treatment consisted of a combination of two bacteria and a combination of three bacteria. Tests were carried out using a completely randomized design (CRD) with eight treatments and four replications of petridishes, so that there were thirty-two experimental units. The treatments are, P1: *B. mycoides* + *Pseudomonas* sp., P2: *B. mycoides* + *A. faecalis*, P3: *Erwinia sp.* + *Pseudomonas* sp., P4: *Clostridium* sp. + *Pseudomonas* sp., P5: *Clostridium* sp. + *A. faecalis*, P6: *B. mycoides* + *Pseudomonas* sp. + *A. faecalis*, P7: *Clostridium sp.*, *Pseudomonas sp.* + *A. Faecalis*.

2.4 Antagonist test from combination bacteria against *F. oxysporum* f.sp. *cepae* in vitro

Tests were carried out on a combination of indigenous bacteria against *Foc* on NA media with a modified multiple culture method in one confrontation plate (Herliyana et al., 2013). Combination bacterial isolates were tested in vitro for growth inhibition of the soil-borne phytopathogenic fungi *Foc.* Briefly, two loops of each bacterial culture (10^8 $CFUml^{-1}$) were dipped in sterile aquades, put filter paper in it, and homogenize them. Take the filter paper, drain it for about 2 hours, then inoculate it in plate with Nutrient Agar media with a distance of 3 cm from the pathogen.

Mycelial agar plug of 5 mm diameter from a 7-day-old culture of *Foc* grown on PDA plate was placed in the 3 cm from the test bacterium. Plates were incubated at 27◦C for 7 day. Antagonistic activity was assessed by relating mycelia diameter on plates inoculated with bacteria to mycelia diameter on control plates and computing percentage of Growth Inhibition (GI%). The mycelia width was measured using a caliper. Observations every day by measuring the width of mycelia *Foc* in each experimental unit. At the end of the observation, the percentage of the effectiveness of inhibition of the combination of bacteria was also evaluated. The best inhibition criteria were selected with a percentage of inhibition value of more than 50%. The effectiveness of the inhibition was obtained from the following equation (Marwoto et al., 2012):

$$GI\% = \frac{Control - Treatment}{Control} x\ 100\%$$

Note: GI%= effectivity of antagonistic bacteria, Control= diameter of the untreated *Foc* colony, Treatment= diameter of the treated of *Foc* colony.

2.5 Data analysis

The data of the diameters of the *Foc* colonies and the inhibition of *Foc* colony growth were analyzed using the SPSS Statistics 21. The observed data showed significant differences followed by Duncan's multiple range test at the 5% level.

3. Results and Discussion

The results of *Foc* inoculation showed the same symptoms as fusarium wilt disease. The treatment of *Foc* pathogens caused the plants to become twisted, collapsed, and wilted. While the control treatment did not cause disease symptoms (Figure 1). This is in accordance with the statement of Wiyatiningsih et al., (2009), reported that common symptoms of the disease are twisted leaves, the color of the leaves is pale green, but does not wither. When diseased plants are removed, the tubers appear smaller, fewer than healthy ones.

Figure 1. Result pathogenicity test, (left): control, (right): *Foc* inoculation

Table 1. Effect of combined indigenous antagonistic bacteria on *Foc* mycelium

Treatments	*Foc* mycelium diameter (cm)			
	Day 2	Day 4	Day 6	Day 7
Control	3.32 ± 0.45^{a}	6.81 ± 0.71^{a}	8.71 ± 0.55^{a}	9.25 ± 0.00^{a}
P1	2.18 ± 0.36^{d}	2.67 ± 0.56^{c}	2.87 ± 0.50^{c}	3.06 ± 0.51^{c}
P2	2.30 ± 0.32^{cd}	2.56 ± 0.48^{c}	2.97 ± 0.49^{c}	3.01 ± 0.46^{c}
P3	2.41 ± 0.49^{bcd}	3.89 ± 1.81^{bc}	4.41 ± 1.83^{bc}	4.57 ± 1.84^{bc}
P4	3.33 ± 0.49^{a}	3.84 ± 0.71^{bc}	4.38 ± 0.95^{bc}	4.47 ± 0.90^{bc}
P5	3.00 ±0.49^{abcd}	4.95 ± 1.06^{b}	5.32 ± 1.31^{b}	5.38 ± 1.32^{b}
P6	3.10 ± 0.31^{abc}	5.50 ± 1.61^{ab}	6.08 ± 0.83^{b}	6.34 ± 0.81^{b}
P7	3.21 ± 0.96^{abc}	5.12 ± 1.61^{ab}	5.81 ± 1.96^{b}	5.99 ± 1.84^{b}

Remarks: Data represent an average of four replications ± SD. the letter differences show significant differences based on the Duncan's multiple range test ($p < 0.05$). P1: *B. mycoides* + *Pseudomonas* sp., P2: *B. mycoides* + *A. faecalis,* P3: *Erwinia* sp. + *Pseudomonas* sp., P4: *Clostridium* sp. + *Pseudomonas* sp., P5: *Clostridium sp.* + *A. faecalis,* P6: *B. mycoides* + *Pseudomonas* sp. + *A. faecalis,* P7: *Clostridium* sp., *Pseudomonas sp.* + *A. faecalis*

The results shows that all treatments with bacterial combination resulted in various inhibition effectiveness values from 31,51 – 67,46% (Figure 3). This result is quite different from the first study which had more than 70% inhibition in a single treatment by *Pseudomonas* sp. in inhibiting the same pathogen (Dinata, Ariani, et al., 2021).

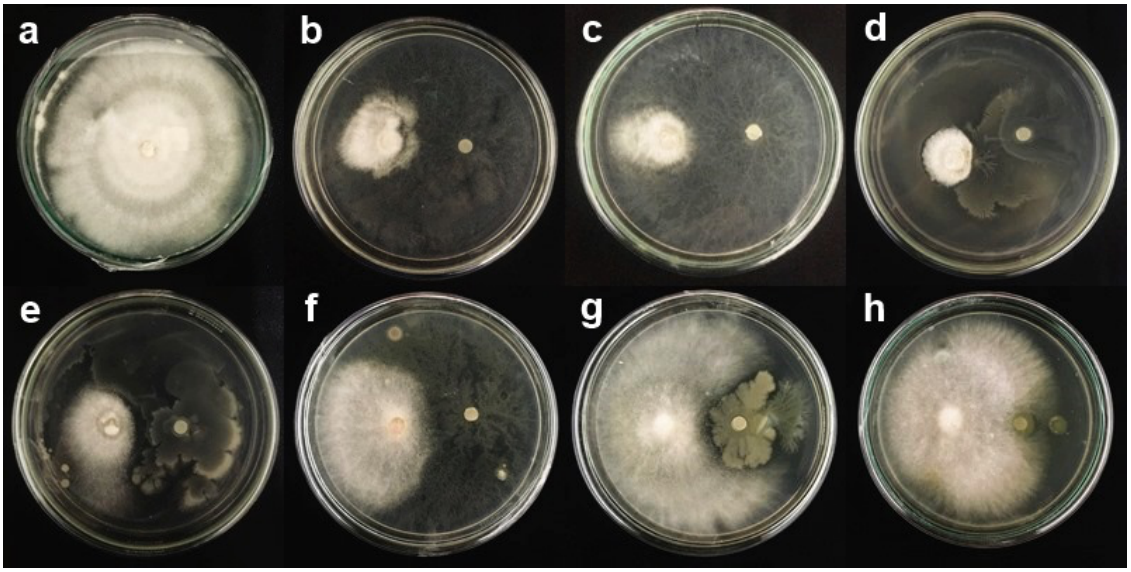

Figure 2. Indigenous antagonistic bacteria combination against *Foc* on the 7th day, (a): control, (b): *B. mycoides* + *Pseudomonas* sp., (c): *B. mycoides* + *A. faecalis*, (d): *Erwinia* sp. + *Pseudomonas* sp. (e): *Clostridium* sp. + *Pseudomonas* sp., (f): *Clostridium* sp. + *A. faecalis*, (g): *B. mycoides* + *Pseudomonas* sp. + A. faecalis (h): *Clostridium* sp. dan *Pseudomonas* sp. dan *A. faecalis.*

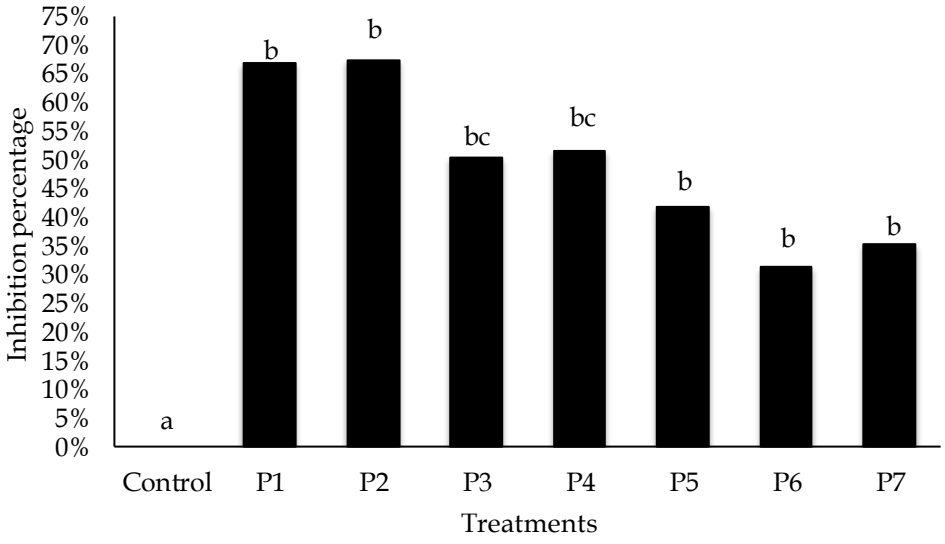

Figure 3. Inhibition effectiveness of indigenous bacteria combination against *Foc* on day 7. Data represent an average of four replications ± SD. the letter differences above each bar show significant differences based on the Duncan's multiple range test test ($p < 0.05$). P1: *B. mycoides* + *Pseudomonas* sp., P2: *B. mycoides* + *A. faecalis,* P3: *Erwinia* sp. + *Pseudomonas* sp., P4: *Clostridium* sp. + *Pseudomonas sp.*, P5: *Clostridium* sp. + *A. faecalis,* P6: *B. mycoides* + *Pseudomonas* sp. + *A.faecalis,* P7: *Clostridium* sp., *Pseudomonas* sp. + *A. faecalis.*

P3 and P4 was seen that the combined bacteria secreted antibiotic compounds as antagonists. These compounds were seen around the Foc mycelia forming a clear zone. This renders the *Foc* mycelia unable to spread widely. The difference in inhibition may be caused by differences in the number of bacterial populations and the virulence of

the antagonistic bacteria. Antagonistic bacteria have different metabolites and can antagonize each other. Competition between bacteria can be influenced by the production of these toxic substances and producer strains are benefited compared to non-producing or sensitive strains by dominating the niche in which they are located (Khare & Tavazoie, 2015).

The combination consisting of two bacteria was more effective in suppressing the growth of *Foc* mycelia than the composition of three bacteria. The results differed from the other combinations, P6 and P7 had lower and significantly different from the other treatments after being analyzed by Duncan's multiple range test. This treatment was only able to inhibit the growth of *Foc* between 31.51 – 35.30%. The smallest percentage of inhibition occurred in the treatment of the combination *B. mycoides*, *Pseudomonas sp.* and *A. faecalis* which provided 31.51% inhibition. This is because the bacteria did not synergize in providing significant resistance to pathogens, and under certain conditions these bacteria would inhibit each other. This is supported by the statement of (Deng & Wang, 2016), that bacteria will antagonize each other when carbon sources are available in limited quantities, so that bacteria will mutually grab the substrate. Several microbes often exist in complex multispecies communities in the environment, but the molecular mechanisms of these communities are still developing, although there are significant antagonistic interactions between species that are still not well understood (Wong et al., 2016). Kai et al, (2016) showed that bacteria also produce other metabolites such as volatile compounds and broad-spectrum antibiotics. Recent research has shown that this compound, when secreted by bacteria, can fight other microorganisms. It is important to define antagonistic bacteria as strains capable of producing toxic compounds that inhibit the growth of other antagonistic bacteria (Russel et al, 2017)

In this study, the antagonist test used Nutrient Agar (NA). Because it is proven that using NA media, fungi and bacteria will grow optimally, then the selection of planting media can have a high impact on the inhibition shown (Yang et al., 2011). The inhibition of *Foc* was influenced by the media used for the in vitro test. The in vitro method of testing the antagonism of the coffee litter bacteria combination against pathogenic fungi provides a fast way to select early candidates for biological control based on antibiotics and competition.

From this research, a new potential has been found, the combination of *B. mycoides* and *A. faecalis* which has the highest inhibition of all treatments at 67.46%. *B. mycoides* and *A. faecalis* are thought to release compounds that can damage the cell walls of *Foc*. *B. mycoides* is a Gram-positive antagonistic bacteria that produces endospores measuring 2.78 – 2.82 µm (Dinata et al., 2021b). Several studies have informed that *B. mycoides* has benefit for biological control. *Bacillus mycoides* has a cell-free supernatant that can reduce the growth of fungal mycelium of the *C. gloeosporioides* species group isolated from avocado by 65% (Guerrero-Barajas et al., 2020). *B. mycoides* strain isolated from rice rhizosphere was shown to produce biosurfadctants, suppress zoospore formation by *P. aphanidermatum* and 35% damping-off disease in cucumbers (Peng et al., 2017). Research by Meena and Kanwar (2015), shows that *Bacillus* spp, produces antibiotic substances that act as antifungal compounds, such as surfactin, iturin and fengicin. Compound like iturin group is a large family of cyclic heptapeptides with a C14-C17 aliphatic β-amino fatty acid that have strong antifungal activity against pathogens Fusarium (Stoica et al., 2019).

A. faecalis is a Gram-negative bacterial found in soil, water and the environment. *A. faecalis* has potential as a biostimulant and bioprotectant. Bacteria of the genus Alcaligenes are usually non-pathogenic but opportunistic infections can occur occasionally in humans. There are several studies related to *A. faecalis* as plant control. In certain subspecies studies such as *A. faecalis* subsp. *Phenolicus* MB207 has a useful compound, namely fusaric acid which can inhibit the growth of pathogens. Research by Basharat et al., (2018), found the potential possessed by *A. faecalis* subsp. *Phenolicus* MB207 which has several useful proteins related to drug resistance such as fusaric acid resistance protein and antibiotic resistance protein. *A. faecalis* is a beneficial bacterium that can live in extreme environments. *A. faecalis* subsp. *Phenolicus* MB207 has many xenobiotic degrading enzymes, which allow it to tolerate and thrive in environments where anthropogenic and toxic compounds are present

Pseudomonas sp. have been identified to play a vital role in controlling diseases. Several studies on *Pseudomonas* have been reported, *P. fluorescens* strain BC8 produces bacteriocin fluoricin-BC8 which inhibits *P. solanacearum* under in vitro conditions, *Pseudomonas aeruginosa* RsB29 causes suppression of Fusarium wilt and rot of chickpeas (Sindhu et al, 2016). Some Pseudomonas inhibit some phytopathogens such as Xanthomonas spp. (Garza-Ramos et al., 2015), and produced a variety of bacteriocins that have been isolated and characterized (Cesa-Luna et al., 2020). The P. fluorescens BC8 strain produced bacteriocin fluoricin-BC8 which inhibited P. solanacearum under in vitro conditions; Pseudomonas aeruginosa RsB29 inhibits Fusarium and chickpea rot (Sindhu et al., 2016). P. protegens CHA0 produces various secondary metabolites including hydrogen cyanide, 2,4-diacetylphloroglucinol, pyoluteorin, and pyrrolnitrin to inhibit several pathogens such as Thievaloviopsis basicola and Pythium ultimum in tobacco and cucumber (Jousset et al., 2014; Sindhu et al., 2016).

The development of a bacterial consortium formula for biological control is still being developed. Although many bacterial inoculants have been designed, formulations of bacterial combination, especially those capable of inhibiting the growth of various types of pathogens, are still being developed (Cesa-Luna et al., 2020). Combination bacteria have been reported and appear to be more effective. The application of a microbial combination consisting of several microbes, such as arbuscular mycorrhizal fungi (AMF), plant growth promoting rhizobacteria (PGPR), and Actinobacteria can improve agricultural soil recovery, improve the quality and health of agricultural crops (Aguilar-Paredes et al., 2020). In addition, a synergy test among the microbial strains of the combination must be carried out, because some antagonistic effects can occur between bacteria (Molina-Romero et al., 2017).

4. Conclusion

In this present investigation, new indigenous antagonistic bacteria combination was developed comprising of two antagonistic coffee litter bacteria as well as efficient inhibit *Foc*. The treatments of P1 (*B. mycoides* + *Pseudomonas* sp.), P2 (*B. mycoides* + *A. faecalis),* P3 (*Erwinia* sp. + *Pseudomonas* sp.), P4 (*Clostridium* sp. + *Pseudomonas* sp.) were able to inhibit *Foc* in vitro by more than 50% and have a significantly different from all treatments.

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