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Potency of the Consortium of Pseudomonad fluorescent pf-142 and *Bacillus mycoides* Isolates Against Bacterial Wilt Disease *In-Vitro*

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Abstract

Bacterial wilt disease caused by *Ralstonia solanacearum* is a disease in chili pepper plants (*Capsicum annuum*) that is difficult to control. One technique to prevent bacterial wilt disease is consortium of antagonistic bacteria such as Pseudomonad fluorescent and *Bacillus mycoides*. This study aimed to determine whether the consortium of Pseudomonad fluorescent pf-142 and *Bacillus mycoides* can inhibit *R. solanacearum* better than a single application in-vitro. This study was conducted in a Completely Randomized Design (CRD) with four treatments (Pseudomonad fluorescent pf-142, *B. mycoides*, Pseudomonad fluorescent pf-142 + *B. mycoides*, and control) in six replications, resulting in 24 experimental units. The variables observed were the symptoms of attack and pathogenicity of *R. solanacearum*, compatibility and inhibitory rate of the consortium of Pseudomonad fluorescens pf-142 and *B. mycoides* against *R. solanacearum*. Based on the study, it was known that *R. solanacearum* caused wilting in chili pepper plants with high virulence. Pseudomonad fluorescent pf-142 and *B. mycoides* did not produce an inhibition zone, indicating that both were compatible. The consortium of Pseudomonad fluorescent pf-142 and *B. mycoides* provided the widest inhibition zone, indicating strong antagonistic ability against *R. solanacearum*.

Keywords: Antagonist, *Capsicum annuum*, *decay*, *Ralstonia solanacearum*, zone of inhibition

Introduction

Chili (*Capsicum annuum*) is a horticultural commodity with high economic value and significant development potential, with demand in Indonesia projected to increase and increase community consumption (Maramis, 2018; Chesaria and Syukur, 2018). Chili peppers are rich in nutrients and vitamins, including calories, protein, fat, carbohydrate, calcium, and vitamins A, B1, and C. Chili pepper, in particular, has a higher vitamin C content than red chili and various fruits such as mango, orange, pineapple, apple, tomato, and star fruit (Egra et al., 2019). Chili can be used as a seasoning, consumed directly, and used as a raw medicine material in the pharmaceutical field. Traditionally, chili has been used to treat pain, edema, and toothache and as an appetite

enhancer with antifungal and antibacterial properties. Additionally, it has antidiabetic, antihypertensive, and anti-obesity effects (Sanati et al., 2018).

Chili production in 2021 decreased by 8.09% (121.96 thousand tons) compared to the previous year (Irjayanti et al., 2021). The factors causing the decline in chili pepper production include low soil fertility, high water evapora-tion due to air temperature, and attacks by Plant Pest Organisms (PPO). *Ralstonia solanacearum*, is one of the diseases in chili that is difficult to control (Muliani et al., 2019), causing yield losses of up to 90% (Palupi and Yulianah, 2015).

R. solanacearum has a wide host range and distribution area, and it can survive in soil and the rhizosphere layer of non-host plants. The bacteria infect plants through the roots and reproduce in the xylem tissue, interfering with water transportation in plants (Nabila & Asri, 2021), the plant lacks water due to the blockage of mass of bacteria, eventually causing the plant to wither, leading to wilting and, eventually, plant death. A characteristic of a bacterial wilt pathogen attack is the appearance of a solid white bacterial mass when the stem of an infected plant is cut and placed in water (Simanjuntak, 2014; Istiqomah, 2018).

Various efforts have been made to control bacterial wilt disease that attacks crop areas. One alternative solution to this problem is controlled with biological agents, for example, *Bacillus* spp and Pseudomonad fluorescent. *Bacillus* spp. has been widely used as a biological agent because of its ability as a biological fertilizer, secreting lysis enzymes, inducing systemic resistance (ISR), producing several antibiotic compounds such as bacitracin, bacillus, bacillus B, difidine, oxidative fidine, and lecithinase, degrading AHL group QS signal molecules through AHL-lactonase enzymes, thereby inhibiting the QS mechanism and reducing the virulence of pathogenic bacteria (Permanti et al., 2018). *Bacillus mycoides* can reduce necrosis symptoms in tobacco plants caused by *R. solanacearum* by up to 20% (Prasetyawati et al., 2023).

On the other hand, Pseudomonad fluorescent is an antagonistic-bacteria widely used as a biological agent against plant pathogenic fungi and bacteria. Pseudomonad fluorescent can control soil-borne plant pathogens and can survive long in the rhizosphere and phyllosphere. Advinda (2022) noted that Pseudomonad fluorescent provides a defense system by releasing antimicrobial compounds, including chitinase, 1,3-glucanase, hydrogen cyanide (HCN), siderophores, salicylic acid, antibiotics, Indole Acetic Acid (IAA), phosphate solubilizing compounds, and 2,4-diacetyl phloroglucinol (DAPG), which inhibit the growth of pathogenic microorganisms. Istiqomah (2018) showed that Pseudomonad fluorescent UB-PF5 and UB-PF6 had an effectiveness of 60% in suppressing *R. solanacearum* disease. However, Pseudomonad fluorescent pf-142 had a disease suppression index of 66.67% (Hariyanto et al., 2022).

A bacterial consortium is a collection of several bacteria in a medium that functions in a complementary environment. Bacterial consortia, both naturally and artificially formed, have the advantage of having complementary metabolic functions in an ecosystem. Pseudomonad fluorescent pf-142 and *Bacillus* spp. have different antagonistic mechanisms to complement each other. The antagonistic mechanism of Pseudomonad fluorescent bacteria mainly involves root colonization and the production of siderophores and cyanide, with relatively low antibiotic production. In contrast, *Bacillus* spp. primarily relies on its ability to produce antibiotics as its antagonistic mechanism.

Agustina (2021) stated that the *Bacillus* spp. consortium and Pseudomonad fluorescent bacteria can inhibit the growth of *Fusarium* sp. by 69.98% in vitro. This study aimed to determine whether *Bacillus mycoides* and Pseudomonad fluorescent pf-142 consortium can inhibit *R. solanacearum* better than single-application in-vitro.

Methods

This research was conducted at the Plant Health Laboratory of Universitas Pembangunan Nasional "Veteran" Jawa Timur, East Java, Indonesia, in September 2023.

Method

This study employed *in vitro* testing to assess the inhibition of antagonistic bacteria, specifically Pseudomonad fluorescent pf-142 and *B. mycoides*, against the growth of *R. solanacearum*. This research was carried out in a Completely Randomized Design (CRD) with four treatments (Pseudomonad fluorescent pf-142, *Bacillus mycoides*, *Pseudomonad fluorescent* pf-142 + *B. mycoides*, and control) in six replications, resulting 24 experimental units.

Isolation of *R. Solanacearum*

R. solanacearum was isolated by collecting plants exhibiting symptoms of bacterial wilt. The initial symptoms observed included wilting on some branches while the leaves remained green. Vinda et al. (2014) added that symptoms of bacterial wilt also include the appearance of adventitious roots on the stem when environmental conditions are less favorable for the pathogen and bacterial ooze, which appears grayish or like fine white threads coming out of the cut stem.

Chili plants displaying symptoms of bacterial wilt were selected, washed with sterile water, and disinfected with 70% alcohol. Subsequently, the base of the stem was trimmed about 2-3 cm, the outer skin was peeled, and the stem was cut into small pieces. These pieces were then placed into test tubes containing 10 ml of sterile distilled water (Irfanti et al. 2021). After waiting approximately 5-10 minutes until a yellowish-white bacterial mass emerges, the bacterial suspension was streaked onto Yeast Peptone Glucose Agar (YPGA) media in petri dishes using an ose needle. The dishes were then incubated at room temperature for 48 hours. The pathogen *R. solanacearum* was isolated and purified from the YPGA media after incubation. Morphological observations of *R. solanacearum* colonies are subsequently conducted.

Pathogenicity Test

The pathogenicity test was conducted by inoculating a bacterial suspension with a population density of 10^8 cells/ml, administering 10 ml per plant. Before inoculation, the roots of chili plants were wounded by cutting with a sterile scalpel (Guttara et al., 2017). Subsequently, the suspension was poured onto the roots and covered with soil. The development of disease symptoms was observed daily for two weeks, recording when bacterial wilt symptoms appeared.

Compatibility Test

The compatibility test was conducted to determine whether the two bacteria tested were compatible. It was carried out by growing *B. mycoides* bacteria on NA media and Pseudomonad fluorescent pf-142 on King's B media, then incubating the bacteria for 48 hours. The next step involved plating NA on a petri dish. Subsequently, previously grown *B. mycoides* and Pseudomonad fluorescent pf-142 bacteria were cut using a 0.5 mm cork-borer and placed on NA media with a distance of 0.5 cm for each bacterium. As depicted in Figure 1, this is called the co-

cultured plate method (Ansari & Ahmad, 2019). The isolates were then incubated for 24-48 hours. Isolates are compatible if there is no inhibition zone in the area where the two isolates meet and incompatible if there is an inhibition zone.

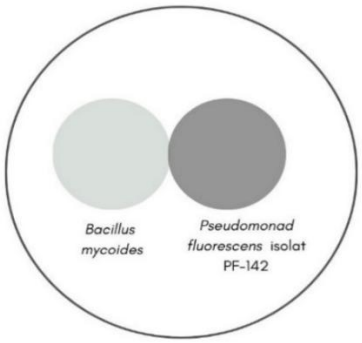


Figure 1. Pitting method in compatibility test between *B. mycoides* and Pseudomonad fluorescent pf-142

Antagonism Test

Antagonism tests against *R. solanacearum* were conducted on bacterial consortia and single bacteria. The tests involved preparing a liquid suspension, into which Whatman paper with a diameter of 0.5 cm was dipped, drained, and dried. Subsequently, the paper was positioned on NA media and incubated for 24 hours at 30°C. Meanwhile, the single bacterial antagonist test followed a similar procedure but was performed separately for each bacterium without combining them.

Upon completion of the incubation period, the petri dish was inverted, and 1 ml of chloroform was added, left to stand for 2 hours at room temperature, and then turned back to its original position. Following this, 200 µl of 10^8 CFU/ml *R. solanacearum* suspension was poured into 4 ml of 0.6% water agar at 30°C. The suspension was subsequently applied onto NA media previously cultured with *B. mycoides* and Pseudomonad fluorescent pf-142. The culture was then incubated for 24 hours at room temperature to observe the inhibition zone. Saputra and Riau (2015) emphasized that isolates producing inhibitory compounds could be identified by the presence of an inhibition zone around the colonies of antagonistic bacteria after the incubation.

Result

Morphology of *Ralstonia solanacearum*

R. solanacearum that was grown on YPGA medium, it appeared milky white, mucoid, and irregularly shaped (Figure 2). This *R. solanacearum* isolate was also fluidal, convex, and opaque. This is consistent with results of Sreedevi et al. (2013), who stated that *R. solanacearum* exhibits irregular shapes and forms white mucoid colonies. *R. solanacearum* bacterial isolate also has a distinctive characteristic of sloughing off when grown on a slant medium.

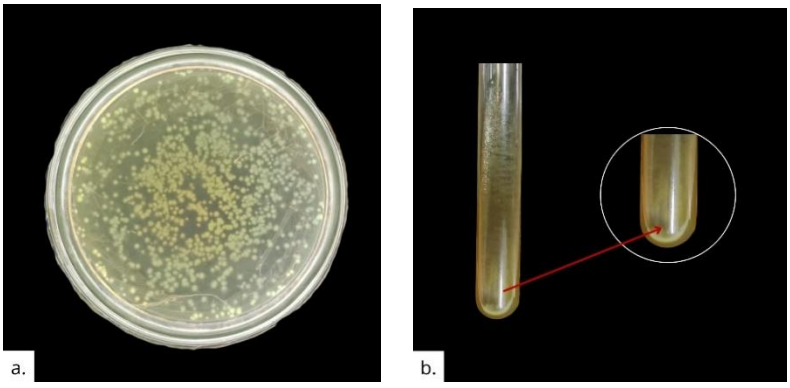


Figure 2. Morphology of *R. solanacearum* on Yeast Pepton Glukosa Agar (YPGA), (a) Morphology on petridish; (b) Morphology on slant agar

Pathogenicity Test of *R. solanacearum*

The results revealed wilting in chili pepper plants due to infection by the pathogen *R. solanacearum*, which has characteristic pale leaves, drooping petioles, and leaf loss (Figure 3). This aligns with Raihanah (2023) that plants attacked by the *R. solanacearum* pathogen will exhibit wilting, particularly in young leaves or shoots, and the wilt will subsequently spread to all parts of the plant. Vascular tissue of the lower stem of the plant will be damaged, and the roots will be brownish.



Figure 3. Chili plants affected by *R. solanacearum* disease; left. 7 days old, right. 14 days old

Compatibility Between Pseudomonad fluorescent pf-142 and *Bacillus mycoides*

The compatibility test between *Bacillus mycoides* and Pseudomonad fluorescent pf-142 showed that the two isolates were compatible, where no visible inhibition zone or clear zone was observed in the intersecting scratches after incubating for 24 hours at 30°C (Figure 4).

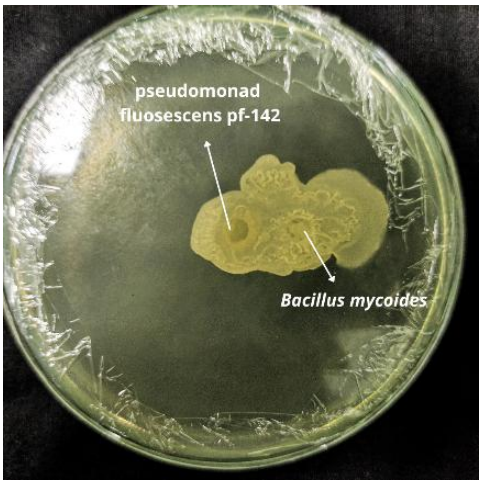


Figure 4. Compatibility beetwen Pseudomonad fluorescent Pf-142 ***and*** *B. mycoides* test showed no clear zone.

Antagonism Test of Antagonistic Bacteria Against *R. solanacearum*

The antagonism test of antagonistic bacteria against *R. solanacearum* involved calculating the clear zone around the colony of antagonistic bacteria. The results of the inhibition zone were obtained after incubating the bacteria for 24 hours and are presented in Table 1.

Table 1. Antagonism test of antagonistic bacteria against *R. solanacearum in vitro*

Treatment	Zone of Inhibition (mm)
B0 (Without antagonistic bacteria)	0
B1 (*B. mycoides*)	5.25
B2 (Pseudomonad fluorescent isolat pf-142)	0
B3 (*B. mycoides* + Pseudomonad fluorescent Pf-142)	7.50

The antagonism test of biological agent bacteria against *R. solanacearum* involved measuring the clear zone around the colony of antagonistic bacteria. The results demonstrated that the antagonistic bacteria B1 (*B. mycoides*) and B3 (*B. mycoides* + Pseudomonad fluorescent Pf-142) were able of suppressing the growth of *R. solanacearum*. Bacteria code B3 (*B. mycoides* + Pseudomonad fluorescent Pf-142) exhibited an average inhibition zone of 7.5 mm, the average inhibition zone for bacteria B1 (*B.*

mycoides) was 5.25 mm, and for bacteria B2 (Pseudomonad fluorescent Pf-142), it was 0 mm. The resulting clear zone provides a spatial separation between the agent and pathogenic bacteria (Figure 5), indicating the inhibitory mechanism produced by biological agent bacteria against the growth of *R. solanacearum*. These biological agents produce a variety of enzymes and antibiotic substances that hinder the growth of pathogenic bacteria. According to (Wijayanto et al., 2022) the inhibitory mechanism by antagonistic bacteria involves the production of siderophores, ß-1,3-glucanase, chitinase, antibiotics, and cyanide, which collectively contribute to inhibiting the growth of pathogenic colonies.

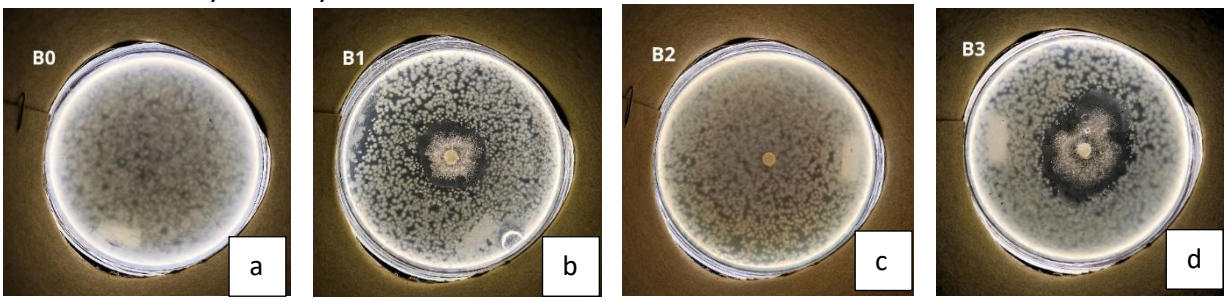


Figure 5. Inhibition of antagonistic bacteria against *R. solanacearum in-vitro*. (a) Control, (b) *Bacillus mycoides*, (c) Pseudomonad fluorescent pf-142, (d) Consortium of bacteria

Discussion

Compatibility is an essential factor for bacteria to function effectively together. Bacterial compatibility refers to the association between two genera or species of certain bacteria that do not interfere. Their activities are mutually beneficial, they can share nutrient sources on the same living media. The relationship between compatible bacteria leads to synergies, resulting in higher breakdown efficiency, and these bacteria do not interfere with each other under sufficient substrate conditions.

There was no zone of inhibition produced by the Pseudomonad fluorescent PF-142 and *B. mycoides* in the compatibility test (Figure 4). This indicates that no antagonistic activity occurred between the bacteria *B. mycoides* and the pseudomonad fluorescent PF-142. Compatible bacteria are those that can coexist and benefit each other. According to Arora (2015), the compatibility or synergistic nature of two or more bacteria is essential for them to work together effectively. Moreover, in a biofertilizer consortium, synergistic properties are crucial for supporting plant growth without any antagonism within the consortium, as the consortium will produce compounds that support and complement each other.

A pathogenicity test was conducted to evaluate the virulence of *R. solanacearum* bacteria based on visible symptoms. Test results showed that *R. solanacearum* isolates could cause wilting when tested on chili pepper plants (Figure 3). Test results show that the pathogen *R. solanacearum* can cause wilt in chili plants with an incubation period of 3 days. The intensity of bacterial wilt disease was calculated 7 days after the pathogenic bacteria were applied and the result was 100%.

Treatment B1 (*B. mycoides*) exhibits a large inhibition zone with an average of 5.25 mm (Figure 5). This inhibition zone may be formed due to the antibiosis mechanism produced by *B. mycoides*, which interferes with the growth of the pathogenic bacterium *R. solanacearum*, preventing its growth within

the area of *B. mycoides*. Flori et al, (2020) noted that Bacillus sp. itself produces several antibiotic compounds, including basitrasin, bacillin, bacillomycin B, diphidine, oxidificidin, and lecithinase. The inhibition zone surrounding the growth of the biological agents inhibits the growth of *R. solanacearum*. Additionally, (Irfanti et al., 2021) explained that this occurs because Bacillus sp. bacteria can secrete antibiotic substances through secondary metabolites such as surfactin, fengisin, iturin, and subtilosin A.

Treatment B2 (Pseudomonad fluorescent pf-142) did not produce an inhibition zone (0 mm) in *in vitro* testing against the pathogenic bacteria R. solanacearum (Figure 5). The absence of an inhibition zone can be influenced by various factors, which can reduce the effectiveness of the antagonism of the fluorescent pseudomonad pf-142. According to Haryani et al. (2015), the width of the inhibition zone formed is influenced by environmental conditions, test bacterial media, temperature, incubation time, and the age of the bacteria.

Treatment B3 (Pseudomonad fluorescent pf-142 + *B. mycoides*) had the largest average zone of inhibition, measuring 7.5 mm (Figure 5). The presence of a clear inhibition zone indicates that the *B. mycoides* isolate and the fluorescent pseudomonad pf-142 were able to inhibit the growth of R. solanacearum on NA media. Microbial consortia have better inhibitory rate than individual biological agent bacterial isolates. This occurs due to a mutually supportive mechanism between the biological agent pseudomonad fluorescent pf-142 and *B. mycoides* bacteria to inhibit the development of the pathogenic bacteria *R. solanacearum*. *B. mycoides* bacteria can inhibit the growth of the pathogen *R. solanacearum* through an antibiosis mechanism. This aligns with research by Prasetyawati et al. (2023), which showed that *B. mycoides* can suppress necrotic symptoms in tobacco plants caused by *R. solanacearum* bacteria to 20%, compared to controls, which had necrotic symptoms up to 60%. This is also supported by Syofiana and Masnilah (2019), who stated that *Bacillus* spp. can act as biological agents against plant pathogens through an antibiosis mechanism by producing inhibitory compounds (antimicrobial compounds), including antibiotics, peptides, phenolic compounds, enzymes, alkaloids, and siderophores. The pseudomonad fluorescent pf-142 is also capable of producing siderophores, which are iron-chelating compounds. According to Kiriho et al. (2017), Pseudomonad fluorescent can chelate the Fe element, making it unavailable to *R. solanacearum* bacteria. The pseudomonad fluorescent pf-142 has also been tested on a field scale and can suppress the growth of *R. solanacearum* bactericidally, with a disease index of 66.67% (Wuryandari et al., 2008). In this way, competition for nutrients occurs, leading to the inhibition of the growth of *R. solanacearum*, as indicated by the presence of an inhibition zone.

The inhibition zone test was then followed by the examination of the inhibitory mechanism of antagonistic bacteria *B. mycoides* and consortium bacteria. Based on the conducted tests, *B. mycoides* and consortium bacteria are identified as bacteriostatic. They are described as bacteriostatic because they can inhibit the growth of pathogenic bacteria without killing them. This is evidenced by the presence of *R. solanacearum* bacterial growth on day 6 on YPGA medium. At the end of the observation period on day 14, there were 41 colonies of *R. solanacearum* bacteria in treatment B1 (*B. mycoides*) and 65 colonies in treatment B3 Pseudomonad fluorescent pf-142 + (*B.*

mycoides). According to Syofiana and Masnilah (2019), bacteriostatic refers to the property of antibiotics that can only inhibit the growth of pathogenic bacteria.

Conclusion

The compatibility test between Pseudomonad fluorescent pf-142 and *Bacillus mycoides* did not produce an inhibition zone, indicating compatibility. In the pathogenicity test, *R. solanacearum* bacteria caused wilting in chili plants, displaying high virulence, making these pathogenic bacteria suitable as consortium. Based on the results of the in-vitro antagonism test, it can be concluded that bacteria B3 (Pseudomonad fluorescent pf-142 + *B. mycoides*) exhibited the widest inhibition zone, showcasing strong antagonistic properties against the pathogen *R. solanacearum*.

Declaration

Author contribution

Yosua Nathanael Itona Sagala is the main contributor to this article, Endang Triwahyu Prasetyawati is corresponding author, and Yenny Wuryandari is with co-authors. All authors have read and approved the final layout and appearance of the article.

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Competing interest

The authors declare that they have no financial conflicts of interest or personal relationships that could influence the work reported in this article.

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