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In Vitro Evaluation of Bacillus subtilis and Pseudomonas fluorescens Against Klebsiella variicola in Kepok Banana

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Abstract

Bacterial wilt represents a major constraint in the cultivation of Kepok banana (*Musa paradisiaca* L.) in East Nusa Tenggara, and recent identification of *Klebsiella variicola* as the causal agent indicates a potential emerging threat to production sustainability. This study aimed to evaluate the antagonistic potential of *Bacillus subtilis* and *Pseudomonas fluorescens* against *K. variicola* under in vitro conditions. The experiment was arranged in a Completely Randomized Design with five treatments, including control groups, single antagonistic bacteria, and their combination, using a dual culture assay with the paper disc method. The results demonstrated that *Bacillus subtilis* exhibited the highest inhibitory activity, followed by the combination treatment and *Pseudomonas fluorescens*, while no inhibition was observed in the negative control. The superior performance of *Bacillus subtilis* is associated with its ability to produce antibacterial metabolites, particularly lipopeptides that disrupt pathogen cell structures. In contrast, *Pseudomonas fluorescens* inhibited pathogen growth through siderophores and toxic compounds, although with lower effectiveness. The combination treatment did not produce a synergistic effect, likely due to microbial competition. These findings suggest that *Bacillus subtilis* holds strong potential as an environmentally friendly biological control agent. Further in vivo studies are required to validate its effectiveness under field conditions.

Keywords

Bacillus Subtilis, *Bacterial Wilt*, Kepok Banana, *Klebsiella Variicola*, *Pseudomonas Fluorescens*.

1. Introduction

Bacterial wilt is one of the major constraints in the cultivation of Kepok banana (*Musa paradisiaca* L.) in East Nusa Tenggara Province, particularly in Manggarai and East Manggarai Regencies. To date, this disease has generally been associated with *Ralstonia syzygii* subsp. *celebesensis*. However, recent molecular identification has revealed that the pathogen responsible for bacterial wilt in this region is *Klebsiella variicola*. This finding indicates a possible shift or variation in the causal pathogen, which may complicate disease management strategies. *Klebsiella variicola* has been reported as a multihost plant pathogen capable of causing both systemic and localized infections, depending on the infection route and the plant tissues colonized (Zhang et al., 2023; Han et al., 2023; American Phytopathological Society, 2024). Furthermore, this pathogen has been identified as the causal agent of soft rot, rhizome rot, pseudostem rot, and corm rot in banana and carrot plants in China and India (Fan et al., 2016; Chandrashekar et al., 2018; Fulton et al., 2020; Loganathan et al., 2021; Sun et al., 2024). Therefore, the presence of *Klebsiella variicola* in Kepok banana plants in Manggarai and East Manggarai represents a potential new threat to the sustainability of local banana production.

Control efforts for bacterial wilt have predominantly relied on synthetic chemical agents. Although effective in the short term, this approach poses several drawbacks, including the risk of chemical residues, the development of pathogen resistance, and the disruption of soil microbial balance. These limitations highlight the need for more environmentally friendly and sustainable alternatives. One promising approach is the use of biological control agents based on antagonistic microorganisms. Bacteria such as *Bacillus subtilis* and *Pseudomonas fluorescens* have been widely reported to suppress pathogen growth through multiple mechanisms, including antibiosis, nutrient competition, and the induction of systemic resistance in plants (Ramamoorthy et al., 2001; Van Loon et al., 2008). *Bacillus subtilis* is known to produce various antibacterial secondary metabolites, such as surfactin, iturin, and fengycin, which disrupt pathogen cell membranes and inhibit cell wall synthesis (Stein, 2005; Ongena & Jacques, 2008). Meanwhile, *Pseudomonas fluorescens* produces siderophores, phenazine compounds, pyrrolnitrin, and Hydrogen Cyanide (HCN), which inhibit pathogen growth through nutrient competition and the production of toxic compounds (Haas & Défago, 2005; Weller, 2007).

Despite the well-documented potential of *Bacillus subtilis* and *Pseudomonas fluorescens* as biological control agents in various agricultural systems, studies focusing on their effectiveness against *Klebsiella variicola* as the causal agent of bacterial wilt in Kepok banana remain very limited, particularly for isolates originating from Manggarai and East Manggarai. This limitation highlights a clear research gap, as differences in pathogen characteristics may significantly influence the effectiveness of biological control agents. Moreover, there is still a lack of studies specifically examining the antagonistic interactions of these bacteria against local isolates of *Klebsiella variicola* under in vitro conditions. Additionally, *B. subtilis* and *P. fluorescens* show isolate-dependent antagonism mediated by secondary metabolites (Saranraj et al., 2023).

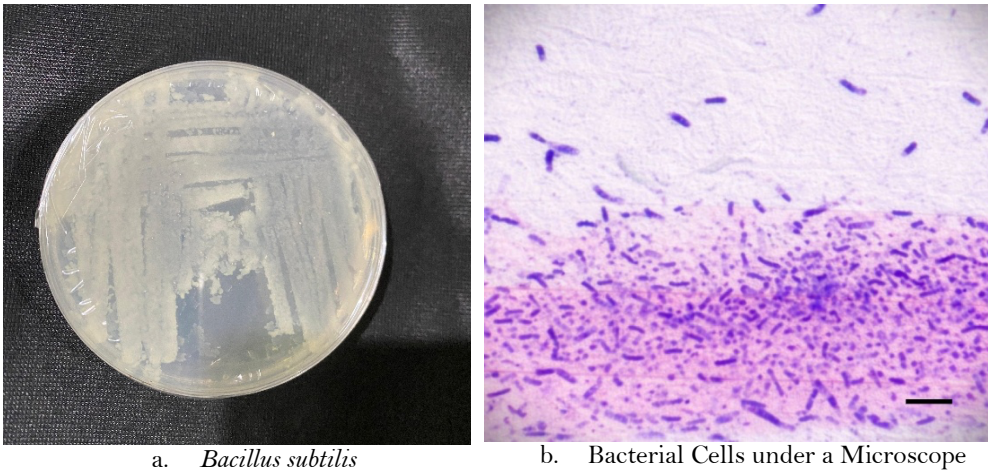
Accordingly, the novelty of this study lies in evaluating the antagonistic potential of *Bacillus subtilis* and *Pseudomonas fluorescens* against *Klebsiella variicola* isolated from Kepok banana in Manggarai and East Manggarai, which has not been widely reported. This study also provides an initial approach to understanding the effectiveness of biological control agents against a relatively newly identified pathogen associated with bacterial wilt in the region. The objective of this study is to evaluate the ability of *Bacillus subtilis* and *Pseudomonas fluorescens* to inhibit the growth of *Klebsiella variicola* in vitro. The findings are expected to contribute scientifically by providing a basis for the development of environmentally friendly,

sustainable, and practical biological control strategies, as well as supporting efforts to enhance the productivity and sustainability of Kepok banana cultivation in the Manggarai region and surrounding areas.

2. Literature Review

2.1. Bacillus Subtilis

The potential of *Bacillus subtilis* as a biological control agent has been documented by Prihatiningsih et al. (2015), who demonstrated that *Bacillus subtilis* isolates act through antibiosis and can induce systemic resistance in plants. Peptide compounds produced by *Bacillus subtilis*, such as bacilomycin, mycobacillin, and fungistatin, are classified as antibiotics that exhibit toxicity toward soil-borne pathogenic fungi (Awais et al., 2010). In addition, *Bacillus subtilis* is known to synthesize plant growth–promoting hormones, including ethylene, auxins, and cytokinins (Gnanamanickam, 2007). As an antagonistic bacterium, *Bacillus subtilis* can suppress the growth of pathogenic fungi through antifungal activity, leading to degradation of the pathogen's cell wall and consequently inhibiting fungal cell development.



a. *Bacillus subtilis* b. Bacterial Cells under a Microscope

Figure 1. Macroscopic and Microscopic Views of *Bacillus subtilis*

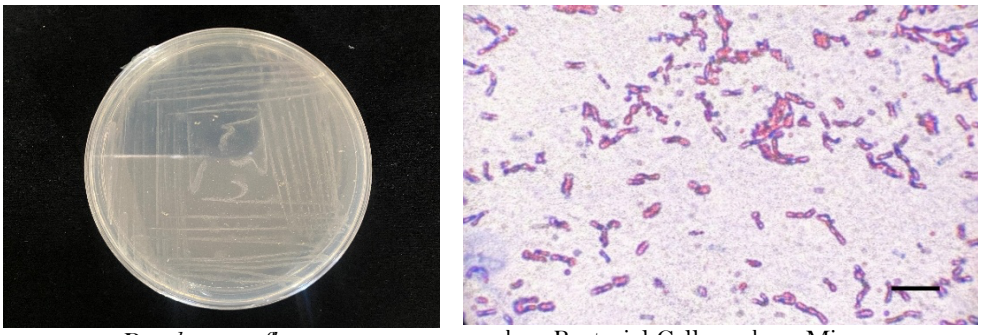
Figure 1 illustrates the macroscopic and microscopic characteristics of *Bacillus subtilis*. At the macroscopic level, colonies of *Bacillus subtilis* grown on NA medium appear milky white with a relatively smooth surface and a spreading form, indicating active bacterial growth. At the microscopic level, observation after Gram staining at 100× magnification reveals rod-shaped (*bacilli*) cells that appear purple, indicating that the bacterium is Gram-positive. The relatively dense distribution of cells with a fairly uniform size, along with the presence of a 10 μm scale bar, provides a clear depiction of the cellular morphology, supporting the identification of *Bacillus subtilis* based on both colony structure and cellular characteristics (Awais et al., 2010).

2.2. Pseudomonas Fluorescens

Pseudomonas fluorescens is a rhizosphere bacterium that inhabits the root zone and actively interacts with plant roots and surrounding soil. This bacterium plays an important role in suppressing disease development while simultaneously promoting the growth of patchouli (*Pogostemon cablin*) plants (Khaeruni et al., 2014). After successfully colonizing the root surface, Pseudomonas fluorescens stimulates plants to enhance the synthesis of secondary metabolites, such as salicylic acid and phytoalexins, which function in plant defense mechanisms (Soesanto et al., 2014). In addition, this bacterium produces various plant growth regulators, including auxins, gibberellins, cytokinins, and Indole-3-Acetic Acid (IAA), which contribute to

improved plant growth (Soesanto et al., 2011; Rahni, 2012). The production of salicylic acid by *Pseudomonas fluorescens* has also been reported to induce resistance in tomato plants against leaf blight caused by *Phytophthora infestans* (Yan et al., 2002).

Pseudomonas fluorescens exhibits high adaptability and is capable of utilizing a wide range of substrates as nutrient sources. Its growth rate, which is faster than that of pathogenic bacteria, enables it to maintain a stable population within the plant rhizosphere. Its population is known to increase with plant age, and it functions effectively as a rhizobacterium capable of both inducing plant resistance and promoting plant growth (Manikandan et al., 2010; Meena, 2011; Meena & Marimuthu, 2012). Various isolates of *Pseudomonas fluorescens* obtained from the rhizosphere of tomato, peanut, chili, and cucumber plants have been reported to enhance both growth and yield in these crops (Sutariati & Safuan, 2012; Khabbaz & Abbasi, 2014).



a. *Pseudomonas fluorescens* b. Bacterial Cells under a Microscope

Figure 2. Macroscopic and Microscopic Views of *Pseudomonas fluorescens*

Figure 2 illustrates the macroscopic and microscopic characteristics of *Pseudomonas fluorescens*. On NA medium, the bacterial colony appears smooth, circular, and pale to whitish with a glossy surface. Microscopic observation following Gram staining at 100× magnification reveals rod-shaped (*bacilli*) bacterial cells dispersed across the field, with a 10 µm scale bar.

3. Methods

This study employed a laboratory-based experimental approach using an in vitro design to evaluate the antagonistic activity of bacteria against plant pathogens. This design was selected to allow controlled conditions, minimize environmental variability, and ensure precise observation of bacterial interactions, thereby improving the internal validity of the results. The experiment was arranged in a Completely Randomized Design (CRD) consisting of five treatments: a negative control without antagonistic bacteria (P0), a positive control using the antibiotic chloramphenicol (P1), *Bacillus subtilis* (P2), *Pseudomonas fluorescens* (P3), and a combination of *Bacillus subtilis* and *Pseudomonas fluorescens* (P4), each with four replications. The selection of antagonistic bacteria was based on their known mechanisms, such as antibiosis and nutrient competition (Haas & Défago, 2005; Weller, 2007). The study was conducted from October to December 2025 at the Plant Disease Laboratory, Plant Protection Concentration, Faculty of Agriculture, Udayana University.

The population in this study consisted of pathogen and antagonistic bacterial isolates used in the experiment. The pathogen isolate, *Klebsiella variicola*, was obtained from Kepok banana (*Musa paradisiaca* L.) plants exhibiting bacterial wilt symptoms, originating from Manggarai and East Manggarai Regencies, East Nusa Tenggara Province. The antagonistic bacteria used were *Bacillus subtilis* and *Pseudomonas fluorescens*, which were rejuvenated on NA medium and incubated at 28–

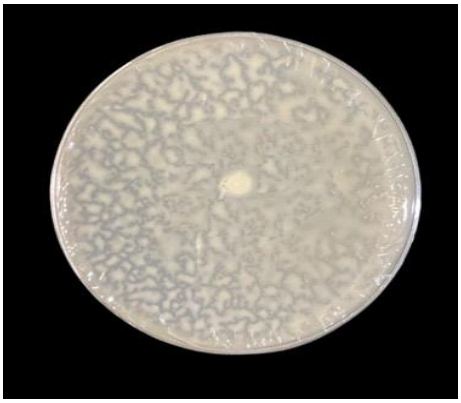
30 °C for 24 hours prior to testing. These isolates were selected due to their reported effectiveness in suppressing plant pathogens through multiple mechanisms.

Evaluating antagonistic activity using the dual culture assay with the paper disc method following Ramesh et al. (2009). Pure cultures of 48-hour-old *Bacillus subtilis* and *Pseudomonas fluorescens* were suspended in sterile distilled water. Sterile filter paper discs (5 mm in diameter) were immersed in the bacterial suspension for approximately 1 minute, then drained and air-dried for 2 hours before being placed on the surface of NA medium in Petri dishes. After 24 hours of incubation, chloroform was applied to the inverted lid of the Petri dishes for 1 hour to stop the growth of antagonistic bacteria. Subsequently, the medium surface was sprayed with a suspension of *Klebsiella variicola*. In the control treatment, filter paper discs were soaked only in sterile distilled water. All treatments were incubated for an additional 48 hours, and the diameter of the inhibition zones formed was measured using a caliper (Istiqomah & Kusumawati, 2018). This analytical approach was used to evaluate the effectiveness of each treatment in inhibiting the growth of *Klebsiella variicola* and to compare the antagonistic activity among *Bacillus subtilis*, *Pseudomonas fluorescens*, and their combination.

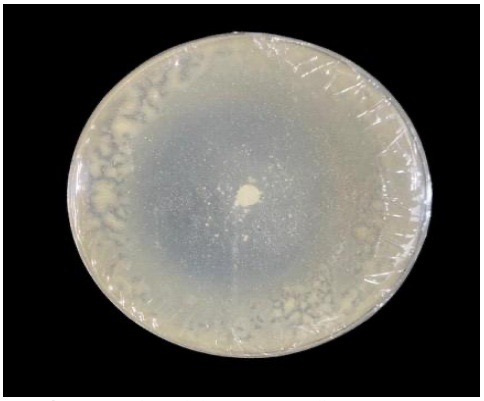
4. Results

To assess the antagonistic activity of *Bacillus subtilis* and *Pseudomonas fluorescens* against *Klebsiella variicola*, an in vitro inhibition assay was performed using multiple treatment groups. This approach enables a direct evaluation of bacterial interactions under controlled laboratory conditions, allowing for clear observation of inhibitory effects. Each treatment was designed to compare the effectiveness of individual and combined antagonistic bacteria in suppressing the growth of the pathogen. The level of inhibition was indicated by the formation of clear zones around the tested bacteria, reflecting their ability to produce antimicrobial compounds or compete for nutrients (Backer et al., 2018). The outcomes of this assay are presented visually to facilitate comparison of the inhibition zones produced under each treatment condition, thereby providing a clearer interpretation of the relative antagonistic performance across treatments.

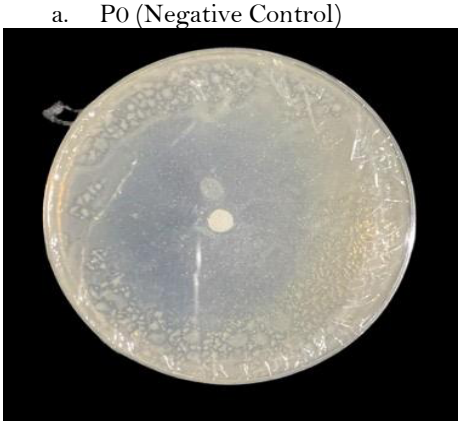
Figure 3 illustrates the differential inhibitory effects of the applied treatments, including both control groups and antagonistic bacteria, thereby providing a clear representation of their relative effectiveness in suppressing the growth of *Klebsiella variicola*. The results of the antibacterial activity test were observed across several petri dishes subjected to different treatments: P0 as a negative control, P1 as a positive control, P2 containing *Bacillus subtilis*, P3 containing *Pseudomonas fluorescens*, and P4 representing a combination of *Bacillus subtilis* and *Pseudomonas fluorescens*. Distinct differences in colony growth patterns and the formation of clear zones around the inoculation points were evident, indicating varying levels of inhibitory activity against the test microorganism. The negative control (P0) exhibited uniform and unrestricted bacterial growth, confirming the absence of inhibitory effects, whereas the positive control (P1) demonstrated a pronounced inhibition zone due to the presence of the antibiotic. Treatments involving antagonistic bacteria (P2, P3, and P4) showed noticeable variations in inhibition zones, suggesting differences in their antibacterial effectiveness. In particular, the combination treatment (P4) tended to exhibit a broader or more distinct inhibition zone compared to single-bacteria treatments, indicating a potential synergistic interaction between *Bacillus subtilis* and *Pseudomonas fluorescens* in suppressing the growth of *Klebsiella variicola*. These visual observations provide preliminary evidence of the antagonistic potential of the tested bacteria and support further quantitative analysis of inhibition zone diameters (Backer et al., 2018).



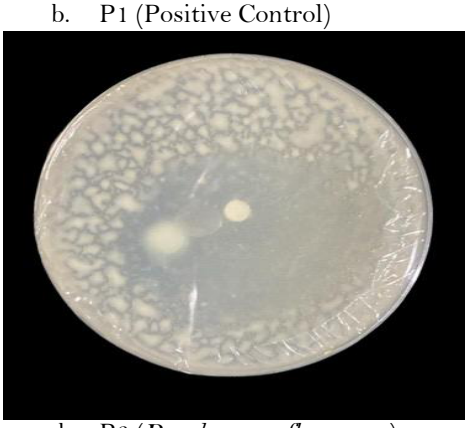
a. P0 (Negative Control)



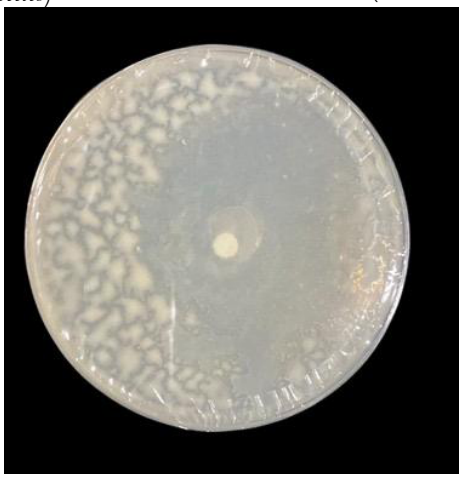
b. P1 (Positive Control)



c. P2 (*Bacillus subtilis*)



d. P3 (*Pseudomonas fluorescens*)



e. P4 (Combination of *Bacillus subtilis* and *Pseudomonas fluorescens)*

Figure 3. Inhibition Test of *Klebsiella variicola*

Table 1. Inhibition Test Results in 48 Hours After Incubation

Treatment	Mean Inhibition Zone of Antagonistic Bacteria Against ***Klebsiella variicola*** **(mm)**
P0	0a
P1	82.9b
P2	109.32c
P3	93.57bc
P4	98.17bc

The results of the inhibition assay presented in Table 1 demonstrate significant differences among treatments in suppressing the growth of *Klebsiella variicola*. In the

negative control (P0), no inhibition zone was observed (0 mm), indicating that in the absence of control agents, whether antagonistic bacteria or antibiotics, *K. variicola* was able to grow optimally on the medium. This condition highlights the pathogen's high colonization ability when not constrained by external factors, thereby increasing its potential to cause more extensive infections in host plants.

In contrast, the positive control (P1), which utilized the antibiotic chloramphenicol, produced an average inhibition zone of 82.9 mm and was significantly different from the negative control. This finding confirms the effectiveness of chloramphenicol in inhibiting the growth of *K. variicola*, as expected from its well-established broad-spectrum antibacterial properties. The presence of a clear and consistent inhibition zone indicates strong suppression of bacterial proliferation under in vitro conditions. However, when compared with treatments involving antagonistic bacteria, the inhibitory effect of the positive control was lower than that of certain treatments, particularly *Bacillus subtilis*. This suggests that specific biological control agents may exhibit comparable or even superior effectiveness to synthetic antibiotics under in vitro conditions. Such results highlight the potential of beneficial bacteria as sustainable and environmentally friendly alternatives to chemical control methods, especially in the context of plant disease management (Chowdhury et al., 2015).

The *Bacillus subtilis* treatment (P2) showed the most prominent result, with an average inhibition zone of 109.32 mm, significantly different from all other treatments. This high level of inhibition indicates that *B. subtilis* possesses strong antagonistic activity against *K. variicola*. This capability is likely attributed to the production of antibacterial secondary metabolites such as surfactin, iturin, and fengycin, which can disrupt pathogen cell membranes and inhibit cell wall synthesis. Additionally, nutrient competition may also contribute to suppressing pathogen growth, further enhancing the effectiveness of *B. subtilis* as a biological control agent (Ongena & Jacques, 2008).

The *Pseudomonas fluorescens* treatment (P3) resulted in an average inhibition zone of 93.57 mm. Statistically, this value was not significantly different from the positive control (P1) or the combination treatment (P4), but it was lower than that of *B. subtilis*. This indicates that *P. fluorescens* also exhibits antagonistic activity against *K. variicola*, although its effectiveness is not as high as that of *B. subtilis*. The inhibitory activity of *P. fluorescens* is likely associated with its ability to produce antimicrobial compounds such as siderophores, phenazines, and hydrogen cyanide, which suppress pathogen growth through nutrient competition and the production of toxic substances (Sudistina et al., 2025).

In the combination treatment of *Bacillus subtilis* and *Pseudomonas fluorescens* (P4), the average inhibition zone was 98.17 mm. This treatment was not significantly different from P1 and P3, and it did not surpass the effectiveness of *B. subtilis* when applied individually. This suggests that the combination of the two bacteria did not produce a significant synergistic effect in inhibiting the growth of *K. variicola*. It is possible that interactions between the two bacterial species were neutral or even competitive, thereby limiting the overall inhibitory effect. Furthermore, differences in their mechanisms of action and nutrient requirements may have influenced the effectiveness of the combination treatment (Stein, 2005).

The results of this study indicate that the use of antagonistic bacteria, particularly *Bacillus subtilis*, has considerable potential as a biological control agent in suppressing the growth of *K. variicola*. The high effectiveness observed under in vitro conditions provides an initial indication that this bacterium could be further developed as an environmentally friendly alternative for controlling bacterial wilt disease (Eljounaidi et al., 2016). However, further evaluation under field conditions (in vivo) is necessary to ensure consistency in its effectiveness across more complex environmental settings.

5. Discussion

In the positive control treatment (P1), a clear inhibition zone was observed, indicating inhibitory activity against both test bacteria. Treatment P2, using *Bacillus subtilis*, produced a larger inhibition zone compared to other treatments against both *Klebsiella variicola* and *Kosakonia cowanii*, suggesting strong antagonistic activity. Treatment P3, with *Pseudomonas fluorescens*, also showed inhibition zones, although smaller than those in P2. Meanwhile, treatment P4 (a combination of *Bacillus subtilis* and *Pseudomonas fluorescens*) resulted in relatively large inhibition zones, particularly against *Kosakonia cowanii*, indicating enhanced inhibitory effects from the combined antagonistic bacteria.

The differences in inhibitory activity against *Klebsiella variicola* indicate that the use of *Bacillus subtilis* as a single treatment provides higher effectiveness compared to its combined application with *Pseudomonas fluorescens*. This condition is closely related to the physiological characteristics of *Klebsiella variicola*, which possesses a capsular polysaccharide as one of its main virulence factors. This capsule functions as a protective layer that can inhibit the diffusion of antimicrobial compounds and potentially bind active molecules before they reach their primary targets on the cell membrane. As a result, a higher concentration of antibacterial agents is required to achieve optimal inhibitory effects (Ongena & Jacques, 2008).

The use of *Bacillus subtilis* in a single culture allows the optimal production of secondary metabolites in the form of lipopeptides, such as surfactin, iturin, and fengycin, without competition from other microorganisms (Stein, 2005). These compounds act by increasing membrane permeability, disrupting ion balance, and causing leakage of intracellular components, ultimately leading to cell death. High concentrations of lipopeptides support gradual penetration through the capsule layer, enabling antibacterial compounds to reach the cell membrane of *Klebsiella variicola* and function effectively.

The combined treatment with *Pseudomonas fluorescens* does not show a significant increase in inhibitory effectiveness due to competitive interactions among microorganisms within the consortium. This competition includes the struggle for nutrients, particularly iron (Fe), which is essential for microbial metabolism. *Pseudomonas fluorescens* has a high capacity to produce siderophores, making it more competitive in binding Fe (Chin-A-Woeng et al., 2003). This condition can suppress the growth and reduce the lipopeptide biosynthesis capacity of *Bacillus subtilis*. Additionally, the production of metabolites such as phenazines and hydrogen cyanide (HCN) by *Pseudomonas fluorescens* may inhibit the activity of *Bacillus subtilis*, further reducing antibacterial compound production.

The lower effectiveness of combined treatments against *Klebsiella variicola* is due to interspecies competition, including quorum-sensing disruption, nutrient and space competition, and energy diversion, which reduce antibacterial metabolite production (Haas & Défago, 2005). As a result, the concentration of active compounds is insufficient to penetrate the capsular layer, indicating that effectiveness depends more on high metabolite production than on the number of agents; thus, single cultures of *Bacillus subtilis* are more optimal. In contrast, the combination of *B. subtilis* and *Pseudomonas fluorescens* is more effective against *Kosakonia cowanii*, a biofilm-forming Gram-negative bacterium with high metabolic adaptability.

The combined interaction of *Bacillus subtilis* and *Pseudomonas fluorescens* demonstrates high effectiveness in inhibiting *Kosakonia cowanii* because both bacteria can simultaneously target the pathogen's key physiological requirements. *Kosakonia cowanii* is a Gram-negative bacterium that strongly depends on iron (Fe) availability, organic nutrients, and biofilm formation for survival and virulence. The presence of *Pseudomonas fluorescens* plays a crucial role in competing for iron through the production of siderophores, Fe^{3+}-chelating molecules with high affinity for

environmental iron ions. This condition creates an iron-limited environment, disrupting basic metabolism, growth, and biofilm formation in *Kosakonia cowanii* (Saha et al., 2016).

Pseudomonas fluorescens contributes not only through nutrient competition but also by producing antibacterial compounds such as phenazines, pyrrolnitrin, and hydrogen cyanide, which disrupt respiration, inhibit enzymes, and induce toxic reactive oxygen species, damaging proteins, lipids, and DNA (Chin-A-Woeng et al., 2003; Saha et al., 2016). This effect is enhanced by *Bacillus subtilis*, whose lipopeptides (surfactin, iturin, fengycin) damage membrane integrity and increase cell susceptibility (Compant et al., 2005; Ongena et al., 2021). Meanwhile, their combination also disrupts biofilms through iron limitation and matrix degradation, weakening embedded cells (Köhl et al., 2019; Ab Rahman et al., 2022). This synergy represents a multi-targeted attack, encompassing nutrient competition, metabolic disruption, membrane damage, and biofilm destruction, that increases inhibitory effectiveness against *Kosakonia cowanii* (Compant et al., 2019; Ongena et al., 2021).

6. Conclusion

Based on the results of this study, *Bacillus subtilis* demonstrated the most effective antagonistic activity in inhibiting the growth of *Klebsiella variicola* compared to *Pseudomonas fluorescens* and their combined treatment. This finding indicates that *Bacillus subtilis* has strong potential as a biological control agent against bacterial wilt pathogens, likely due to its ability to produce antimicrobial compounds and compete effectively for nutrients. The consistently larger inhibition zones observed in this treatment further support its superior performance under in vitro conditions. The implication of this result is the opportunity to develop more environmentally friendly and sustainable disease management strategies as alternatives to synthetic chemical agents, thereby reducing potential negative impacts on soil health and microbial diversity. However, the lack of enhanced effectiveness in the combined treatment also highlights the importance of considering ecological compatibility and interaction dynamics among microorganisms in biocontrol applications. This suggests that not all microbial combinations result in synergistic effects, and careful selection and evaluation are required to optimize their practical application in agricultural systems.

Nevertheless, this study has limitations, as it was conducted under in vitro conditions that do not fully represent the complexity of field environments. Factors such as interactions with other soil microorganisms, environmental variability, and host plant responses were not addressed. Therefore, future research should focus on in vivo evaluations under greenhouse and field conditions to assess the consistency of *Bacillus subtilis* effectiveness and to determine optimal formulation and application methods. Furthermore, a deeper investigation into microbial interactions and the molecular mechanisms underlying antagonistic activity is necessary to support the development of more effective and applicable biological control technologies.

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