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Potential of *Bacillus* sp., *Pseudomonas fluorescens*, and *Trichoderma* sp. Consortium as an Alternative Soybean Growth Medium Against *Ralstonia solanacearum* in Vitro

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Abstract

Background: *Ralstonia solanacearum is a bacterium highly adaptive to tropical and subtropical environments and can survive for long periods in the soil. This pathogen has a wide host range and is capable of causing severe damage to tomato plants, even leading to total crop failure until 100%. Therefore, appropriate control of these pathogens using biological agents is necessary. Soybean media is considered suitable for propagating Bacillus sp., Pseudomonas fluorescens, and Trichoderma sp. due to its adequate nutritional content and economic value. Previous studies have addressed microbes or media separately, such as PDA or NA, whereas this study combines microbial consortia and alternative media in a single integrated test. This study aims to examine the compatibility and antagonistic ability of a consortium of Bacillus sp., P. fluorescens, and Trichoderma sp. cultured on a soybean-based alternative medium against the bacterium Ralstonia solanacearum under in vitro conditions.* **Methodology:** *Observations were made on microbial compatibility and inhibition ability against R. solanacearum using thee double layer pepper disk diffusion methode.* **Findings:** *The compatibility test results showed that the microbial consortium was compatible and grew optimally on the soybean-based alternative medium. The highest compatibility index (CI) was observed in the Bacillus sp. + P. fluorescens treatment (0.402), while the lowest CI was found in the Trichoderma sp. × Bacillus sp. + P. fluorescens combination (0.873). In the antagonism test, the combination of Trichoderma sp. + Bacillus sp. × P. fluorescens produced an average inhibition zone of 17.2 mm, categorized as strong. Meanwhile, the Bacillus sp. + P. fluorescens treatment had the lowest average inhibition zone of 9.3 mm, categorized as moderate.* **Contribution:** *This study indicate that the consortium of Bacillus sp., P. fluorescens, and Trichoderma sp. maintains compatibility and antagonistic activity against R. solanacearum when grown on alternative soybean-based media, which has generally been tested on conventional media. This study recommends the utilization of soybean-based media as an alternative for developing microbial consortia of antagonists in the control of bacterial wilt disease*

Keywords: *Bacillus* sp.; *In Vitro; P. Fluorescens; R. solanacearum; Trichoderma* sp.

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INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most important horticultural commodities in Indonesia, with high economic value and significant potential for export. According to Navitasari (2021), national demand for tomatoes continues to increase at an average annual rate of 4.34 %. However, the increase in production has not always been able to meet market demand due to various technical and non-technical factors in the cultivation process. According to Yusriadi (2020) one of the main constraints faced by farmers is bacterial wilt disease, caused by *Ralstonia solanacearum*. This soil-borne pathogen infects plants through the root system and spreads through the xylem.

Ralstonia solanacearum is a bacterium highly adaptive to tropical and subtropical environments and can survive for long periods in the soil. *R. solanacearum* is an obligate aerobic organism; pathogenic strains have minimum, optimal, and maximum temperatures of 10 °C, 35 °C, and 41 °C, respectively. This bacterium is aerobic, and its colonies on solid media are small, irregularly round, white in reflected light and brown in transmitted light (Setiawan, 2019). This pathogen has a wide host range and is capable of causing severe damage to tomato plants, even leading to total crop failure until 100 % (Davis et al., 2018). Infections generally occur during the early growth stages of the plant, significantly affecting productivity (Kalpage & De Costa, 2019). Conventional control of this disease still largely relies on synthetic pesticides, which, although effective in the short term, pose risks such as pathogen resistance, environmental pollution, loss of natural enemies, and the presence of harmful residues in agricultural products (Navitasari et al., 2020).

As a more sustainable and environmentally friendly solution, the utilization of biological control agents has been increasingly developed. Several microorganisms such as *Bacillus* sp., *Pseudomonad fluorescens*, and *Trichoderma* sp. have been proven to possess the potential to suppress the development of *R. solanacearum* through various mechanisms, including competition, antibiosis, and induction of plant resistance (Fachrezzy et al., 2022; Ambar et al., 2021; Suanda & Ratnadi, 2019). The use of microbial consortia, which involves combining several synergistic and antagonistic microbes, is considered more effective than using single microbes because it produces stronger inhibitory effects and enhances the stability of the microbial ecosystem in the growth medium (Hayati, 2018).

In addition to microbial effectiveness, the availability of economical growth media that support microbial development is also an important factor. Main media such as PDA and NA are considered too expensive for farmers and will increase control costs. One promising alternative is the use of soybean boiling water, which is rich in nutrients. Processed soybean boiling water contains 0.11 % carbohydrates, 0.42 % protein, 0.13 % fat, 4.55 % iron, 1.74 % phosphorus, and 98.8 % water (Yuliarti & Nuherti, 2019). Several studies, such as Fadlillah et al. (2023); Andayani et al. (2022); and Nurwahidah (2022), have shown that boiling soybean water can support the high-density growth of antagonistic microbes, making it a potential alternative medium for the mass production of biological control agents. Previous research on alternative media has only focused on single-propagation methods.

Previous studies on alternative growth media have mainly focused on the propagation of single microorganisms and were generally evaluated using conventional media such as PDA or NA. Information regarding the compatibility and antagonistic activity of microbial consortia developed on soybean-based alternative media against *Ralstonia solanacearum* remains limited. Therefore, this study was conducted to evaluate the compatibility and antagonistic potential of a consortium consisting of *Bacillus* sp., *Pseudomonad fluorescens*, and *Trichoderma* sp. grown on soybean boiling water-based media as an alternative to conventional media.

METHOD

Time and Place

This research was carried out from February to May 2024 at the Plant Health Laboratory, Faculty of Agriculture, Universitas Pembangunan Nasional "Veteran" East Java, Surabaya. All *in vitro* testing activities were conducted in the laboratory's aseptic and incubation rooms to maintain sterile conditions throughout the experimental process.

Instrument

The materials used in this study included The *Bacillus* sp. The Bth-21 isolate used is the property of Dr. Arika Purnawati, M.P, and was also used in the study by Purnawati et al., (2025) against *R. solanacearum*, with an infection rate of 17%-18% compared to the control at 23%. Similarly, the *Trichoderma* sp. used also belongs to her, which was previously tested by Mahfud et al., (2025). In a 24-hour rice seed-soaking treatment with *Trichoderma sp.*, the infection rate was 8.33%. Meanwhile, the *P. fluorescens* isolate used is a collection from the Plant Pest and Disease Laboratory at Brawijaya University, Malang. It was previously tested in Aprilia & Aini (2022) study against Fusarium wilt in shallots with an efficacy level of 50%. As well as the pathogen *Ralstonia solanacearum* isolated from collection of Laboratory of Plant Protection UPN "Veteran" Jawa Timur (Rahayu, 2024). The microbial growth media consisted of Potato Dextrose Agar (PDA), Nutrient Agar (NA), King's B agar for rejuvenation, and a soybean boiling water-based alternative medium. Additional chemical materials used included sterile distilled water, 70% alcohol, and 1% NaOCl solution for sterilization. The main equipment used included an autoclave, laminar air flow cabinet, Petri dishes, Erlenmeyer flasks, micropipettes, inoculating needles, an incubator, and a binocular microscope (Olympus CX33).

Research Design

This study employed a Completely Randomized Design (CRD) with one factor, seven treatments, and three replications. The treatments were as follows: code T = *Trichoderma* sp., code B = *Bacillus* sp., code P = *P. fluorescens*, code TB = *Trichoderma* sp. + *Bacillus* sp., code TP = *Trichoderma* sp. + *P. fluorescens*, code BP = *Bacillus* sp. + *P. fluorescens*, and code TBP = *Trichoderma* sp. + *Bacillus* sp. + *P. fluorescens*.

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Microbial Multiplication

The soybean extract was prepared by boiling 1 kg of thoroughly washed soybeans in 3500 mL of distilled water until the liquid turned yellowish-brown. The mixture was filtered to obtain the extract, to which 20 g of sugar was added, then cooled. The propagation of *Bacillus* sp., *P. fluorescens*, and *Trichoderma* sp. was carried out using a simple aerator system, followed by the determination of their population and spore densities. The following diagram illustrates the supporting installation design.

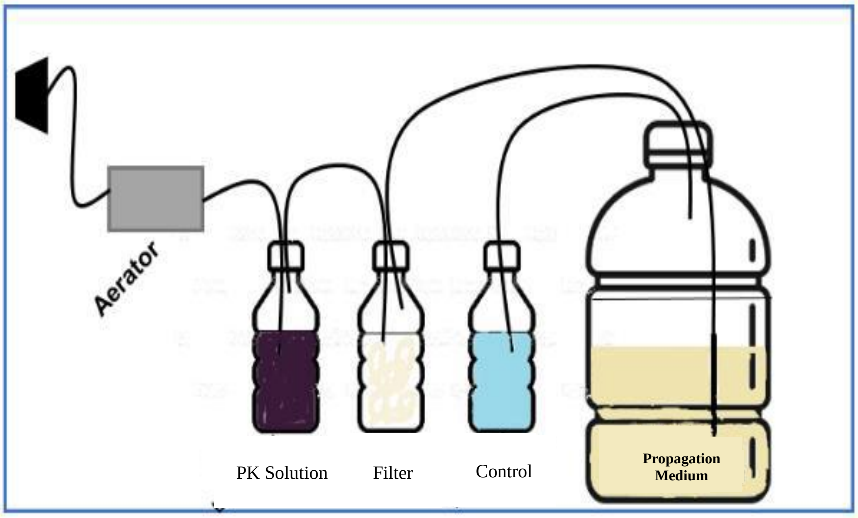

Figure 1. Aerator Installation Design for the Propagation of Antagonistic Microbes by Fadlillah et al., (2023)

Incubation was carried out at room temperature (28 ± 2 °C) with incubation periods adjusted according to each microorganism. The pH of the soybean boiling water-based medium ranged from 6.5 to 7.0 prior to sterilization. All media and equipment were sterilized using an autoclave at 121 °C for 15 minutes. The initial concentration of bacterial and fungal inoculum was standardized to approximately 10^5 CFU/ml or spores/ml before use.

Compatibility Test

For the compatibility test, following Hanudin et al., (2012), soybean agar medium was used as the growth medium. Filter papers with a diameter of 0.5 mm were dipped into the suspension of antagonistic microbes according to the treatment, then placed on the soybean agar medium, covered, sealed with plastic wrap, and stored on the incubation rack. This process was carried out under aseptic conditions and incubated for 14 days. Incubation was carried out of room temperature (28 ± 2 °C).

Antagonism Test

The antagonism test was performed using the double-layer method with the paper disk diffusion technique (Sagala, 2024) on soybean agar medium. Filter papers with a diameter of 0.5 mm were dipped into the antagonistic microbial suspension according to the treatment, placed in Petri dishes, and incubated at room temperature for 24 hours. After incubation, the Petri dishes were inverted, and 1 ml of chloroform solution was added and the dishes were left for 2 hours until the solvent evaporated. Once evaporation was complete, the dishes were inverted again. A Warm, molten agar medium was then inoculated with *R. solanacearum* suspension at a population density of 10^7 CFU/ml and poured into the petri dishes containing the antagonistic microbial cultures using the pour plate method. The plates were gently shaken until homogeneous, stored at room temperature, and left for 24 hours (Navitasari et al., 2021).

Research Parameter

Population/Spore Density

The Population or Spore Density is calculated using the formula by (Eni, 2020),

$$\text{Population/Spore Density} = \text{Number of colonies} \times \frac{1}{Dilution\ factor} \quad \text{.......................} \quad (1)$$

Meanwhile, the liquid suspension of *Trichoderma* sp. is obtained by harvesting *Trichoderma* sp. isolates from the propagation media and then directly counting them using a hemocytometer and the following formula (Naibaho et al., 2023).

$$S = \frac{X}{L\ x\ t\ x\ d} \times 10^3 \quad \text{...} \quad (2)$$

Notes :
S : Spore density
X : Average spores in the counting box
L : Hemocytometer counting box area (0.2 mm^2)
t : depth of the hemocytometer counting chamber (0,1 mm)
d : Dilution factor
10^3 : Calculated suspension volume (1 ml : 10^3 mm^3)

Compatibility Index

The compatibility observation of antagonistic microbes was carried out after 7 days of incubation (Hanudin et al., 2012). The assessment was performed by observing the presence or absence of an inhibition zone between two interacting bacterial isolates. According to Silitonga et al. (2013), compatible isolates are characterized by the absence of an inhibition zone. Istifadah et al., (2014) further stated that isolates are considered compatible when no inhibitory zone forms at the point of interaction between the two isolates, and are considered incompatible when an inhibitory zone appears in that interaction area. The compatibility of antagonistic microbes was determined based on the compatibility index (CI) using a formula adopted and modified from Hamilton & Attia (1997),

$$CI = \frac{Single\ Antagonism\ Microbe\ Growth}{Consortium\ Antagonism\ Microbes\ Growth} \quad \text{.............................} \quad (3)$$

Note :
$CI \leq 1$ = The antagonistic microbial combination is compatible;
$CI > 1$ = The antagonistic microbial combination is incompatible

Clear Zone

The calculation of inhibition zone parameters was carried out in accordance with the method described by Pormes et al., (2016),

$$Obstacle\ zone = \frac{Dv+Dh}{2} \quad (4)$$

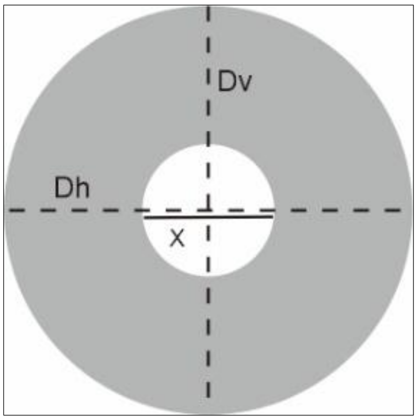

Figure 2. Inhibition Zone Calculation Plan Refers to (Pormes, *et al.* 2016)
Description: Dv = Vertical diameter; Dh = Horizontal diameter; X = Filter paper diameter (5mm); Grey area = Clear Zone

Data collection

Bacterial colony counts were observed using a Total Plate Count (TPC), while spore density was calculated using a hemocytometer. Compatibility index and clear zone area were measured using a ruler and expressed in millimeters (mm).

Data analysis

The research data were analyzed using the Analysis of Variance (ANOVA) method to determine whether each treatment had a significant effect. If the analysis showed a significant difference between treatments, further testing was conducted using the Duncan Multiple Range Test (DMRT) at a 5% confidence level to determine the differences between treatments more specifically.

RESULT AND DISCUSSION

Microbial Multiplication

Based on the results of antagonistic microbial propagation using a simple aerator device, the population/spore density ranged from 3×10^7 to 7.2×10^7 CFU/ml (Table 1), with the highest density observed in *P. fluorescens* at 7.2×10^7 CFU/ml, and the lowest in *Bacillus* sp. at 3×10^7 CFU/ml. These values meet the requirements of SNI 8027.3:2014, which specifies a range of 2.70×10^7 CFU/ml to 6.73×10^7 CFU/ml. During the 7-day incubation process, a slightly acidic fermentation odor resembling that of *tape* (fermented cassava) was detected. This observation is consistent with the statement of Fadlillah et al., (2023), who noted that during incubation, a distinctive mildly acidic fermentation aroma similar to *tape* is produced. No contamination was observed, as indicated by the absence of any foreign particles in the distilled water bottles (Figure 4).

Figure 3. Microbial Multiplication Series Using an Aerator
Description: A. PK Solution; B. Glasswool; C. Microbes in Soybean Boiled Water; C. Aquadest

According to Joe (2011), soybeans, or soy beans, are a type of legume that is the basic ingredient in many East Asian foods such as milk, soy sauce, tofu, and tempeh. The resulting soybean broth has a brownish-yellow color, smells like boiled soybeans, and is frothy. However, the liquid waste from boiling soybeans contains the nutrients Phosphorus (P), Nitrogen (N), and Potassium (K), which are essential for plant growth. The processed soybean broth contains 0.11% carbohydrates, 0.42% protein, 0.13% fat, 4.55% iron, 1.74% phosphorus, and 98.8% water. Insufficient protein intake from the culture medium can reduce the ability of spores to germinate, thus decreasing viability. Vitamins function as additives or supplements so that mushroom growth becomes better. Minerals are micronutrients that are useful as a complement to mushroom growth (Kalsum et al., 2011). Syahnen et al., (2014), stated that high spore density or meeting standards will be an indicator of the ability of biological control agents to suppress pathogen infections.

Table 1. Population Density/Spore Count

Isolate	Population Density/Spore Count
Trichoderma sp.	4.5 x 10^7 Spora/ml
Bacillus sp.	3.0 x 10^7 CFU/ml
Pseudomonad fluorescens	7.2 x 10^7 CFU/ml

Compatibility Test

The compatibility test was conducted to determine whether the microbes in the consortium were compatible, indicated by the absence of inhibition zones, and the compatibility index was calculated. The absence of inhibition zones signifies that no antagonistic activity occurred between the antagonistic microbes (Arora, 2015). Table 2 shows that all antagonistic microbial consortium treatments, both fungal and bacterial, exhibited compatible reactions, as indicated by compatibility index values of less than 1 (<1). The highest compatibility index (CI) was observed in the *Bacillus* sp. × *P. fluorescens* treatment (0.402), while the lowest CI was found in the *Trichoderma* sp. × *Bacillus* sp. × *P. fluorescens* treatment (0.873). This is in accordance with the findings of Marwoto (2012), namely that the *Bacillus* sp. and Pseudomonas sp. bacteria groups, when combined, will be incompatible, meaning that when combined, these two antagonistic bacteria will inhibit each other.

Biologically, a lower compatibility index (CI) value indicates a more harmonious interaction among antagonistic microorganisms, characterized by minimal inhibitory effects and efficient coexistence within the same growth medium. Conversely, CI values approaching 1 suggest the presence of mild competition, although the microbial combination remains compatible.

Table 2. Inter-Microbial Compatibility Test Reaction

Treatments	Diameter Colony (cm)	Compatibility Indeks (CI)	Reaction
T (single)	4.40	-	-
B (single)	1.80	-	-
P (single)	1.50	-	-
T + B	4.65	0.666	Compatible
T + P	4.05	0.728	Compatible
B + P	4.10	0.402	Compatible
T + B + P	2.93	0.873	Compatible

Description: - = Compatibility reaction not tested; Code T= *Trichoderma* sp.; Code B= *Bacillus* sp.; Code P= *P. fluorescens*

Antagonism Test

The results of the *in vitro* antagonism test showed that the TB and TBP isolate codes represented treatment combinations with the highest average clear zone diameter, measuring 17.1 mm (Table 2). Meanwhile, the treatment with the lowest average clear zone was the BP code, with an average diameter of 9.33 mm. The formation of the clear zone occurs due to the inhibitory mechanisms produced by antagonistic microbes that suppress the growth of *R. solanacearum*.

Antagonistic microbes produce various enzymes and antibiotic compounds that can inhibit the growth of pathogenic bacteria. According to Luz (2001), the inhibition mechanism by antagonistic microbes involves the production of siderophores, β-1,3-glucanase, chitinase, antibiotics, and cyanide, which play a role in suppressing the growth of pathogenic colonies. According to Gravel et al., (2017), the mechanism of pathogen inhibition by *Trichoderma* sp. secondary metabolites occurs through protein denaturation, both in structural and functional proteins of pathogen cells. Active compounds in secondary metabolites are able to break disulfide bonds between polypeptides in the cell walls and membranes of pathogen cells. Zalila et al., (2016) sayed one of the suppression mechanisms by strains of the genus *Bacillus* is antibiosis, which is indicated by the formation of inhibition zones in *Bacillus* sp. cultures grown on a layered medium with pathogenic bacteria.

Table 3. Average Clear Zone

Isolate Code	Average Clear Zone (mm)
T	15.0
B	12.7
P	14.2
TB	16.3
TP	12.8
BP	9.3
TBP	17.2

Description: Code T= *Trichoderma* sp.; Code B= *Bacillus* sp.; Code P= *P. fluorescens*

Table 4. DMRT Clear Zone Further Test

Treatments	Average
T	15.00 ab
B	12.66 ab
P	14.16 ab
TB	16.33 b
TP	12.83 ab
BP	9.33 a
TBP	17.16 b

Description: The same letters in the same column indicate no significant difference in the Duncan test with a 5% confidence level. Code T= *Trichoderma* sp.; Code B= *Bacillus* sp.; Code P= *P. fluorescens*

The results of further testing using the DMRT (Duncan Multiple Range Test) also supported these findings. The mean data obtained from each treatment showed no significant differences. The average inhibition zone formed in each treatment is presented in Table 4.2. The mean inhibition zone diameters ranged from 9.3 mm to 17.2 mm. The TBP treatment exhibited the highest average value of 17.2 mm while the BP treatment showed the lowest average of 9.3 mm. Although there were descriptive variations in the data, these differences were not large enough to be considered statistically significant. These results indicate that although all antagonistic microbes possess the potential to inhibit the growth of *R. Solanacearum*, none of the treatments proved to be the most effective statistically. According to Jamilatun et al., (2020), all treatments were categorized as strong, with average inhibition zones ranging between 10–20 mm, except for the BP treatment, which was classified as moderate (>10 mm) because it had an average inhibition zone of only 9.3 mm.

CONCLUSION

This study demonstrated that the consortium of *Bacillus* sp., *Pseudomonad fluorescens*, and *Trichoderma* sp. Was biologically compatible on soybean-based alternative media, as indicated by compatibility index values below 1, and exhibited antagonistic activity against *Ralstonia solanacearum* with inhibition zones ranging from 9.3 to 17.2 mm. Scientifically, these findings provide insight into the stability and functionality of microbial consortia developed on non-conventional growth media. From an applicative perspective, soybean boiling water shows potential as an alternative medium for the propagation of antagonistic microorganisms at laboratory to semi-mass production scales. However, since this study was conducted under in vitro conditions, further research under greenhouse and field conditions is necessary to validate the effectiveness of the consortium under more complex environments.

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