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The Potential of Soil Bacteria and nanochitosan in inhibiting *Colletotrichum* sp. Fungi

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Abstract. *Colletotrichum* sp. is the main pathogen causing anthracnose in horticultural crops, especially chili peppers, which causes losses in yield and production quality. The use of synthetic fungicides still dominates the control of this disease, but causes problems with residues, pathogen resistance, and environmental impact. Environmentally friendly alternatives based on biological agents and nanomaterials are needed for sustainable solutions. The methods used in this study evaluated the potential of soil bacteria as antagonistic agents and nanochitosan in inhibiting the growth of *Colletotrichum* sp. Bacterial isolates were obtained from the rhizosphere of healthy plants, selected based on antifungal activity, and tested for antagonism in vitro using the dual culture method. Nanochitosan was applied at concentrations of 0.2–1.5% to observe its effect on mycelium growth. The results showed that several soil bacterial isolates were able to produce significant inhibition zones with an inhibition percentage of >30%. Nanochitosan treatment effectively slowed colony growth and disrupted hyphal morphology. The combination of antagonistic bacteria with nanochitosan produced a synergistic effect with a higher inhibition rate than either treatment alone. Therefore, the integration of soil bacteria and nanochitosan has great potential to be developed as an innovative, environmentally friendly, and sustainable biofungicide formulation for controlling anthracnose disease in horticultural crops

Keywords: *Anthracnose; Biofungicide; Colletotrichum sp.; Nanochitosan; Soil Bacteria*

1. Introduction

Colletotrichum sp can cause significant damage to plants and has the potential to drastically reduce crop yields and threaten production sustainability [1], [2] Anthracnose disease caused by *Colletotrichum* sp is known to affect various parts of plants, from leaves to fruits, with symptoms such as brown spots on fruits that can spread rapidly, affecting the quality and quantity of chili yields [3]. Control of this disease often faces challenges, especially when relying on chemical fungicides that can cause resistance in pathogens [4], [5]. Therefore, it is important to develop control methods that are not only effective but also sustainable, utilizing a combination of antagonistic bacteria and carriers. The development of methods for controlling

selected bacterial isolates should be promising. In soil, bacterial diversity plays an important role in maintaining fertility [6], thus playing an important role in improving plant development and soil quality [7]. Bacteria play a very important role in agriculture to improve nutrient exchange for plants and reduce the use of chemical fertilizers and pesticides as much as possible. [8], [9].

Several bacteria that have been isolated from soil, such as *Pseudomonas* spp., have beneficial applications like in agriculture and environmental management through plant growth promotion, pathogen suppression, pollutant degradation, and support of biotechnological process [10], [11], [12]. *Pseudomonas* spp. can enhance plant growth through several mechanisms, such as increasing the availability and absorption of mineral nutrients through phosphate solubilization, root growth through phytohormone production, or by increasing tolerance to abiotic stress [13]. They can also be applied as biofungicides. The potential of bacteria to be used as biofungicides stems from their various mechanisms for protecting plants from pathogen infection. They can use one or a combination of mechanisms to prevent or reduce plant diseases, interacting directly or indirectly with pathogens [14]. Bacteria can interact directly with pathogens through the secretion of antimicrobial compounds, disrupting pathogen virulence, and competing for nutrients and space. Some bacteria can synthesize and release metabolites such as lipopeptides, bacteriocins, antibiotics, biosurfactants, cell wall-degrading enzymes, or microbial volatile compounds that have antimicrobial activity by reducing the growth or metabolic activity of pathogens.

Biofungicide activities require a suitable carrier to facilitate application with the bacteria used. A suitable carrier for microbial growth will maintain high viability and effectiveness during storage, ensuring the survival and effectiveness of the microbes within it from biotic and abiotic factors [15]. Bacteria-mediated nanotechnology is emerging as a promising approach in plant disease management. Nano-based carriers have high compatibility with bacterial agents, thereby supporting microbial growth and enabling them to withstand changes in the external environment, and are useful for increasing activity in degrading organic contaminants [16]. Inappropriate carriers can cause toxicity to microorganisms, inhibit the release of active substances, or even cause phytotoxicity in plants. Various types of carriers can be made using natural polymers such as cyclodextrin, starch, chitosan, carboxymethylcellulose, ethylcellulose, and silica, due to their wide availability and biodegradability [17]. Nanochitosan was selected as the carrier material because its particle size and surface charge facilitate effective bacterial absorption. To evaluate its potential, in vitro tests were conducted to examine the interaction between bacteria and nanochitosan in inhibiting *Colletotrichum* sp. fungi.

2. Research Methods

2.1. Location of Sampling and isolation method

Soil was collected from three locations, namely forest plants, bamboo plants, and chili plants in the Sibolangit area. The collected soil was taken from the root zone of each plant with a depth of 0-20 cm. Soil samples weighing 1 g were collected and repeated three times. The samples were then diluted to 10-6 and cultured using the spread plate method on NA medium, incubated for 24 hours at 37°C. Bacterial colonies that grew were isolated on Nutrient Agar medium [18].

2.2. Morpological characterization and identification

The results of the soil bacterial isolation were then subjected to morphological characterization based on the shape, edge, elevation, and color of the colonies obtained. Then different colonies were separated and pure cultures were made. Furthermore, biochemical characterization was carried out using Gram staining and biochemical tests such as catalase, Simon Citrate Agar (SCA), sugar fermentation (Triple Sugar Iron Agar) / TSIA, catalase, starch, and gelatin. The reaction was considered positive (+) if there was a color change from green to blue on the medium. The reaction is considered negative (-) if there is no color change.

Aseptically, the TSIA (Triple Sugar Iron Agar) test is performed by inoculating bacteria into the TSIA medium. It is then incubated at 37°C for 24 hours. The reaction is considered positive if a yellow color appears and negative if a red color appears (Saimin et al. 2020). The catalase test is performed by making a smear on a glass slide and then adding 2 drops of 3% H2O2. A positive reaction is indicated by the formation of gas bubbles due to the degradation of H2O2 caused by the secretion of the catalase enzyme (Khatoon et al. 2022). The starch test is performed by inoculating bacteria into starch medium at 37°C for 24 hours. After the bacteria grow, iodine/lugol solution is dripped around the bacterial colonies. If an area forms around the colonies, then starch hydrolysis occurs due to amylase enzyme secretion (Algofar et al. 2021). The gelatin test was performed by inoculating bacterial isolates into nutrient agar medium at 37°C for 24 hours, then stored in an incubator at 40°C for 30 minutes. Positive results indicate that gelatin remains liquid even when stored at 4°C [19].

2.3. Nanochitosan Synthesis

Nanochitosan synthesis was carried out by dissolving 0.2 g of 0.3% acetic acid in 50 ml of each beaker glass. The size reduction was carried out using a magnetic stirrer, homogenizer, and ultrasonicator. Each 50 ml of chitosan solution was reduced for 30 minutes, until the solution was clear. Nanoparticle formation was carried out through an emulsification step with the addition of 50 microliters of 0.1% Tween while continuously stirring for 1 hour. Subsequently, the nanoparticles were stabilized with 7 ml of 0.1% sodium tripolyphosphate solution while homogenizing for 1 hour.

2.4. *Test formulation preparation*

Prepare bacteria that have inhibitory ability against Colletotrichum sp, were propagated on Nutrient Agar (NA) media and incubated for 48 hours. The growing bacterial colonies were made into a suspension by adding 5 ml of sterile distilled water and harvested using an L rod to obtain a bacterial suspension. Next, the bacteria were transferred to Nutrient Broth so that the bacterial cells could grow in the liquid media to produce a bacterial suspension with a more homogeneous number of cells and high concentration by shaking for 13 hours. For bacterial testing with nano particles, the bacteria taken were a density of 10^7 cfu/ml. [20].

2.5. *Bacterial suspension and fungal antagonist test*

An antagonism test against *Colletotrichum* sp. was conducted in vitro to evaluate the ability of a bacterial suspension formulated with nanochitosan as a carrier to inhibit the growth of the pathogenic fungus. Nanochitosan formulations at concentrations of 0.2%, 0.5%, 1.0%, and 1.5% were mixed with the bacterial suspension. A 5-mm-diameter piece of *Colletotrichum* sp. mycelium was placed in the center of a 9-mm-diameter Petri dish containing PDA medium. Next, a 5-mm-diameter sterile paper disc was placed on the opposite side of the fungal inoculum, and the bacterial suspension–nanochitosan mixture was dropped onto the disc. Fungal growth was observed and recorded for 14 days after inoculation to determine the inhibitory capacity of each treatment. Each treatment was performed in triplicate.

2.6 *Data Analysis*

Data on the diameter of the *Colletotrichum* sp. inhibition zone were analyzed using analysis of variance (ANOVA). The analysis was conducted to determine the effects of treatment, concentration, and the interaction between these two factors on the diameter of the inhibition zone. If the ANOVA results showed significant differences at the 5% level ($p < 0.05$), Duncan's Multiple Range Test (DMRT) was conducted at the 5% significance level to compare the means among treatments.

3. Results and Discussion

3.1. Bacterial Morfology and Biochemical Test

The results of isolation from soil obtained morphological characterization and biochemical tests as well as physiological properties of potential bacteria, based on observations of colonies, edges, elevation, color, and biochemical tests of SCA, TSIA, Catalase, starch hydrolysis, and gelatinase (Table 1).

Table 1. Morphological Characteristics, Biochemical Properties, and Gram Reaction of Bacterial Isolates

Code	Shape	Edge	Elevation	Color	SCA	TSIA	Catalase	Starch	Gelatine	Gram
B 3	Circular	Entire	Convex	Yellowish White	-	M/K	+	+	+	+
B 6	Circular	Entire	Convex	Transparant Yellow	-	M/K	+	+	+	+
B9	Circular	Curled	Convex	Creamy	-	M/K	-	+	+	+
B10	Irregular	Undulate	Umbonate	Creamy White	+	M/K	-	+	+	+
B13	Circular	Undulate	Umbonate	Creamy	+	M/K	+	+	+	+
B19	Circular	Undulate	Convex	Creamy	+	M/K	-	+	+	+
B 20	Circular	Undulate	Convex	Creamy	+	M/K	-	+	+	+
T 2	Irregular	Undulate	Umbonate	Yellowish White	-	M/M/M/K	+	+	+	+
T 4	Circular	Entire	Convex	Yellowish White	+	M/K	+	+	+	+
T 6	Irregular	Undulate	Raised	Yellowish White	+	K/K ((+) black sediment	-	+	+	+
T 18	Irregular	Undulate	Umbonate	Creamy	-	M/K	-	+	+	-
T20	Circular	Undulate	Umbonate	Creamy	-	M/K	-	+	+	+
T 21	Circular	Entire	Convex	Yellowish White	-	M/K	+	+	+	+
T 22	Circular	Entire	Convex	Yellowish White	-	M/K	+	+	+	+
S P 1	Irregular	Curled	Umbonate	Creamy	-	M/K	+	+	+	+
SP 4	Irregular	Lobate	Umbonate	Yellowish White	-	M/K	+	+	+	+
SP 5	Rhizoid	Filamentous	Umbonate	Yellowish White	-	M/K	+	+	+	+
SP 6	Irregular	Undulate	Umbonate	Creamy	+	M/K	+	+	+	-
SP 10	Irregular	Undulate	Convex	Yellowish White	-	M/K	+	+	+	+
SP 11	Circular	Entire	Convex	White	-	M/K	+	+	+	+
SP 12	Irregular	Lobate	Umbonate	Yellowish White	+	M/K	+	+	+	-

From Table 1, in general, isolates show morphological variation in colonies, both in shape (circular, irregular, rhizoid), edges (entire, undulate, lobate, curled), elevation (convex, umbonate, raised), and colony color (white, cream, pale yellow, transparent yellow). This variation reflects the genetic and physiological diversity among isolates obtained from the plant rhizosphere. Isolates B6, B3, and T21 have circular colony shapes, entire edges, and convex elevation with yellowish to transparent colors. These characteristics are often found in the genus Bacillus, which is known as an effective PGPR. Isolates with irregular or lobate colony shapes, such as SP4, SP6, and T2, exhibit the characteristic morphology of *Pseudomonas* spp., which generally form irregular and slimy colonies. Isolate SP5, with its rhizoid shape and

filamentous edges, indicates high motility, which is often found in bacteria that produce natural surfactants. The variation in bacterial shape and elevation reflects genetic differences and the adaptive abilities of each isolate to the growth medium [22]. Biochemical testing showed that in the SCA (Simmon's Citrate Agar) test, several isolates such as B10, B13, B19, B20, T4, T6, SP6, and SP12 showed positive (+). In the SCA test, which indicates the ability to use citrate as a single carbon source. This ability reflects high metabolic flexibility, supporting bacteria in root colonization in environments with limited carbon availability. In the TSIA (Triple Sugar Iron Agar) test, most isolates showed an M/K (red/yellow) reaction, indicating glucose fermentation without excess gas production. A K/K reaction with black precipitation (e.g., T6) indicates the ability to produce H_2S, which can play a role in microbial competition and the inhibition of pathogenic fungi. In the Catalase Test, all isolates showed positive (+) results in the catalase test, except for a few that were partially negative. Catalase enzyme activity indicates the ability of bacteria to break down H_2O_2 into H_2O and O_2, enabling them to survive oxidative stress. This is important for the survival of bacteria in the rhizosphere, which is rich in reactive oxygen due to plant root activity. Starch Hydrolysis Test All isolates showed the ability to hydrolyze starch (+), indicating the production of amylase enzymes. This enzyme plays a role in the degradation of soil organic matter and helps make carbon sources available to other bacteria and plants. Gelatinase Test Most isolates showed positive results (+). In the gelatinase test, it showed the ability to degrade complex proteins. Gelatinase enzymes also play a role in tissue penetration and colonization. Gram staining Most isolates showed a Gram-positive reaction, consistent with the general characteristics of Bacillus sp., Paenibacillus sp., and other endospore-forming soil bacteria. However, some isolates, such as SP6 and T18, showed Gram-negative variations, which may belong to the Pseudomonas or Enterobacter spp. group. From this study, two isolates—b6 and sp1—were selected to test their ability to inhibit the fungus Colletotrichum sp. using the following treatments:

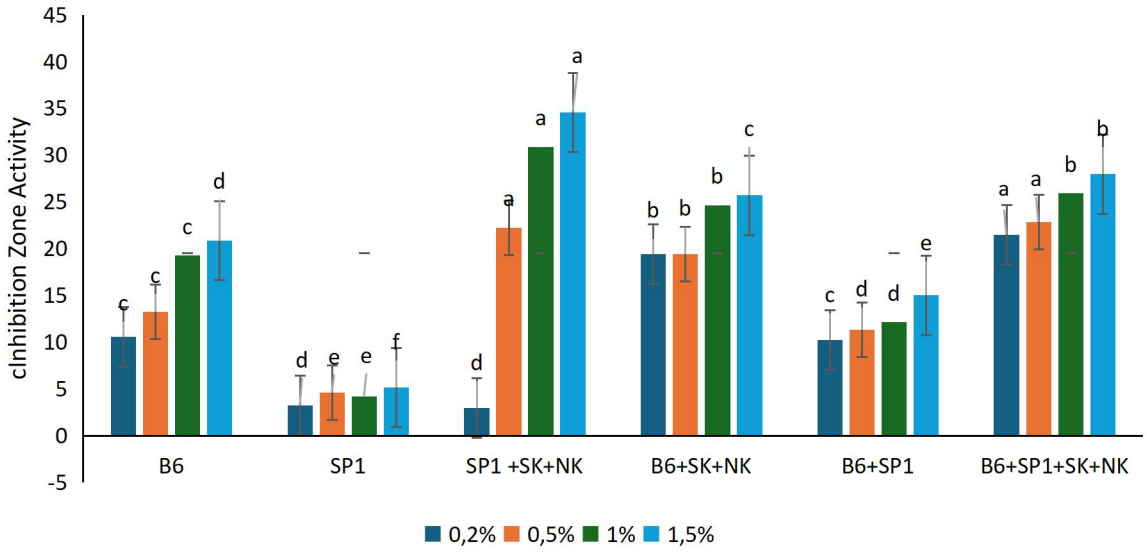

Figure 1. inhibition zone of treatment with pathogenic fungal growth

The results of the inhibitory activity test showed that the combination of bacteria and nanochitosan had a significant effect on the growth of *Colletotrichum* sp. The results of the analysis of variance (ANOVA) showed that the treatment, concentration, and interaction between treatment and concentration had a highly significant effect on the observed fungal inhibition zones ($p<0.001$). The very high calculated F-values for the treatment factor (13,111.94), concentration (6,614.76), and the treatment × concentration interaction (1,356.03) indicate that the variation observed in the inhibition zones was primarily due to the effects of

the treatment and concentration applied. The highly significant effects of the treatments indicate that each antagonistic agent has a different ability to inhibit the growth of pathogenic fungi. These differences may be due to the ability of each bacterial isolate to produce antifungal secondary metabolites, such as antibiotics, siderophores, hydrolytic enzymes, and volatile compounds. These compounds are capable of inhibiting fungal mycelial growth through mechanisms such as cell wall destruction, inhibition of protein synthesis, and disruption of fungal cell metabolism.

In addition, concentration also has a very significant effect on the inhibition zone. The higher the concentration of the bacterial suspension or metabolite used, the greater the likelihood of contact between the active compound and the pathogenic fungus, thereby increasing the resulting inhibitory effect. An increase in concentration leads to a greater amount of available antimicrobial compounds, resulting in higher effectiveness in inhibiting fungal growth.

The highly significant interaction between treatment and concentration indicates that the response of each treatment varies at different concentration levels. In other words, the effectiveness of an antagonistic agent is influenced by the concentration used. Some treatments may show a significant increase in the inhibition zone at high concentrations, while others yield a relatively lower response. Based on the bar chart, it can be seen that an increase in nanochitosan concentration from 0.2% to 1.5% consistently increased the diameter of the inhibition zone in most treatments. This indicates that the higher the concentration of nanochitosan used, the greater the antifungal activity produced by the combination of bacteria and nanochitosan. A single treatment using bacterial isolate B6 produced a relatively high inhibition zone compared to SP1, with an increase from 10.58 mm at a concentration of 0.2% to 20.86 mm at 1.5%. Meanwhile, the SP1 isolate alone showed a lower inhibition zone (2.25–5.16 mm), indicating that its antifungal ability is weak when not combined with nano materials. The combination of the two bacterial isolates (B6 + SP1) showed a moderate increase in the inhibition zone (10.23–15.00 mm), indicating a complementary effect but not yet optimal without the help of nano carriers. The above data shows that nanochitosan not only acts as a carrier but also actively contributes to antifungal activity [23]. The most notable results were seen in the combination of SP1 + SK + NK, which showed a significant increase from 2.98 mm at 0.2% to 34.55 mm at 1.5%. This formulation produced the highest inhibitory power compared to other treatments. This synergistic effect is thought to occur because nanochitosan acts as a carrier that enhances the stability of the antifungal metabolites produced by SP1. The combination of B6 + SK + NK also showed a similar increase, with a maximum value of 25.69 mm at a concentration of 1.5%. In addition, the combined treatment of two isolates with nanomaterials (B6 + SP1 + SK + NK) resulted in a significant increase in inhibition power, reaching 27.97 mm. This shows that combining two bacteria with a nanochitosan-based carrier system can strengthen the fungal inhibition mechanism. This combination allows for more efficient metabolic interactions between bacteria, such as increased production of siderophores, HCN, chitinase enzymes, and other antifungal compounds that can damage fungal cell walls.

In general, the results of this study show that nanochitosan plays an important role as a carrier and protector of bioactive compounds produced by bacteria. The synergy between nanomaterials and microbes indicates a potential approach in the development of environmentally friendly biofungicide formulations. The combination formulation of SP1 + SK + NK at a concentration of 1.5% can be recommended as the most effective treatment in suppressing the growth of Colletotrichum sp. This confirms that a formulation system with two PGPR strains and nanomaterials can enhance antifungal activity through multi-target mechanisms, such as increasing fungal cell wall permeability, inhibiting hyphal growth, and disrupting nitrogen metabolism in pathogens [24]. This mechanism is consistent with previous reports that nanochitosan can penetrate fungal membranes and stimulate the production of chitinase enzymes by bacteria [25].

4. Conclusions

The bacterial isolates exhibited considerable morphological and biochemical diversity, indicating broad physiological potential. Most isolates displayed colony characteristics typical of Bacillus and Pseudomonas, with circular to irregular shapes, convex or umbonate elevations, and creamy to yellowish pigmentation. Nearly all isolates showed positive catalase, amylase, and gelatinase activities, reflecting their capacity to produce antioxidant and hydrolytic enzymes that support root colonization and suppress pathogenic fungi. Most isolates were also Gram-positive, utilized citrate, and partially fermented glucose (M/K pattern), consistent with the characteristics of Plant Growth-Promoting Rhizobacteria (PGPR). Among them, isolate SP1 was identified as the most promising candidate due to its stable morphology, high enzymatic activity, and strong inhibition of Colletotrichum sp., especially when combined with nanochitosan. This combination produced a synergistic antifungal effect by enhancing the stability of bioactive compounds and proteolytic enzyme activity, demonstrating its potential as an eco-friendly biofungicide.

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