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Diversity of bacterial isolates as biocontrol agents against *Fusarium oxysporum* f. sp. *lycopersici*

IRWANDHI[1,♥], NUR PRIHATININGSIH[2], SILVA ABRAHAM[3], MUHSON ISRONI[4], RIZKA GITAMI SATIVA[5], NADIA NURANIYA KAMALUDDIN[1], FIQRIAH HANUM KHUMAIRAH[1], HARIS MAULANA[6], EMMA TRINURANI SOFYAN[1], TUALAR SIMARMATA[1,♥♥]

[1]Department of Soil Science and Land Resources, Faculty of Agriculture, Universitas Padjadjaran. Jl. Raya Bandung Sumedang KM 21, Sumedang 45363, West Java, Indonesia. Tel.: +62-22-84288890, ♥email: irwandhi23001@mail.unpad.ac.id; ♥♥tualar.simarmata@unpad.ac.id
[2]Department of Plant Protection, Faculty of Agriculture, Universitas Jenderal Soedirman. Jl. DR. Soeparno No. 63, Banyumas 53122, Central Java, Indonesia
[3]Directorate of Laboratory Management, Research Facilities, Science and Technology Areas, Deputy for Research and Innovation Infrastructure, National Research and Innovation Agency. Jl. M.H. Thamrin No. 8, Jakarta Pusat 10340, Jakarta, Indonesia
[4]Research Center for Applied Microbiology, National Research and Innovation Agency. Jl. M.H. Thamrin No. 8, Jakarta Pusat 10340, Jakarta, Indonesia
[5]Research Center for Agroindustry, National Research and Innovation Agency. Jl. M.H. Thamrin No. 8, Jakarta Pusat 10340, Jakarta, Indonesia
[6]Research Center for Horticultural and Estate Crops, National Research and Innovation Agency. Jl. M.H. Thamrin No. 8, Jakarta Pusat 10340, Jakarta, Indonesia

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Abstract. *Irwandhi, Prihatiningsih N, Abraham S, Isroni M, Sativa RG, Kamaluddin NN, Khumairah FH, Maulana H, Sofyan ET, Simarmata T. 2024. Diversity of bacterial isolates as biocontrol agents against* Fusarium oxysporum *f. sp.* lycopersici. *Biodiversitas 25: 3403-3411. Fusarium oxysporum* f. sp. *lycopersici* (FOL) is a soil-borne pathogen causing fusarium wilt in tomato plants, leading to significant crop losses worldwide. The severity of this disease is likely to increase with climate change, as rising temperatures and soil salinization create more favorable conditions for the pathogen. This study focused on characterizing bacterial isolates that could become biocontrol agents resilient to climate stress and identifying them through molecular techniques. Biochemical assays assessed the bacteria's ability to fix nitrogen and produce protease enzymes, siderophores, and hydrogen cyanide (HCN), which are essential for antagonizing FOL. The biological efficacy of these isolates was determined through antagonism assays, followed by molecular identification of the most effective isolates. A Completely Randomized Design (CRD) was used, involving six bacterial isolates and a control group with four replications for each treatment. All six isolates demonstrated the ability to fix nitrogen and produce protease enzymes, siderophores, and HCN, successfully inhibiting the growth of FOL. Among these, isolate R18 exhibited the strongest inhibition zone (55.55%), significantly reducing the mycelium weight to 0.0417 g, and showed tolerance to both temperature and salinity stress. Through 16S rRNA sequencing molecular identification, R11 was identified as *Bacillus megaterium*, and R18 was identified as *Bacillus albus*. These results highlight the potential of these bacterial isolates, especially R18, as effective biocontrol agents for managing fusarium wilt in tomato plants under changing climate conditions.

Keywords: Antagonistic bacteria, biocontrol agents, climate resilience, fusarium wilt, tomatoes

INTRODUCTION

Tomatoes (*Lycopersicon esculentum* Mill.) are the most widely consumed vegetable due to their affordability and high nutritional content, including vitamin C, potassium, oxalic acid, and folate (Rahman et al. 2024). However, attacks are susceptible to these plants by pathogens such as fungi, viruses, and bacteria (Pengproh et al. 2023). The presence of diseases is a threat to the plants and food security components, including availability, quality, production, nutritional value, and distribution (Arkhipov et al. 2023). According to Abro et al. (2022), diseases threatening tomato plants include anthracnose, tomato wilt, leaf blight disease, verticillium wilt, bacterial wilt, and bacterial wilt disease. One of the dangerous pathogens attacking the tomato plant is *Fusarium oxysporum* f.sp. *lycopersici.*

Fusarium oxysporum f.sp. *lycopersici* (FOL) is a soil-borne pathogen that poses a severe threat to tomato plants. It can persist in the soil and plant debris for several years as chlamydospores (McGovern et al. 2015). It infects tomato plants by entering through the regions where lateral roots form and at the root tips (Pazarlar et al. 2022). Once inside the roots, FOL obstructs the xylem vessels with its polysaccharides, spores, or mycelium, leading to the wilting, yellowing leaf, and eventual death of the tomato plants (Singh et al. 2017). The damage inflicted by this disease is not just biological but also economical, as Arsih et al. (2015) reported that it results in 20-30% losses in tomato crops. The current climate changes will exacerbate this condition.

Global climate change, characterized by accelerated global warming and increased extreme weather events (Sembiring et al. 2020), leads to rising sea levels and increased soil salinity (Clermont-Dauphin et al. 2010). This could greatly reduce crop yields and degrade agricultural land. These changes, including more severe droughts, higher humidity levels, and a greater prevalence of plant pests, worsen the situation (Skendžić et al. 2021). The rise in

temperatures is driving the development of more virulent pathogen strains, which have more detrimental effects on crop productivity (Velásquez et al. 2018). Drought stress also accelerates the development of diseases caused by *Fusarium* spp. (Wakelin et al. 2018).

The efforts to combat FOL often involve resistant plant varieties. However, this approach can unintentionally trigger the development of new pathogens that adapt to these resistant strains (Biju et al. 2017). Another standard method is pesticide application to manage pathogens. Although pesticides can effectively eliminate and control these threats, their excessive use may result in environmental and health risks and the emergence of pesticide-resistant pathogen strains (Al-Askar et al. 2021). The overreliance on chemical pesticides has also contributed to the development of pesticide-resistant weeds (Paramanandham et al. 2017). On the other hand, biocontrol-based strategies offer an eco-friendly alternative, leaving no harmful residues and promoting plant growth (Prihatiningsih et al. 2015).

Biological control of pathogens using biocontrol agents is a promising approach to protect plants, as it involves the production of nutrient-solubilizing compounds and toxic substances and inducing plant resistance (Mugiastuti et al. 2022). Antagonistic bacteria serve as biocontrol agents through mechanisms such as antibiosis, production of cell wall-degrading enzymes, competition, resistance induction, and growth promotion (Prihatiningsih et al. 2015). According to Wang et al. (2018), bacteria have many abilities to mitigate plant diseases by enhancing nutrient uptake and producing hydrocyanic acid, siderophores, and antifungal compounds. Bubici et al. (2019) demonstrated the high effectiveness of bacterial biocontrol agents in managing plant diseases in greenhouse and field conditions, reassuring them about their potential against pathogens. This research is dedicated to characterizing bacteria isolates as biocontrol agents capable of withstanding climate change and identifying bacterial isolates through molecular techniques. The results of this research are the first step in developing plant disease biocontrol products resistant to environmental stress amidst current and future climate change.

MATERIALS AND METHODS

Location

The research was carried out collaboratively between the Plant Protection Laboratory of Universitas Jenderal Soedirman and the Bacteriology and Mycology Laboratory of BRIN. The study utilized six bacterial isolates: A8, C1TR7, and TSB2, which were obtained from the rhizosphere of bamboo plants on Burung Island, Riau, and R11, R18, and R20 collected from the rhizosphere of rice plants in Banyumas, Central Java. The *Fusarium oxysporum* f.sp. *lycopersici* (FOL) isolate used in this study was supplied by the Plant Protection Laboratory of Universitas Jenderal Soedirman (Figure 1).

Characterization of bacterial isolates

Gram staining

The inoculation of fresh bacterial isolates onto glass slides and spread them by adding physiological saline. The slides were then air-dried and heat-fixed. A crystal violet solution was applied for 2 minutes, followed by rinsing with water. Lugol's solution is added for 1 minute before another rinse and drying. Decolorization was achieved by applying 96% alcohol for 15 to 20 seconds, after which the slides were rinsed and dried. Safranin was added for 30 seconds, followed by a final rinse and examination under a microscope. Isolates that appear blue or purple with bacterial isolates were Gram-positive, while those that appear pink were Gram-negative (Ulucay et al. 2022).

Catalase test

The catalase test was performed by placing bacterial isolates onto a microscope slide and then applying a drop of 3% hydrogen peroxide. The presence of air bubbles around the isolate indicates a positive catalase reaction (Reiner 2010).

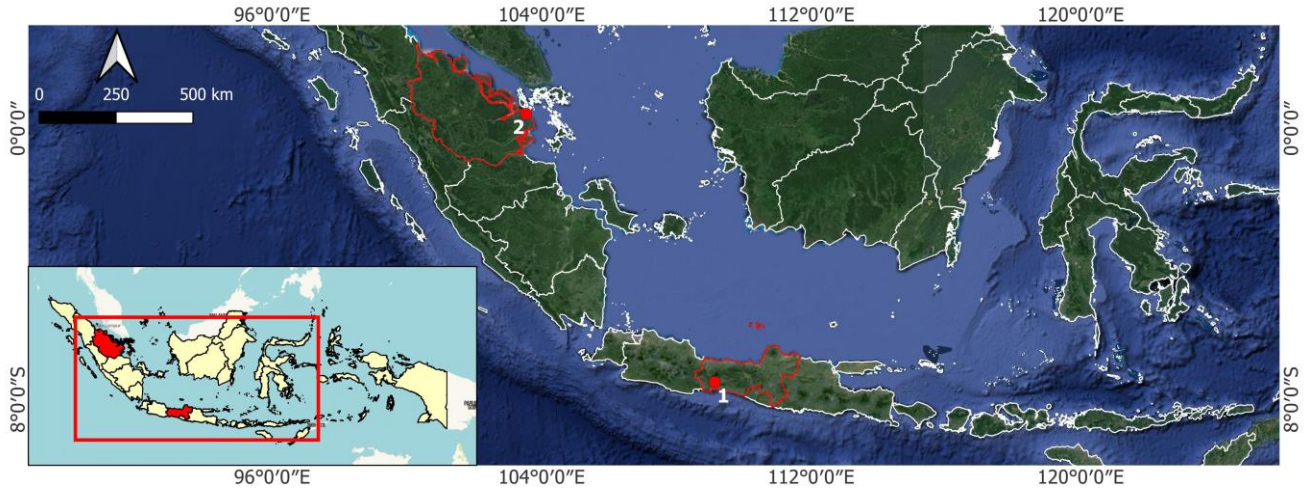

Figure 1. Locations for bacterial isolate sampling from 1: Banyumas, Central Java; 2: Burung Island, Riau, Indonesia

Starch hydrolysis test

Bacterial cultures, aged 16-18 hours, were inoculated onto Starch agar medium and incubated for 24-48 hours. After incubation, Gram's iodine solution was applied to the agar surface. Clear zones around the colonies identified a positive result (+), while a negative result (-) showed no clear zones (Lal and Cheeptham 2012).

Physiological properties

The physiological properties of the isolates, specifically their temperature and salinity tolerance, were assessed at various growth stages following the modified method by Ulucay et al. (2022). Next, to test resistance to temperature stress, bacterial isolates were incubated at 4°C, room temperature, and 40°C. NaCl was added to the growth media for salinity stress resistance at concentrations of 0%, 3%, 5%, and 7%. Bacterial growth was determined by monitoring changes in the turbidity of the growth medium. The results were considered positive (+) if the medium became turbid and negative (-) if it remained clear.

Nitrogen fixation ability test

Bacterial isolates were introduced to Jensen's medium and left to incubate at room temperature for 48 hours. The growth of bacterial isolates on Jensen's medium suggests their capability to fix nitrogen (Pambudi et al. 2016).

Antagonistic test of bacteria against FOL

Istiqomah et al. (2022) describe the antagonism test of bacterial isolates against the FOL pathogen using the spot method. In this approach, FOL colonies and bacterial isolates were cultured on PDA medium facing each other with three replicates and incubated at 30°C for 5 days. The inhibition zones produced by each isolate were then observed and compared to assess the antagonistic potential of each bacterial isolate. The procedure for evaluating bacterial antagonism against FOL growth is illustrated in Figure 2. The calculation of inhibition zones was carried out using the following formula:

$$P = (R_1\text{-}R_2) : R_2 \times 100\%$$

Where:

P : Inhibition rate (%)

R_1 : Fungal growth without bacterial antagonism/ control (cm)

R_2 : Distance of antagonistic inhibition (cm)

E : Bacterial isolate

C : Pathogenic fungal isolate

FOL mycelium weight

Each FOL mycelium from the antagonism test was collected and placed on pre-weighed Whatman No. 1 filter paper (initial weight). The filter paper with the mycelium was then treated with 10 ml of 1% HCl and placed in a water bath until the paper dried. Afterward, the paper was dried in an oven at 40°C until it reached a constant weight. The weight of the dry mycelium was calculated by subtracting the empty filter paper's initial weight from the filter paper's final weight plus the mycelium.

Protease enzyme production test

Each bacterial isolate was cultured on Skim Milk Agar medium using the streak method and incubated for two 24-hour periods at 37°C; clear zones around the colonies after incubation signify protease activity (Masi et al. 2021).

Siderophore production test

The test involved mixing 0.5 mL of a 2% $FeCl_3$ solution with 0.5 ml of cell-free culture supernatant; a color shift from yellow to reddish-brown or orange signals the production of siderophores (Jangir et al. 2018).

HCN production test

The bacterial isolates were cultured on a slanted NA medium. A filter paper, soaked in a solution for HCN detection (prepared by dissolving 2 g of picric acid and 8 g of sodium carbonate in 200 mL of distilled water), was placed at the top of the reaction tube. The bacterial cultures were then incubated at room temperature. The presence of HCN was detected by observing a color change on the filter paper. A consistent yellow color suggests that the bacterial isolate does not produce HCN, while shades of light brown, dark brown, or brick red indicate increasing levels of HCN production (El-Rahman et al. 2019).

Identification of bacteria based on 16S rRNA gene

Genomic DNA was extracted from each bacterial isolate using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). The DNA samples were then sent to 1st Base in Singapore for 16S rRNA sequencing. The resulting bidirectional sequencing data were assembled with BioEdit 7.2 software. Therefore, to identify the species, nucleotide sequences were analyzed using BLASTn at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Additional analysis was conducted with MEGA-11 software. The assembly of the 16S rRNA sequences was performed using BioEdit software (Retnowati et al. 2024).

Data analysis

The impact of bacterial isolate treatments on antagonism was evaluated using ANOVA through SPSS software. Differences in means were assessed using the Least Significant Difference (LSD) Test, with a significance level of $p<0.05$.

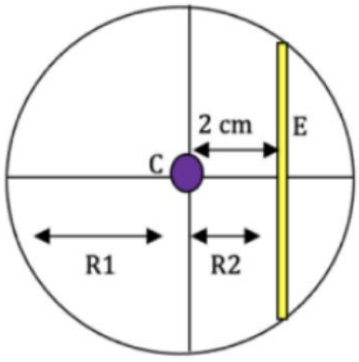

Figure 2. Method for testing the antagonistic activity of bacterial isolates

RESULTS AND DISCUSSION

Characterization of bacterial isolates

Bacterial traits

The bacterial characterization results detailed in Table 1 were obtained through a rigorous research methodology. These results, which are of significant importance, were obtained through macroscopic observations that assess the shape, color, and edges of bacterial colonies. Therefore, to identify bacterial cell shape and Gram type, staining of the isolates was performed and examined microscopically. The Gram staining results revealed that all isolates were Gram-positive, as evidenced by their purple coloration. Tripathi and Sapra (2021) explain that Gram-positive bacteria appear purple, whereas Gram-negative bacteria appear red. This difference arises from the varying cell wall compositions: Gram-positive bacteria possess a thick peptidoglycan layer, while Gram-negative bacteria have a thick lipid layer.

In the catalase test (Table 1), all bacterial isolates were found to produce the catalase enzyme. The appearance of air bubbles indicated a positive result for the catalase test after applying Hydrogen Peroxide (H_2O_2) to the isolates. This reaction, catalyzed by the catalase enzyme, decomposes H_2O_2 into water and oxygen (Reiner 2010). Bacteria producing catalase use this enzyme to protect themselves from the toxic effects of H_2O_2 (Murali and Patel 2017). The presence of catalase in bacteria clearly indicates their ability to manage environmental stress. Importantly, bacterial catalase plays a significant role in enhancing plant immune systemic resistance (ISR) to pathogens (Saeed et al. 2023), and the catalase activity in *Bacillus* strains aids in the detoxification of hydrogen peroxide in plants (Babiker et al. 2016).

Bacterial isolates R18, R20, C1TR7, and TSB2 demonstrated starch hydrolysis by developing clear zones around their colonies (Table 1). This finding aligns with the study by Kim et al. (2016), where the starch hydrolysis test revealed that isolates APEC136 and APEC170 also showed positive results, marked by clear zones around their colonies. These zones suggest that the bacteria can produce the α-amylase enzyme, which breaks down starch into glucose.

All bacterial isolates can produce protease enzymes (Table 1), breaking down proteins into peptides and amino acids (Masi et al. 2021). Protease, chitinase, and β-glucanase are extracellular enzymes produced by bacteria that are essential for cell wall degradation. Their ability to inhibit the growth of pathogenic fungi is a significant aspect, providing reassurance about their protective role. The more bacteria secrete these enzymes, the more effective they are at preventing the growth of pathogenic fungi (Khairah et al. 2023). Additionally, *Bacillus subtilis* strains produce protease that contributes to their antagonistic activity against pathogenic bacteria and fungi in vitro (Basurto-Cadena et al. 2012).

All isolates were able to survive incubation at 40°C and room temperature, but at 4°C, the growth of all bacterial isolates was inhibited (no growth occurred). Incubation of bacteria at low temperatures affects increased regeneration time and inhibits cell growth. Under salinity stress conditions, the isolates R11, R18, R20, C1TR7, and TSB2 were able to tolerate up to 7% salinity, while A8 could only survive up to 5% salinity stress. As shown in Table 1, the inhibitory effect of higher NaCl concentrations on bacterial growth is significant. Higher NaCl concentrations cause the water within bacterial cells to exit, thus inhibiting bacterial growth or causing plasmolysis. Furthermore, elevated NaCl concentrations also inhibit biofilm formation, bacterial motility, and oxidative resistance (Li et al. 2021).

Nitrogen fixation ability test

All bacterial isolates can fix nitrogen, as shown by their growth on the Jensen medium (Figure 3). This nitrogen-fixing ability is essential for supplying the nutrients needed to boost plant growth and yield (Islam et al. 2013). This function is linked to the bacteria's production of nitrogenase enzymes, which convert atmospheric nitrogen (N_2) into ammonia (NH_3) (Olanrewaju et al. 2017). Importantly, the increased nitrogen fixation in the atmosphere significantly improves nitrogen availability in the soil for plants (Timofeeva et al. 2023).

Table 1. Traits of bacterial isolates as biocontrol agents

Variable	Bacterial isolates					
	R11	**R18**	**R20**	**A8**	**C1TR7**	**TSB2**
Colony morphology	Circular	Circular	Rhizoid	Circular	Circular	Circular
Colony color	Yellow	Yellow	White	White	White	White
Colony edge	Entire	Entire	Rhizoid	Entire	Entire	Entire
Cell shape	Bacilli	Bacilli	Coccus	Bacilli	Bacilli	Bacilli
Gram staining	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Starch hydrolysis	-	+	+	-	+	+
Protease	+	+	+	+	+	+
Temperature tolerance						
4°C	-	-	-	-	-	-
Room temperature	+++	+++	+++	+++	+++	+++
40°C	+++	+++	+++	+++	+++	+++
NaCl tolerance						
0%	+++	+++	+++	+++	+++	+++
3%	+++	+++	+++	++	+++	+++
5%	+++	+++	+++	+	+++	+++
7%	++	++	++	-	++	++

Antagonistic test of bacteria against FOL

The in vitro application of bacterial isolates R11, R18, R20, A8, C1TR7, and TSB2 significantly inhibited the growth of FOL (Table 2). The inhibitory effect produced by each treatment of bacteria ranged from 36.07% to 55.55%. The bacterial isolate treatment of R18 performed the best with the largest inhibitory effect compared to other treatments of bacterial isolates, which was 55.55%. This is further supported by Rafanomezantsoa et al. (2022), who discovered that *Bacillus* spp. can inhibit FOL growth by 44.32-61.36%. According to Zhu et al. (2020), the variances observed in the inhibitory effects exerted by biocontrol agents may be attributed to variances in the chemical composition of antibiotics produced by each isolate, including bacillomycin, mersacidin, surfactin, bacilysin, and fengycin.

Antagonistic treatment against bacteria primarily reduced FOL mycelium weight (Table 2). This is in line with the ability of each bacterial isolate to suppress FOL growth, leading to potential inhibitory effects and lower fungal biomass. Conversely, effective fungal growth and development can lead to a significant increase in biomass (Saputri et al. 2020). These findings have significant implications for the fields of microbiology and agriculture, underlining the importance of this research.

Mechanism of bacteria antagonism

The FOL hyphae treated with antagonistic bacterial isolates (R11, R18, R20, A8, C1TR7, and TSB2) show irregular growth compared to the control FOL hyphae, presenting as discontinuous hyphae (Figure 4). This abnormal growth is likely due to lysis caused by antifungal compounds and cell wall-degrading enzymes produced by the bacterial isolates. These enzymes and compounds can damage the cell wall and hyphae of FOL. It aligns with Zhu et al. (2020), who noted that pathogenic hyphae's abnormal growth or malformation is attributed to the presence of such antifungal compounds and degrading enzymes. These antifungal compounds include tasA, fengycin, bacilysin, mersacidin, bacillomycin, and surfactin.

Each positive bacterial isolate produces siderophores, indicated by a color change from yellow to reddish-brown or orange (Figure 5). Siderophore production by bacteria helps address environmental iron (Fe) limitations (Sarwar et al. 2020). These siderophores can significantly impact plant growth using iron (Fe^{3+}) in the rhizosphere (Islam et al. 2013). By binding Fe^{3+} that plants could use, siderophores make it less accessible to phytopathogens, thereby indirectly benefiting plant health (Wani and Khan 2013).

Table 2. Test of bacterial antagonism and its impact on the mycelial weight of *Fusarium oxysporum* f.sp *lycopersici* (FOL)

Bacterial isolates	Inhibition (%)	Mycelium weight (mg)
Control	00.00a	116.2b
R11	43.76bc	60.5a
R18	55.55c	41.7a
R20	36.07b	56.2a
A8	37.28b	54.0a
C1TR7	40.06bc	52.7a
TSB2	37.71b	52.3a

Notes: The numbers sharing the same letter within a column and treatment group signify no significant difference, as confirmed by the LSD test at a 5% significance level

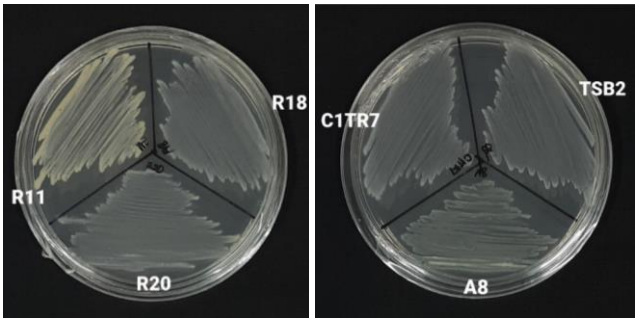

Figure 3. The growth of bacterial isolates on the Jensen medium demonstrates the nitrogen-fixing capability of bacteria

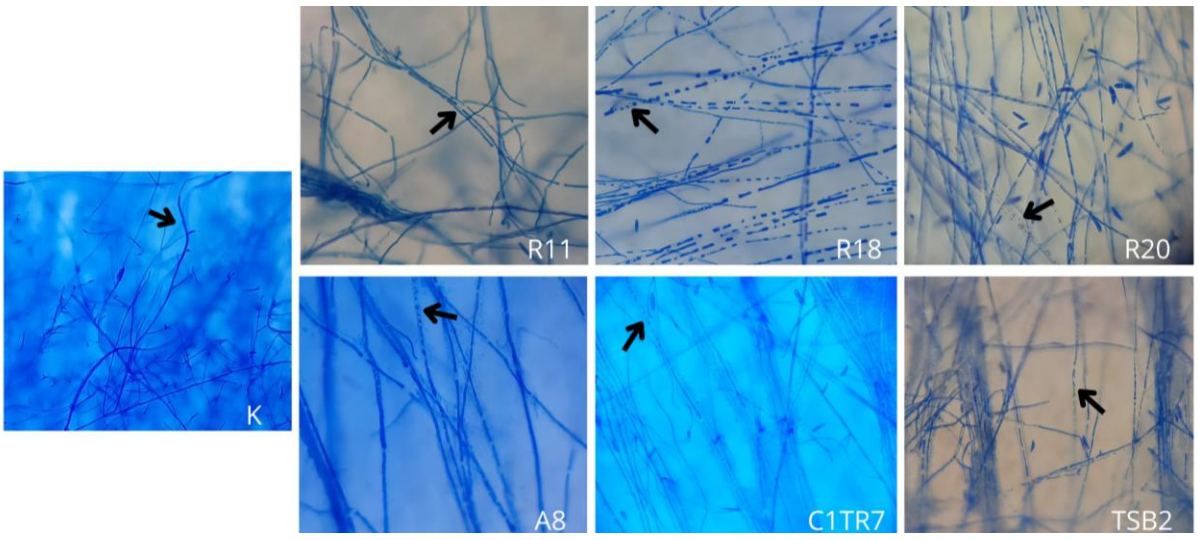

Figure 4. The study examined structural changes in *Fusarium oxysporum* f.sp. *lycopersici* following antagonistic treatment with bacterial isolates. The treatments resulted in alterations to the hyphal structure, showing fragmented hyphae, whereas the control exhibited continuous hyphal structures (indicated by an arrow)

Table 3. Determining isolated bacterial species through analysis of 16S rRNA sequences

Code	Description	Per. ident	Accession
R11	*Bacillus megaterium* strain ROA024 16S rRNA gene, partial sequence	100%	MT510154.1
R18	*Bacillus albus* strain 1Z2D15 16S rRNA, partial sequence	99,35%	MZ470060.1

The production of HCN, as indicated by a color change from yellow to red on filter paper in the bacterial isolates (Figure 6), was a significant finding. This color shift confirms that each bacterial isolate that tested positive produces HCN. HCN is a compound involved in antibiosis mechanisms. The potential of HCN production to revolutionize biocontrol strategies is quite promising, as biocontrol agents can generate one or more compounds that function in these mechanisms (Sivasakthi et al. 2014). This aligns with the findings of Olanrewaju et al. (2017), who noted that bacterial-produced HCN works synergistically with other biocontrol strategies, such as producing antibiotics or enzymes that degrade cell walls.

The results of the bacterial antagonism tests against FOL, a pivotal aspect of this research, demonstrate that bacterial isolates utilize antibiosis as a control mechanism. Prihatiningsih et al. (2015) identified several biological control methods, including antibiosis, competition, mycoparasitism, cell wall-degrading enzymes, resistance induction, growth promotion, and rhizosphere colonization. Bhagat et al. (2014) emphasized the role of HCN production by biocontrol agents in inducing systemic resistance, a finding that enlightens us about the mechanisms of biocontrol. HCN production is significant in biocontrol as it promotes plant resistance. R11 and R18 are the isolates with the most significant potential for controlling FOL in tomato plants.

Bacterial identification based on 16S rRNA gene

Visualization of gel results from 16S rRNA sequence amplification of two bacterial isolates (R11 and R18) in Figure 7 shows the presence of PCR reaction products of approximately 1500 bp long. Species identification results using BLASTn analysis (Table 3) revealed that both isolates belonged to the genus *Bacillus*. Isolate R11 was identified with accession number MT510154.1 as *Bacillus megaterium*, while isolate R18 was identified with accession number MZ470060.1 as *Bacillus albus*. The results of the phylogenetic analysis conducted with alignment (MUSCLE) and constructed using the Maximum Likelihood statistical method were observable in Figure 8.

Bacillus megaterium is a Gram-positive bacterium, aerobic, spore-forming commonly found in diverse environments like soil, seawater, sediment, and paddy fields (Lee et al. 2016). It forms pale yellow colonies and can grow between 7°C and 45°C, with a tolerance to NaCl concentrations of up to 5%. It uses glucose and lactose as carbon sources but does not ferment them. It also produces several enzymes, including cellulase, amylase, and protease (Nascimento et al. 2020).

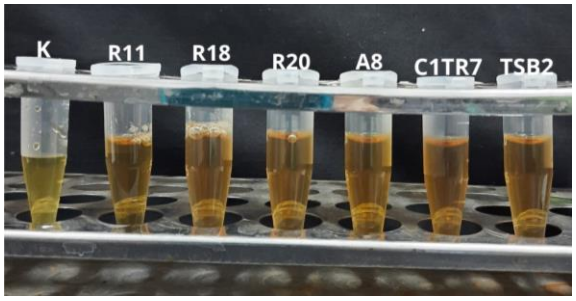

Figure 5. Siderophore production test for bacterial isolates. The production of siderophores is signified by a color shift from yellow to reddish-brown or orange

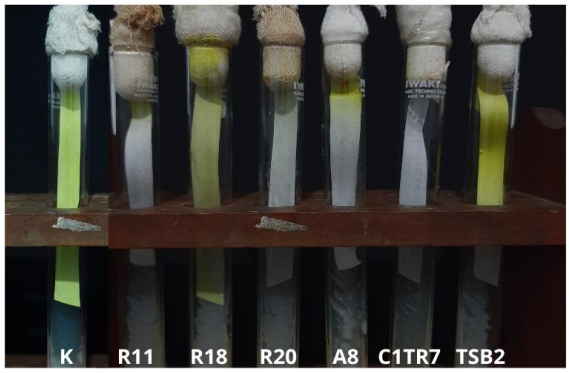

Figure 6. Hydrogen cyanide (HCN) production test for each bacterial isolate. HCN production was identified by a color change in the filter paper, transitioning from yellow to light brown, dark brown, and red

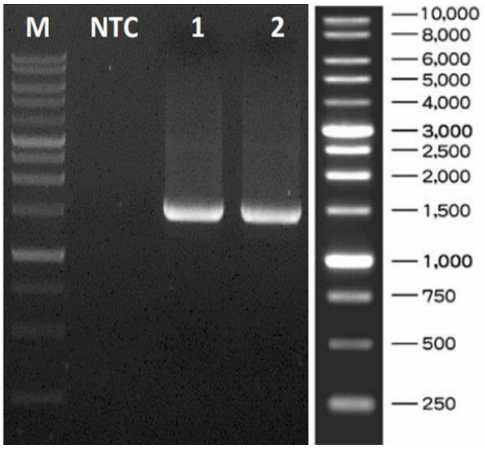

Figure 7. Visualization of 16S rRNA sequences from isolates R11 and R18 showing PCR reaction products of approximately 1500 bp long. Legend: 1: R11; 2: R18; M: 1 Kbp DNA ladder

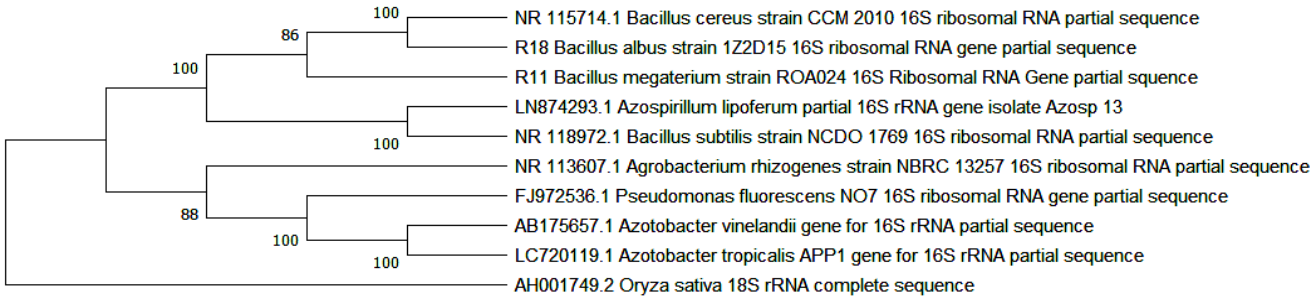

Figure 8. The phylogenetic tree obtained the sequence of R18 *Bacillus megaterium* (MT510154.1) with the closest *Bacillus cereus* (NR115714.1), and the sequence obtained from *Bacillus albus* (MZ470060.1) with the closest sequence from *Bacillus cereus* (NR115714.1) stored in GenBank

In agriculture, *B. megaterium* has been explored as a plant growth promoter, biofertilizer, bio-fungicide, and a means to reduce soil-borne plant pathogens (Kamal et al. 2021). Its antibacterial substances can be applied to soil to boost plant growth and decrease plant pathogens (Hu et al. 2013). *Bacillus albus*, another bacterium in the *Bacillus* genus, features round-white colonies (Abada et al. 2021). It is Gram-positive, rod-shaped, facultatively anaerobic, non-motile, and endospore-forming. *B. albus* tests positive for catalase, oxidase, arginine dihydrolase, citrate utilization, Voges-Proskauer, and gelatinase activities (Kishor et al. 2021).

In conclusion, our research has made significant strides in identifying potential biocontrol agents. We have identified six bacterial isolates with promising potential, demonstrating beneficial characteristics such as nitrogen fixation, production of protease enzymes, siderophores, and hydrogen cyanide (HCN). All treatments with these bacteria effectively inhibited *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and reduced the weight of FOL mycelium. The R18 treatment had the highest inhibition zone at 55.55%, with a mycelium weight of 0.0417 g. The primary antagonistic mechanism of these isolates was antibiosis; this isolate also shows tolerance to stress from temperature and salinity. Molecular identification identified two promising biocontrol agents: R11 (*Bacillus megaterium*) and R12 (*Bacillus albus*). The results indicated that R11 and R12 could be developed as biological control agents against FOL in tomatoes. Further studies are needed to assess the effectiveness of these isolates as biocontrol agents in field conditions, but our findings have laid a strong foundation for future studies in this area.

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