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Evaluation of antifungal activity against *Corynespora cassiicola* by bacteria isolated from soil in the root zone of cucumber plants

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Abstract. *Duy LQ. 2023. Evaluation of antifungal activity against* Corynespora cassiicola *by bacteria isolated from soil in the root zone of cucumber plants. Biodiversitas 24: 6584-6591.* Target leaf spot caused by *Corynespora cassiicola* poses a major disease threat to cucumber cultivation worldwide. Reliance on chemical fungicides has raised resistance to environmental and health concerns. As cucumber production expands globally, sustainable solutions are needed. Plant Growth-Promoting Rhizobacteria (PGPR) show promise but remain underexplored against *C. cassiicola* in cucumber. This study aimed to assess the potency of rhizobacteria in antagonistic activity against *C. cassiicola*, causing cucumber target leaf spots under in vitro conditions. The present study isolated bacterial strains from cucumber soil rhizosphere and screened for antifungal activity against *C. cassiicola* using dual culture assays. Using quantitative assays, selected antagonistic strains were further assessed for key biocontrol traits, including β-1,3-glucanase, chitinase, and siderophore production. Cell-free supernatants were tested for direct fungal growth inhibition using the environmental toxicity. Isolated PGPR strains showed robust *C. cassiicola* inhibition from 39.8% to 62.6% growth reduction. Most antagonistic isolates produced β-1,3-glucanase, chitinase, and siderophores and suppressed pathogen growth via metabolites in their supernatants. These results demonstrate multiple biocontrol mechanisms, including enzymatic degradation of fungal cell walls, iron competition through siderophores, and antimicrobial compound secretion. *Bacillus siamensis* strain TV16 exhibited the strongest overall antagonism against *C. cassiicola*. These findings reveal the biocontrol potential of PGPR for managing cucumber-target leaf-spot disease, offering a sustainable alternative to chemical pesticides. With further optimization and field testing, PGPR-based biopesticides can be integrated into cucumber production to improve crop health while reducing fungicide reliance.

Keywords: Antifungal activity, *Bacillus siamensis*, *Corynespora cassiicola*, PGPR, target leaf spot

INTRODUCTION

Cucumber (*Cucumis sativus*) is an economically vital vegetable crop extensively cultivated worldwide for fresh consumption, processed into pickles, or preserved in marinades and spices (Sharma et al. 2020). In Vietnam specifically, cucumber harvesting covered over 11,000 hectares in 2021 and generated average yields of 43.8 tons per hectare (GSO 2022). This represents a substantial increase from just 34.9 tons/ha in 2011, highlighting the steady growth and importance of continued advancements in cucumber agriculture to Vietnam's economy (GSO 2022).

However, cucumber productivity is severely threatened by destructive diseases caused by fungal, bacterial, and viral plant pathogens prevalent in tropical and subtropical growing regions. *Corynespora cassiicola* is among the most widespread and damaging cucumber pathogens globally, causing target leaf spot disease (Figure 1) (Dixon et al. 2009; Chau et al. 2022). *C. cassiicola* is a pathogenic fungus with an extensive host range, capable of infecting hundreds of plant species besides cucumbers, such as tomato, soybean, rubber, cotton, and many others (Lu et al. 2021; Ribeiro et al. 2021; Zhao et al. 2021; Fischer et al. 2022; Khadka et al. 2023).

Chemical fungicides have traditionally been used to control cucumber-target leaf spot disease. However, sole dependence on pesticides has led to several issues including accelerated pathogen resistance, environmental pollution, and contamination of agriculture products with potentially harmful residues (Rondon and Lawrence 2021). There is an urgent need to develop sustainable integrated pest management (IPM) strategies to protect cucumber yields from *C. cassiicola* while minimizing negative impacts on agricultural and environmental health.

Biological control using beneficial microorganisms offers promising alternative disease management approach compatible with IPM principles and ecological sustainability (Tariq et al. 2020; Collinge et al. 2022). Plant Growth-Promoting Rhizobacteria (PGPR) are naturally occurring soil bacteria that can suppress plant diseases through antibiosis, induced systemic resistance, and competition with pathogens for nutrients and space (Lugtenberg and Kamilova 2009). Prior studies have demonstrated the potential effectiveness of PGPR applications against various major cucumber pathogens, including *Fusarium oxysporum*, *Colletotrichum orbiculare*, and *Pseudoperonospora cubensis* (Abo-Elyousr et al. 2022; Ganphung et al. 2022; Liu et al. 2023). However, there is still limited research exploring the utilization of PGPR as a biocontrol agent specifically targeting *C. cassiicola* in cucumbers.

This study aims to thoroughly investigate the potential of PGPR application as a component of an integrated pest management strategy for managing cucumber-target leaf spot disease caused by *C. cassiicola*. Novel PGPR strains will be isolated from the cucumber rhizosphere and rigorously screened for broadly effective antifungal activity under

controlled in vitro conditions. Molecular identification techniques and in-depth characterization of key PGPR biocontrol traits, including enzyme production, antimicrobial metabolites, and induced systemic resistance, will be leveraged to identify elite isolates with the highest potential as cucumber biopesticides (Brzezinska et al. 2020; Harish et al. 2023). Formulation, application methodologies, shelf life stability, and rhizosphere colonization behaviors will be optimized based on analyses of PGPR biofilm formation and plant root adhesion factors. Controlled environment greenhouse experiments will evaluate the efficacy of developed PGPR-based biopesticide formulations against cucumber-target leaf spot disease. Multi-season field testing will then provide essential data on biocontrol effectiveness under commercial large-scale growing conditions.

The successful development and commercialization of effective PGPR-based biopesticides integrated into cucumber IPM programs significantly reduce reliance on synthetic chemical fungicides, promoting more sustainable crop production. With optimized application protocols, PGPR products can play a key role in minimizing yield losses from *C. cassiicola* while protecting agricultural and environmental health. Widespread adoption of ecologically sustainable practices, including biological control, will be a key part of the multifaceted approach necessary to ensure cucumber productivity meets rising domestic and global food demand. This research will also expand fundamental knowledge on PGPR-pathogen interactions in the cucumber phytosphere and deliver impactful solutions to enhance biologically based integrated crop protection strategies. Overall, introducing PGPR biopesticides facilitates transitioning from chemical-intensive agriculture to more regenerative farming systems aligned with Vietnam's sustainable development goals.

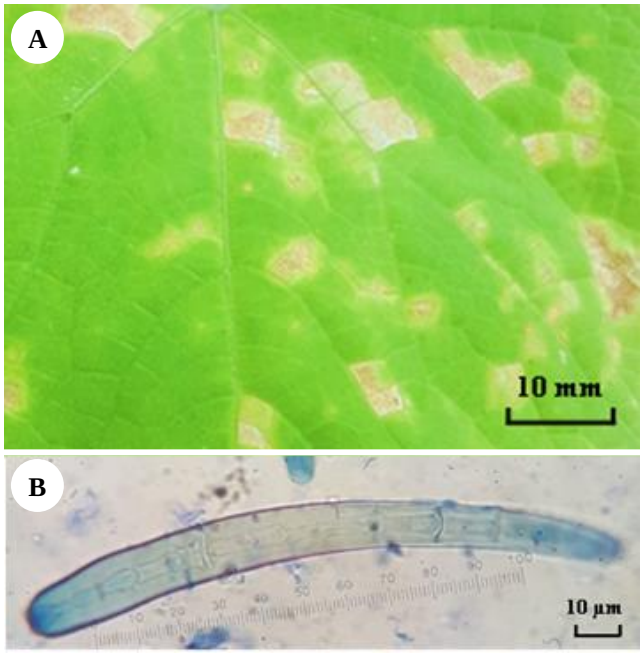

Figure 1. A. Target leaf spot lesions on cucumber leave. B. *Corynespora cassiicola* spore

MATERIALS AND METHODS

Isolation of bacteria

Soil samples from the root zone of cucumber plants were collected from cucumber fields in various provinces/ cities, including Can Tho, Vinh Long, and Tra Vinh, Vietnam. The soil samples were placed in sterile nylon bags, stored in containers with gel ice packs, and transported quickly to the laboratory. The soil samples were cleaned of debris and unwanted material, and 10 g of soil was added to Erlenmeyer flasks containing 90 mL of sterile physiological saline solution. The flasks were shaken vigorously for 20 minutes, and then the samples were serially diluted to concentration of 10^{-6}.

Diluted samples (100 µL) were evenly spread onto Trypticase Soy Agar (TSA) plates and incubated at 37°C for 48 hours. A single bacterial colony was streaked on a fresh TSA plate, and streaking was performed until homogeneous bacterial colonies were obtained. Isolated bacterial strains were preserved in Trypticase Soy Broth with 25% glycerol at -20°C for further research.

Assessment of antagonistic activity against *C. cassiicola*

Preparation of Bacterial Inoculum - Isolated bacterial strains were cultured in 10 mL of Trypticase Soy Broth (TSB) and incubated at 30°C on a shaker at 100 rpm for 24 hours. The bacterial population was adjusted to 10^8 cells/mL by counting using an improved Neubauer counting chamber.

Antagonistic Assay - The method was conducted following the description by Phu et al. (2023) with some modifications. A fungal agar plug of *C. cassiicola* (6 mm in diameter) was taken from the periphery of actively growing fungal colonies. Potato dextrose agar plates were divided into two halves. A sterile filter paper disc inoculated with 5 µL of bacterial suspension was placed similarly but opposite the fungal plug. A sterile filter paper disc inoculated with 5 µL TSB was used as a control treatment instead of the bacterial inoculum. As positive control, a fluconazol disc (25 µg/disc) (Oxoid, UK) was placed similarly but opposite the fungal plug. The test plates were incubated in dark conditions at 30°C for 7 days.

The Percentage of Growth Inhibition (GI%) was calculated by assessing the growth on two types of plates: the control plate (r1) and the plate containing the antagonistic bacterial isolate (r2) (Phu et al. 2023). This experiment was repeated three times for accuracy. The GI% was determined using the following formula:

$$GI\% = \frac{r1 - r2}{r1} \times 100 \quad (1)$$

The GI% value measures how much the antagonistic bacteria inhibit the growth of the target organism compared to the control, expressed as a percentage.

Assessment of β-1,3-Glucanase, chitinase, and siderophore production

Preparation of Enzyme Extracts - Bacterial strains showing antifungal activity were cultured in 10 mL of Trypticase Soy Broth (TSB) and incubated on a shaker at 100 rpm at 30°C. The bacterial culture was centrifuged at 14,000 rpm for 10 minutes at 4°C after 24 hours of

incubation. The resulting supernatant was used as the crude enzyme extract.

β-1,3-Glucanase Assay - A crude enzyme extract (0.1 mL) was added to 0.9 mL of laminarin substrate (0.05% in 0.05 M sodium maleate buffer, pH 5.8) (Isaac and Gokhale 1982). The mixture was incubated at 37°C for 15 minutes. The blank samples were prepared with TSB and laminarin substrate, and the water bath at 100°C for 5 minutes stopped the reaction. The amount of reducing sugar produced from the reaction was determined using the 3,5-dinitrosalicylic acid method described by Ramírez et al. (2004). Next, 1 mL of 1% 3,5-dinitrosalicylic acid (dissolved in 30% sodium potassium tartrate in 2 N NaOH) was added to the mixture and shaken. The mixture was incubated for 10 minutes in boiling water in a water bath and then cooled to room temperature. The mixture adsorption was measured at 540 nm using a NanoDrop spectrophotometer. Glucose (0-10 µmol/mL) was used as a standard. β-1,3-glucanase activity was expressed as µmol min^{-1} mL^{-1} (Isaac and Gokhale 1982).

Chitinase Assay - Colloidal chitin was prepared based on Ramírez et al. (2004) description. A crude enzyme extract (2 mL) was mixed with 2 mL of colloidal chitin substrate (0.1% in 0.05 M sodium maleate buffer, pH 5.8) (Isaac and Gokhale 1982). The blank samples were prepared with TSB and colloidal chitin substrate. The mixture was incubated for 2 hours at 37°C. The reaction was then stopped by placing the samples in a water bath at 100°C for 5 minutes. The mixture was centrifuged at 7,000 rpm for 5 minutes. The amount of reduced sugar in the supernatant was determined using the DNS method described above. N-acetyl-D-glucosamine (0-10 µmol/mL) was the standard, and chitinase activity was expressed as µmol min^{-1} mL^{-1} (Isaac and Gokhale 1982).

Siderophore Assay - The chrome azurol S (CAS) assay was prepared following Schwyn and Neiland's (1987) description. The supernatant (500 µL) was mixed with CAS reagent (500 µL). The control samples were prepared with TSB (500 µL) and CAS reagent (500 µL). The mixture was incubated in the dark for 20 minutes. The absorbance of the mixture was measured at a wavelength of 630 nm (Senthilkumar et al. 2020). Siderophore activity was calculated using the formula:

$$\text{Siderophore Production (\%)} = \frac{\text{Ar} - \text{As}}{\text{Ar}} \times 100 \quad (2)$$

Where: Ar: Absorbance of the control (CAS reagent + TSB), As: Absorbance of the sample (CAS reagent + supernatant).

Assessment of inhibition of *C. cassiicola* by Cell-Free Supernatant (CFS) obtained from antagonistic bacterial strains

Preparation of Cell-Free Supernatant (CFS) - Bacterial strains exhibiting antifungal activity were cultured in 50 mL of Trypticase Soy Broth (TSB) and incubated on a shaker at 100 rpm at 30°C. After 48 hours of incubation, the bacterial culture was centrifuged at 14,000 rpm for 10 minutes at 4°C, and a 0.22 µm syringe filter was used to filter the supernatant. The CFS was stored at -20°C until use.

Assessment of Fungal Inhibition - The experiment was conducted following the description by Phu et al. (2023). CFS was mixed with molten Potato Dextrose Agar (PDA) to achieve a final concentration of 20%, and the mixture was distributed into Petri dishes. In the control experiment, TSB was used instead of CFS. *C. cassiicola* fungal agar plugs were placed at the center of the test medium plates. Next, the test plates were incubated at 30°C for 7 days. The percentage of inhibition of *C. cassiicola* growth was calculated using the formula:

$$\text{Percentage Growth Inhibition (\%)} = \left(1 - \frac{\text{d2}}{d1}\right) \times 100 \quad (3)$$

Where "d1" is the diameter of fungal colonies in the control experiment, and "d2" is the diameter of fungal colonies in the experiment with the presence of CFS.

Identification of antagonistic bacterial strain

The bacterial strain exhibiting the highest inhibition of *C. cassiicola* growth was identified at the species level by analyzing the 16S rRNA gene region.

Isolation of DNA - Total DNA from the bacterial strain was extracted following the TRIzol Reagent protocol (Invitrogen, UK). Next, a NanoDrop spectrophotometer was used to assess the DNA quality by measuring the absorbance values at 260 nm and 280 nm (Le et al. 2021).

Amplification of the 16S rRNA Gene - The 16S rRNA gene region was amplified using the following primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) as the forward primer and 1492R (5′-GGCTACCTTGTTACGTA-3′) as the reverse primer (Lane 1991). The PCR reaction mixture included 5 µL of DreamTaq DNA polymerase buffer (5×), 1 µL of each forward and reverse primer (10 µM), 1.25 U of DreamTaq DNA polymerase, 2 µL of total DNA, and double-distilled water to a final volume of 25 µL. The PCR amplification procedure was conducted as follows: an initial denaturation step of 5 min at 94°C, next by 30 cycles of denaturation at 94°C for 1 min, then annealing at 58°C for 1 min, next the extension at 72°C for 2 min and a Bio-Rad automatic thermal cycler was used as the final extension step for 10 min at 72°C.

Sequencing of the 16S rRNA Gene - The PCR products were sent for sequencing at Integrated DNA Technologies, Inc. (IDT, USA). The 16S rRNA gene region sequences were checked for homology in the NCBI database using the BLAST tool. The Neighbor-joining method with the Jukes-Cantor model algorithm bootstrap analysis with 1000 replicates using Molecular Evolution Genetics Analysis (MEGA) software version 6.0.6 was used to construct a phylogenetic tree on the highly similar strains.

Several morphological and biochemical characteristics of the antagonistic bacterial strain were assessed. Cell morphology and motility were observed under a light microscope at a magnification 1000x. Growth Range Temperature - The ability to grow at 30°C to 55°C was evaluated by culturing the bacterial strain on TSA (Trypticase Soy Agar) medium and incubating it at different temperatures for assessment. Gram Reaction and Endospore Formation - The Gram reaction and the ability to produce endospores were determined for the bacterial cells. This was done by culturing the bacterial strain on TSA at 37°C for 48 hours.

The Gram reaction and endospore staining were performed following the instructions in the Gram stains kit and Schaeffer-Fulton's spore kit (HiMedia).

Data analysis

The data collected were processed and visualized using Microsoft Excel 2019. The IBM SPSS Statistics version 22 (IPM SPSS version 22) was used to evaluate the statistical analysis, which involved various statistical tests and techniques in assessing the significance of the results, determining trends, and drawing conclusions based on the experimental data.

RESULTS AND DISCUSSION

Results of isolation and evaluation of *C. cassiicola* inhibition

Therefore, 73 bacterial strains were isolated from soil samples collected from the cucumber plants' root zone. The number of bacterial strains isolated from each sampling region was 23 (Soc Trang), 26 (Vinh Long), and 24 (Tra Vinh). The soil samples from the cucumber root zones exhibited a high bacterial density, and the isolated bacterial strains displayed diversity.

There was variation in the ability to isolate antagonistic bacteria among the soil samples. Out of the 73 isolated strains, 25 can inhibit the growth of *C. cassiicola*. The growth of the fungal pathogen was restricted in the presence of these antagonistic bacteria. The percentage of growth inhibition (GI%) ranged from 39.8% to 62.6%. Strain TV16 exhibited the highest antifungal activity with a GI% of 62.6% ($p<0.05$), followed by strain ST7 with a GI% of 61.2%. Detailed results are presented in Figure 2.

Assessment of β-1,3-Glucanase, chitinase, siderophore production, and inhibition of *C. cassiicola* by CFS

All antagonistic bacterial strains demonstrated the ability to produce β-1,3-glucanase and chitinase. Strain TV16 exhibited the highest β-1,3-glucanase activity, reaching 0.5 µmol glucose min^{-1} mL^{-1} (Figure 3). Strain ST14 showed the highest chitinase activity, reaching 0.217 µmol N-acetyl-D-glucosamine min^{-1} mL^{-1} (Figure 4).

Among the 25 bacterial strains surveyed, 20 showed the ability to produce siderophores. Strains TV15, VL4, and VL9 displayed the highest siderophore production, with percentages of siderophore units being 28.0%, 28.0%, and 26.5%, respectively (Figure 5). These results indicate that the isolated strains produce secondary metabolites that inhibit the growth of *C. cassiicola*. CFS obtained from strain ST14 showed the highest percentage of growth inhibition (GI%) at 47.7% (Figure 6).

These findings demonstrate that the antagonistic bacterial strains also inhibit the growth of *C. cassiicola* and produce various secondary metabolites, including β-1,3-glucanase, chitinase, and siderophores, which play a role in disease suppression and plant protection.

Strain TV16 identification

Strain TV16 exhibited strong inhibition of the growth of the fungal pathogen *C. cassiicola*, with the highest percentage of growth inhibition (%GI) compared to the other strains, and this difference is statistically significant. The 16S rRNA gene sequence of strain TV16 was edited using BioEdit (version 7.0) to remove overlapping regions and chimeras. Furthermore, BLAST compared the resulting sequence against reference strains in the NCBI GenBank database. TV16 showed over 97% sequence similarity to strains of *Bacillus siamensis*, *Bacillus velezensis*, *Bacillus methylotrophicus*, *Bacillus amyloliquefaciens*, and *Bacillus subtilis*. The phylogenetic analysis generated a tree confirming the close positioning of TV16 to *B. siamensis* strain KTCC 13613, with a 79% bootstrap support value based on 1000 replicates. These molecular techniques identified TV16 as belonging to *Bacillus siamensis* species (Figure 7).

Strain TV16 possesses rod-shaped cells, motility, Gram-positive staining, spore formation, and thermophilic growth (able to grow up to 50°C) (Figure 8). These characteristics align with those described for *Bacillus siamensis* by Sumpavapol et al. (2010). Based on these results, it can be concluded that strain TV16 belongs to the species *B. siamensis*. Strain TV16's ability to inhibit the growth of *C. cassiicola* and its identification as *B. siamensis* suggests its potential for use in plant disease management and biocontrol strategies.

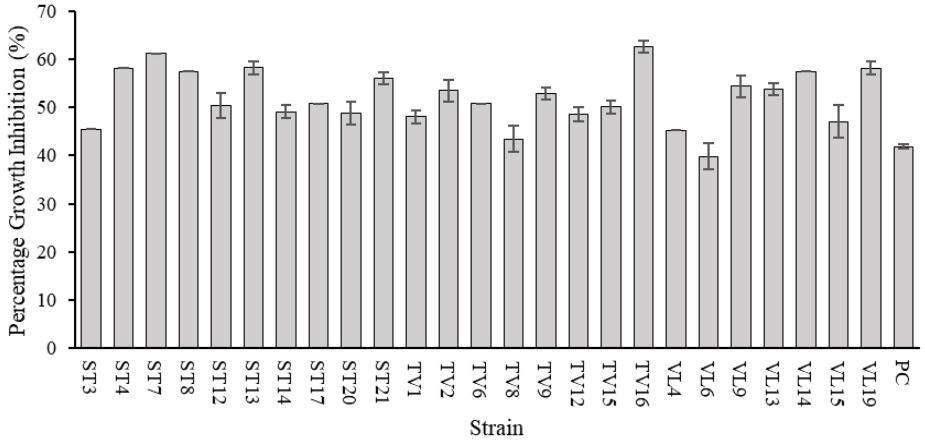

Figure 2. Evaluation results of the inhibitory ability against *Corynespora cassiicola* by isolated bacterial strains

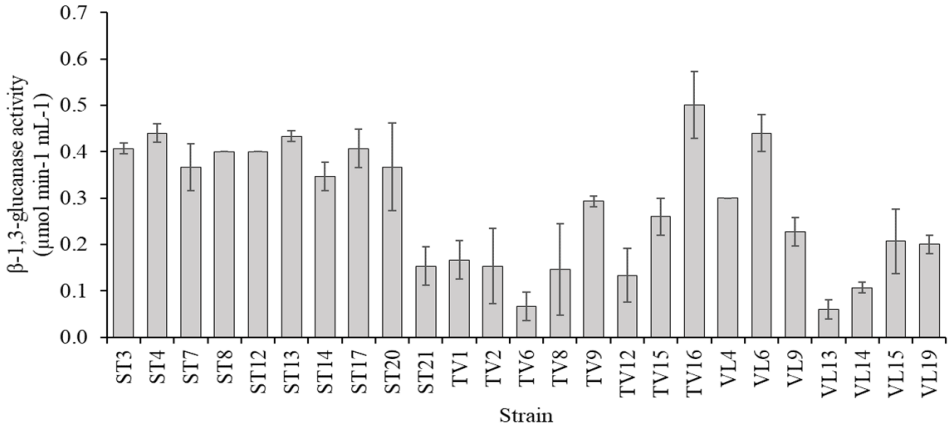

Figure 3. β-1,3-glucanase activity results of isolated bacterial strains

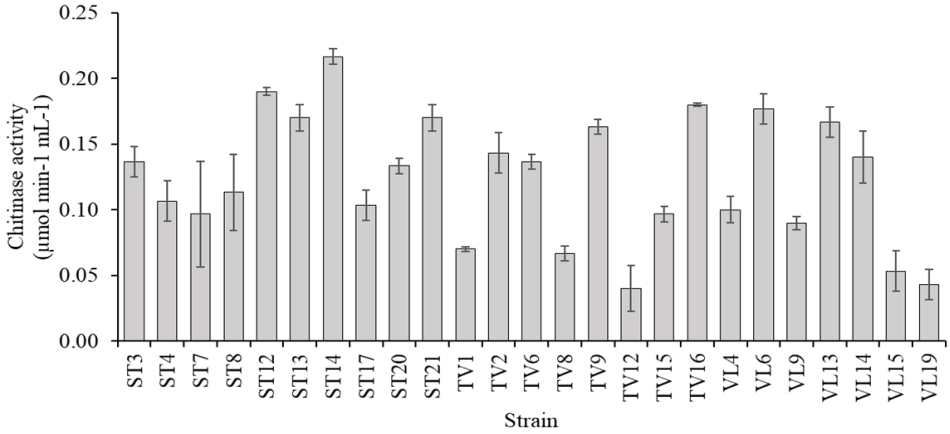

Figure 4. Chitinase activity results of isolated bacterial strains

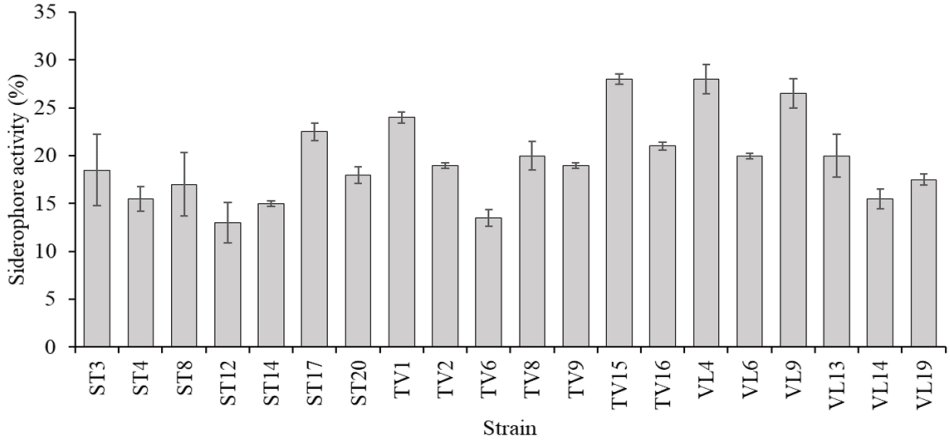

Figure 5. Siderophore activity results of isolated bacterial strains

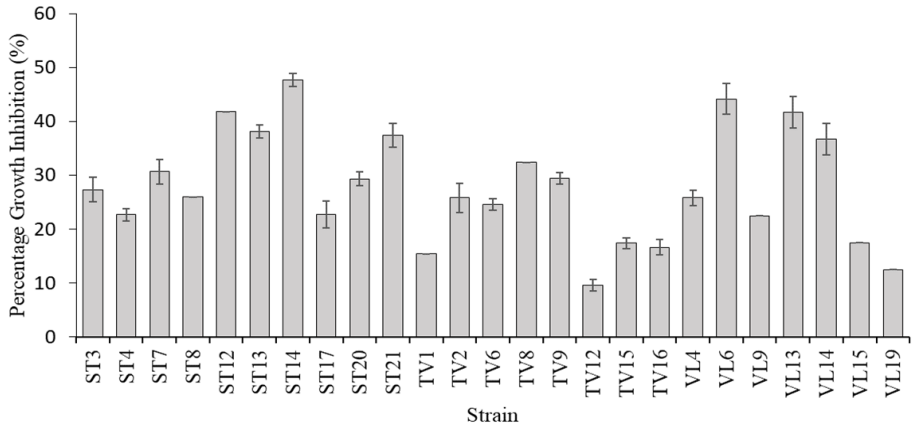

Figure 6. Results of inhibition ability against *Corynespora cassiicola* by CFS of isolated bacterial strains

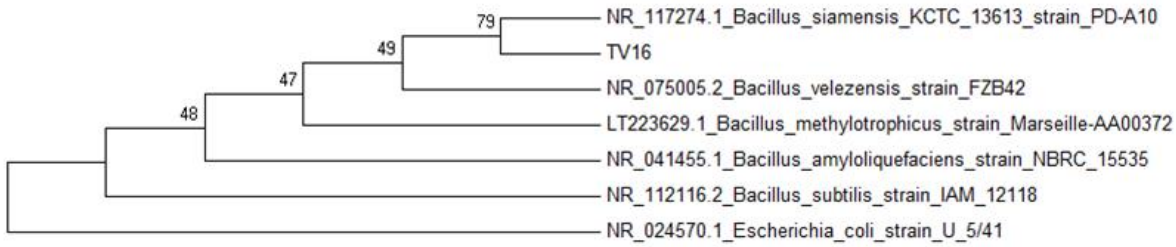

Figure 7. The phylogenetic tree was constructed based on the 16S rDNA region of strain TV16 and closely related species

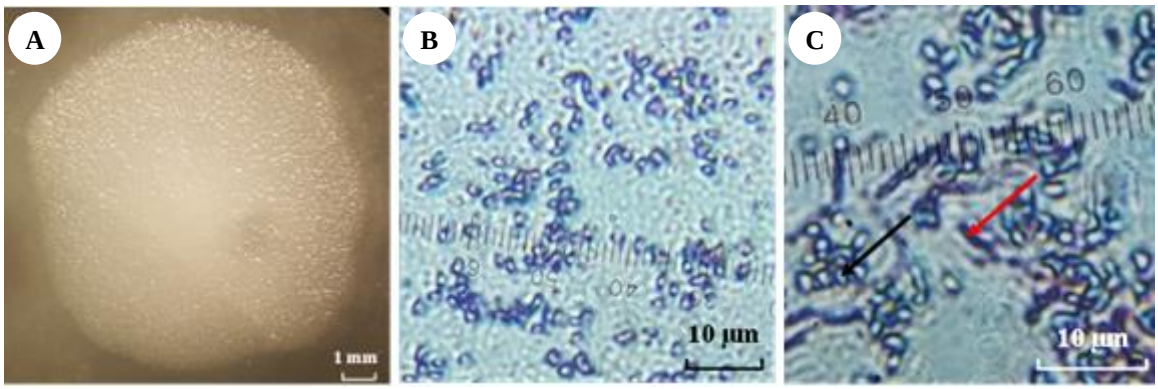

Figure 8. Strain TV16. A. Colony, B. Gram reaction, C. Endospore formation. Red arrow: vegetative cell, black arrow: endospore

Discussion

Target leaf spot on cucumber causes significant damage (Gao et al. 2020) and is highly concerning due to its rapid spread (Liu et al. 2018). This study aimed to select potential bacterial strains with strong inhibitory effects on the growth of *C. cassiicola*. The antagonistic bacterial strains were identified for their ability to produce several antifungal compounds, such as hydrolytic enzymes, siderophores, and non-volatile organic compounds.

The bacterial strains isolated from the root zone soil of cucumber plants demonstrated strong inhibitory effects on the growth of the *C. cassiicola* fungus. In various other studies, root-associated bacteria have been reported to control airborne-transmitted pathogens such as *Pseudoperonospora cubensis* effectively (Zheng et al. 2017) and *Phytophthora infestans* (Yan et al. 2020). Plants can release compounds to attract beneficial bacteria to colonize their roots, helping them compete for space with pathogenic microorganisms in the soil (Liu et al. 2016). The relationship between root-associated bacteria and plants enhances the plants' resistance to various biotic and abiotic stresses through multiple mechanisms (Lugtenberg and Kamilova 2009). The results indicate the potential of

root-associated bacteria in mitigating harmful diseases in cucumber plants.

Root-associated bacteria have significant potential to combat various types of plant pathogens using their diverse weaponry, such as antibiotics, volatile organic compounds, and lytic enzymes, and indirectly activating the plant's natural immune system (Maung et al. 2022). The isolated bacterial strains have demonstrated the ability to produce chitinase, β-1,3-glucanase, and siderophores and inhibit *C. cassiicola* through cell-free supernatants (CFS). The activity level of each mechanism depends on the specific bacterial strain. The combination of multiple mechanisms contributes to the resistance capabilities of these bacterial strains against *C. cassiicola*. Fungal cell walls are composed of a matrix of different polymer molecules with diversity in composition and structure for each fungal species (Gow et al. 2017). The cell wall protects and maintains the cell's shape (Roncero and Vázquez de Aldana 2020). Cell wall degradation of the pathogenic fungus is one of the essential mechanisms that lead to the antifungal activity of these bacteria. β-(1,3)-glucan and chitin are essential components with a central structural role in the fungal cell wall (Roncero and Vázquez de Aldana 2020). Brzezinska et al. (2020) reported chitinase obtained from *Bacillus* spp., showed antifungal activity against some fungal phytopathogens. The ability to produce chitinase helps antagonistic bacteria degrade fungal cell walls, leading to antifungal activity. β-1,3-glucanase-mediated glucan degradation can weaken fungal cell walls, thereby inhibiting fungal growth. The mycolytic activity of β-1,3-glucanase could differ between the enzyme sources and the substrates in fungal cell walls (Takashima et al. 2023).

Root-associated bacteria can produce siderophore molecules with a high affinity for iron. Strong iron uptake can deplete iron in the environment, thereby inhibiting fungal growth. Priyanka et al. (2017) reported that most *Pseudomonas* spp. strains resistant to phytopathogens produce potent siderophores. Metabolites from bacteria with antifungal activity have been reported to include organic acids (Jung et al. 2018; Liu et al. 2016), and cyclic lipopeptide (Tanaka et al. 2014).

Numerous biological control agents are widely used, including genera such as *Trichoderma*, *Bacillus*, *Streptomyces*, and *Pseudomonas*. *Bacillus* spp. is commonly employed as a biological control agent due to its ability to suppress pathogens and withstand harsh conditions by producing endospores (Albayrak 2019). The strain *B. siamensis* TV16 has demonstrated strong inhibition of *C. cassiicola;* its capacity to produce hydrolytic enzymes and secondary metabolites could enhance its ability to control synthetic pests on cucumber plants. In several studies, *B. siamensis* has also been reported for its disease-control capabilities and growth-promoting effects on crops (Hossain et al. 2019; Gorai et al. 2021; Gorai et al. 2023).

In conclusion, this study successfully identified 25 bacterial strains demonstrating antifungal activity against *C. cassiicola*, the causative agent of target leaf spot in cucumbers. Among these strains, *B. siamensis* TV16 exhibited the highest antagonistic potential, with a growth inhibition percentage of 62.6%. The antagonistic bacterial strains could produce hydrolytic enzymes (β-1,3-glucanase, chitinase), siderophores, and other secondary metabolites with antifungal properties. Such studies would help exploit bacterial strains, especially *Bacillus*, to manage target leaf spot disease in cucumber. Further studies are required on applying the potential antagonistic isolates to increase crop yield and achieve high-quality products.

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