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Isolation of bacteria displaying potent antagonistic activity against fungi causes anthracnose disease in chili

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Abstract. *Tram TTN, Quang HT, Vu NQH, Nguyen PTT, Thi TNM, Phuong TTB, Thi PTD. 2023. Isolation of bacteria displaying potent antagonistic activity against fungi causes anthracnose disease in chili. Biodiversitas 24: 4919-4926.* Anthracnose is a common disease on many crops caused by *Colletotrichum* species, and it leaves serious consequences, especially on chili. Beta-1,3 glucanase is a semi-constitutive hydrolytic enzyme that is involved in the biological regulation of plant pathogens. Its main function is the breakdown of β-1,3-glucan, a major constituent of the fungal cell wall. In a study conducted in Vietnam's Quang Tri and Thua Thien Hue Provinces, researchers isolated five bacterial strains from soil where chili plants were grown. To induce the production of beta-1,3 glucanase, these isolates were cultivated in a nutrient-rich medium supplemented with 1% laminarin. The activity of beta-1,3-glucanase in the bacterial strains ranged from 0.31 to 1.72 U mL^{-1}, and antagonistic activity against *Colletotrichum* spp. causes of anthracnose reached from 50.68 to 69.04%. Strain AT4 had strong antagonistic activity against *C. scovillei* HUCL1, *C. siamensis* PV6, and CL3 of 76.44, 62.65, and 68.04%, respectively. Molecular identification based on 16S-rRNA sequences showed that the strains were *Paenibacillus polymyxa* AT4, *B. siamensis* ML3, *B. tequilensis* ML4 and ML6, and *B. velezensis* GL7. AT4 and ML6 are potential strains for the control of anthracnose diseases caused by *Colletotrichum* spp. on chili peppers.

Keywords: β-1,3-glucanase, antagonistic bacteria, anthracnose, chili pepper, Vietnam

INTRODUCTION

Chili (*Capsicum* spp.) is a popular and favorite cultivated crop all over the world because of its color, flavor, and nutritional value. In Vietnam, chili has high economic value in plant conversion strategies and crop rotation between seasons in many provinces. The acreage for the planting of chili in the Central Provinces is sharply increasing because its economic efficiency is higher than that of other kinds of plants. Besides, it requires low investment, cultivated technique, and well-unrequited care. However, annual chili yield and production are affected due to plant diseases caused by various fungal and bacterial pathogens. On chili, fungal diseases such as stem rot (caused by *Sclerotium rolfsii*) (Naresh et al. 2023), blight (*Phytophthora capsici*) (Majid et al. 2016), anthracnose (*Colletotrichum* spp.), white leaf spot (*Cercospora capsici*) (Adedire et al. 2019) are common diseases. Among all the above diseases, anthracnose is the most common and harmful fungal disease in plants including chili (Cannon et al. 2012; Gautam 2014a, b). In a recent study, our team identified the presence of *Colletotrichum scovillei*, *C. siamense*, *C. fructicola*, and *C. brevisporum* in chili anthracnose fungus samples from North Central Vietnam (Vu et al. 2023, unpublished data). When an anthracnose outbreak occurs, almost all farmers often use different chemicals to prevent disease development, which have a heavy impact on the environment. Using bioproducts with strong antagonistic activity in plant disease prevention, especially anthracnose, is a useful alternative to chemicals and contributes to environmental protection and human health.

Biological control of chili anthracnose with antagonistic organisms offers an effective method of managing pathogens in chili crop. Several reports show that bateria was already used as an anthrace pathogen for fungal antagonism caused by *Colletotrichum* spp., for instances, *Bacillus amyloliquefaciens* and *B. velezensis* inhibition *C. scovillei* (Wei et al. 2023), *B. tequilensis* against *C. acutatum* (Kwon et al. 2022). *B. velezensis* against *C. gloeosporioides* (Kim et al. 2021), *B. siamensis* against *C. higginsianum* (Lee et al. 2018) and *Paenibacillus polymyxa* inhibited *C. gloeosporioides* and *C. acutatum* (Kim et al. 2016). *In vitro* research has proven that exposure of certain phytopathogenic fungi to antibiotics and fungal hydrolyzing enzymes such as chitinases, proteases, cellulases, or glucanases, peroxidase, and phenylalanine ammonia lyase can inhibit the fungal pathogens by degrading most fungal cell walls (Gomaa 2012).

According to Fesel and Zuccaro (2016), chitin, glucans and mannoproteins are main compounds of fungi cell wall structure. Chitin is situated in proximity to the cell membrane, β-1,3- and β-1,6-glucans are positioned next to the chitin fibers. Beyond them, mannoproteins form the outermost layer of the cell wall. In the majority of fungi, the most abundant cell wall polysaccharide is β-glucan,

chitin represents only a rather low percentage of the total cell wall mass. Glucans are polysaccharides consisting of glucose units. The most abundant β-glucan found in fungal cell walls is β-1,3-glucan, constituting about 65% to 90% of the total β-glucan content. Within this structure, β-1,3-glucan chains are linked by 1,6-glycosidic side branches, making up around 3-10% of all glucan connections and forming a rigid network. The synthesis of β-glucan takes place in filamentous fungi at the hyphal tip and along the sides of cell growth and branching (Bowman and Free 2006; Fesel and Zuccaro 2016). Thus, high production of chitinase and β-glucanase organisms has the potential for phytopathogen fungal antagonism.

In the previous study, we found that chitinase (60 U/mL) from *Trichoderma asperellum* PQ34 inhibited nearly completely *in vitro* growth of *Colletotrichum* sp., chitinase can also prevent anthracnose that is caused by *Colletotrichum* sp. on both mango and chili fruits up to 72 h after enzyme pre-treatment at 40 U/mL (Loc et al. 2020). To develop an effective bioproducts (chitinase and 1,3-glucanase) for the prevention and treatment of anthracnose on chili peppers in Vietnam, this research was conducted to isolate antagonic bacteria strains with anthracnose fungus disease as well as releasing glucanase enzymes.

MATERIALS AND METHODS

Materials

The anthracnose fungus *Colletotrichum scovillei* HUCL1, *C. siamense* PV6 and HUCL3 was procured from the Laboratory of Gene Technology, Institute of Biotechnology, Hue University, Vietnam. Fungal pathogens were maintained on Potato Dextrose Agar at room temperature.

Methods

Isolation and screening of enzyme-producing bacteria

Topsoil and soil samples from the rhizosphere of healthy chili with a depth of 5-25 cm in chili plantation areas in Quang Tri and Thua Thien Hue provinces (Vietnam) were collected for the isolation and selection of enzyme biosynthesis bacteria. Collected soil samples are stored in sample storage bags and brought to the laboratory for isolation. Next, carry out cultures on the isolates.

For the isolation of enzyme-producing bacteria, a serial dilution technique was used considering different aqueous dilutions (10^{-1} to 10^{-4}) using phosphate buffered saline (PBS, pH 7.2). A sample from each dilution was then spread on a nutrient agar plate amended with 1% laminarin and incubated at 37°C for 24 h. The nutrient agar medium contains (per liter): 0.65 g K_2HPO_4, 2.5 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 2.5 g NaCl, 0.12 g $MgSO_4$.$7H_2O$ and 1.5 g yeast extract (Dewi et al. 2016). After incubation at 37°C for 24 hours in darkness, the colonies that displayed a halo with Lugol stain were collected for antifungal activity (Gomaa 2012). A enzymatic index (EI) was defined and calculated according to the following equation (Edison and Pradeep 2020):

$$EI = \frac{Clear\ zone\ diameter}{colony\ diameter}.$$

The colonies were streaked several times to create pure strains, cultivated separately in a liquid medium, and stored at -40°C.

Screening of high enzyme-producing isolates

As a growth substrate, 1% laminarin was added to 50 mL of liquid media to prepare the inoculum, which was then incubated on a shaker at 37ºC and 130 rpm agitation until the optical density achieved OD_{600} = 1. Furthermore, 1% inoculum (v v^{-1}) was cultivated in a 250-mL Erlenmeyer flask containing 100 mL of production media supplemented with 1% laminarin at pH 7.0 (Dewi et al. 2016). After 24 hours, the culture was harvested, and a crude extract of the enzyme β-1,3-glucanase was obtained by centrifuging the mixture for 15 minutes at 10.000 rpm. For later usage, the bacterial supernatant was kept in a freezer at 4°C. The activity of the crude 1,3-glucanase was then assessed.

The screening of isolated bacteria with high β-1,3-glucan degrading activity was performed by agar diffusion tests. The primary screening starts with inoculating 100 µL of each culture filtrate bacterial inoculum into pre-punched holes of equal sizes (6 mm) on the assay plate containing 1% laminarin, at 37°C for 24 hours in the chamber. After incubation, Lugol solution is applied to detect substrate hydrolysis (Carrazco-Palafox et al. 2018). To estimate the quantity of β-1,3-glucanase in each culture filtrate, the value of *D-d* (where *D* represents the diameter of the clear zone from the center and *d* is the diameter of the pre-punched hole used for enzyme loading) is utilized (Loc et al. 2020).

Total β-1,3-glucanase activity assay

By detecting reducing sugar production using a colorimetric technique, the standard activity assay for β-1,3-glucan degradation was performed (Wu et al. 2018). Total 500 mL of the strains' culture filtrates were combined with 500 mL of 0.5% (w v^{-1}) laminarin in a buffer containing 100 mM sodium acetate (pH 7.0). The reaction was carried out for 60 minutes at 50ºC before being heated for 5 minutes at 100ºC to end it. The reaction solution was then mixed with 2 mL of 1% 3,5-dinitro salicylic acid (DNS), and the mixture was heated for 10 minutes to develop the color. The combination was put in an ice bath, and the spectrophotometer was used to measure it at 540 nm (Spectro UV-2650, Labomed, Los Angeles, CA, USA). Glucose (Sigma-Aldrich) was used as a standard to determine β-1,3-glucanase activity with an estimated regression equation of y = 2.043x+0.2786 (R2 = 0.9998), where y is glucose content and x is the absorbance at 540 nm. According to the standard assay conditions, one unit (U) of activity was defined as the amount of enzymes that can liberate 1 µmol of glucose in one minute.

In vitro antifungal activity

The antifungal activity of strains was determined using an *in vitro* dual culture assay by Fokkema (1973) based upon inhibition of hyphal growth of the phytopathogenic fungus *C. siamense* and *C. scovillei*. A petri dish (Φ = 9 cm) containing 1/2 potato dextrose agar broth (PDA) was

co-cultured with an isolated strain and fungus. Each fungal inoculum (~ 2 mm) was placed opposite each other at a distance from the fungal pathogen of at a minimum 3 cm on PDA medium, while mycelial discs without a bacterial streaked line served as blank controls. For six days, the culture was kept at 28°C to monitor the fungus' progress. The diameter of the inhibited zone served as the basis for calculating the antifungal activity. The inhibition of fungal pathogen growth by bacteria was calculated according to the following equation: $I = \frac{R1-R2}{R1}$ x 100%, where R1 is the length of radial growth towards the plate edge and R2 is the length of radial growth towards bacteria (Shih et al. 2023). The bacteria with the best antagonism ability were selected for further work.

Species identification

Isolated bacterial strains were identified based on the sequencing of 16S rDNA genes. Genomic DNA of bacterial strains was extracted by the TopPURE® genomic DNA extraction kit (ABT, Vietnam). The total DNA was used as a template for the 16S rDNA amplification using the universal primer pair of 27F (5'-AGAGTTTGATCCTG GCTCAG-3') and 1492R (5'-GGTTACCTTGTTACG ACTT-3'). The PCR reaction was conducted on a PCR machine according to the following thermal cycle: 95ºC for 10 minutes; 30 cycles of 95ºC for 30s, 55ºC for 1 min and 72ºC for 1 min 30s; and 72ºC for 10 minutes (Quang et al. 2020). PCR products are purified and sequenced at First Base Company (Malaysia). The nucleotide sequences of 16S rDNA were used for species identification with the BLAST tool on Genbank. The Phylogenetic tree was built with MEGA11 software (Tamura et al. 2021).

Statistical analysis

The experiments were conducted with three replications per treatment. The data are represented as the mean of the repeats; the means were compared using one-way ANOVA (Duncan's test at p = 0.05).

RESULTS AND DISCUSSION

Isolation of potential microorganisms in antifungal biocontrol

From soil samples in the anthracnose-infected chili-planted region in Thua Thien Hue and Quang Tri Provinces, we obtained 5 indigenous bacterial strains with a halo after Lugol staining. The strains were named as AT4, ML3, ML4, ML6, and GL7. Among the isolates, strains AT4, ML3, ML4, and ML6 had large resolution halos, while strain GL7 had the smallest clear zone. The glucanlytic index ranged from 3.13 to 4.25, with the highest being the AT4 and ML6 strains (Table 1).

The potential bacterial isolates were screened through a qualitative hydrolytic plate assay to test β-1,3-glucanase. Diffusion studies on an agar plate revealed that all 5 strains could degrade laminarin by 1%, as evidenced by the formation of large clear zones on the agar plate. Notably, the largest clear zone areas were observed for ML6 and AT4 (Figure 1). The presence of colorless areas on the agar plate due to application in the decomposition of laminarin indicates the action of the enzyme β-1,3-glucanase, confirming that the isolated strains possess strong extracellular β-1,3-glucanase production, making them suitable for further investigations.

Following the identification of preliminary enzyme-producing strains, we proceeded to cultivate them in a liquid medium to determine their activities. The results of the β-1,3-glucanase activity assessment revealed that the strains exhibited activities ranging from 0.31 to 1.72 U mL^{-1}. Among these, strain ML6 displayed the highest activity; however, there was no statistically significant difference between strains AT4 and ML6 (Table 1). The small clear zone observed in Figure 1 for strain GL7 was consistent with its minimal β-1,3-glucanase activity. Hence, it is likely that AT4, ML3, ML4, and ML6 strains may have employed the β-1,3-glucanase enzyme in their antagonistic effects, while the GL7 strain might have relied on another agent that may have been associated with the chitinase enzyme.

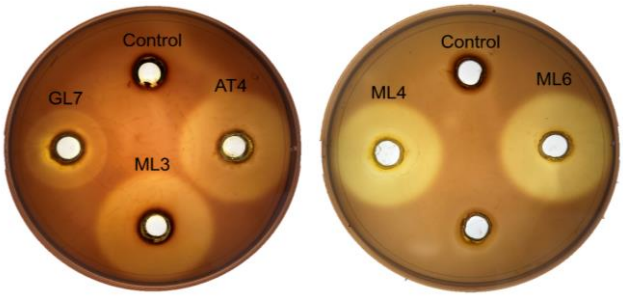

Figure 1. β-1,3-glucanase-producing ability of bacterial isolates AT4, ML3, ML4, ML6, and GL7, respectively, on agar plates containing 1% laminarin was indicated by a zone of clearance around inoculum wells and was compared to a control well added with distilled water

Table 1. β-1,3-glucanase activities of isolated bacterial strains

Strains	Location of isolation	EI	*D-d (cm)*	β-1,3-glucanase activity (U mL^{-1})
AT4	Cam Tuyen commune, Cam Lo district, Quang Tri province	4.25^{a}	2.04^{a}	1.65^{ab}
ML3	Huong So Ward, Hue City, Thua Thien Hue province	3.75^{b}	1.86^{b}	1.50^{b}
ML4	Huong So Ward, Hue City, Thua Thien Hue province	4.00^{ab}	1.75^{b}	1.52^{b}
ML6	Huong So Ward, Hue City, Thua Thien Hue province	4.25^{a}	2.47^{a}	1.72^{a}
GL7	Gio Linh district, Quang Tri province	3.13^{c}	0.98^{c}	0.31^{c}

Note: Different letters (a, b, c, …) in each column indicate significantly different means (Duncan's test, $p < 0.05$)

Table 2. The inhibition rates of bacterial isolates against *Colletotrichum* spp.

Strains	Inhibition ratio (%)			Average (%)
	C. siamense PV6	*C. siamense* HUCL3	*C. scovillei* HUCL1	
AT4	62.65[b]	68.04[a]	76.44[a]	69.04[a]
ML3	56.53[c]	47.42[d]	67.22[b]	57.06[b]
ML4	44.98[d]	56.35[c]	50.71[c]	50.68[c]
ML6	67.79[a]	63.09[b]	56.61[c]	62.50[ab]
GL7	53.14[c]	71.33[a]	73.95[a]	66.14[a]

Note: Different letters (a, b, c, …) in each column indicate significantly different means (Duncan's test, $p < 0.05$)

Antagonistic activity against *Colletotrichum* spp.

After the preliminary selection process, 5 strains with high of enzyme production abilities were chosen to represent fungi in the antagonistic ability experiment, and isolated bacterial strains (Figure 2).

Disease management from hydrotic enzymes released by indigenous bacterial strains is a potential biotechnological application because it not only controls pathogens but also protects crops (El-Shora et al. 2021). So, the antagonistic ability of glucanolytic bacteria against common *Collectrotrichum* spp. strains was tested by the dual culture technique (Figure 2). Overall, all of the isolates are capable of impeding the mycelial growth of *C. scovillei* HUCL1, *C. siamense* PV6 and HUCL3 compared to the control plates. The biocontrol of the same phylogenetical fungus was indicated in many studies in advance. The mycelial growth inhibition rates of biocontrol bacterial isolates on *Collectrotrichum* spp. ranged from 50.68 to 69.04%, with the highest inhibition rate observed for AT4 (69.04%). The weakest fungistatic impact was observed in ML4 (Table 2).

Thus, AT4 has broad-spectrum antifungal biocontrol potential with the ability to inhibit the growth of tested filamentous fungi strains (*C. scovillei* HUCL1, *C. siamense* PV6 and HUCL3), followed by GL7 and ML6.

Species identification

We conducted 16S rDNA gene sequence analysis to identify high β-1,3-glucanase biosynthetic and vital fungus-inhibited bacteria trains. The results of the 16S rDNA sequence alignment by utilizing BLAST software showed that the AT4 strain was *Paenibacillus polymyxa*, the ML3 was *Bacillus siamensis*, the GL7 strains were *Bacillus velezensis*, and the ML4 and ML6 strains were *Bacillus tequilensis* (Table 3). Except for the AT4 strain, all strains of the genus *Bacillus* have a similarity of 99.72 or more to the published standard strain (Figure 3).

Discussion

Recent research has led to a shift in the understanding of how soil microbes play a role in supporting plant health. The focus has now turned to the rhizosphere microbiome, which has been identified as a crucial element in this context. Plant growth-promoting rhizobacteria (PGPR) have gained considerable attention for their favorable effects on crop growth and the ecosystem within the rhizosphere microbiome (Jeong et al. 2019). Moreover, by degrading fungal cell walls, pathogenesis-related (PR) proteins such as chitinases and glucanases contribute significantly to plant defense against pathogens. These PR proteins are widely used as plant protection and antifungal agents (Li et al. 2015).

Biological control with antagonistic bacteria offers an effective method of managing plant pathogens and raising crop productivity. Degrading the mycelial cell wall, spore germination inhibition, bursting of spores and hyphal tips, and germ tube elongation to eliminate fungal pathogens were reported in recent studies (Gomaa 2012; Ueki et al. 2020). Together with chitin, glucans are key structural components of fungal cell walls, and their presence is widely distributed throughout all fungal phyla (Ruiz-Herrera and Ortiz-Castellanos 2019). Glucanolytic enzymes, namely 1,3-glucanase, are significant for the biological control of fungal pathogens because of their ability to degrade pathogen cell walls that contain 1,3-glucan. So, β-1,3-glucanase and antifungal biocontrol bacterial strains are promising candidates for conventional antifungals (Gadallah et al. 2023).

In this study, *Bacillus siamensis*, *B. tequilensis*, *B. velezensis*, and *Paenibacillus polymyxa* were isolated from chili-cultivated soil, they exhibit high β-1,3-glucanase activity and fungi antagonism capacity against *Colletotrichum* spp. (Table 3).

To our knowledge, *Bacillus* spp. was already used as an anthrace pathogen for fungal antagonism caused by *Colletotrichum* spp. (Table 4). For example, *Bacillus amyloliquefaciens* and *B. velezensis* isolated from pepper leaves displayed growth inhibition rates of 79 and 80% against *C. scovillei* mycelium (Wei et al. 2023). According to Kwon et al. (2022), *B. tequilensis* GYUN-300 was used for antagonization of the chili anthracnose fungus caused by *C. acutatum* in Korea. When red pepper fruits were treated with GYUN-300, the preventive and curative effects were 66.6 and 38.3% in wounded fruits, respectively. *Bacillus tequilensis* A3 isolated from avocado rhizospheric soil significantly diminished *C. gloeosporioides* mycelial fungal growth by 25% after 3 days post-inoculation (Guerrero-Barajas et al. 2020).

Table 3. Homology of 16S rDNA gene sequencing with type strains

Strains	Species	Type strain	Identity (%)
AT4	*Paenibacillus polymyxa*	ATCC 842	98.67
ML3	*Bacillus siamensis*	KCTC 13613	100
ML4	*Bacillus tequilensis*	KCTC 13622	99.93
ML6	*Bacillus tequilensis*	KCTC 13622	99.72
GL7	*Bacillus velezensis*	NPRL B-41580	99.79

Figure 2. *In vitro* antifungal activity of bacterial isolates against *Colletotrichum* spp. by co-cultured tests

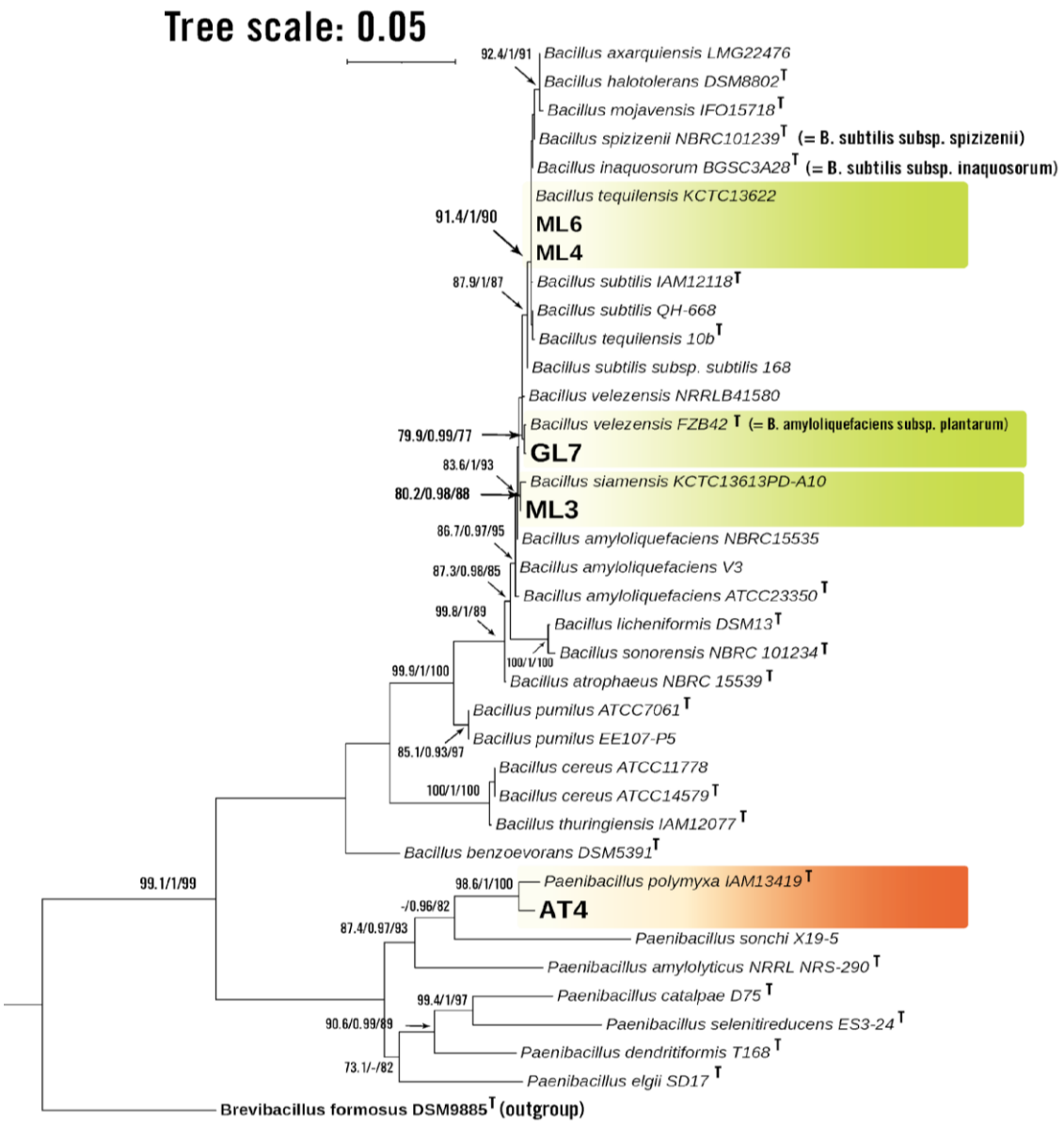

Figure 3. Phylogenetic analysis of isolates based on 16S rDNA sequences, with reference sequences from NCBI using MEGA 11

Table 4. Antagonist activity to pathogenic fungi *Colletotrichum* spp.

Bacterial species	Pathogens	Antagonist factors/enzymes	Percentage of inhibition (%)	References
Bacillus velezensis AK-0	*C. gloeosporioides*	-	80.70	Kim et al. (2021)
B. velezensis CE 100	*C. gloeosporioides*	Chitinase, protease, β-l,3-glucanase	33.60	Choub et al. (2021)
B. velezensis L3-5	*C. scovillei*	-	73.39	Wei et al. (2023)
B. amyloliquefaciens L1-7	*C. scovillei*	-	80.60	Wei et al. (2023)
B. siamensis C3.8	*C. gloeosporioides*	Bis(2-ethylhexyl) phthalate	17.68	Masrukhin et al. (2021)
B. siamensis H30-3	*C. higginsianum*	Chitinase, protease, cellulase	-	Lee et al. (2018)
B. tequilensis GYUN-300	*C. acutatum*	-	-	Kwon et al. (2022)
B. tequilensis A3	*C. gloeosporioides*	-	60	Guerrero-Barajas et al. (2020)
Bacillus subtilis SAHA	*C. gloeosporioides*	β-1,3-glucanase	-	Dewi et al. (2016)
Paenibacillus polymyxa APEC128	*C. gloeosporioides* and *C. acutatum*	-	83.6 and 79	Kim et al. (2016)
P. polymyxa C1	*C. scovillei*	-	100	Suprapta (2022)
P. polymyxa CP7	*C. musae*	β-1,3-1,4-glucanase	-	Wen et al. (2010)

Bacillus velezensis AK-0 isolated from Korean ginseng rhizospheric soil was found to exhibit antagonistic activity against apple bitter rot caused by *C. gloeosporioides* (Kim et al. 2021). The crude enzyme from *B. velezensis* CE 100 degraded the cell wall of *C. gloeosporioides*, inhibiting spore germination and mycelial growth by 99.3% and 33.6% at 100 µL mL^{-1}, respectively (Choub et al. 2021). *Bacillus siamensis* C3.8 has inhibitory activity against phytopathogenic fungi such as *C. gloeosporioides* (Masrukhin et al. 2021). *B. siamensis* H30-3 demonstrated antifungal activities *in vitro* against *C. higginsianum*, thereby preventing anthracnose disease in Chinese cabbage (Lee et al. 2018).

The genus *Paenibacillus* was also used as a pathogen for fungal antagonism. For instance, *P. polymyxa* A21 isolated from the snow-covered high-altitude area in Tibet (China) has high antagonistic activity against *Botrytis cinerea* (Li et al. 2015). Several reports showed that *P. polymyxa* was used as an inhibition factor for anthracnose fungus antagonism. *Paenibacillus polymyxa* APEC128 isolated from the apple rhizosphere inhibited the mycelial growth of *C. gloeosporioides* and *C. acutatum* (Kim et al. 2016). Suprapta (2022) reported that *P. polymyxa* C1 effectively controls the anthracnose disease of the chili pepper cultivar Cabe Besar caused by *C. scovillei* in Indonesia. Research on fungus antagonism factors in *P. polymyxa* showed that most were β-1,3-1,4-glucanase. The different antagonist activity against pathogenic fungi may be due to the specificity of the enzyme constitution and substrate of β-1,3-1,4-glucanase in different strains (Li et al. 2015).

Bacillus genus produces several secondary metabolites with biocontrol abilities against various phytopathogens (Kim et al. 2021). The fungus-inhibited factors of *Bacillus* spp. were very abundant. Important antagonistic traits, such as siderophore production, solubilization of insoluble phosphate, and production of lytic enzymes (cellulase, protease, and amylase) were observed in *Bacillus tequilensis* GYUN-300 (Kwon et al. 2022). *Bacillus velezensis* CE 100 exhibited chitinase, protease, and β-l,3-glucanase activity (Choub et al. 2021).

As shown in Table 4, β-l,3-glucanase was a popular fungus-inhibited factor of *Bacillus* spp. while β-1,3-1,4-glucanase was found in *Paenibacillus polymyxa*. These enzymes degraded β-glucan in the fungi's cell wall and inhibited the growth of the phytopathogen fungus. In our research, five isolated exhibited strong β-glucanase activity, among them, ML6 had the highest β-glucanase activity with total activity and the clear zone (*D-d*) of 1.72 U mL^{-1} and 2.47 cm, respectively. ML6 was identified as *Bacillus tequilensis* ML6.

Paenibacillus polymyxa is capable of releasing β-1,3-glucanase, hydrolytic enzymes and volatile organic compounds to control fungal pathogens (Zhai et al. 2021). In our study, *P. polymyxa* AT4 exhibited the best antagonistic capacity against *Colletotrichum* spp., the total β-1,3-glucanase activity obtained in *P. polymyxa* AT4 was 1,65 U mL^{-1}, with a glucanlytic index of 4,25. More importantly, *C. scovillei* HUCL1, *C. siamensis* PV6 and CL3 were controlled by the AT4 strain with an inhibition rate of 76.44, 62.65 and 68.04%, respectively, similar to before research subjects.

Taken together, the above results indicate that *P. polymyxa* AT4 and *B. tequilensis* ML6 have a crucial prospective approach to the prevention and inhibition of anthracnose diseases and are capable of controlling different fungal problems in chili.

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