HUE UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY
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STUDY ON THE BIOLOGICAL ANTAGONISM AGAINST *Aspergillus* sp. CAUSING COLLAR ROT OF GROUNDNUT (*Arachis hypogaea*) IN QUANG TRI PROVINCE AND HUE CITY

Major: Biology
Code: 9420101

DOCTORAL THESIS IN BIOLOGY

HUE, 2026

The project was completed at: **Institute of Biotechnology, Hue University**

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INTRODUCTION

1. RATIONALE FOR THE STUDY

Groundnut (*Arachis hypogaea*) is an important upland crop in Central Vietnam, contributing to the improvement of sandy soils and increased household income. However, groundnut productivity is severely affected by diseases, particularly collar rot caused by *Aspergillus niger*, which results in plant wilting, high mortality rates, and substantial economic losses. Some strains of *A. niger* can also produce ochratoxin A, posing potential food safety risks. Currently, disease management relies primarily on chemical fungicides, which may contribute to environmental pollution and the development of fungicide resistance. Therefore, the study entitled "Biological Antagonism Against *Aspergillus* sp. Causing Collar Rot of Groundnut (*Arachis hypogaea*) in Quang Tri Province and Hue City" aims to develop safe and effective biological control strategies for sustainable groundnut production.

2. RESEARCH OBJECTIVES

To select and investigate indigenous microorganisms exhibiting antagonistic activity against *Aspergillus* sp. causing collar rot of groundnut, with particular emphasis on the role of β-1,3-glucanase in antagonistic activity, thereby providing a scientific basis for biological disease control in Quang Tri Province and Hue City.

3. RESEARCH CONTENT

Content 1: Isolation and identification of *Aspergillus* sp. as the causal agent of collar rot of groundnut in Quang Tri Province and Hue City.

Content 2: Isolation and selection of indigenous microbial strains exhibiting antagonistic activity against *Aspergillus* sp., extracellular enzyme activities, and compliance with the biosafety criteria evaluated in this study.

Content 3: Determination of the effects of selected factors on β-1,3-glucanase activity and production by selected microbial strains; evaluation of the effects of extracellular enzyme fractions exhibiting β-1,3-glucanase activity on *Aspergillus* sp. under *in vitro* conditions.

Content 4: Evaluation of the efficacy of selected microbial strains in controlling collar rot of groundnut under greenhouse and field conditions.

4. RESEARCH SUBJECTS

- Strains of *Aspergillus* sp. causing collar rot of groundnut in Quang Tri Province and Hue City.

- Microbial strains exhibiting antagonistic activity and producing extracellular enzymes (chitinase, β-1,3-glucanase, and cellulase), isolated from groundnut-cultivated soils in Quang Tri Province and Hue City.

- The locally cultivated groundnut variety L14.

5. SCOPE OF THE STUDY

5.1. Research period

From December 2021 to June 2025.

5.2. Research locations

Field surveys, sampling, and biological control trials were conducted in groundnut-growing areas of Quang Tri Province and Hue City. Microbial isolation, screening, enzyme characterization, *in vitro* assays, and greenhouse experiments were performed at the Institute of Biotechnology, Hue University. Scanning electron microscopy (SEM) was conducted at the Materials Science Laboratory, University of Science and Education, University of Danang.

6. NOVEL CONTRIBUTIONS OF THE DISSERTATION

6.1. Three indigenous *Bacillus* strains, namely *B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137, were selected based on their antagonistic activity against *A. niger* and their ability to produce extracellular enzymes (chitinase, β-1,3-glucanase, and cellulase).

6.2. The effects of environmental factors on β-1,3-glucanase activity and culture conditions affecting β-1,3-glucanase production by the three selected indigenous *Bacillus* strains were determined. Furthermore, evidence was provided supporting the important role of extracellular enzyme fractions exhibiting β-1,3-glucanase activity in antagonism against *A. niger* under *in vitro* conditions.

6.3. The efficacy of a mixture of culture broths from three indigenous *Bacillus* strains (*B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137), combined at a volumetric ratio of 1:1:1 with a cell density of 10^8 CFU/mL, in controlling collar rot of groundnut was evaluated through greenhouse experiments and field trials at multiple locations in Quang Tri Province and Hue City. The treatments maintained low disease-associated plant mortality rates (1.16–3.57%) and increased actual groundnut yields by 24.90–30.86% compared with the untreated control.

7. SCIENTIFIC AND PRACTICAL SIGNIFICANCE OF THE DISSERTATION

7.1. Scientific significance

- The dissertation provides additional systematic scientific data on the biological characteristics of *Aspergillus* sp., the fungal causal

agent of collar rot of groundnut, and indigenous microorganisms exhibiting antagonistic activity against this pathogen in Quang Tri Province and Hue City.

- The selection and investigation of three indigenous *Bacillus* strains provide data on their antagonistic activity and extracellular enzyme activities, including chitinase, β-1,3-glucanase, and cellulase. The study also elucidates certain characteristics of β-1,3-glucanase and factors influencing its production. Findings on the effects of extracellular enzyme fractions exhibiting β-1,3-glucanase activity on fungal growth and hyphal morphology provide further evidence supporting the involvement of extracellular enzymes in antagonistic activity against *A. niger*.

- The evaluation of the efficacy of the mixture of culture broths from three *Bacillus* strains under *in vitro*, greenhouse, and field conditions contributes to the scientific basis for combining *Bacillus* strains in the biological control of collar rot of groundnut.

7.2. Practical significance

- The dissertation identifies three indigenous bacterial strains, *B. siamensis* 101*, B. siamensis* 112, and *B. velezensis* 137, which exhibit antagonistic activity against *A. niger* and meet the biosafety criteria evaluated in this study.

- The mixture of culture broths from the three bacterial strains (*B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137) was evaluated under greenhouse conditions and at groundnut-growing sites in Quang Tri Province and Hue City, demonstrating its potential for the biological control of collar rot of groundnut and improvement of groundnut yield.

- The findings provide a practical basis for further research aimed at refining application protocols and developing biological control products from the selected indigenous *Bacillus* strains for the management of collar rot of groundnut.

Chapter 1
LITERATURE REVIEW

Groundnut is an economically important crop in coastal Central Vietnam, but its productivity is constrained by collar rot, caused by *Aspergillus* sp., a soilborne fungal pathogen that can persist in soil for extended periods and infect plants through their roots, making disease management challenging. Current chemical control measures pose potential risks of environmental contamination and the development of

fungicide resistance; therefore, biological control is considered a sustainable alternative. Antagonistic microorganisms, such as *Bacillus* and *Trichoderma*, can inhibit fungal pathogens through the production of extracellular enzymes, particularly β-1,3-glucanase, which can degrade β-1,3-glucans in fungal cell walls. However, studies addressing local ecological conditions in Quang Tri Province and Hue City remain limited. Therefore, selecting indigenous antagonistic microorganisms and elucidating the involvement of β-1,3-glucanase in biological antagonism are necessary and have both scientific and practical significance.

Chapter 2
RESEARCH METHODS

- Collection, isolation, identification, and pathogenicity verification of *Aspergillus* sp. causing black mold collar rot according to Koch's postulates.
- Isolation, screening, and identification of indigenous microorganisms antagonistic to *Aspergillus* sp. and producing extracellular enzymes.
- Evaluation of *in vitro* antagonistic activity, determination of MIC and MFC, and assessment of fungal growth inhibition and the effects of extracellular enzyme fractions exhibiting β-1,3-glucanase activity on hyphal morphology.
- Investigation of factors affecting β-1,3-glucanase activity and production.
- Assessment of selected biosafety parameters, IAA production, and compatibility among *Bacillus* strains.
- Evaluation of the efficacy of a three-strain *Bacillus* mixture against black mold collar rot under greenhouse and field conditions.
- Statistical analysis: Data (≥3 replicates) were analyzed using SPSS 26.0 and Excel 2016 and expressed as mean ± SD. Analyses included ANOVA, Duncan's test, Fisher's LSD, Bonferroni correction, Levene's test, and linear regression (R^2), with significance set at $p < 0.05$.

Chapter 3
RESEARCH CONTENT AND RESULTS

3.1. Isolation and identification of *Aspergillus* sp. causing collar rot of groundnut

Isolation and identification of fungal pathogens: Nine representative fungal isolates were selected from diseased groundnut plants collected in Quang Tri Province and Hue City for morphological characterization, identification, and determination of the causal agent of collar rot of groundnut (Figure 3.2).

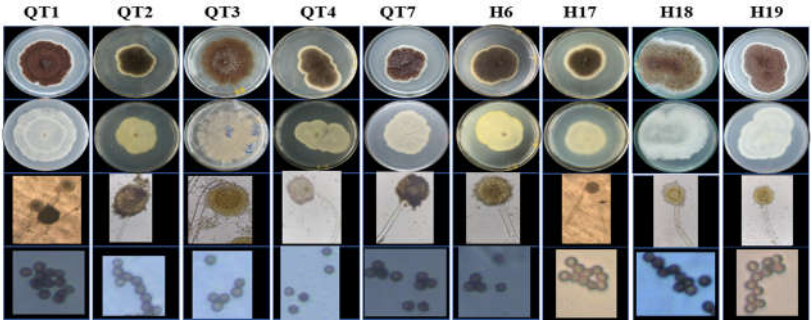


Figure 3.2. *Morphological characteristics of fungal isolates causing collar rot of groundnut on potato dextrose agar (PDA) medium.*

Verification of pathogenicity according to Koch's postulates and selection of highly pathogenic fungal isolates: All nine fungal isolates were pathogenic (100%), with disease symptoms appearing 3–6 days after inoculation. Plant mortality rates ranged from 85.7% to 100%, with isolates QT1 and H17 causing the highest mortality rates and showing statistically significant differences.

Molecular identification of fungal pathogens: Isolates QT1 and H17 were identified based on a combination of morphological characteristics and ITS1–ITS4 sequence analysis, showing 100% sequence similarity to *Aspergillus niger*. Accordingly, both isolates were identified as *A. niger* within the scope of this study (Figure 3.5).

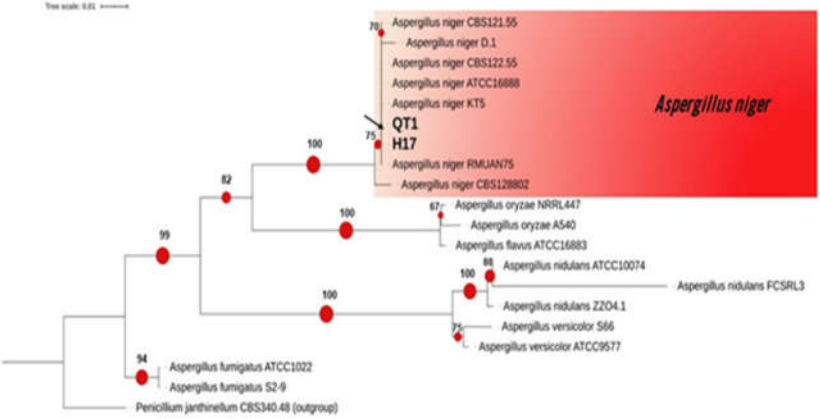


Figure 3.5. *Phylogenetic tree showing the positions of A. niger QT1 and A. niger H17.*

3.2. Selection of indigenous microbial strains exhibiting antagonistic activity against *A. niger*

3.2.1. Isolation and preliminary screening of microbial strains exhibiting antagonistic activity against *A. niger*

A total of 144 microbial isolates exhibiting antagonistic activity against *A. niger* QT1 and H17 were obtained from 12 soil samples collected from groundnut-growing areas affected by collar rot in Quang Tri Province and Hue City. Among these, 16 promising isolates were selected for further evaluation of their antagonistic activity *in vitro* (Table 3.5; Figure 3.6).

Table 3.5. *Inhibition rates of microbial isolates against A. niger QT1 and A. niger H17 after 6 days of incubation.*

No.	Isolated strain	Inhibition rate (%)	
		QT1	H17
1	H1	45,20 ± 0,46 ^{gh}	45,21 ± 0,35 ^{d}
2	**K1**	**61,60 ± 0,05** ^{b}	**45,50 ± 0,35** ^{d}
3	K2	49,86 ± 0,13 ^{ef}	41,15 ± 0,18 ^{e}
4	**B8**	**55,38 ± 0,18** ^{d}	**45,61 ± 0,08** ^{d}
5	45K	41,05 ± 0,19 ^{i}	46,44 ± 0,20 ^{dc}
6	46K	45,10 ± 0,03 ^{h}	45,54 ± 0,08 ^{d}
7	**101**	**58,60 ± 0,18** ^{c}	**54,56 ±** 0,12 ^{b}
8	**112**	**52,10 ± 0,04** ^{e}	**52,28 ±** 0,07 ^{b}
9	118	46,90 ± 0,06 ^{gh}	42,63 ± 0,12 ^{e}
10	**137**	**57,14 ± 0,13** ^{cd}	**57,63 ±** 0,15 ^{a}
11	VL1	37,04 ± 3,40 ^{k}	35,82 ± 3,36 ^{f}
12	Đak3	46,67 ± 2,23 ^{gh}	40,83 ± 2,28 ^{e}
13	**HL6**	**77,41 ± 1,28** ^{a}	**48,30 ± 1,22** ^{c}
14	PhĐ3	47,78 ± 2,94 ^{fg}	27,64 ± 2,61 ^{g}
15	TPH4	50,37 ± 2,31 ^{e}	26,91 ± 2,18 ^{g}
16	PhV1	61,48 ± 0,64 ^{b}	23,28 ± 2,31 ^{h}

Different letters within the same column indicate statistically significant differences among means at $p < 0.05$ (Duncan's test). M ± SD

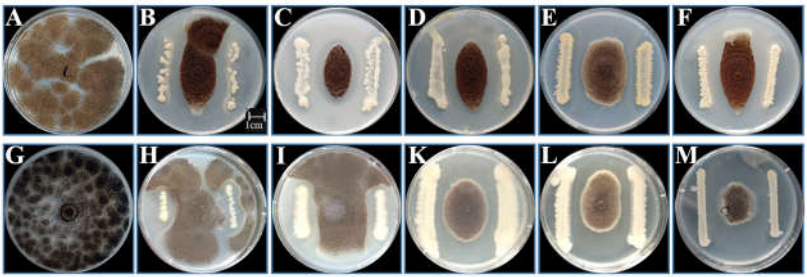


Figure 3.6. *Antagonistic activity of bacterial isolates against fungal pathogens on potato dextrose agar (PDA) after 6 days of incubation (A–F: antagonistic activity against QT1; G–M: antagonistic activity against H17; bacterial isolates: B8, K1, 101, 112, and 137).*

3.2.2. Selection of antagonistic microbial strains producing extracellular enzymes

Further screening of extracellular enzyme activities among isolates exhibiting strong antagonistic activity, based on the diameters of hydrolysis zones on agar plates, resulted in the selection of 11 isolates. The results are presented in Table 3.6 and Figure 3.9.

Table 3.6. *Extracellular enzyme activities of antagonistic microbial isolates, expressed as hydrolysis zone diameters (mm).*

No.	Microbial strain	Hydrolysis zone diameter (mm)		
		1,3- β -glucanase	Chitinase	Cellulase
1	H1	18,50 ± 0,20 ^{d}	20,47 ± 0,15 ^{c}	17,70 ± 0,62 ^{d}
2	K1	24,97 ± 0,15 ^{ab}	25,83 ± 0,15 ^{a}	25,47 ± 0,06 ^{a}
3	K2	22,77 ± 0,25 ^{bc}	23,30 ± 0,20 ^{b}	24,03 ± 0,15 ^{ab}
4	B8	26,17 ± 0,42 ^{a}	26,10 ± 0,10 ^{a}	25,77 ± 0,25 ^{a}
5	45K	20,07 ± 0,31 ^{cd}	19,37 ± 0,15 ^{cd}	18,73 ± 0,21 ^{cd}
6	46K	22,23 ± 0,38 ^{bc}	22,93 ± 0,21 ^{bc}	22,97 ± 0,15 ^{b}
7	101	23,20 ± 0,36 ^{b}	22,53 ± 0,06 ^{bc}	21,23 ± 0,31 ^{bc}
8	112	23,93 ± 0,31 ^{b}	22,87 ± 0,23 ^{bc}	21,90 ± 0,79 ^{bc}
9	118	21,03 ± 0,15 ^{c}	18,17 ± 0,15 ^{d}	20,10 ± 0,10 ^{c}
10	137	21,03 ± 0,15 ^{c}	20,47 ± 0,06 ^{c}	20,67 ± 0,31 ^{c}
11	HL6	18,30 ± 0,18 ^{d}	18,45 ± 0,15 ^{d}	17,68 ± 0,20 ^{d}

Different letters within the same column indicate statistically significant differences among means at $p < 0.05$ (Duncan's test). M ± SD

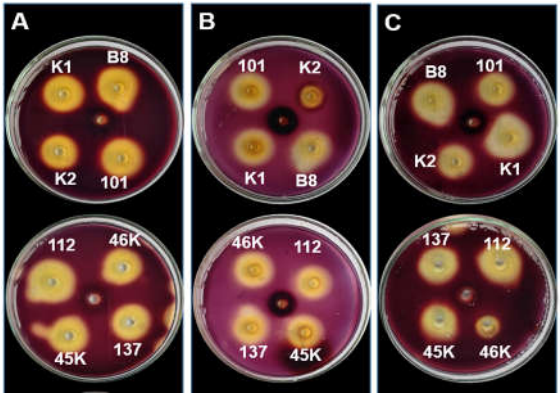


Figure 3.9. *β-1,3-Glucanase (A), chitinase (B), and cellulase (C) activities of selected microbial isolates.*

3.2.3. Identification of microbial strains selected based on antagonistic activity and extracellular enzyme activities

Six microbial isolates exhibiting strong antagonistic activity and high extracellular enzyme activities were identified using a combination of morphological and molecular methods. The five bacterial isolates belonged to the genus *Bacillus* and were identified as *B. siamensis* B8, *B. siamensis* 101, *B. siamensis* 112, *B. velezensis* K1, and *B. velezensis* 137 (Figure 3.11). The fungal isolate HL6 was identified as *T. asperellum* (Figure 3.13).

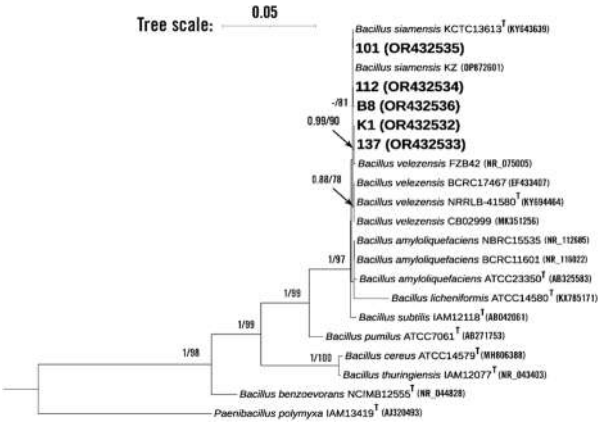


Figure 3.11. *Phylogenetic tree based on 16S rRNA gene nucleotide sequences of isolates B8, K1, 101, 112, and 137.*

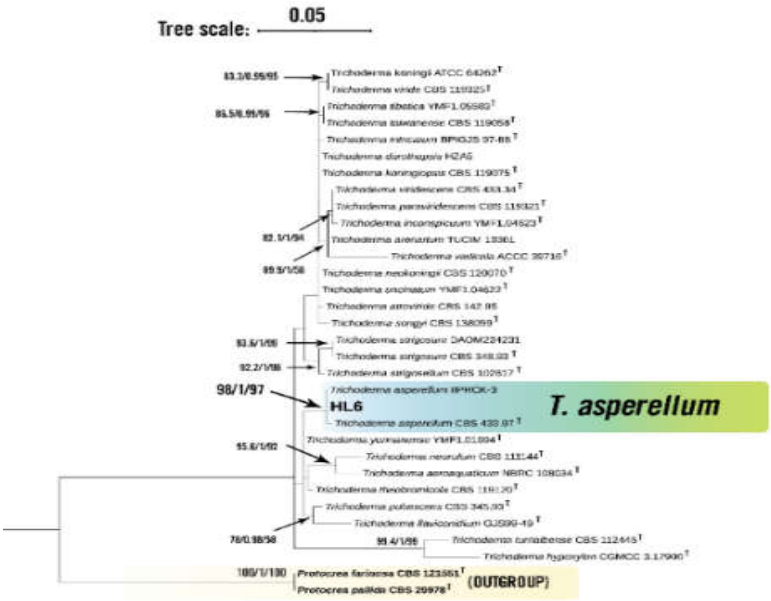


Figure 3.13. *Phylogenetic tree based on the ITS sequence of fungal isolate HL6.*

3.2.4. Effects of antagonistic microbial strains on groundnut seed germination

Treatment of L14 groundnut seeds with the six selected microbial strains resulted in high germination percentages (98.33–99.67%), with no statistically significant differences compared with the control.

3.2.5. Efficacy of individual antagonistic microbial strains in controlling collar rot of groundnut under greenhouse conditions

Greenhouse experiments evaluating the control of collar rot of groundnut caused by *A. niger* isolates QT1 and H17 showed that *B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137 exhibited more consistent disease control effects than *T. asperellum* HL6, *B. siamensis* B8, and *B. velezensis* K1 under the same experimental conditions. Based on these findings, three strains, *B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137, were selected for further investigation.

3.2.6. Compatibility among the selected *Bacillus* strains

Cross-streak assays on Luria–Bertani (LB) agar over 48 hours showed that all three strains, *B. siamensis* 101, *B. siamensis* 112, and

B. velezensis 137, exhibited normal growth, with no zones of growth inhibition observed at the interfaces between the strains (Figure 3.14).



Figure 3.14. *Compatibility among three Bacillus strains, B. siamensis 101, B. siamensis 112, and B. velezensis 137, on Luria–Bertani agar (LBA) after 48 hours of incubation.*

3.2.7. Antagonistic activity of *Bacillus* strains against *A. niger* and the role of extracellular enzyme fractions exhibiting β-1,3-glucanase activity under *in vitro* conditions

3.2.7.1. Antagonistic activity against A. niger and inhibition of fungal growth on solid medium

Table 3.12. *Inhibitory activity of crude cell-free culture supernatants against A. niger.*

Bacterial strain	Diameter of growth inhibition of pathogenic fungal strains (mm)	
	***A.niger* QT1**	***A.niger* H17**
B. siamensis 101	26,6± 0,54 [c]	21,1± 0,44 [c]
B. siamensis 112	25,4±0,46 [c]	20,3±0,46 [c]
B. velezensis 137	28,3±0,61 [b]	23,5±0,53 [b]
Mixture of three *Bacillus* strains	**30,8±0,58 [a]**	**26,8±0,66 [a]**
Control	0,0±0,00 [d]	0,0±0,00 [d]

3.2.7.2. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

The individual extracellular enzyme fractions exhibiting β-1,3-glucanase activity from *B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137 each showed an MIC and MFC of 0.4 U/mL against

both *A. niger* QT1 and H17. When the extracellular enzyme fractions exhibiting β-1,3-glucanase activity from these three *Bacillus* strains were combined at a ratio of 1:1:1, their antifungal activity increased markedly, with MIC and MFC values of 0.1 and 0.2 U/mL, respectively, against QT1, and 0.2 and 0.4 U/mL, respectively, against H17. The MFC/MIC ratio was 2 for both fungal isolates (Table 3.13).

Table 3.13. *Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC).*

Enzyme source	Pathogen control *A. niger* QT1			Pathogen control *A. niger H17*		
	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC
B. siamensis 101	0,4	0,4	1	0,4	0,4	1
B. siamensis 112	0,4	0,4	1	0,4	0,4	1
B. velezensis 137	0,4	0,4	1	0,4	0,4	1
Mixture of three Bacillus strains	0,1	0,2	2	0,2	0,4	2

MFC was defined as the β-1,3-glucanase activity level in the microwell at which no fungal growth was observed after 48 hours of incubation following transfer onto potato dextrose agar (PDA).

3.2.7.3. Reduction of A. niger biomass in liquid medium

The effects of individual bacterial strains and their mixture on fungal biomass are presented in Tables 3.14 and 3.15 and Figures 3.18 and 3.19.

Table 3.14*. Effects of extracellular enzyme fractions exhibiting β-1,3-glucanase activity on the dry biomass of A. niger QT1.*

Source of extracellular enzyme fractions	β-1,3-Glucanase activity (U/mL)				
	0	0,2	0,4	0,8	1,6
B. siamensis 101	**39,19±1,22**	36,33±1,15 ^{dA}	23,22± 2,17 ^{cB}	18,13±1,05 ^{bC}	13,50±0,68 ^{aC}
B. siamensis 112	**39,19±1,22**	36,00±1,01 ^{dA}	22,80±1,79 ^{cB}	17,27±0,82 ^{bC}	12,62±1,37 ^{aBC}
B. velezensis 137	**39,19±1,22**	35,72±1,80 ^{dA}	21,14±0,38 ^{cB}	13,79±0,60 ^{bB}	10,59±0,44 ^{aB}
Mixture of three *Bacillus* strains	**39,19±1,22**	**35,29±1,23** ^{dA}	**16,68±1,55** ^{cA}	**10,46±0,42** ^{bA}	**6,43±0,71** ^{aA}

Two-way ANOVA results

Source of variation	df	F	p
Enzyme source (Factor 1)	3; 32	52,632	< 0,001
Enzyme activity (Factor 2)	3; 32	1007,792	< 0,001
Interaction (enzyme source × enzyme activity)	9; 32	4,383	0,001

Data are presented as mean ± standard deviation (M ± SD; n = 3). Within the same row, values followed by different lowercase letters

(a–d) indicate statistically significant differences among enzyme activity levels; within the same column, values followed by different uppercase letters (A–C) indicate statistically significant differences among enzyme sources (p < 0.05; Bonferroni-adjusted pairwise comparisons). Two-way ANOVA was performed on 16 combinations of enzyme source × enzyme activity, excluding the common control at 0 U/mL. Levene's test revealed no statistically significant differences in variances among groups (p = 0.201).

Table 3.15. *Effects of extracellular enzyme fractions exhibiting β-1,3-glucanase activity on the dry biomass of A. niger H17.*

Source of extracellular enzyme fractions	**β-1,3-Glucanase activity (U/mL)**				
	0	0,2	0,4	0,8	1,6
B. siamensis 101	**31,93±2,00**	30,60±1,62 ^{cA}	22,54±1,25 ^{bB}	16,34±1,10 ^{aB}	13,94±0,77 ^{aB}
B. siamensis 112	**31,93±2,00**	30,14±2,03 ^{cA}	21,34±2,19 ^{bB}	15,19±1,23 ^{aB}	12,88±0,98 ^{aB}
B. velezensis 137	**31,93±2,00**	29,85±1,26 ^{cA}	19,44±3,19 ^{bB}	13,23±1,03 ^{aBA}	10,63±1,57 ^{aBA}
Mixture of three *Bacillus* strains	**31,93±2,00**	**29,68±2,28** ^{cA}	**15,40±1,93** ^{bA}	**10,30±1,10** ^{aA}	**7,53±1,00** ^{aA}

Two-way ANOVA results			
Source of variation	df	F	p
Enzyme source (Factor 1)	3; 32	22,075	< 0,001
Enzyme activity (Factor 2)	3; 32	307,487	< 0,001
Interaction (enzyme source × enzyme activity)	9; 32	1,784	0,110

Data are presented as mean ± standard deviation (M ± SD; n = 3). Within the same row, values followed by different lowercase letters (a–c) indicate statistically significant differences among enzyme activity levels; within the same column, values followed by uppercase letters sharing no common letter (A–B) indicate statistically significant differences among enzyme sources (p < 0.05; Bonferroni-adjusted pairwise comparisons). Two-way ANOVA was performed on 16 combinations of enzyme source × enzyme activity, excluding the common control at 0 U/mL. Levene's test revealed no statistically significant differences in variances among groups (p = 0.262).

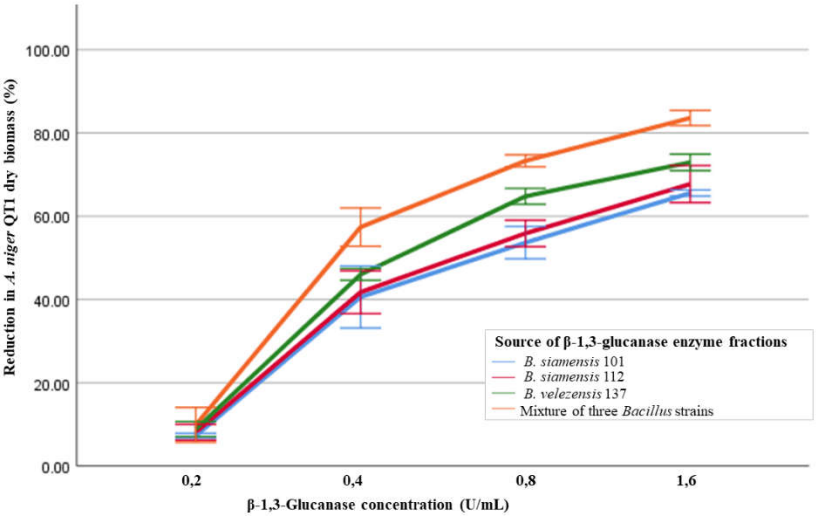


Figure 3.18. *Percentage reduction in dry biomass of A. niger QT1 following treatment with extracellular enzyme fractions exhibiting β-1,3-glucanase activity.*

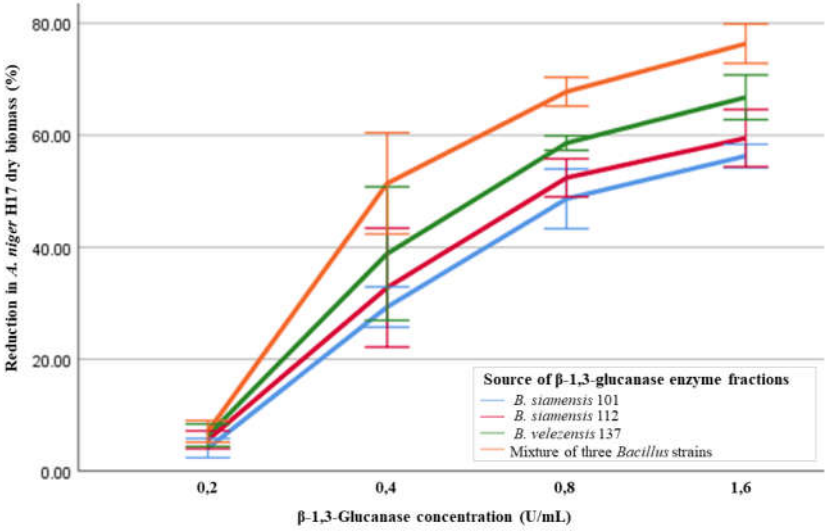


Figure 3.19. *Percentage reduction in dry biomass of A. niger H17 following treatment with extracellular enzyme fractions exhibiting β-1,3-glucanase activity.*

3.2.7.4. Correlation between β-1,3-glucanase activity and the diameter of the inhibition zone against A. niger

Table 3.16. *Inhibitory activity of mixtures of extracellular enzyme fractions exhibiting β-1,3-glucanase activity against A. niger at different enzyme activity levels on agar plates.*

Concentration (U/mL)	Diameter of growth inhibition of pathogenic fungal strains (mm)	
	***A.niger* QT1**	***A.niger* H17**
0 (Control)	0,0± 0,00	0,0± 0,00
0,2	ND	ND
0,4	23,5±0,49 [c]	20,7±0,43 [c]
0,8	26,4±0,17 [b]	25,2±0,17 [b]
1,6	33,3±0,19 [a]	30,3±0,31 [a]

ND: Not determined; different letters indicate significant differences within columns (Duncan's test, $p < 0.05$); M ± SD.

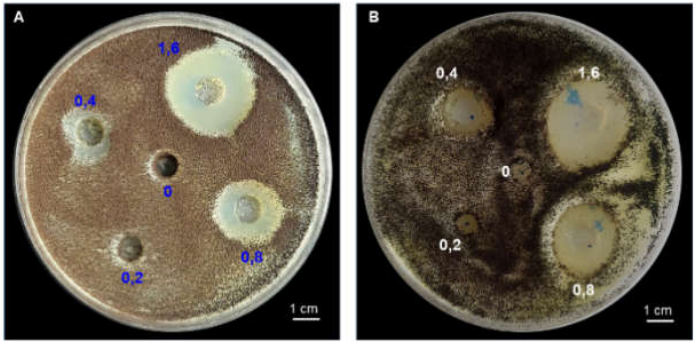


Figure 3.20. *Inhibitory activity of a mixture of extracellular enzyme fractions exhibiting β-1,3-glucanase activity from three Bacillus strains against A. niger QT1 (A) and A. niger H17 (B).*

Table 3.17. *Linear regression parameters for the relationship between β-1,3-glucanase activity and the diameter of inhibition zones against A. niger.*

Pathogenic fungal strain	Regression equation (y = ax + b)	Coefficient of determination (R^2)	Significance level (p)
A. niger QT1	y = 18,609x + 6,843	0,634	0,002
A. niger H17	y = 17,191x + 6,157	0,641	0,002

The results presented in Tables 3.16 and 3.17 and Figure 3.20 provide additional experimental evidence supporting the involvement of β-1,3-glucanase in antagonistic activity against *A. niger*.

3.2.7.5. Effects of extracellular enzyme fractions exhibiting β-1,3-glucanase activity on the hyphal morphology of A. niger

Scanning electron microscopy (SEM) observations showed that the control samples exhibited normal hyphal morphology, whereas treatment with β-1,3-glucanase caused hyphal deformation, cracking, rupture, and pore formation in the hyphal cell walls.

3.2.8. Selected characteristics related to biosafety and plant growth-promoting potential of the selected *Bacillus* strains

Hemolytic activity on blood agar: None of the three bacterial strains, *B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137, exhibited hemolytic activity on blood agar plates under the experimental conditions.

Antibiotic susceptibility: All three bacterial strains, *B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137, were susceptible to six antibiotics: tetracycline (30 µg), chloramphenicol (30 µg), trimethoprim (25 µg), levofloxacin (Lv-5 µg), gentamicin (Ge-10 µg), and streptomycin (Sm-10 µg).

Acute toxicity to earthworms: The three bacterial strains, *B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137, applied individually or in combination (10^7–10^9 CFU/g dry soil), showed no evidence of acute toxicity to the earthworm *Eisenia fetida* after 14 days of exposure. Mortality rates (5.00–7.50%) and biomass reductions (10.29–10.99%) did not differ significantly from those of the control ($p > 0.05$), consistent with the assessment criteria specified in TCVN 5961:1995 and ISO 11268-1:2012.

IAA production: All three bacterial strains, *B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137, were capable of producing IAA. IAA concentrations increased with cultivation time, reaching their highest levels after 72 hours, ranging from 0.025 to 0.027 mg/mL, with no substantial differences among the strains. These findings suggest their potential to promote plant growth (Figure 3.24).

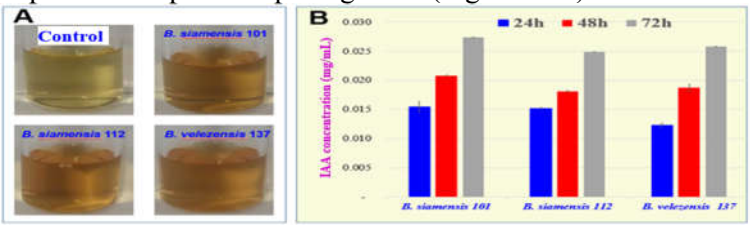


Figure 3.24. *Qualitative analysis of Indole-3-acetic acid (IAA) production capacity by selected Bacillus strains (A) and quantification of IAA content (B)*

3.3. FACTORS AFFECTING β-1,3-GLUCANASE ACTIVITY AND BIOSYNTHESIS BY THE SELECTED *BACILLUS* STRAINS

3.3.1. Changes in β-1,3-glucanase activity over cultivation time

The optimal time for enzyme harvesting was 20–24 hours for all three bacterial strains: *B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137 (Figures 3.25, 3.26, and 3.27).

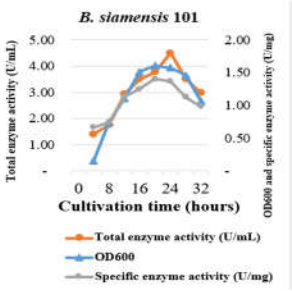


Figure 3.25. *Cumulative curve of beta-1,3-glucanase production by B. siamensis 101*

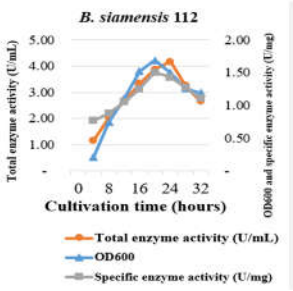


Figure 3.26. *Cumulative curve of beta-1,3-glucanase production by B. siamensis 112*

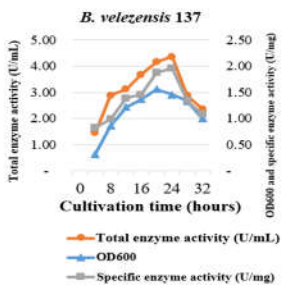


Figure 3.27. *Cumulative curve of beta-1,3-glucanase production by B. velezensis 137*

3.3.2. Effects of selected factors on β-1,3-glucanase activity

Effect of temperature: β-1,3-Glucanase activity of the three *Bacillus* strains reached its maximum at temperatures ranging from 50 to 60°C. Maximum activity was observed at 50°C for *B. siamensis* 101 and at 60°C for *B. siamensis* 112 and *B. velezensis* 137 (Figure 3.28).

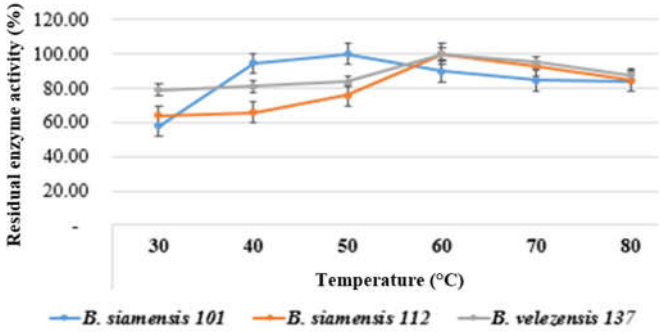


Figure 3.28*. Effect of temperature on β-1,3-glucanase activity of the three selected Bacillus strains.*

Effect of pH: β-1,3-Glucanase from all three *Bacillus* strains exhibited optimal activity at pH 5.0, with a marked decrease in activity under highly acidic or alkaline conditions.

Effects of metal ions and chemical compounds: Mn^{2+} and Co^{2+} increased β-1,3-glucanase activity of *B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137 to 132–202% of the control level, whereas EDTA, urea, and Tris markedly reduced enzyme activity (70–91%) (Figure 3.30).

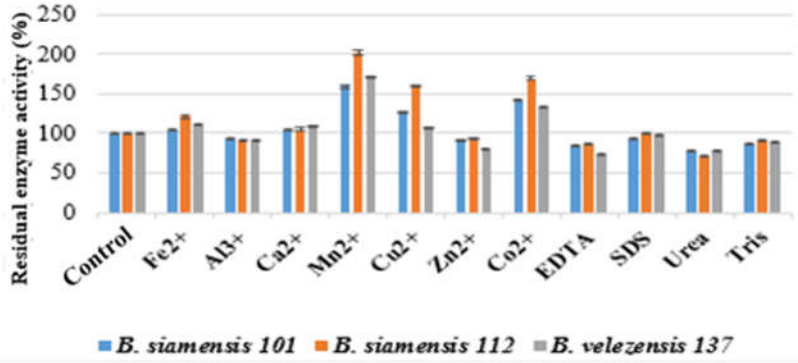


Figure 3.30. *Effects of metal ions and chemical compounds on β-1,3-glucanase activity of the three selected Bacillus strains.*

3.3.3. Effects of selected factors on β-1,3-glucanase biosynthesis

Effect of carbon sources: Corn flour and curdlan as carbon sources resulted in comparable β-1,3-glucanase production after 24 hours of cultivation, with higher levels than those obtained using barley and wheat.

Effect of culture media: LB medium was identified as the most suitable medium for cultivating the *Bacillus* strains among the four media evaluated (LB, MPB, NB, and NBY).

Effect of supplementary nitrogen sources: Yeast extract was the most suitable nitrogen source among those evaluated (yeast extract, tryptone, and peptone).

Effect of inoculum size: An inoculum size of 7% yielded optimal results under the experimental conditions (Table 3.23).

Table 3.23. *Effect of inoculum size on growth and β-1,3-glucanase biosynthesis by the Bacillus strains.*

Inoculum size (%)	*B. siamensis* 101		*B. siamensis* 112		*B. velezensis* 137	
	Enzyme activity (U/mL)	Biomass (× 10^9 CFU/mL)	Enzyme activity (U/mL)	Biomass (× 10^9 CFU/mL)	Enzyme activity (U/mL)	Biomass (× 10^9 CFU/mL)
1	0,93±0,44^c	1,51±0,21^b	1,70±0,20^c	2,40±0,41^b	1,88±0,51^c	1,81±0,34^b
5	3,89±0,23^b	**7,20±0,61^a**	**3,92±0,21^a**	**8,40±0,31^a**	3,90±0,11^b	**7,33±0,54^a**
7	**4,98±0,11^a**	**7,71±0,43^a**	**4,32±0,20^a**	**8,50±0,22^a**	**4,39±0,41^a**	**8,80±0,41^a**
10	**3,92±0,12^a**	**7,42±0,41^a**	3,72±0,24^b	**8,11±0,63^a**	3,80±0,40^b	**8,32±0,32^a**

Different letters within the same column indicate statistically significant differences at $p < 0.05$ (Duncan's test). Data are presented as mean ± standard deviation (M ± SD). Culture conditions: 1% (w/v) corn flour, 1% (w/v) yeast extract, 30°C, pH 7.0, 150 rpm, and 24 hours.

Effect of cultivation time: Within the investigated range, a cultivation period of 24 hours was the most suitable for growth and β-1,3-glucanase biosynthesis by all three *Bacillus* strains (Table 3.24).

Table 3.24. *Effect of cultivation time on growth and β-1,3-glucanase accumulation by the Bacillus strains.*

Time (hours)	*B. siamensis* 101		*B. siamensis* 112		*B. velezensis* 137	
	Enzyme activity (U/mL)	Biomass (× 10^9 CFU/mL)	Enzyme activity (U/mL)	Biomass (× 10^9 CFU/mL)	Enzyme activity (U/mL)	Biomass (× 10^9 CFU/mL)
0	0,19±0,10^c	**0,00002±0,3^a**	2,60±0,13^b	**0,00002±0,51^a**	0,58±0,33^c	**0,000015±0,50^a**
24	**5,15±0,40^a**	**7,52±0,51**^{a}	**4,40±0,21^a**	**8,94 ±0,90**^{a}	**4,41±0,51^a**	**8,44±0,53**^{a}
36	3,66±0,52^b	**6,34±0,53^a**	3,92±0,24^b	**7,72 ±0,91^a**	**3,90±0,14^a**	**7,34±0,81^a**
48	1,63±0,53^c	**3,62±1,14^a**	1,96±0,41^c	**4,22 ±0,54^a**	2,08±0,93^b	**3,82 ±1,14^a**

Different letters within the same column indicate statistically significant differences at $p < 0.05$ (Duncan's test). Data are presented as mean ± standard deviation (M ± SD). Culture conditions: 1% (w/v) corn flour, 1% (w/v) yeast extract, 7% inoculum size, 30°C, pH 7.0, and 150 rpm.

Effect of cultivation temperature: Within the investigated range, 30°C was the most suitable temperature for growth and β-1,3-glucanase biosynthesis by all three *Bacillus* strains (Table 3.25).

Table 3.25. *Effect of cultivation temperature on growth and β-1,3-glucanase biosynthesis by the Bacillus strains.*

t°C	*B. siamensis* 101		*B. siamensis* 112		*B. velezensis* 137	
	Enzyme activity (U/mL)	Biomass (× 10⁹ CFU/mL)	Enzyme activity (U/mL)	Biomass (× 10⁹ CFU/mL)	Enzyme activity (U/mL)	Biomass (× 10⁹ CFU/mL)
25	3,01±0,62[b]	0,33±0,12[b]	**3,06±0,63[a]**	0,40±0,34[b]	3,17[b]±0,73	0,50±0,21[b]
30	**5,24±0,44[a]**	**7,42±0,23[a]**	**4,49±0,11[a]**	**9,11±0,52[a]**	**4,52±0,54[a]**	**8,24±0,41[a]**
35	3,9±0,42[b]	**7,02±0,60[a]**	**3,66±0,32[a]**	**8,82±0,43[a]**	**3,52±0,52[a]**	**8,52±0,54[a]**
37	2,91±0,60[b]	**7,04±0,51[a]**	**2,79±0,44[a]**	**8,14±0,31[a]**	2,59±0,40 [b]	**7,91±0,72[a]**

Effect of culture medium pH: Within the investigated range, pH 7.0 was the most suitable condition for growth and β-1,3-glucanase biosynthesis by all three *Bacillus* strains (Table 3.26).

Table 3.26. *Effect of culture medium pH on growth and β-1,3-glucanase biosynthesis by the Bacillus strains.*

pH	*B. siamensis* 101		*B. siamensis* 112		*B. velezensis* 137	
	Enzyme activity (U/mL)	Biomass (× 10⁹ CFU/mL)	Enzyme activity (U/mL)	Biomass (× 10⁹ CFU/mL)	Enzyme activity (U/mL)	Biomass (× 10⁹ CFU/mL)
5,0	3,01±0,91[c]	0,16±0,3 [c]	2,80±0,50[b]	0,52±0,4 [b]	2,85±0,41 [c]	0,23±0,62[c]
6,0	3,51±0,32 [c]	0,31±0,62[c]	3,62±0,44 [b]	**0,71±0,41[a]**	3,64±0,10 [b]	0,50±0,31[c]
6,5	4,21±0,50 [b]	**7,12±0,74[a]**	3,67±0,32 [b]	**8,40±0,82[a]**	3,74±0,34 [b]	8,11±0,50[ab]
7,0	**5,43±0,34 [a]**	**7,63±0,82[a]**	**4,53±0,10[a]**	**8,84±0,53[a]**	**4,52 ± 0,51 [a]**	**8,51±0,62[a]**
7,5	4,17±0,11 [b]	4,51±0,50[b]	3,50±0,42 [b]	**6,40±0,33[a]**	3,04±0,62 [b]	7,01±0,22[ab]
8,0	2,86±0,21 [c]	2,30±0,44[b]	3,21±0,50 [b]	3,82±0,60[b]	2,24±0,63 [c]	5,23±0,44 [b]

Different letters within the same column indicate statistically significant differences at $p < 0.05$ (Duncan's test). Mean ± SD. Culture conditions: 1% (w/v) corn flour; 1% (w/v) yeast extract; 7% inoculum size; 30°C; 150 rpm; and 24 h of incubation.

Effect of shaking speed: Within the investigated range, a shaking speed of 150 rpm was the most suitable condition for growth and β-1,3-glucanase production by all three *Bacillus* strains.

Culture conditions for β-1,3-glucanase production: Liquid LB medium; yeast extract as a supplementary nitrogen source; 7% inoculum size; 24 h of incubation; incubation temperature of 30–35°C; initial culture medium pH of 7.0; and shaking speed of 150 rpm.

3.4. Efficacy of a mixture of culture broths from three *Bacillus* strains in controlling collar rot of groundnut under greenhouse and field conditions

3.4.1. Disease control efficacy under greenhouse conditions

In the negative control treatment (CT1), the groundnut plant mortality rate remained at 0.00% throughout the 45-day experimental period. Following inoculation with *A. niger* QT1 at a concentration of 10^6 spores/mL, the pathogen-inoculated control treatment (CT2) exhibited a plant mortality rate of 26.67% at 5 days post-inoculation, with typical symptoms of collar rot. The mortality rate increased to 60.00% at 7 days post-inoculation and reached 100% at 14 days post-inoculation. In contrast, treatment CT3, in which seeds and seedlings were treated with a mixture of culture broths from three *Bacillus* strains (*B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137) at a concentration of 10^8 CFU/mL, showed no symptoms of collar rot and maintained a plant mortality rate of 0% throughout the 45-day period following pathogen inoculation.

Similarly, following inoculation with *A. niger* H17 at a concentration of 10^6 spores/mL, the pathogen-inoculated control treatment (CT4) exhibited a plant mortality rate of 26.67% at 5 days post-inoculation, with typical symptoms of collar rot. The mortality rate increased to 60.00% at 14 days post-inoculation and reached 100% at 21 days post-inoculation. In treatment CT5, seeds and seedlings were treated with a mixture of culture broths from three *Bacillus* strains (*B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137) at a concentration of 10^8 CFU/mL, resulting in a disease control efficacy of 73.33% throughout the 45-day period following pathogen inoculation (Figure 3.31). Statistically significant differences were observed between the treatments and the controls ($p < 0.05$).

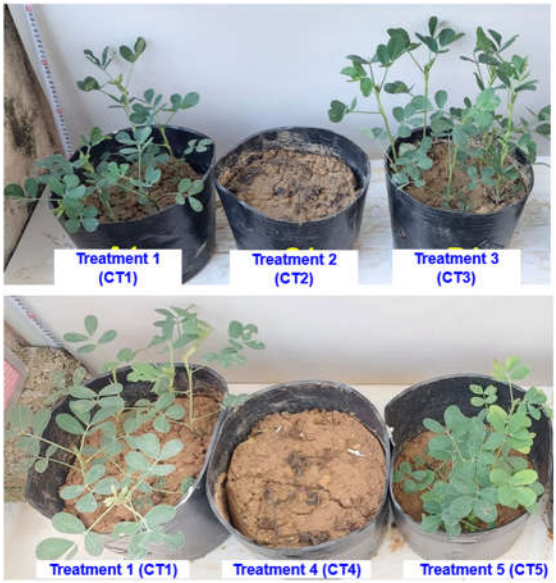


Figure 3.31. *Efficacy of a mixture of culture broths from three Bacillus strains (B. siamensis 101, B. siamensis 112, and B. velezensis 137) in controlling collar rot of groundnut under greenhouse conditions. CT1: Not inoculated with the pathogen and treated with sterile water (negative control); CT2: Inoculated with A. niger QT1 and treated with sterile water (disease control); CT3: Inoculated with A. niger QT1 and treated with a mixture of culture broths from three Bacillus strains; CT4: Inoculated with A. niger H17 and treated with sterile water (disease control); CT5: Inoculated with A. niger H17 and treated with a mixture of culture broths from three Bacillus strains.*

3.4.2. Efficacy under Field Conditions

Effect on main stem height: At all four experimental sites (Phu Vang, Huong Tra, Nam Hai Lang, and Trieu Phong), treatment with a mixture of culture broths from three *Bacillus* strains increased the main stem height of L14 peanut plants compared with the negative control.

Effect of the *Bacillus* mixture on collar rot of groundnut: At all four experimental sites (Phu Vang, Huong Tra, Nam Hai Lang, and Trieu Phong), plant mortality rates caused by collar rot of groundnut were significantly lower in the treatment receiving a mixture of culture

broths from three *Bacillus* strains (CT1) than in the negative control treatment (CT3), according to the $LSD_{0.05}$ criterion (Table 3.32).

Table 3.32. *Effect of a mixture of culture broths from three Bacillus strains on plant mortality caused by collar rot of groundnut in peanut cultivar L14 at the experimental sites (%).*

Treatment	Phu Vang, Hue	Huong Tra, Hue	Hai Lang, Quang Tri	Trieu Phong, Quang Tri
Mixture of culture broths from three strains	**3,57 ±0,33 b**	**1,16 ± 0,06 b**	**1,38 ± 0,11 b**	**2,11 ± 0,20 b**
Trichoderma (CT2)	4,08 ± 0,31 b	1,75 ± 0,11 b	1,78 ± 0,14 b	2,21 ± 0,28 b
Control (CT3)	**10,71±1,05 a**	**6,57 ± 0,62 a**	**4,13 ± 0,35 a**	**8,16 ± 0,69 a**
LSD 0.05	*2,61*	*3,05*	*1,07*	*2,13*

Different letters within the same column indicate statistically significant differences among treatment means ($p < 0.05$).

Effect of the mixture of culture broths from three *Bacillus* strains on peanut yield: At all four experimental sites, treatment of peanut cultivar L14 with a mixture of culture broths from three *Bacillus* strains (CT1) resulted in actual yields ranging from 2.95 to 3.18 t/ha, compared with 2.28–2.53 t/ha in the negative control, corresponding to yield increases of 0.63–0.75 t/ha (24.9–30.86%) (Figure 3.32).

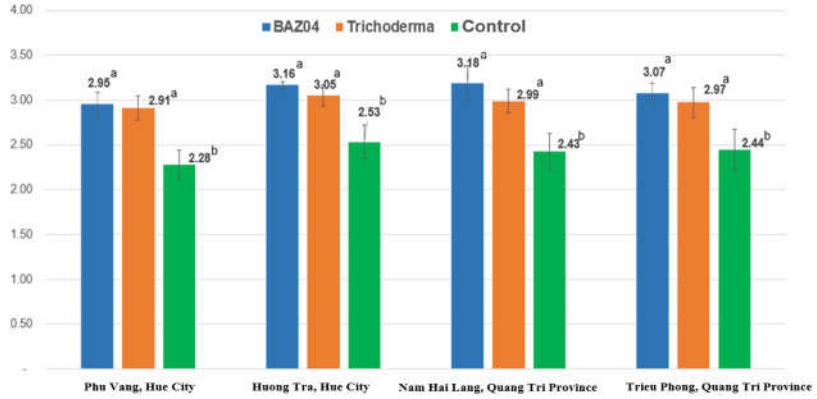


Figure 3.32. *Peanut yield at the experimental sites in Huong Tra, Phu Vang, Trieu Phong, and Nam Hai Lang. Different letters within the same experimental site indicate statistically significant differences among treatment means ($p < 0.05$; Duncan's multiple range test). Data are presented as mean ± standard deviation (M ± SD).*

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Based on the research findings, the following conclusions were drawn:

1. A total of 60 fungal isolates were obtained from peanut plants exhibiting symptoms of collar rot in Quang Tri Province and Hue City. Pathogenicity tests based on Koch's postulates and virulence assessments of nine selected isolates revealed plant mortality rates ranging from 85.7% to 100%. Based on morphological characteristics and ITS rDNA sequence analysis, the two representative isolates with the highest pathogenicity (QT1 and H17), each causing 100% plant mortality, were provisionally identified as *Aspergillus niger* and designated *A. niger* QT1 and *A. niger* H17.

2. Three indigenous bacterial strains (*B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137) exhibiting stable antagonistic activity against both fungal pathogens, *A. niger* QT1 and *A. niger* H17, were selected. These three *Bacillus* strains exhibited extracellular chitinase, β-1,3-glucanase, and cellulase activities, produced indole-3-acetic acid (IAA), and met the investigated biosafety criteria, including the absence of hemolytic activity, no observed toxicity to earthworms at 10^9 CFU/g dry soil, susceptibility to six antibiotics (tetracycline, chloramphenicol, trimethoprim, levofloxacin, gentamicin, and streptomycin), and no adverse effects on peanut seed germination.

3. In single-factor experiments, extracellular enzyme fractions exhibiting β-1,3-glucanase activity from the three bacterial strains (*B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137) showed maximum activity at pH 5.0 and reaction temperatures of 50–60 °C. Enzyme activity was enhanced by Mn^{2+} and Co^{2+} but strongly inhibited by EDTA and urea. Suitable culture conditions for obtaining extracellular enzyme-containing broths with β-1,3-glucanase activity from the three *Bacillus* strains included liquid LB medium supplemented with 1% corn flour and 1% yeast extract, an initial medium pH of 7.0, an incubation temperature of 30–35 °C, a shaking speed of 150 rpm, a 7% inoculum size, and an incubation period of

24h. Combining the extracellular enzyme fractions exhibiting β-1,3-glucanase activity at a volumetric ratio of 1:1:1 reduced the minimum inhibitory concentration (MIC) to 0.1–0.2 U/mL and the minimum fungicidal concentration (MFC) to 0.2–0.4 U/mL. Scanning electron microscopy (SEM) revealed deformation and cracking of the cell walls of *A. niger* QT1 and H17.

4. A mixture of culture broths from the three bacterial strains (*B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137), combined at a volumetric ratio of 1:1:1 with a cell density of 10^8 CFU/mL, exhibited greater inhibitory efficacy against *A. niger* QT1 and *A. niger* H17 than the individual strains under *in vitro* conditions. The mixture also helped maintain low plant mortality rates caused by collar rot of groundnut (1.16–3.57%) and increased actual yields by 24.90–30.86% across the four field experimental sites compared with the negative control.

Recommendations

Further studies should evaluate the efficacy of the mixture of culture broths from the three bacterial strains (*B. siamensis* 101, *B. siamensis* 112, and *B. velezensis* 137) in controlling collar rot of groundnut over multiple growing seasons and under different cultivation conditions, as well as investigate its effects on beneficial soil microorganisms. In addition, further research should focus on formulation development, viable cell density, stability, storage duration and conditions, and efficacy after storage to provide a basis for assessing its potential application in controlling collar rot of groundnut in peanut cultivation in Central Vietnam./.

LIST OF PUBLISHED WORKS

[1]. Nguyen Xuan Hieu, Nguyen Thi Minh Nga, Nguyen Duc Huy, Nguyen Quang Co, Cao Thi Thuyet, Pham Thi Thuy Hoai, Nguyen Thi Thu Thuy (**2023**). **Identification and characterization of *Aspergillus niger* causing collar rot of groundnut (*Arachis hypogaea*)**. *Biodiversitas*, *24*(5). 2556-2562.

https://doi.org/10.13057/biodiv/d240507

[2]. Nguyen Thi Minh Nga, **Nguyen Xuan Hieu**, Nguyen Tien Long, Nguyen Duc Huy and Nguyen Thi Thu Thuy (2024). **Isolation and characterization of antagonistic bacterial strains for biocontrol of collar rot (*Aspergillus niger*) in groundnut (*Arachis hypogaea* L.).** *Chiang Mai J Sci 2024* 51(2): e2024022.

https://doi.org/10.12982/CMJS.2024.022

[3]. Nguyen Xuan Hieu, Nguyen Duc Huy, Nguyen Tien Long, Cao Thi Thuyet, Pham Thi Thuy Hoai and Nguyen Thi Thu Thuy (**2024**). **Controlling soil-borne fungus *Aspergillus niger* in groundnut by optimizing the function of isolated *Bacillus* bacteria**. *Malaysian Applied Biology*, 53(2), 134-144.

https://doi.org/10.55230/mabjournal.v53i2.3012

[4]. Nguyen Xuan Hieu, Nguyen Đuc Huy, Ton Đuc Hong, Nguyen Tien Long, Thai Thi Huyen, Nguyen Thi Thu Thuy (**2024**). **Evaluation of *Trichoderma asperellum* HL6 against *Aspergillus niger* Van Tieghem causing collar rot in groundnut.** Hue University Journal of Science: *Agriculture and Rural Development*, 133(3C), 41-55.

https://doi.org/10.26459/hueunijard.v133i3C.7496