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VIỆN CÔNG NGHỆ SINH HỌC

DANH MỤC CÁC BÀI BÁO, CÔNG TRÌNH LIÊN QUAN ĐẾN LUẬN ÁN

Tên luận án: Nghiên cứu đối kháng sinh học nấm *Aspergillus* sp. gây bệnh héo rũ gốc mốc đen trên cây lạc (*Arachis hypogaea*) ở tỉnh Quảng Trị và thành phố Huế

Ngành: Sinh học
Mã số: 9420101

NGHIÊN CỨU SINH **NGƯỜI HƯỚNG DẪN KHOA HỌC**

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DANH MỤC CÁC BÀI BÁO, CÔNG TRÌNH LIÊN QUAN ĐẾN LUẬN ÁN

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Identification and characterization of *Aspergillus niger* causing collar rot of groundnut (*Arachis hypogaea*)

XUAN HIEU NGUYEN[1,2], THI MINH NGA NGUYEN[1], DUC HUY NGUYEN[1], QUANG CO NGUYEN[1], THI THUYET CAO[3], THI THUY HOAI PHAM[4], THI THU THUY NGUYEN[5,♥]

[1]Laboratory of Enzyme and Protein, Institute of Biotechnology, Hue University. Thua Thien Hue 49000, Vietnam
[2]Department of Education and Training of Quang Tri province. 136 National Highway No.9, Ward 1, Dong Ha City, Quang Tri 48000, Vietnam
[3]Department of Economic, Faculty of Rural Development, University of Agriculture and Forestry. Hue University Thua Thien Hue 49000, Vietnam
[4]MienTrung Institute of Scientific Research, Vietnam National Museum of Nature, Vietnam Academy of Science and Technology (VAST). Thua Thien Hue 49000, Vietnam
[5]Department of Plant Protection, Faculty of Agronomy, University of Agriculture and Forestry, Hue University. Thua Thien Hue 49000, Vietnam.
Tel.: +84-234-948829130, Fax.: +84 234.3524.923, ♥email: nguyenthithuthuy@huaf.edu.vn; nttthuy@hueuni.edu.vn

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Abstract. *Nguyen XH, Nguyen TMN, Nguyen DH, Nguyen QC, Cao TT, Pham TTH, Nguyen TTT. 2023. Identification and characterization of* Aspergillus niger *causing collar rot of groundnut* (Arachis hypogaea). *Biodiversitas 24: 2556-2562.* Groundnut (*Arachis hypogaea* L.) is an economically important legume crop in Vietnam and many other countries worldwide. Groundnut cultivation is affected by many biotic and abiotic stresses. Among biotic stresses, groundnut is attacked by many fungal, bacterial and viral pathogens. The most harmful fungal diseases are rust, stem rot, collar rot and other soil borne diseases. Collar rot caused by *Aspergillus niger* is one of the most important disease of groundnut extensive in Vietnam and worldwide. In this study, symptomatic infected plant samples were collected from nine different locations in Quang Tri and Thua Thien Hue provinces, Vietnam Isolated fungal species were identified on the basis of morphological characterization, and molecular level. The pathogenicity of fungal species was confirmed on the basis of Koch's postulates. The result showed that a total of nine fungal strains were isolated from infected groundnut samples. Morphological analysis results showed that all isolates exhibited *A. niger* characteristics, including black colony, biseriate conidia, hyaline conidiophores and sterigmata. Pathogenicity test showed that all nine *A. niger* isolates caused collar rot disease in groundnuts. Further molecular identification based on ITS1-4 region nucleotides comparison indicated that selected two isolates, namely QT1 and H17 belonged to *A. niger*. Further research should be done to find antagonistic microorganisms isolates for the bio-control of *A. niger*.

Keywords: *Aspergillus niger*, groundnut, morphological characterization, pathogenicity, phylogenetic analysis

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an annual legume grown in more than 80 countries in tropical, subtropical and warm temperate regions (Desmae et al. 2021; Bajaya et al. 2022). Groundnut seeds contain many nutrients, such as protein, carbohydrates, calcium, and mineral as a source of food for humans, raw materials for industry, and animal feed (Arya et al. 2016; Janila et al. 2016; Desmae et al. 2021). Economically, it is ranks 13th among food crops in the world and tops the list of oilseed crops in both area and production. In 2019, the worldwide groundnut cultivation area reached 29.6 million hectares and the yield reached 48.76 million tons/year. In Vietnam, among short-term industrial crops, groundnut has been grown for a long time and is considered the most important oil crop in terms of production area, yields and export, contributing greatly to the total export value of agricultural products of the country. In 2019, groundnut was grown on 177.040 hectares with an estimated total production of 438.860 ton (FAO 2021).

Groundnut cultivation is affected by a wide range of diseases, such as leaf spot, rust, stem rot, seedling diseases, limb and pod rot, nematode and viral diseases (Janila et al. 2013; Lora and Begum 2019). Among these, groundnut collar rot disease is one of the most serious, destructive diseases causing great yield losses in the many countries such as Ethiopia, Chine, India and Vietnam (Mohammed and Chala 2014; Le et al. 2018a; Lora and Begum 2019; Nathawat et al. 2021). *Aspergillus niger* van Tieghem is a soil-borne pathogen associated with black collar rot disease of groundnut (Rohtas et al. 2016; Dighule et al. 2018; Bajaya et al. 2022). The fungus causes rotting in seed, seedling and mature plants, and thereby drastically reduces plant growth (Kumar et al. 2020). In moist soil, seeds may be attacked and killed due to rotting. Seeds collected from infected soil typically show black sooty cover. The infected areas of seedlings are covered with black fungal spores. Symptom in seedling is usually rapid desiccation of the entire plant. The entire collar infected region becomes dark brown and is subsequently covered with black fungal spores. In infected mature plants, lesions develop on the stem just below the soil surface and then spread upward along the branches. This results in permanent wilting of branches of the entire plant. Dead and dried branches are easily detached from the disintegrated collar region and

infected pods reveal patches of black sooty spores (Kumar et al. 2020; Shricharan et al. 2020). Annual world yield loss caused by collar rot disease is more than 10% (Kumar et al. 2020) and is more prevalent in soils with low moisture content and high temperature (i.e., approximately 30°C) (Guchi et al. 2014). In Vietnam, collar rot disease is the most devastating diseases in many regions. Plant mortality due to collar rot ranges from 9 to 30%, because most of groundnut cultivars are susceptible to the disease. The disease is emerging as a major and wide spread problem in the central provinces of Vietnam (Nguyen et al. 2004; Le et al. 2018a; Le et al. 2018b). Therefore, accurate identification of fungus species that causing collar rot disease of groundnut can provide a useful guide to effective control this disease.

Fungal identification can be carried out using morphological and molecular characterizations. However, morphological identification by comparing the characteristics of isolates has certain limitations in species identification. Currently, molecular identification may be performed by sequencing the Internal Transcribed Spacer (ITS). ITS sequencing has been widely utilized in fungal molecular characterization at the species level (Schoch et al. 2012; Xu et al. 2015; Hawaladar et al. 2022). The purpose of this study was to identify the fungal species causing black collar rot disease of groundnut based on morphological characterization, and ITS sequence analysis.

MATERIALS AND METHODS

Isolation and morphological identification of fungi

Symptomatic plant of black collar rot disease of groundnut (Figure 1.A) were collected from the groundnut fields in Quang Tri and Thua Thien Hue (Figure 1.B) provinces, Vietnam. Samples were collected from five different fields in Quang Tri and four different fields in Thua Thien Hue. For each field, five symptomatic plants were collected into paper bags and transported to the laboratory and then stored at 4°C until used. Plant samples were surface sterilized in 70% ethanol for 1 min, and then rinsed thoroughly with sterile distilled water for 1 min. Finally, samples were dried on sterile blotting paper inside the laminar airflow chamber. Collar measuring of about 3 mm in diameter were then cut out and inoculated onto Petri plates containing Potato Dextrose Agar (PDA) medium supplemented with streptomycin sulfate (50 µg/mL). All plates were incubated at 25 ± 2°C for 7 days and the growth of mycelia was analyzed daily. Mycelia tips growing out of the collar fragments were transferred to new PDA, and then transferred to PDA slants, sub-cultured, and stored at 4°C for further use.

Fungal isolates were identified based on comparison of morphological characteristics (hyphae, conidiophore, conidia, and vesicle) of fungi with descriptions available in the literature (Samson et al. 2014; Xu et al. 2015; Abdelghany et al. 2017; Bajaya et al. 2022).

Pathogenicity test

A total of nine fungal isolates were tested for their pathogenicity. The pathogenicity test was conducted using 14 days-old seedlings of groundnut (variety L14) in the net house at the Institute of Biotechnology, Hue University. The pathogenicity test was carried out using modified method of Kumar et al. (2020). Groundnut seeds were grown in plastic pots (35×30×30 cm) containing 5 kg of sterilized soil. Three seeds were planted in each sterilized pot and irrigated with tap water. The experiment was conducted in a randomized complete block design with five replicates of each fungal isolates. Nine isolates were cultured on PDA and incubated for seven days at 25 ± 2°C. Inocula were prepared by pouring sterilized distilled water into the plates with the fungal cultures and scraping the colonies with a sterile inoculating loop. The colony suspensions were filtered through sterile cheesecloth, and spore suspensions were counted with a Neubauer chamber under a light microscope and adjusted with sterile water to the final concentration of 1×10^5 spores mL^{-1}. Fungal suspension was sprayed into the soil around the root base of soil inoculation technique and each pot was inoculated with 20 mL of fungal suspension. Inoculation pots of different fungal isolates were spaced 50cm apart to avoid cross-contamination. The pots were then maintained in a net house. Control plants were sprayed with 20 mL of distilled water without fungal inoculum. Disease incidence (DI) and disease severity (DS) at 14 days after inoculation (DAI) were calculated as follows:

DI was calculated based on the formula:

DI= [diseased plants ÷ total number of plants] x 100

DS was calculated based on the formula:

Where: DS = [(1× number of plants rated as scale 1) + (2 × number of plants rated as scale 2) + (3 × number of plants rated as scale 3) + (4 × number of plants rated as scale 4)] × 100 ÷ (4 × total number of plants).

DS was rated on a scale from 0 to 4 with 0: No disease symptoms, 1: Disease symptoms without visible outgrowth of the fungus, 2: Disease symptoms with visible outgrowth of the fungus, 3: Partial wilting of the plant, and 4: Complete wilting and plant death.

Molecular characterization

DNA extraction, PCR amplification and sequencing

Pure fungal isolates were cultured on a PDA medium, incubated at room temperature for seven days, and used as inoculum source. The fungal mycelia grew on PDA medium was cut and transferred onto the PDB medium (Potato Dextrose Broth), and the cultures were shaken on a shaker at 125 rpm for 3 days. Subsequently, the growing mycelia of each fungal isolates were harvested for DNA isolation. Total fungal DNA extraction was performed using the TopPURE Genomic DNA extraction KIT (ABT Biological Solution Company Limited, Viet Nam) following the manufacturer's protocol.

The fungal ITS regions were amplified using universal primer pairs ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR reaction was performed in a total volume of 60 µL, with 30 µL of 2× Go Taq® Green Master Mix (M7502, Promega, USA), 5 µL of ITS1 (10 µM), 5 µL of ITS4 (10 µM), 2.5 µL of template DNA and 17.5 µL of sterile double-distilled water. The PCR cycling protocol consisted of initial denaturation at 95°C for 10 min and 30 cycles of denaturation, annealing and elongation at 94°C for 1 min, 53°C for 30s, and 72°C for 1 min, respectively which was followed by a final elongation step of 72°C for 10 min. After amplification, an aliquot was analyzed by running it on a 1% (w/v) TAE agarose gel stained with SafeView™ Classic Nucleic Acid Stain (Applied Biological Materials Inc., Canada). The amplified PCR products were sent to Firstbase Company (Malaysia) for sequencing.

The complete sequences were assembled using the DNA Baser V.4 program. Then, a search for homologous complete sequences against nucleotide database on NCBI was performed using BLAST analysis (http://ncbi.nlm.nih.gov/BLAST). The highest similarity nucleotide sequences including *A. niger* CBS121.55 (MH857404), *A. niger* CBS122.55 (MH857405), *A. niger* D.1 (GU183162), *A. niger* ATCC16888 (NR111348), *A. niger* RMUAN75 (MT541882), *A. niger* CBS 128802 (MH865152), *A. niger* KT 5 (LC105682) and other less relative nucleotide sequences including *A. oryzae* NRRL447 (NR135395), *A. oryzae* A540 (KX462996), *A. flavus* ATCC16883 (MW513940), *A. nidulans* ATCC10074 (KC146354), *A. nidulans* FCSRL3 (ON383939), *A. versicolor* S66 (KU318417), *A. versicolor* ATCC 9577 (AY373880), *A. fumigatus* ATCC1022 (NR121481), and *A. fumigatus* S2-9 (JX537971) obtained as search results were used as references species to construct phylogenetic tree of the 2 isolates using MEGA software (Ver. 7.0). *Penicillium janthinellum* CBS 340.48 (GU981585) was used as outgroup control. The robustness of the internal branches was also assessed with 1000 bootstrap replications.

Data analysis

All quantitative data were statistically analyzed using Analysis of Variance (ANOVA). Statistical significant differences ($p < 0.05$) between treatments were analyzed by ANOVA followed by the Duncan multiple range tests using statistical software SPSS Statistics, Chicago.

RESULTS AND DISCUSSION

Isolation and identification of fungi

In this study, a total of nine fungal species were isolated from different infected groundnut plants grown in various agro-ecological zones of Quang Tri and Thua Thien Hue provinces (Table 1). The fungal isolates grew rapidly on PDA medium and colonies had reached 9 cm six days after inoculation. Macroscopic observation of colonies on PDA were initially in white color, which quickly became black and the edge of colony was occasionally yellowish white color. The reverse side was white or pale yellow with radial fissures in agar (Figure 2). Collar rot fungus produced white hyphae, but changed to brown after a few days (Figure 3.A). The conidiophores were 300-1000 µm long, smooth and hyaline. The conidiophores were dark color at the apex and produced a globose vesicle which was 130-145 µm in diameter (Figure 3.B). The conidia were globose, rough texture, dark brown color with a diameter of 2-4 µm (Figure 3.E and 3.F). These features were consistent with the description of *A. niger*. Therefore, all isolates were identified as *A. niger* based on cultural and morphological characteristics.

The similar specifications of *Aspergillus niger* was reported by (Gautam and Bhadauria 2012; Kasfi et al. 2018; Romsdahl et al. 2018; Rodriguez et al. 2019; Kumar et al. 2020; Šimonovicová et al. 2021). Previously, Santoso et al. (2021) identified *A. niger* based on cultural and morphological characteristics, such as black colony, hyaline conidiophore and sterigmata, biseriate conidia. Mahammed and Chala (2014) examined *Aspergillus* species differentiation based on their cultural features like colony color, texture and margins, as well as the size of conidia and conidiophores. Villalba et al. (2018) observed that conidiophores of *A. niger* are smooth-walled, hyaline or turning dark towards the vesicle; conidia are brown, then become dark brown to black and rough-walled. *Aspergillus niger* isolates were identified morphologically, based on unique characteristics, including slender conidiophores bearing small brown spherical vesicles with black pigmented spore heads containing numerous large, globose conidia on phialides and metulae.

Table 1. Morphological characterization of nine fungal species isolated from symptomatic groundnut plants

Diseases	Origin	Isolates	Host and varieties	Symptoms
Collar rot	Trieu Phong, Quang Tri	QT1	Groundnut, L14	Seedling was covered with black fungal spores, dried and dead plant
Collar rot	Darkrong, Quang Tri	QT2	Groundnut, L16	Yellowing leaves, collar rot
Collar rot	Cam Lo, Quang Tri	QT3	Groundnut, TB15	Plant was covered with black fungal spores, dried and dead plant
Collar rot	Hai Lang, Quang Tri	QT4	Groundnut, Sen Lai	Plant was covered with black fungal spores, dead plant
Collar rot	Dong Ha, Quang Tri	QT7	Groundnut, L14	Yellowing leaves, collar rot
Collar rot	Phong Dien, Thua Thien Hue	H6	Groundnut, TK10	Plant was covered with black fungal spores, dried and dead plant
Collar rot	Quang Dien, Thua Thien Hue	H17	Groundnut, L16	Yellowing leaves, collar rot
Collar rot	Huong Tra, Thua Thien Hue	H18	Groundnut, L14	Plant was covered with black fungal spores, dead plant
Collar rot	Phu Vang, Thua Thien Hue	H19	Groundnut, Ran	Plant was covered with black fungal spores, dried and dead plant

Pathogenicity test

In the net house, nine *A. niger* isolates were inoculated to healthy groundnut plants variety L14 using soil inoculation technique. The results showed that symptoms in artificially inoculated plants were similar to those visualized in naturally infected plants during survey (Figure 4.A). The first symptom was the appearance of brown spots appears on the stem just above the soil level. The affected portion became soft and rotten, leaves and branchesfall off, resulting to death of the whole plant. The dead and dried branches were easily detached from the collar region (Figure 4.B). The infected areas of plants were covered with black fungal spores. The un-inoculated plants (control) were free from any diseased symptoms (Figure 4.A). Symptoms developed on the plants were recorded and fungus was re-isolated from diseased plants thus pathogenicity of respective fungal isolates was confirmed. All the nine isolates were able to cause collar rot symptoms to varying degrees over the period of investigation. The time for the first symptoms to appear ranged from 3-6 days and no statistically significant differences ($p>0.05$) in disease incidence were found between the isolates. However, the two isolates QT1 and H17 showed the highest disease severity (100%) and were statistically different from other isolates ($p\leq0.05$) (Table 2). Several studies emphasized the importance of using combined morphological characteristics and pathogenicity assays for accurate identification of *Aspergillus* species (Rani et al. 2018; Kumar et al. 2020).

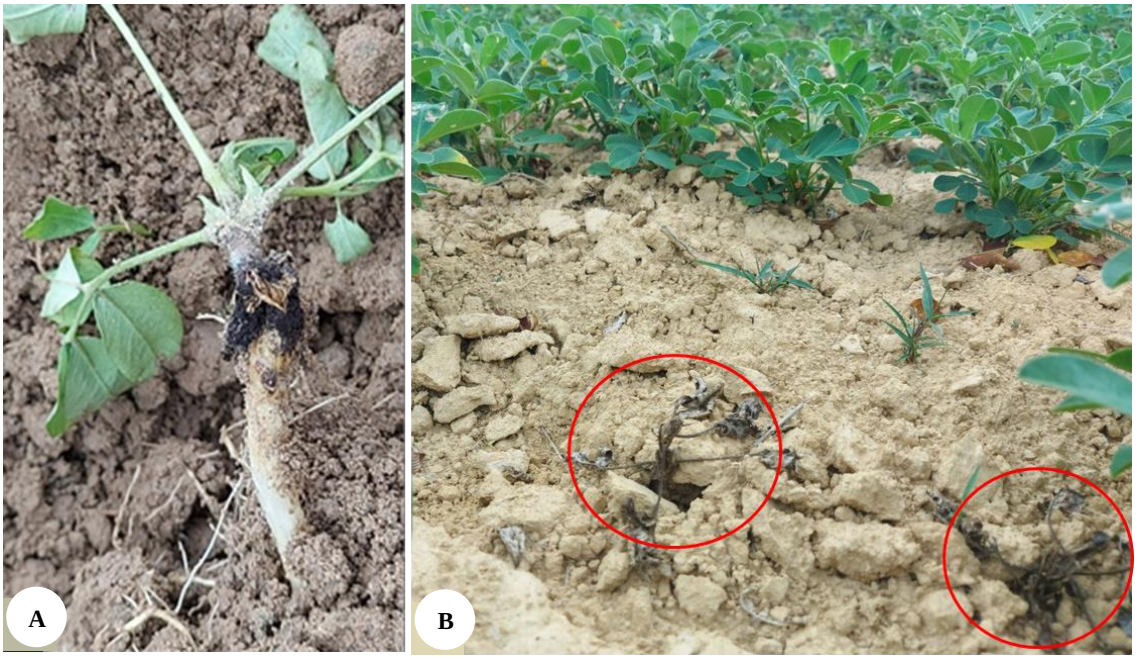


Figure 1. Collar rot symptoms observed on L14 cultivar groundnut on the field. A. A close-up image of plant exhibiting rot symptom on collar, B. Plant exhibiting rot symptoms on collar in Quang Tri province (Red circle indicates diseased plant with collar rot disease of groundnut)

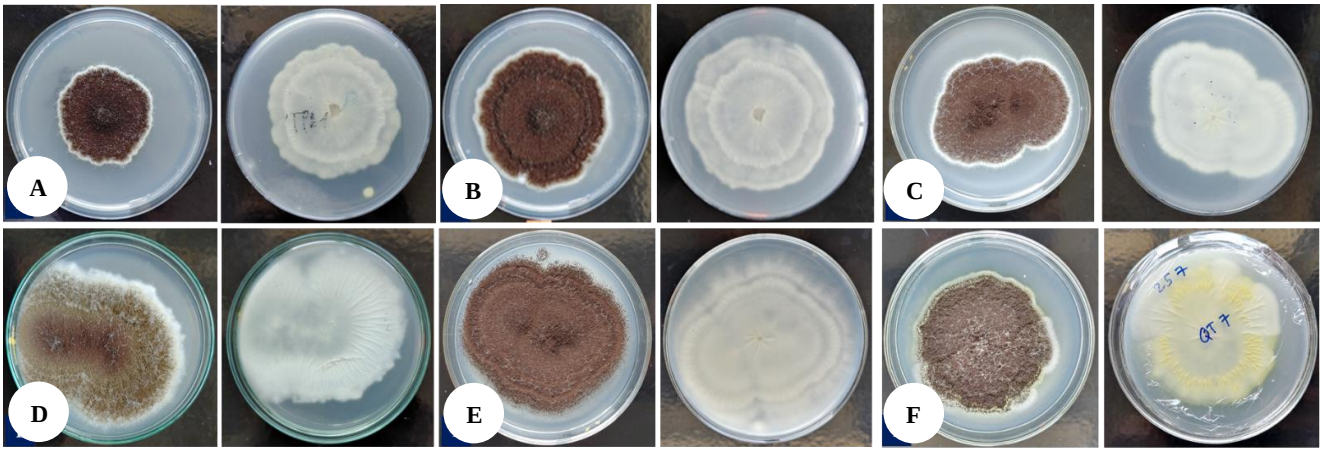


Figure 2. Cultural characteristics of *Aspergillus niger* isolates: (A-F) Colonies on PDA medium

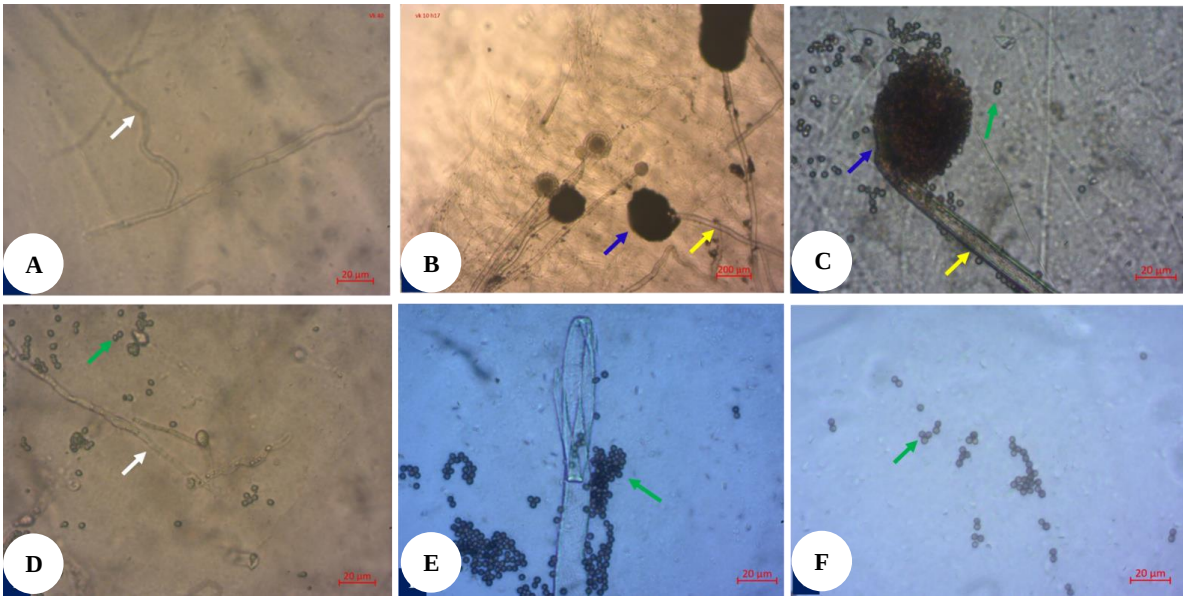


Figure 3. Morphological characteristics of *Aspergillus niger* isolates A-F: Microscopic observations (x 40 magnification). White arrows indicate fungal hyphae, blue arrows indicate vesicle, yellow arrows indicate conidiophores, green arrows indicate conidia, and red bars indicate size references (scalebar = 20 µm)

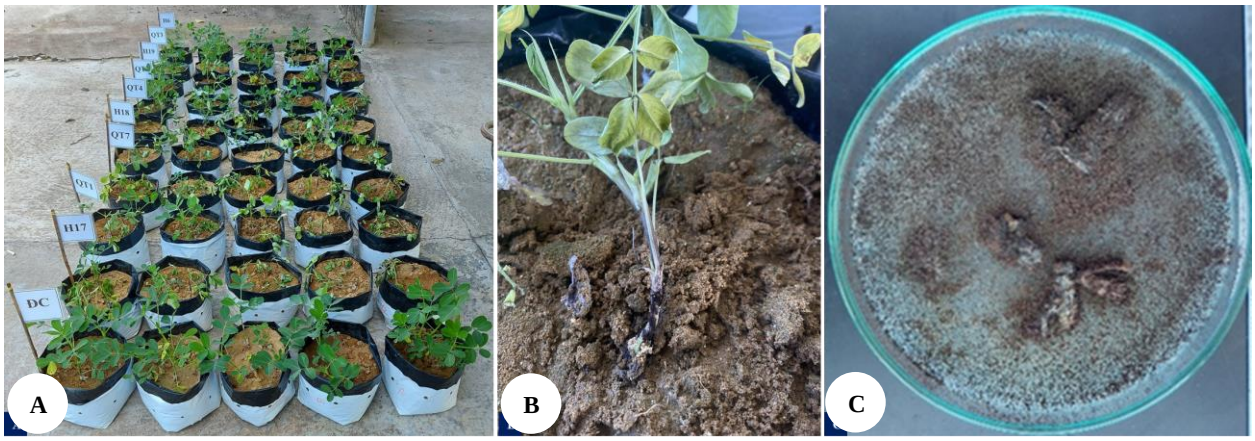


Figure 4. Pathogenicity test on healthy groundnut plants. A. Inoculation of nine fungal isolates on healthy plants (10 days after inoculation), B. Plant exhibiting rot symptom on collar after fungal inoculation, C. Re-isolation of *A. niger* from infected plants

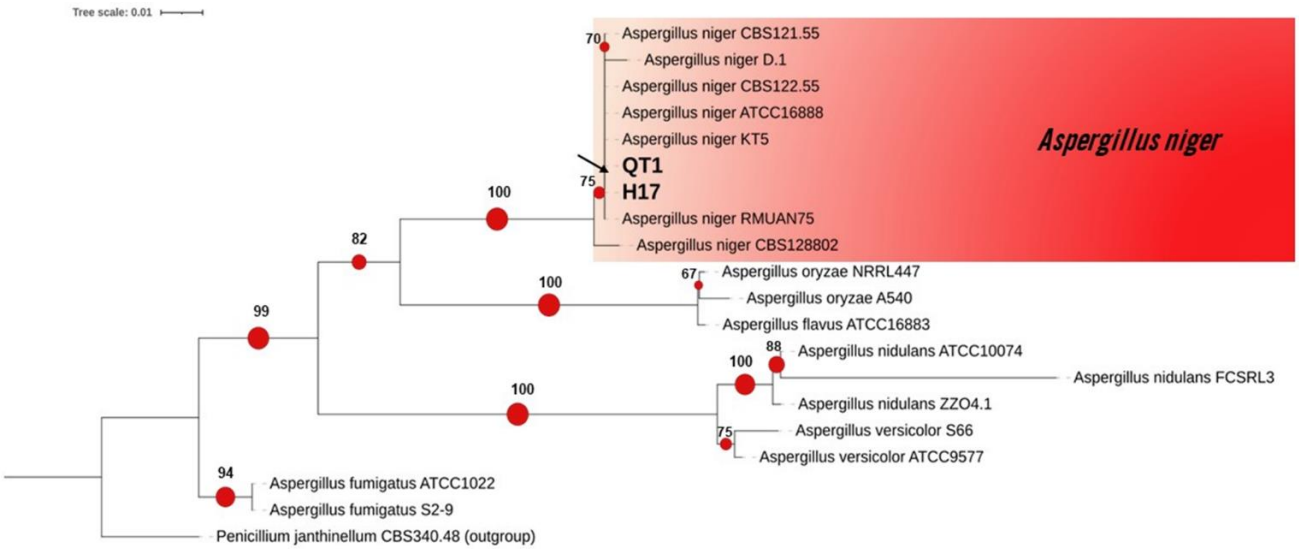


Figure 5. Phylogenetic tree based on ITS sequences of two *Aspergillus niger* isolates (QT1 and H17)

Table 2. Disease Incidence (DI) and Disease Severity (DS) of collar rot on groundnut plants in pathogenicity experiment at 14 DAI

Inoculation with isolate codes	The time of the first symptoms appeared (day)	Disease incidence (%)	Disease severity (%)
QT1	3-4	100.0[a]	100.0[a]
QT2	3-4	100.0[a]	95.6[b]
QT3	5-6	100.0[a]	86.0[c]
QT4	3-4	100.0[a]	97.7[ab]
QT7	3-4	100.0[a]	98.0[ab]
H6	4-6	100.0[a]	85.7[c]
H17	3-4	100.0[a]	100.0[a]
H18	4-5	100.0[a]	97.8[ab]
H19	4-5	100.0[a]	95.6[b]
Control	0	0.0[b]	0.0[d]

Note: Averages of five replications. For each column, different letters indicate a statistically significant difference between the treatments (p = 0.05, Duncan Multiple Range Test)

Molecular characterization

Results showed that nine tested fungal isolates had similar morphological characteristics and also proved their pathogenicity. For molecular characterization, only two isolates, namely QT1 and H17, were selected because they showed the highest disease incidence and severity. DNA from two isolates were successfully amplified using universal primer pairs ITS1 and IST4 targeting the ITS region. The total size of ITS1 and ITS4 regions, including the 5.8S rDNA gene of the isolates studied was 599 bp. The ITS sequences for *Aspergillus* isolates QT1 and H17 were compared against the sequences in the NCBI nucleotide database using BLAST tool to confirm the identity of fungal species. The BLAST results indicated that two isolates were identified as *A. niger.* Phytogenetic tree of the fungal isolates revealed that only two isolates were clustered into group of resemblance (Figure 5). More specifically, *A. niger* H17 and QT1 strains were most closely related to the reference sequence *A. niger* strain KT5, RMUAN75, ATCC16888, CBS 122.55 and D1 from NCBI database. The query cover of isolates identity was 100% homology to that of *A. niger.* This indicates that both isolates belonged to *A. niger* species. The nucleotide sequences of ITS1-4 regions had been deposited in the NCBI database as ascension numbers of OQ561194 and OQ561195 for *A. niger* H17 and *A. niger* QT1, respectively.

Morphological approaches can be used for species identification, however this method is not appropriate because the high degree of accuracy is required at the species level where most isolates of the same genus are nearly identical. In addition, species *Aspergillus* is one of the most complex and difficult to classify and identify (Šimonovicová et al. 2013; Rodriguez et al. 2019). *Aspergillus niger* species are very similar morphologically and in many cases, are phenotypically indistinguishable and can only be reliably identified by molecular characterization (Susca et al. 2016; George and Ramteke 2019). Therefore, consistent and reliable pathogen identification is an essential indicator in plant disease epidemiology and the development of management strategies (Singha et al. 2016; Umesha et al. 2016; George and Ramteke 2019; Alshehri and Palanisamy 2020; Ezeonuegbu et al. 2022). Currently, molecular methods focusing on genotypic characteristics are used to confirm identification method and accelerate detection of species (Loeffler et al. 2000; Singha et al. 2016; Lin et al. 2018; Rodriguez et al. 2019; Guo et al. 2021; Ezeonuegbu et al. 2022). Dania et al. (2021) successfully identified 12 strains of *A. niger* causing white sweet potato (*Dioscorea rotundata* Poir) postharvest rot based on morphology, molecular identification and phylogenetic analysis. Similarly, Guo (2021) used morphological characterization along with PCR amplification and genome sequencing of ITS regions to identify *Aspergillus tubingensis* causes the rotten fruit of pomegranate (*Punica granatum* L.). In conclusion, the results of present study revealed that all nine isolates were capable of causing collar disease in groundnut. Molecular results showed that two isolates, namely QT1 and H17 showed 100% similarity with *A. niger*. Accurate information about collar disease is needed to develop an effective disease management strategy. Further research should be done to find antagonistic microorganisms isolates for the bio-control of *A. niger*.

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Research Article

Isolation and Characterization of Antagonistic Bacterial Strains for Biocontrol of Collar Rot (*Aspergillus niger*) in Groundnut (*Arachis hypogaea* L.)

Nguyen Thi Minh Nga [a], Nguyen Xuan Hieu [a,b]*, Nguyen Tien Long [a], Nguyen Duc Huy [a] and Nguyen Thi Thu Thuy [c]*

[a] Institute of Biotechnology, Hue University, Thua Thien Hue 49000, Vietnam

[b] Department of Education and Training of Quang Tri province. 136 National Highway No.9, Ward 1, Dong Ha City, Quang Tri 48000, Vietnam

[c] Department of Plant Protection, Faculty of Agronomy, University of Agriculture and Forestry, Hue University, Thua Thien Hue 49000, Vietnam

*Author for correspondence; e-mail: nguyenthithuthuy@huaf.edu.vn; nttthuy.huaf@hueuni.edu.vn; xhieunhl@gmail.com

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ABSTRACT

Aspergillus niger is a pathogenic fungus causing collar rot disease in groundnut (*Arachis hypogaea* L.). The species is currently recognized as one of the most severe threats to groundnut worldwide, negatively impacting on productivity and quality of groundnut. This study was conducted to isolate bacterial strains capable of inhibiting the pathogenic *A. niger* QT1 strain as well as the production of some extracellular cell wall hydrolytic enzymes. A preliminary screening resulted in a total of 138 bacterial isolates exhibiting endo-**β-1**,3-glucanase activity. The second screening resulted in 10 isolates showing strong antagonistic activities against *A. niger* QT1 of 41.05 to 61.6% inhibitory ability. Among these, five isolates produced high cell wall hydrolytic enzymes, including endo β-1,3-glucanase, chitinase, and cellulase. Using the nucleotide sequence of 16s rRNA as a marker, it was revealed that these isolates were identified as *Bacillus velezensis* (2 strains), and *Bacillus siamensis* (3 strains). The *in vivo* evaluation of the potential application for treatment of *A. niger* QT1 demonstrated that all five strains effectively controlled collar rot disease of groundnuts. The highest efficacy was observerd in the treatment supplemented with *B. velezensis* 137 with no groundnut death. The present results strongly supported for the application of bacteria as biocontrol agents in order to replace the use of chemical plant fungal pesticides.

Keywords: *Aspergillus niger*, collar rot disease, groundnut, antagonistic activity, inhibition

1. INTRODUCTION

The groundnut (*Arachis hypogaea*), also known as the peanut, is a species of plant in the legume family (Fabaceae), planted from South America to Europe, Africa and Asia [1]. Groundnut is an important oil seed crop worldwide and has been used extensively for feed and food [2-3]. Groundnut is considered a rich source of protein, vitamin, and lipid oil and is among the most globally consumed

oilseeds. In particular, groundnut contains high levels of oleic acid, low levels of saturated fatty acids and exhibits strong antioxidant activities. On the other hand, groundnut also enriches with vitamins, including thiamine, vitamin E, riboflavin, niacin, folate, and vitamin B6 [4].

Moreover, groundnut kernel contains various metal ions such as potassium, magnesium, and bioactive components, including polyphenols and phytosterols, which are associated with decreasing the risk of various diseases [3-4]. Thus, groundnut is one of the most economic importance among food crops worldwide and tops the list of oilseed crops in both area and production. As of 2021, China produced over 18.3 million metric tons of peanuts, while India produced more than ten million metric tons [5]. In Vietnam, groundnut has been cultivated with a long history, and productivity reached 430.4 thousand tons by 2021 [5].

Diseases are the primary threat to the production of groundnut worldwide. The wide range of diseases, such as stem rot, rust, leaf spot, limb and pod rot, seedling diseases, nematode and viral diseases, negatively affected crop productivity [6-7]. For instance, collar rot disease caused by *Aspergillus niger* van Teighem has been reported as the most severe problem, losing an average from 13 to 52% total productivity yield or can be as high as 93.6% in some areas [8-9]. The initial symptom of the disease is brown spots on the stem and roots. Infected plants are stunted and wilt suddenly. In severe infection plants, infected tissues exhibit brown discoloration and rotting, and numerous dark brown and black chalky spores can be seen on the surface of the affected parts. Eventually, the affected plants fell and died [10]. *Aspergillus niger* is also a common contaminant factor in food and agricultural products, causing a loss of economic value and health safety problems. For instance, *A. niger* produces toxic secondary metabolites such as ochratoxin [11-12] and fumonisin [13]. Ochratoxin is toxic to kidneys, carcinogenic and teratogenic in animals, including humans. Meanwhile, mycotoxin causes lowered performance, sickness or death in humans and animals [14]. Thus, the safety food are required to eliminate *A. niger* contamination and prevention of *A. niger* infection in groundnut plays an important role in sustainable cultivation for farmers [15].

Chemical fungicides have been extensively used to control fungi pathogens such as *Rhizoctonia solani*, *Collectotrichum*, *A. niger*, *Fusarium* sp. However, using chemical fungicides with acute or chronic toxicity negatively affects organisms in nature [16]. The extensive use of chemical fungicides also raises concerns for public health and the ecosystem. There has been reported that chemical fungicides caused haematological, histopathological, biochemical, and endocrine-disrupting effects in rats [17]. Furthermore, using chemical fungicides has also developed and formed phytopathogen fungicide resistance. Therefore, numerous fungicides are classified as hazardous chemicals by the World Health Organization (WHO) and are being eliminated in many countries worldwide [16]. Biological control of plant diseases using antagonistic bacteria is a promising alternative to replace chemical management strategies [18]. Some of the antagonistic bacteria have been commercialized. European Commission admitted a group based on the inhibitory capacity of bacteria named biopesticides for disease control [19]. Therefore, exploring the application of antagonist bacteria and their active metabolites attracts researchers to finding a sustainable strategy for management phytopathogenic fungi in agriculture.

The fungal cell wall is one of the most crucial barriers to a resistant mechanism of fungal pathogens [20]. The cell wall of the *Aspergillus* genus is mainly consisted of glucans, chitin and glycoproteins in which 1,3-β-glucans being the most abundant polysaccharide [21]. Therefore, agents showing activity against cell wall synthesis or toward cell hydrolysis can be a good candidate for harmful fungi control [22]. Other microorganisms can efficiently hydrolyze the cell wall of fungi through the action of cell wall hydrolytic enzymes, including chitinase, β-1,3-glucanase, and cellulase

[23]. Thus, identifying bacteria with antagonistic activity associated with extracellular hydrolytic enzyme secretion is a promising strategy for finding a broad range of inhibition against the pathogenic fungi.

2. MATERIALS AND METHODS

2.1 Materials

The pathogenic strain *A. niger* QT1 caused collar rot diseases was provided by the Laboratory of Enzyme and Protein Technology at the Institute of Biotechnology, Hue University. This strain was isolated from peanut plants infected with collar rot disease and was identified as *A. niger* based on morphological characteristics, Koch's postulates, and molecular biology [24]. Groundnut variety L14 was provided by the Institute of Biotechnology, Hue University.

2.2 Methods

2.2.1 Sample collection

Soil samples were collected from groundnut rhizosphere of fifteen farming fields at Quang Tri provinces, Vietnam. Five soil cores (25cm diameter x 25cm in depth) were collected and placed in clean plastic bags for each sampling site. The samples were stored at 4°C before conducting further experiments.

2.2.2 Identify of potential endo-1,3-β-glucanase producing bacteria

To identify the group of potential endo-1,3-β-glucanase producing bacteria, the soil samples were mixed with distilled water in a ratio of 10:90. A serial dilution were conducted, and 100 µL of each dilution ranging from 10^{-5} to 10^{-8} were spreaded on selective medium agar plates. The specific selection medium were prepared with baker's yeast (5 g), $FeSO_4 \cdot 7H_2O$ (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KCl (0.5 g), $NaNO_3$ (3 g), K_2HPO_4 (1 g), agar (20 g), and water to 1 litter [25]. The plates were incubated at 30°C for 48 h in an oven. The clear zone around the colonies was assessed to indicate glucanase activity. The colonies that exhibited the great clearance zone were selected and sub-cultured on LB medium (1% NaCl, 1% tryptone, 0.5% yeast extract, and 1.5% agar).

2.2.3 *In vitro* screening for antagonist activity

The selected endo-1,3-β-glucanase producing bacterial strains were screened for antagonist activity against *A. niger* QT1. On each petri dish, an agar plug of a 3-day-old mycelium of strain *A. niger* QT1 was placed in the center, and bacterial strain was streaked in a straight line approximately 2 cm from the edge of fungal plugs. After incubation for five days at 30°C, the growth of *A. niger* QT1 mycelium was measured and compared with the average growth of the control without treatment [26]. Each treatment was performed in triplicate. Growth inhibition rate (GIR) was calculated using the following equation [27].

$$GIR\ (\%) = (C\text{-}T)/\ C \times 100$$

C: fungal radial hyphal growth of control samples (mm)
T: fungal radial hyphal growth of treatments (mm)

2.2.4 Hydrolytic Enzymes Production

The strong antagonistic isolates were cultivated on liquid LB medium and incubated at 30°C on rotary incubator (180 rpm) for 24 h. The culture supernatant were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. Two wells with a diameter of 5 mm were made by corkborer in the agar medium. Chytinolytic activity was examined on agar plate containing 0.1% colloidal chitin. Fifty microliters of culture supernatant were added into each well. The plates were incubated at 30°C for 48 h and further stained with Lugol's solution to visualize the chitin hydrolytic zone [28]. Meanwhile, the cellulase activity was assessed by evaluating the culture supernatant on agar plate containing 0.1% carboxymethyl cellulose (CMC). The plates were incubated at 30°C for 48 h. The hydrolytic zones were visualized using 1% Congo

red solution and washed using 1M NaCl solution [28]. Finally, endo-1,3-β-glucanase activity was confirmed using curdlan as substrate. The agar plates were prepared by mixing curdlan into a 2% agar solution to final concentration of 0.1%. The wells were made as described above and 50 µl of culture supernatant were loaded into the wells. The plates were incubated at 30°C for 48 h, and enzyme activity was visualized by staining the plates with 0.1% Lugol's solution and observing the clearance zone [28].

2.2.5 Molecular identification

The isolates were cultured in LB liquid medium overnight, and the cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The DNA was extracted using the ToPURE Genomic DNA extraction KIT (ABT, Vietnam) according to the manufacturer's recommendation. The 16S rRNA sequence was amplified using universal primers of 27_Forward: 5'-AGATTTGATCCTGGCTCAG-3' and 1492_ Reverse: 5'-GGTTACCTT GTTACGACTT-3'. PCR reaction consisted 30 µl of 2X Go Taq® Green Master Mix (Promega, USA), 2.5 µl of extracted DNA, 5 µl of 10 µM of each primer, and 17.5 µl of sterile double-distilled water. The PCR cycle was performed with a preheating for 10 min at 95°C, followed by 30 cycles of 1 min at 94°C, annealing at 53°C for 30 s, and extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR amplicons were evaluated on a 1% (w/v) TAE agarose gel stained with SafeView™ Classic Nucleic Acid Stain (Applied Biological Materials, Canada). The amplified PCR products were sent to a commercial sequencing service (Firstbase, Malaysia) for sequencing.

The DNA sequences obtained from both strands were assembled to obtain consensus sequences for each isolate using BioEdit v7.0.5 [29]. Reference 16S rRNA sequences were searched on NCBI database and were selected as nucleotide sequences from type strains or identified strains in published articles. Sequence alignment was manually performed with related reference sequences using ClustalW2 software (http:// www. ebi. ac. uk/Tools/ phylogenecy/ clust alw2- phylo geny/) using default parameters. The aligned nucleotide regions were manually adjusted using MEGA 11 [30]. A phylogenetic tree was constructed according to the maximum likelihood method using IQ-TREE v. 2.1.3 [31]. ModelFinder was used to determine the best fit model [32]. Branch support was determined using 1,000 bootstraps and Bayesian posterior probability support. The resulting trees were plotted using Interactive Tree of Life (iTOL) v.6 [33]. New sequences generated in this study were deposited in GenBank (https://www.ncbi.nlm.nih.gov)

2.2.6 *In vivo* evaluation for antagonistic activity

Healthy groundnut plants were grown on a plastic pot containing 150 kg of clay loam soil collected from a groundnut field mixed with 1 kg CaO and 0.5 kg NPK fertilizer (Dau Trau, Vietnam). The pots were washed thoroughly with tap water and allowed to dry before use. Groundnut seeds were grown in plastic pot (size 35 x 30 x 30 cm) containing 5 kg of sterilized soil. Five seeds were planted in each pot. All pots were placed under net-house conditions at the Institute of Biotechnology, Hue University, Vietnam.

Preparation of *A. niger* QT1 spore for artificial infection: 14 day-old plants (four leaf stage) groundnut plants were inoculated with spore suspensions as described previously in Kumar et al. (2020) [9]. *A. niger* QT1 was cultured on petri dishes with PDA medium and incubated for seven days at 28°C ± 2. Sterilized distilled water was added into the plates and fungal cells were scraped with a sterile inoculating loop. The cells suspensions were filtered through sterile filter papers, and fungal spore were counted with a Neubauer chamber under a light microscope. The fungal cells were adjusted to the final concentration of 1×10^6 spores ml^{-1} for further investigation.

Preparation of antagonistic bacteria for pot infection study: bacterial strains K1, 101, 137, B8, and 112 activated in LB medium and incubated at 30°C for 24h at 180 rpm. The concentration of cell suspension was adjusted according to the 0.5 McFarland scale until reaching approximately 10^7 CFU/ml with sterile distilled water. One-hundred milliliters of cell suspension of each bacterial strain were prepared for the experiment to evaluate the ability to inhibit *A. niger* QT1 of the antagonistic bacterial strains.

The experiment was conducted in a randomized complete with three replications and 5 plants per replication. The treatments were as follows:
T1: Plants were uninfected *A. niger* QT1, and groundnut seeds and plants were treated with sterile water (Uninfected plants)
T2: Plants were infected *A. niger* QT1, and groundnut seeds and plants treated with sterile water (Negative control)
T3: Plants were infected *A. niger* QT1, groundnut seeds and plants were treated 101 strain
T4: Plants were infected *A. niger* QT1, groundnut seeds and plants were treated K1 strain
T5: Plants were infected *A. niger* QT1, groundnut seeds and plants were treated B8 strain
T6: Plants were infected *A. niger* QT1, groundnut seeds and plants were treated 137 strain
T7: Plants were infected *A. niger* QT1, groundnut seeds and plants were treated 112 strain
T8: Plants were infected *A. niger* QT1, and groundnut seeds and plants were treated Ridomil Gold 68WG (Metalaxyl M + Mancozeb) (Syngenta Company, Vietnam) (Positive control)

Groundnut seeds were soaked in 2 ml of cell suspension of antagonism bacteria ($1x10^8$ CFU/ml) for 1 h, then 5 seeds were sown in each pot. For the treatments control, the groundnut seeds were treated sterile water. Fourteen days after sowing (4 leaf stage), each pot sprayed 20 ml of cell suspension of antagonism bacteria at $1x10^8$cfu/ml into the soil around the roots. Then, after 1 day, each pot was inoculated 20 ml of *A. niger* spore suspension $1x10^6$ spores/ml into the soil around the roots. The same solution without fungal inoculum was used to set the control plants. Then place the pots in the net-house.

Observations and recordings were made of the mortality rate (MR) at 7, 14, 21 and 42 days after inoculation (DAI).

MR (%) = (Number of dead plants/ Total number of plants observed)*100

2.3 Statistical Analysis

All experiments were performed in triplicates and the data were expressed as the mean ± standard deviation. The *in vivo* trial were performed in a randomized design. Data were evaluated using analysis of variance (ANOVA) with the SPSS software version 22 (https://www.ibm.com/products/spss-statistics). The significant difference was accessed by the Ducan's test at the level of $p < 0.05$.

3. Results and Discussion

3.1 Isolation of the Potential endo-β-1,3-Glucanase Producing Bacteria

β-1,3-glucans are importance component in structure of cell wall of *A. niger*. Endo-β-1,3-glucanase is a crucial factor contributing to the defense mechanism of bacteria against *A. niger*. Our preliminary screening resulted in a total of 138 bacterial isolates exhibited endo-β-1,3-glucanase activities. These bacteria were purified by sub-cultured in new plates and used for further investigations.

3.2 *In vitro* Evaluation Antagonist Activity of Isolates

Among of 138 bacteria isolates exhibiting endo-β-1,3-glucanase activity, ten isolates showed the best antagonistic activity against *A. niger* QT1 in *in vitro* (**Table 1**). As shown in **Table 1**, ten isolates inhibited the growth of *A. niger* QT1 with percentage ranged from 41.05 to 61.6%. The highest antagonistic activity was isolate K1 while the lowest activity was isolate 45K. Meanwhile, five isolates including K1, 101, 137, B8 and 112

Table 1. Antagonistic activity of selected isolates against *A. niger* QT1 after 6 days of incubation.

Sr. No.	Isolate	Growth inhibition rate (%)
1	H1	45.20 ± 0.46^{f}
2	K1	61.60 ± 0.05^{a}
3	K2	49.86 ± 0.13^{e}
4	B8	55.38 ± 0.18^{c}
5	45K	41.05 ± 0.19^{g}
6	46K	45.10 ± 0.03^{f}
7	101	58.60 ± 0.18^{b}
8	112	52.10 ± 0.04^{d}
9	118	46.90 ± 0.06^{f}
10	137	57.14 ± 0.13^{bc}

*Means ± SD in the same column with different letters are significantly different (P <0,05).

exhibited over 50% inhibitory activity against *A. niger* QT1 (**Figure 1**). There was no significant difference in inhibition activities between isolates H1, 46K and 118 (p>0.05).

3.3 Hydrolytic Enzyme Production

The ability on cell wall hydrolytic enzymes production by the bacterial isolates was investigated. **Table 2** showed that all ten isolates exhibited endo-1,3-β-glucanase, chitinase, and cellulase activities. The β-1,3-glucan hydrolysis zone of crude enzymes ranged from 18.50±0.20 to 26.17±0.42mm, whereas diameter of chitin hydrolytic areas were 18.17±0.15 to 26.10±0.1 mm. Meanwhile, the isolate hydrolyzed CMC at the ranging of 17.70±0.62 to 25.77±0.25 mm. Typically, *A. niger* inhibition ratio by the isolates were positively correlated with the lytic enzymes production in liquid culture. The isolates exhibited strongest antagonistic activities, including isolates K1, 101, 137, B8 and 112 also produced high amounts of endo-1,3-β-glucanase, chitinase, and cellulase. On the other hands, the isolates 45K and H1 secreted the lowest endo-1,3-β-glucanase, chitinase, and cellulase and only exhibited moderate antagonictic activities against *A. niger* QT1. These results demonstrated the cell wall hydrolytic enzyme positively contributes toward the inhibitory ability of biocontrol bacteria.

Due to the negative effects on the use of chemical pesticides, biological control defines as a sustainable alternative to replace chemical pesticides use for fungal plant disease management [34]. In fact, the plant rhizosphere carries different microorganisms producing bioactive compounds which promote the plant growth and control the spread of phytopathogens. The inhibitory abilities of biocontrol microorganisms have been demonstrated in correlation with cell wall hydrolytic enzyme. For example, Zhang et al. (2021) isolated 17 actinomycetes significant exhibited antifungal activity against *Fusarium oxysporum*. Among these, the strain BITDG-11 exhibiting strongest inhibition ability with a broad-spectrum antifungal activity, and also produced high chitinase, β-1,3-glucanase, lipase [35]. Similarly, Ueki et al. (2020) reported a *Clostridium beijerinckii* strain that efficiently inhibits *F. oxysporum* and secretes β-1,3-glucan hydrolyzing enzymes [36]. Besides, Suebsan et al. (2023) reported *Bacillus subtilis* AS80 exhibited high potential against *Fusarium* sp. (61.7%), *Lasiodiplodia* sp. (66.7%) and *Colletotrichum* sp. (74.1%) which caused fruit rot and anthracnose in "Hass" avocado fruit [37]. In the present study, we succesffully isolated ten potential antagonistic bacteria against the fungus

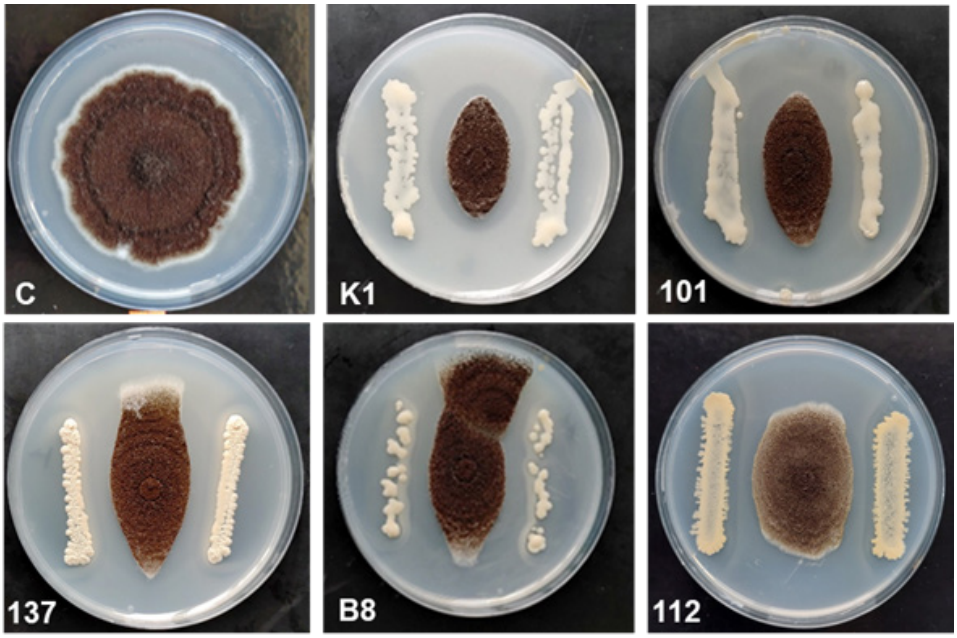


Figure 1. Antagonistic activities of five isolates showing over 50% inhibition toward *A. niger* QT1 after 6 days of inocubation at 30°C. C: Control; K1; 101; 137; B8 and 112: selected isolates.

Table 2. Hydrolytic activities of isolates on different substrates (mm).

C	endo-1,3- β -glucanase	Chitinase	Cellulase
H1	18.50±0.20^{d}	20.47 ±0.15^{c}	17.70±0.62^{d}
K1	24.97±0.15^{ab}	25.83±0.15^{a}	25.47±0.06^{a}
K2	22.77±0.25^{bc}	23.30±0.20^{b}	24.03±0,15^{ab}
B8	26.17±0.42^{a}	26.10±0.10^{a}	25.77±0.25^{a}
45K	20.07±0.31^{cd}	19.37±0.15^{cd}	18.73±0.21^{cd}
46K	22.23±0.38^{bc}	22.93±0.21^{bc}	22.97±0.15^{b}
101	23.20±0.36^{b}	22.53±0.06^{bc}	21.23±0.31^{bc}
112	23.93±0.31^{b}	22.87±0.23^{bc}	21.90±0.79^{bc}
118	21.03±0.15^{c}	18.17 ±0.15^{d}	20.10±0.10^{c}
137	21.03±0.15^{c}	20.47±0.06^{c}	20.67±0.31^{c}

Means ± SD in the same column with different letters are significantly different (P <0,05)

pathogen *A. niger* QT1 which also produced endo-1,3-β-glucanase, chitinase, and cellulase. Our further screening resulted in finding the isolates K1, 101, 137, B8 and 112 significantly inhibited *A. niger* QT1 and produced greatest hydrolytic enzymes amount. Altogether, our result supports the notion that isolation and analysis of microorganisms exhibiting both antagonistic properties and cell wall hydrolytic enzymes may accelerate the development of bio-based products for controlling fungal diseases in crops.

3.4 Identification and Nomemclature of Antagonistic Isolates

The classification of isolates was carried out by analysis the nucleotide sequence of 16S rRNA region that was amplified by primer of 27F and 1492R (see **Methods**). Our analysis resulted in DNA fragments of 1413, 1415, 1396, 1399, and 1415 bp for the isolates 137, K1, B8, 101 and 112, respectively. The phylogenetic tree is shown in **Figure 2**. Generally, all isolates exhibited high similarity to a *Bacillus* supper family including *B. velezensis, B. siamensis* , and *B. amyloliquefaciens*. In particularly, the results revealed that the isolates 137 and K1 were 99,93% similar to *B. velezensis* FZB42 (accession no. NR_075005) [38] and type strains including *B. velezensis* BCRC17467 (accession no. EF433407), and *B. velezensis* NRRLB-41580T (accession no KY694464). Thus, the isolates were named as *B. velezensis* 137 and *B. velezensis* K1. Meanwhile, the isolates B8, 101 and 112 were 100% similar to a *B. siamensis* type strain KCTC 13613 (accession no. KY643639) (**Table 3**). These sequences have been deposited in the NCBI database as accession numbers OR432532, OR432533, OR432534, OR432535, OR432536 for *B. velezensis* K1, *B. velezensis* 137, *B. siamensis* 112, *B. siamensis* 101 and *B. siamensis* B8, respectively.

Up to date, *Bacillus* species are among the most well-known beneficial bacteria to be used as biocontrol agents. These species can reduce the growth of a wide range of fungal plant pathogens [39-42]. The resistance induced by *Bacillus* is involved with the production of defense related enzymes such as peroxidases (POXs),

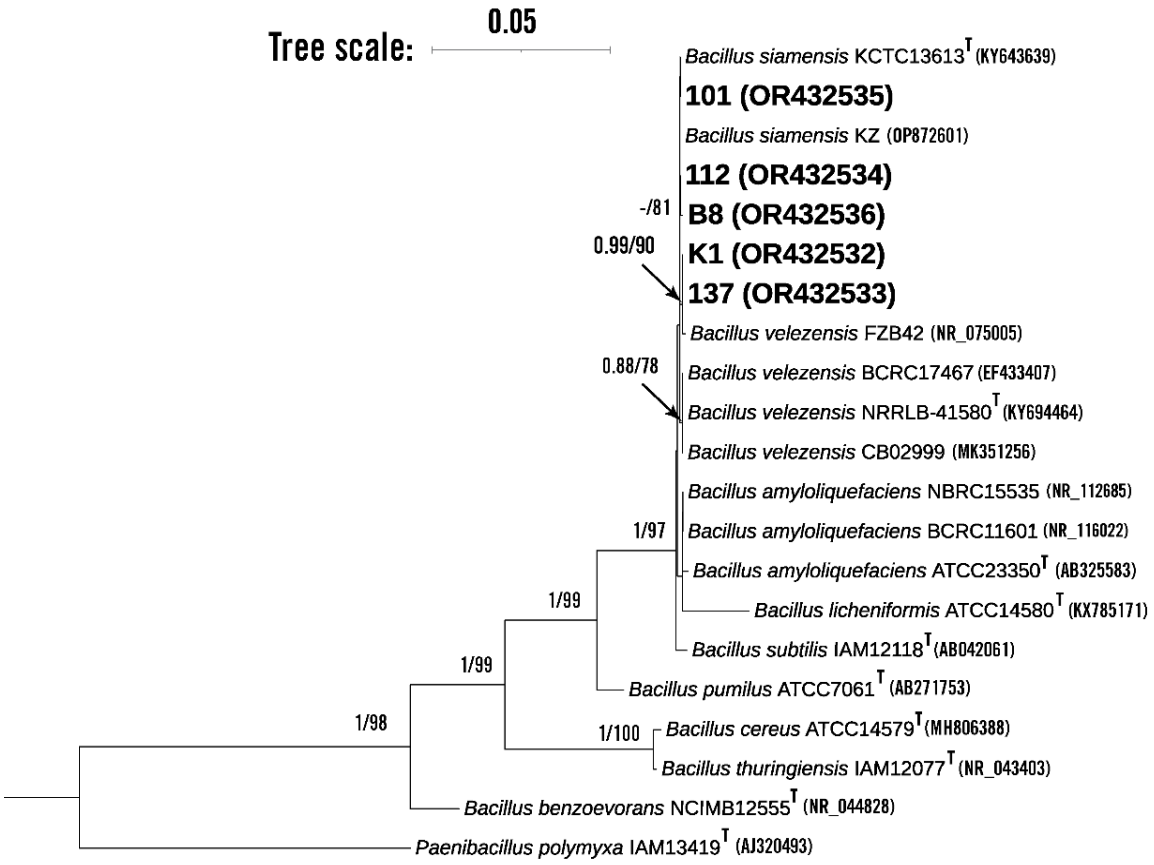


Figure 2. Phylogenetic tree based on nucleotide sequences of 16S rRNA of the potential isolates and reference strains showing the position of the strains among members of the genus *Bacillus*. *Paenibacillus polymyxa* was used as an outgroup control.

Table 3. Similarity index of 16S rRNA regions between isolates and reference strains.

Strains No.	Acession number	Length of 16S rRNA region (Nu)	Number of identical nucleotides	Similarity index (%)	Reference strains
K1	OR432532	1393	1393/1394	99.93	*B. velezensis* FZB42 (NR_075005)
137	OR432533	1393	1393/1394	99.93	*B.velezensis* FZB42 (NR_075005)
112	OR432534	1394	1394/1394	100	*B.siamensis* KCTC 13613 (KY643639)
101	OR432535	1394	1394/1394	100	*B. siamensis* KCTC 13613 (KY643639)
B8	OR432536	1394	1394/1394	100	*B. siamensis* KCTC 13613 (KY643639)

phenylalanine ammonia-lyase (PAL), endo-β-1,3 glucanases, cellulases, lipases, and chitinases [43-45]. *Bacillus* is also well known to secrete various cell wall hydrolytic enzymes [46], and antifungal peptide [47]. Previously, Ashwini and Srividya (2014) showed that *B. subtilis* suppressed the *Colletotrichum gloeosporioides* growth depends on amounts of selected enzymes such as chitinase, glucanase and cellulose [48]. Similarly, Akocak et al. (2015) reported that *B. cereus* and *B. thuringensis* produced chitinase that inhibit the growth of *A. flavus* [49].

3.5 *In vivo* evaluation of Antagonistic Activities

The isolates 101, K1, B8, 137 and 112 were evaluated for their application as bioconcol agents against *A. niger* QT1 using groundnut plants grown in net-house model. The data collected at the day of 7, 14, 21, and 28 after inoculation of plants with *A. niger* QT1, and are presented in **Table 4** and **Figure 3**. The results indicated that five *Bacillus* isolates from our study reduced the collar rot disease development of *A. niger* QT1 on groundnuts. In the negative control group, the mortality rate of groundnuts significantly increased to 60% after 7 days treatment. The groundnuts completed death after 14 day of treatment. Meanwhile, in the uninfected group, groundnut plants grew well and there were no symptom of collar rot disease observed. Among the groundnut plants infected *A. niger* QT1 and then treated with *Bacillus* isolates, the disease symptom developed after 14 days of treatment with a mortality of 6.67%. Thus, the treatment increased the survival rate of groundnuts by inhibiting the growth of *A. niger* QT1. The groundnuts continuously grew well until the day of 28 whereas no death was observered. Interestingly, the supplementation of *B. velezensis* 137 as biocontrol agent completely reduced the effect of *A. niger* QT1 on groundnuts, and all tested groundnuts developed well and compareable to the negative control groundnuts. In contrast, the chemical treatment by plant fungal pesticides (in the positive control group) did not completely prevent the effect of *A. niger* QT1. The groundnuts treated by mixture of Metalaxyl M and Mancozeb exhibited weaker growth compared to those treated with *Bacillus* isolates, in which 33.33% of groundnuts died after 7 day of infection and increased to 46.67% after 14 days of infection. Overall, the lowest mortality rate of groundnuts infected by *A. niger* QT1 treated with *Bacillus* isolates compared to other treatments in our study strongly demonstrated that the antagonistic bacteria are great agents in order to replace commercial pesticide.

In summary, the *in vivo* experiments showed a great enhancement in the development of ground-

Table 4. Effect of five *Bacillus* isolates treatment on mortality rate of groundnut inoculated with *A. niger* QT1 under nethouse condition.

Samples	Mortality rate (%) of groundnut at day after fungal inoculation			
	7	14	21	28
Uninfected plants	0.00[c]	0.00[c]	0.00[c]	0.00[c]
Negative control	60.00[a]	100.00[a]	100.00[a]	100.00[a]
B. siamensis 101	0.00[c]	6.67[c]	6.67[c]	6.67[c]
B. velezensis K1	0.00[c]	6.67[c]	6.67[c]	6.67[c]
B. siamensis B8	0.00[c]	6.67[c]	6.67[c]	6.67[c]
B. velezensis 137	0.00[c]	0.00[c]	0.00[c]	0.00[c]
B. siamensis 112	0.00[c]	6.67[c]	6.67[c]	6.67[c]
Positive control (Metalaxyl M + Mancozeb treatment)	33.33[b]	46.67[b]	46.67[b]	46.67[b]

Value in the same column with different letters are significantly different (P <0,05).



Figure 3. Biological control of collar rot (*A. niger* QT1) in groundnuts by *B. velezensis* 137 under net-house condition with artificial fungal inoculation. A3. Uninfected plants; B3. *B. velezensis* 137 treatment; C3. Negative control; D3. Positive control.

nuts by the *Bacillus* isolates. Among the five *Bacillus* strains tested, we observed that the diseases only affect 6.67% of groundnuts in the presence of *B. siamensis* 101, *B. velezensis* K1, *B. siamensis* B8, and *B. siamensis* 112. Meanwhile, *B. velezensis* K137 completely inhibited the toxic effect of *A. niger*. The supplementation of *Bacillus* isolates as biocontrol agents expressed the higher efficacy than that of chemical fungal pestisides. The results presented here are in accordance with previous reports on using bacteria a biocontrol agents. For instance, previous studies have reported that *B. atitudinis*

JSCX-1 strain improved soybean growth and showed inhibitory activity against *Phytophthora sojae* [50] or *Bacillus* sp. S20D12 inhibited the disease symptoms caused by *Sclerotium rolfsii* and *A. niger* on groundnut *in vivo* [40].

4. Conclusions

In this study we demonstrated that the growth of the grounduts pathogenic fungus *A. niger* QT1 was suppressed by the supplementation of *Bacillus* isolates. These isolates not only expressed the strong inhibition against *A. niger* QT1 but also produced a large amount of cell wall hydrolytic enzymes including endo β-1,3-glucanase, chitinase, and cellulase. The growth of grounduts in the presence of these *Bacillus* isolates, especially *B. velezensis* 137 is comparable to non-disease effection plants. Thus, these bacteria exhibit a great potential for improving plant health and use in developing commercial biocontrol agents for groundnut landfills.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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Research

Controlling Soil-Borne Fungus *Aspergillus niger* in Groundnut By Optimizing The Function of Isolated *Bacillus* Bacteria

Nguyen Xuan Hieu[1], Nguyen Duc Huy[1], Nguyen Tien Long[1], Cao Thi Thuyet[2], Pham Thi Thuy Hoai[3,4], Nguyen Thi Thu Thuy[2]*

1. Institute of Biotechnology, Hue University, Thua Thien Hue 49000, Vietnam
2. University of Agriculture and Forestry, Hue University, Thua Thien Hue 49000, Vietnam
3. MienTrung Institute of Scientific Research, Vietnam National Museum of Nature, Vietnam
4. Academy of Science and Technology (VAST), Thua Thien Hue 49000, Vietnam
*Corresponding author: nguyenthithuthuy@huaf.edu.vn, nttthuy.huaf@hueuni.edu.vn

ABSTRACT

Collar rot is a devastating disease caused by the soil-borne pathogen *Aspergillus niger* that greatly affects groundnut production worldwide. The long-term persistence of the fungus in the soil can reduce the effectiveness of synthetic fungicides. Recently, significant attention has been raised to the use of the biological control method such as the application of antagonistic microorganisms, which potentially decline the number of spores and eradicated *A. niger* from the soil. In the present study, three *Bacillus* strains (*Bacillus siamensis* 101, *B. siamensis* 112 and *B. velezensis* 137) isolated from the rhizosphere soil of groundnut cultivation farms were found to inhibit the growth of *A. niger* mycelia by 53.6% to 60.8% *in vitro*. In pot experiments, the supplementation of this mixture of three bacterial strains (namely BAZ04) strongly reduced the collar rot symptoms of groundnut with a biocontrol efficacy of 100% compared to nil (no treatment). Field trials demonstrated the efficiency of BAZ04 in controlling collar rot disease, which increased the yield by 20.5–22.7% compared to the untreated plots. These results suggest that BAZ04 is a potential biocontrol agent for collar rot disease.

Key words: *Aspergillus niger*, *Bacillus*, biocontrol, collar rot disease, groundnut

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INTRODUCTION

Groundnut (*Arachis hypogaea* L.) or peanuts is a species belonging to the family Fabaceae and ranks second after soybeans as the essential oil crop grown worldwide. Due to its wide adaptability to different climates and soils, groundnuts are widely grown in subtropical and tropical areas with the largest groundnut cultivation in Asia, Africa, North America, and South America. The top countries growing groundnuts include India, China, America, Senegal, Myanmar, Brazil, and Argentina (Arya *et al*., 2016; Le *et al*., 2018; Lora *et al*., 2019; Nathawwat *et al*., 2021). Currently, the areas cultivated by groundnut are expanding in some countries such as Malawi, Sudan, Thailand, and Vietnam. In Vietnam, groundnuts have been widely cultivated and become one of the top groundnut producers in the world, (Fabra *et al*. 2010). In 2020, the total area under cultivation was 177.040 thousand ha, with an average yield of 2.51 tons/ha and a total production of 438.860 tons (Nguyen *et al*., 2023). However, groundnut crops are strongly vulnerable to diseases from plant pathogenic fungi, particularly those originating in the soil such as *Aspergillus spp*., *Sclerotium rolfsii*, *Rhizoctonia solani* (Janila *et al*., 2013; Le *et al*., 2018; Lora *et al*., 2019; Nguyen *et al*., 2023). These fungal diseases cause several damages including plant death, yield loss, decreasing nutrition value, and secreting toxins in seeds that lead to dangerous diseases for humans and livestock (Qaiser *et al*., 2018; Navale *et al*., 2021; Wei *et al*., 2023).

Collar rot is a disease caused by the population of *A. niger* which may lead to significant yield loss globally (Janila *et al*., 2013; Mohammed *et al*., 2014; Le *et al*., 2018; Lora *et al*., 2019; Nguyen *et la*., 2023). For example, annual global yield losses due to this disease account for approximately 13% - 52% (Kumari & Singh, 2017). In Vietnam, the mortality rate of plants by the fungus ranges from 9% to 30% (Nguyen *et al*., 2023). This disease occurs in two phases: pre-emergence and post-emergence. In the pre-emergence phase, the symptom of collar rot may include rotted seeds, thus reducing the emergence rate. In the post-emergence phase, the disease attacks the stem tissues near the ground surface, resulting in rot, wilting, and death of the plant (Harsukh *et al*., 2011).

Collar rot disease in groundnuts has been commonly controlled through cultural practices and fungicide application (Omugo *et al*., 2018; Gikas *et al*., 2022). The use of fungicides often results in chemical residues both in soils and nuts, which risks to human health after consumption (Okigbo, 2016). Moreover, the application of fungicides over time can result in reduced biodiversity, environmental pollution, and increased fungal resistance (Rao & Nnaji, 2017). Due to these concerns, synthetic chemical fungicides have been alerted to minimize their application and/or replaced with biological methods (Li *et al*., 2016).

The use of antagonistic bacteria for biocontrol has become increasingly popular and widespread (Putri *et al*., 2020; Kim *et al*., 2021). Among these, *Bacillus* spp. is a well-known bacteria that can be a biocontrol agent (Kuzina *et al*., 2019). Several reports suggest that Bacillus species can promote plant growth and inhibit the growth of fungal pathogens in plants. In particular, Kim *et al*. (2021) found that *Bacillus* subtilis had a high level of antagonism against five phytopathogenic fungi, including *Sclerotinia minor*, *Fusarium oxysporum, Colletotrichum gloeosporioides, Alternaria solani, Sclerotinia sclerotiorum,* and *Rhizoctonia solani.* Additionally, a study by Xu *et al*. (2016) demonstrated the effectiveness of *Bacillus* in controlling *Magnapothe grisea, R. solani,* and *C. gloeosporioides.* Moreover, a *Bacillus* sp. strain S20D12 produces the lipopeptide surfactin, therefore inhibiting the growth of *Sclerotium* fungus that causes groundnut stem rot (Le *et al*., 2018). However, little information is known about the effectiveness of *Bacillus* strains in controlling groundnut collar rot disease in Central Vietnam.

This study aimed to assess the effectiveness of three *Bacillus* strains previously isolated from peanut growing areas in Central Vietnam in preventing groundnut collar rot disease under *in vitro*, glasshouse, and field conditions.

MATERIALS AND METHODS

Materials

The pathogenic strain: *A. niger* QT1 was supplied by the Laboratory of Enzyme and Protein Technology at the Institute of Biotechnology, Hue University. It was isolated from groundnut plants infected with collar rot disease on variety L14 and identified through morphological characteristics, Koch's postulates, and molecular biology analysis (Nguyen *et al*., 2023).

Antagonist bacteria: Three bacterial strains, *Bacillus siamensis* 101, *Bacillus velezensis* 137, and *Bacillus siamensis* 112 were isolated and identified by our team (Nguyen *et al*., 2024). These strains were stored in glycerol stock at -80 °C until use.

The groundnut variety used in the glasshouse and field experiments, L14, was provided by the Institute of Biotechnology, Hue University.

Methods

Antagonist assay of *Bacillus* strains

Two qualitative methods (Antagonistic assay & diffusion method) were used to test the antagonistic ability of *Bacillus* strains against *A. niger* QT1. Prepare fungus: *A. niger* QT1 was grown on PDA medium in petri dishes and incubated at 28 °C ± 2 for seven days. Sterilized distilled water was added to the plates and fungal cells were scraped with a sterile inoculating loop. The cell suspensions were then filtered through sterile papers, and fungal spores were quantified using a Neubauer chamber under a light microscope. The fungal cell concentration was adjusted to 1×10^6 spores/mL for further study (Nguyen *et al*., 2024). Prepare antagonistic bacteria: Three bacterial strains (*B. siamensis* 101, *B. velezensis* 137 & *B. siamensis* 112) activated in LB (Luria-Bertani) medium and incubated at 30 °C for 24 hr at 180 rpm. The concentration of cell suspension was adjusted according to the 0.5 McFarland scale until reaching approximately 1×10^8 cfu/mL with sterile distilled water (Nguyen *et al*., 2024).

Antagonistic assay was performed using the dual culture technique on the potato dextrose agar PDA, as described by Anith *et al*. (2021). A well with a 5 mm diameter was created in PDA plates using a corkborer. Following this, 50 microliters of bacterial suspension were added to the well. An agar piece

of 3-day-old mycelia of QT1 strain was dropped at the opposite side of the well containing bacterial suspension. An identical set was prepared as the control except for *Bacillus* suspension culture. All plates were incubated at 30 °C until the fungal mycelia in the control plate reached the corners. The growth of *A. niger* QT1 mycelia was measured and compared with the control of average *A. niger* QT1 growth of the control without treatment. The assay was replicated three times. The Growth Inhibition Rate (GIR) was measured as described by Li *et al*. (2016).

$$\% \text{ GIR} = \frac{C-T}{C} \times 100$$

Where,

C: Size of fungal mycelial growth in the control (mm)

T: Size of fungal mycelial growth in treatment (mm)

In the second method, The antifungal activity of *Bacillus* strains against *A. niger* QT1 was tested using the agar diffusion method. Briefly, one milliliter of *A. niger* QT1 spores suspension (1×10^6 spores/mL) was melt-inoculated onto PDA (Potato Dextrose Agar) medium plates. Then, a corkborer was used to make wells with a 5 mm diameter in PDA medium. Fifty microliters of bacterial suspension were added to each well (Kuzina *et al*., 2019). A well added with sterile distilled water was assessed as the control. The results were evaluated after 48 hr of incubation. The antagonistic potential of each bacterium was determined by the diameter of growth inhibited zone on the agar plate. The experiment was done in duplicate and carried out three times to ensure the reproducibility of results.

Glasshouse experiments

The effectiveness of a mixture of three bacterial strains (*B. siamensis* 101, *B. velezensis* 137, and *B. siamensis* 112) in controlling groundnut collar rot disease was tested in the glasshouse at the Institute of Biotechnology, Hue University. To prepare for the infection study: Three bacterial strains (*B. siamensis* 101, *B. velezensis* 137 & *B. siamensis* 112) were grown separately until reaching approximately $1x10^8$ cfu/mL. Then 30 mL of cell suspension containing a mixture of three Bacillus strains was prepared in a 1:1:1 ratio for each replication.

The experiment was conducted in a randomized complete design with three replicates and five plants per replication. The treatments were as follows:

N1: Plants were uninfected *A. niger* QT1, groundnut seeds, and plants were treated with sterile water (uninfected plants);
N2= Plants were infected with *A. niger*, groundnut seeds, and plants treated with sterile water (negative control);
N3 = Plants were infected *A. niger*, groundnut seeds, and plants were treated with a mixture of three *Bacillus* strains;
N4= Plants were infected with *A. niger*, groundnut seeds, and plants were treated with a commercial fungicide, Ridomil Gold 68WG (Metalaxyl M + Mancozeb) (Syngenta Company, Vietnam) (positive control).

In this experiment, methods of infection with *A. niger* fungus and spraying antagonistic bacteria on groundnut plants were carried out as described by Nguyen *et al*. (2024). Groundnut seeds were soaked in 10 mL of mixed bacterial (1×10^8 cfu/mL) for 1 hr, then 5 seeds were sown in each pot. For N1 and N2 treatments, the groundnut seeds were treated with sterile water. Groundnut seeds of treatment N4 were treated with Ridomil Gold 68WG. Then, groundnut seeds were cultivated in a plastic pot measuring 35 × 30 × 30 cm containing 5 kg of sterilized soil. Five seeds were planted in each pot. All pots were placed in a glasshouse condition. Fourteen days after sowing (4 leaf stage), each pot poured 20 mL of mixed bacterial suspension at $1x10^8$ cfu/mL into the soil around the roots. After one day, each pot was inoculated with 20 mL of *A. niger* QT1 spore suspension $1x10^6$ spores/mL into the soil around the roots. The same solution without fungal inoculum was used to set the N1 treatment. For N4 treatment, seeds and plants were treated with Ridomil Gold 68WG as per the manufacturer's instructions.

Mortality rates (MR) were observed and recorded at 5, 7, 14, and 21 days post-infection (dpi) for each treatment. Biocontrol efficacy is calculated according to Yadav *et al*., 2023.

$$\text{Biocontrol effication (\%)} = \frac{\text{MR of control - MR of treatment}}{\text{MR of control}} \times 100$$

Field trials

The mixture of three antagonistic bacteria (*B. siamensis* 101, *B. venelensis* 137, and *B. siamensis*) was hereafter referred to as BAZ04. A field trial was conducted to test the effectiveness of BAZ04 against *A. niger* in L14 variety groundnut. The trials were carried out in two sandy soils, central provinces of Vietnam including Quang Tri (16°51′N 107°06′E) and Thua Thien Hue (16°26′N 107°42′E), where collar rot disease in groundnut was severely affected (Nguyen *et al*., 2023).

The experiment was conducted following a randomized complete block design (RCBD) with two treatments: CT1 (control, the untreated farmer field) and CT2 (treatment with biocontrol product BAZ04). The experiment consisted of three blocks with three replications (a, b, c), and each plot had a size of 30 m^2 (15 m × 2 m). The plants were spaced 10 cm within each row and 30 cm between each row. Before sowing groundnut seeds, BAZ04 product is applied to the beds with a final density of Bacillus of 10^6 cfu/cm^2 of soil.

The treated and untreated groundnut fields were applied with chemical fertilizer (per hectare) of 40kg of nitrogen (N), 60 kg of phosphorus (P_2O_5), 80 kg of potassium (K_2O), and 500 kg of calcium ($Ca(OH)_2$). Before sowing, 100% P2O5, 50% K2O, 50% N and 50% Ca(OH)2 are applied. When the seedlings have 3 - 4 leaves, 50% N and 50% K2O are applied. The remainder of Ca(OH)2 fertilizer is applied at flowering.

The collar rot (*A. niger*), stem rot (*Sclerotium rolfsii*), and root rot (*Rhizoctonia solani*) diseases were monitored weekly by evaluating a square of 10 m² (2 m × 5 m) area in the center of each plot. The plant mortality rate (MR) was counted. The groundnut yield obtained from the experimental plots was used to calculate the yield per hectare.

$$\text{MR (\%)} = \frac{\text{Total dead plants}}{\text{Total number of plants observed}} \times 100$$

Statistical analysis.

The data presented in this study are shown as mean ± SD (standard deviations). The data collected from both glasshouse and field experiments were statistically analyzed by the ANOVA method (SPSS software, version 22). The significant differences (p<0.05) among the treatments were determined by Duncan's test or t-test.

RESULTS

Evaluation of antagonist activity of *Bacillus* strains *in vitro* condition

The results of the antagonistic assay indicated that *Bacillus* strains were effective in inhibiting the mycelium growth of *A. niger* QT1. *B. siamenses* 101 inhibited *A. niger* growth diameter by 56.5%, while the inhibition rate by the treatment of *B. siamenses* 112 and *B. velezensis* 137 were 53.6% and 58.1%, respectively. Meanwhile, the mixture of three bacteria (*B. siamenses* 101, *B. siamenses* 112, and *B. velezensis* 137) increased the inhibition up to 60.8% (Table 1, Figure 1).

Table 1. The antifungal activity of *Bacillus* strains against *A. niger* QT1 by dual culture method

S. No	Isolate	Growth Inhibition Rate (%)
1	*B. siamenses* 101	56.5^c
2	*B. siamenses* 112	53.6^d
3	*B. velezensis* 137	58.1^b
4	Mixture of three bacteria	60.8^a

Means in the identical column with varying letters exhibit significant differences (*P≤0.05*)

In the test measuring the fungal inhibition zone of *Bacillus* strains, the results showed that different *Bacillus* strains reduced the growth rate of *A. niger* QT1 at different degrees. *B. siamenses* 101, *B. siamenses* 112, and *B. velenzesis* 137 had inhibition zones of 26.6, 25.4, and 28.3 mm respectively. The mixture of three bacterial strains yielded the highest inhibition zone diameter of 30.5 mm (Table 2, Figure 2). Overall, the three-strain combination expressed the most effective against *A. niger* QT1, based on both mycelium growth and inhibition zones *in vitro* tests.

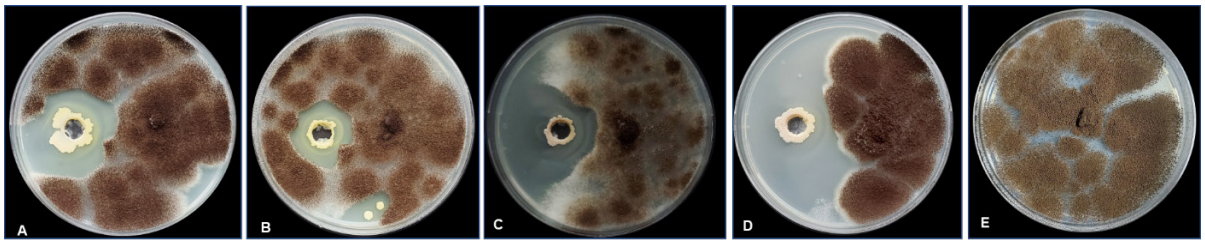


Fig. 1. Antagonist assay of *Bacillus* strains against *A. niger* QT1 by dual culture method on PDA. (A) *Bacillus* sp. 101, (B) *Bacillus* sp. 112, (C) *B. velezensis* 137, (D) Mixture of three bacteria, (E) Control

Table 2. The antifungal activity of *Bacillus* strains against *A. niger* QT1 by agar diffusion method

S. No	Isolate	Diameter of inhibition zones (mm)
1	*B. siamenses* 101	26.6^{c}± 0.54
2	*B. siamenses* 112	25.4^{c}±0.46
3	*B. velezensis* 137	28.3^{b}±0.61
4	A mixture of three bacteria	30.8^{a}±0.58
5	Control	0.0^{d}

Means ± SD in the identical column with varying letters exhibit significant differences (*P≤0.05*)

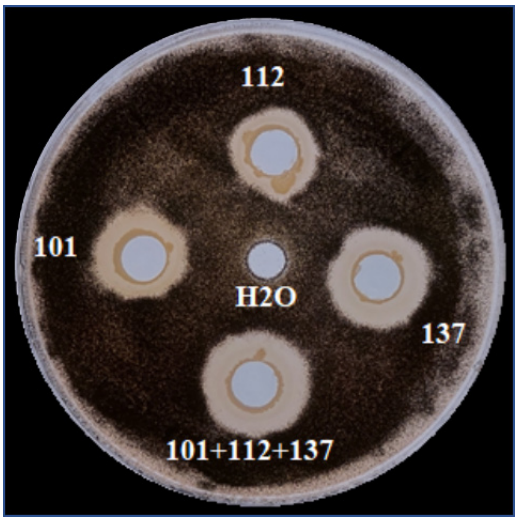


Fig. 2. Antagonist assay of *Bacillus* strains against *A. niger* QT1 by agar diffusion method (101. *B. siamenses* 101; 112. *B. siamenses* 112; 137. *B. velezensis* 137; 101+112+137. Mixture of three bacteria; H2O: Sterile water)

Evaluation of antagonistic activities of *Bacillus* strains under glasshouse conditions

The results showed that the biocontrol agents were successful in suppressing the disease (Table 3). At 5 days after inoculation, the antagonistic bacteria treatment was found to be highly effective in inhibiting the mortality rate of groundnut (MR=0) while the control was severely affected by the disease (MR=26.7%). The chemical fungicide treatment showed a decrease in the mortality rate of plants compared with the control. At 7 days, the mortality rate of plants increased significantly in both the control (MR=60%) and the chemical fungicide treatment (MR=26.67%). However, there were no disease symptoms observed in plants following the application of antagonistic bacteria (Figure 3). At 14 and 21 days, the control had the highest mortality rate (100%), while the lowest mortality rate (0%) was observed in plants inoculated with the mixture of three bacterial strains. In the uninfected group, groundnut plants grew well and there were no symptoms of collar rot disease observed throughout the experiment. From the results above, it was also observed that the mixture of three bacterial strains was the most promising for controlling *A. niger* QT1, with a biocontrol efficacy of 100%.

Table 3. Biocontrol efficacies of *Bacillus* strains against groundnut collar rot under glasshouse condition

Treatment	Mortality rate (%) of groundnut at day post inoculation				Biocontrol Efficacy 21DAI (%)
	5 dpi	7 dpi	14 dpi	21 dpi	
Uninfected plants	0.00^{c}	0.00^{c}	0.00^{c}	0.00^{c}	100.00
Negative control	26.67^{a}	60.00^{a}	100.00^{a}	100.00^{a}	-
Positive control	6.67^{b}	26.67^{b}	46.67^{b}	46.67^{b}	53.33
A mixture of three bacteria	0.00^{c}	0.00^{c}	0.00^{d}	0.00^{d}	100.00

Means in the identical column with varying letters exhibit significant differences (*P<0.05*)

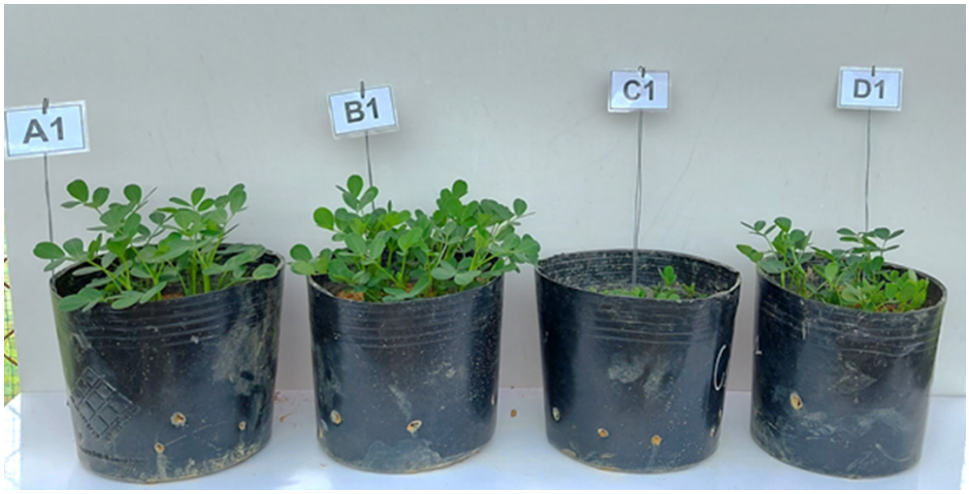


Fig. 3. Biological control of collar rot of groundnut by *Bacillus* strains under net house condition at 7 DAI (A1. Uninfected plants; B1. Mixture of three bacteria treatment; C1. Negative control; D1. Chemical treatment)

Evaluation of biological control of *Bacillus* strains under field condition

The plant mortality rate caused by collar rot (*A. niger*), stem rot (*Sclerotium rolfsii*), and root rot (*Rhizoctonia solani*) diseases differed in the nil and BAZ04 treatment at both experimental sites. In the Quang Trị site, the application of BAZ04 resulted in a decrease of 51.2 to 69.2% in plant mortality rate as compared to the control fields. Similarly, in the Thua Thien Hue site, BAZ04 had a much lower rate of plant mortality compared with nil (Table 4, Figure 4).

The yield of groundnut in the Quang Tri site was 2.44 tons/ha for nil but 3.07 tons/ha for the BAZ04 treatment. there was a significant increase in the yield of groundnut under the supplementation of BAZ04 up to 20.5% compared to the control.

Similarly, in Thua Thien Hue, the yield of groundnut in fields treated with BAZ04 was 2.95 tons/ha compared to 2.28 tons/ha in control fields. BAZ04 was found to significantly increase the yield of groundnut up to 22.7%, which is notably different compared to the untreated field (Figure 5).

Table 4. Biological control of collar rot in groundnut by *Bacillus* strains under farmer field conditions

Disease	Quang Tri				Thua Thien Hue			
	MR (%) control	MR (%) BAZ04	% difference between control and BAZ04	T-test (p)	MR (%) control	MR (%) BAZ04	% difference between control and BAZ04	T-test (p)
Collar rot (*A. niger*)	11.40	3.50	69.2	0.0231	6.5	1.1	83.1	0.0025
Stem rot (*Sclerotium rolfsii*)	8.60	4.20	51.2	0,0462	4.2	1.0	76.2	0.0361
Root rot (*Rhizoctonia solani*)	5.20	2.10	59.6	0.0414	4.0	1.2	70.0	0.0392

DISCUSSION

The present study demonstrated the effectiveness of using a mixture of three isolated Bacillus strains to control the mycelium growth of *A. niger*, suggesting its potential replacement for chemical fungicides against collar rot disease in groundnut. Data from 2 field trials showed that using the biological product BAZ04 (A mixture of three strains of *B. siamenses* 101, *B. siamenses* 112 & *B. velezensis* 137) resulted

in decreased plant mortality rates. Additionally, BAZ04 was found to be beneficial for crop growth and improved the yield of groundnut. Therefore, the mixture of *B. siamenses* 101, *B. siamenses* 112, and *B. velezensis* 137 holds the potential to be developed as agricultural inoculants to prevent phytopathogens and increase crop yield. Our results support previous findings by Xu *et al*. (2016) that antagonists were effective in managing plant diseases. *B. velezensis* by inhibiting the growth of many pathogenic fungi such as *Cylindrocladium quinqueseptatum*, *Helicobasidium purpureum*, and *Cryphonectria parasitica*. In other reports, *B. velezensis* acts as biocontrol agent to inhibit various pathogen fungi such as *Rhizoctonia solani*, *Aspergillus flavus*, and *Fusarium oxysporum* (Chowdhury *et al*., 2013; Cao *et al*., 2018; Chen *et al*., 2019). Additionally, Shereen *et al*. (2023) showed that *B. amyloliquefaciens* RaSh1 expresses excellent biocontrol ability to fungus *Alternaria alternata* and positively increases the growth rate of black pepper. Moreover, *Bacillus* strains can enhance several physiological processes of plants such as emergence, root length, and plant height extension, which contribute to increasing the yield of crops (Sayyed *et al*., 2010; Hashem *et al*., 2019; Dong *et al*., 2023). Dong *et al*. (2023) reported that *B. cereus* DW019 enhanced the productivity of cherry tomatoes and promoted the accumulation of phytochemicals.



Fig. 4. Biological control of collar rot in groundnut by *Bacillus* strains under farmer field condition (The circles indicate the symptoms of plant wilting & death)

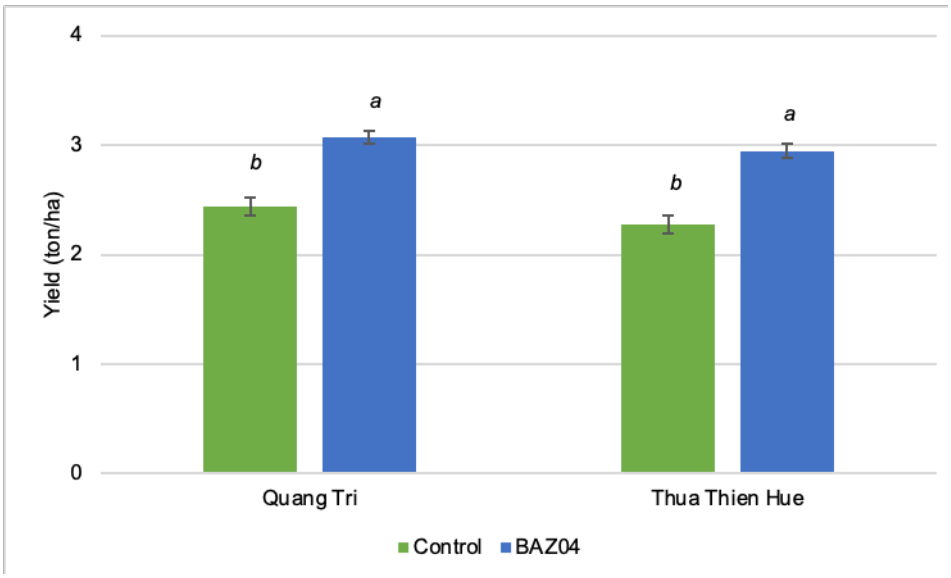


Fig. 5. Effect of Bacillus strains on the yield of groundnut cultivar L14 under field conditions in Quang Tri and Thua Thien Hue provinces, Vietnam. Bars are standard errors. Different letters indicate significant differences between the treatments within the site.

The reduction in plant mortality rates in the presence of the three *Bacillus* strains tested in this study may be related to the ability of these strains to inhibit mycelia growth of *A. niger* QT1. Each bacterial strain had inhibitory effects on *A. niger* (>50%, Table 1) in the PDA medium. The mixture of three bacteria (*B. siamenses* 101, *B. siamenses* 112, and *B. velezensis* 137) increased the growth inhibition by 60.8% compared with that measured in the control. This is consistent with our previous study (Nguyen *et al*. 2024) that these *Bacillus* strains can inhibit the growth of fungus *A. niger* by producing cell wall hydrolytic enzymes (endo-1,3-β-glucanase, chitinase & cellulase).

The application of BAZ04 to soils (a mixture of three *Bacillus* strains) appeared to be more effective and beneficial than the use of a commercial fungicide in controlling collar rot disease. The results from the glasshouse experiment found an immediate effect of Ridomil Gold 68WG (Metalaxyl M + Mancozeb) within 5 days after infection. However, its effectiveness reduced over time, with an increase in MR to 26% after 7 days and 46% after 21 days. The growing reliance on chemical fungicides to protect crops and maximize yields has negative impacts on human health, animal welfare, and the environment (Dadrasnia *et al*., 2020). Meanwhile, using rhizosphere microorganisms proves to be an efficient method for disease control, promoting growth, and ultimately enhancing crop productivity (Huruna *et al*., 2021; Shereen *et al*., 2023). Several studies reported that rhizosphere bacteria such as *Streptomyces* spp., *Pseudomonas* spp., and *Bacillus* spp. act as potential biocontrol agents for various pathogen fungi in replacement of chemical fungicides (Jangir *et al*., 2018; Andric *et al*., 2020; Stefany Castaldi *et al*., 2021; Shereen *et al*., 2023). Importantly, numerous *Bacillus* species have been used as effective biocontrol agents for the inhibition of plant pathogens (Jangir *et al*., 2018; Andric *et al*., 2020). *Bacillus* sp. is a versatile bacterium that employs various direct and indirect mechanisms to increase plant growth rate and control pathogen proliferation. These mechanisms include nitrogen fixation, mineralization and solubilization of nutrients such as phosphorus and potassium, production of hydrolytic enzymes, phytohormones, antimicrobial compounds, and abiotic stress tolerance through competition for nutrients, parasitism, and stimulation of host plant defenses (Zhang *et al*., 2020; Sharma *et al*., 2021; Yánez-Mendizábal *et al*., 2021; Mirskaya *et al*., 2022; Bathke *et al*., 2022; Ajuna *et al*., 2023).

Although the present study observed the effectiveness of using BAZ04 against collar rot disease in groundnut and to increase the yield from field sites, the influence of these bacterial strains on nodulation and nitrogen fixation of groundnut needs further investigation.

CONCLUSION

The results from *in vitro* conditions showed that the mixture of three antagonistic bacteria strains (*B. siamensis* 101, *B. siamensis* 112, *B. venelensis* 137) had a greater ability to inhibit *A. niger* compared to each bacterium alone. In the glasshouse and field conditions, it has been proven that applying the mixture of antagonistic bacteria to soils had the highest effectiveness in reducing plant mortality caused by *A. niger* on groundnut crops grown in two field trials in Quang Tri and Thua Thien Hue provinces, Vietnam. Importantly, our findings suggested that an appropriate application of three antagonistic bacteria strains together could increase the yields in groundnuts by 20.5 - 22.7 %.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ĐÁNH GIÁ KHẢ NĂNG ĐỐI KHÁNG CỦA NẤM *Trichoderma asperellum* HL6 ĐỐI VỚI NẤM *Aspergillus niger* Van Tieghem GÂY BỆNH HÉO RŨ GỐC MỐC ĐEN TRÊN CÂY LẠC

Nguyễn Xuân Hiếu[1, 2], Nguyễn Đức Huy[1], Tôn Đức Hồng[3], Nguyễn Tiến Long[1], Thái Thị Huyền[4], Nguyễn Thị Thu Thủy[4] *

[1] Viện Công nghệ Sinh học, Đại học Huế, Nguyễn Đình Tứ, Huế, Việt Nam
[2] Trường THCS & THPT Cửa Việt, Thị trấn Cửa Việt, huyện Gio Linh, tỉnh Quảng Trị, Việt Nam
[3] Đại học Huế, 3 Lê Lợi, Huế, Việt Nam
[4] Trường Đại học Nông Lâm, Đại học Huế, 102 Phùng Hưng, Huế, Việt Nam

* Tác giả liên hệ: Nguyễn Thị Thu Thủy <nttthuy.huaf@hueuni.edu.vn>
(*Ngày nhận bài: 7-5-2024; Ngày chấp nhận đăng: 8-8-2024*)

Tóm tắt. Bệnh héo rũ gốc mốc đen do nấm *Aspergillus niger* Van Tieghem ảnh hưởng lớn đến sản xuất lạc. Sự tồn tại lâu dài của nấm bệnh trong đất có thể làm giảm hiệu quả của thuốc hoá học diệt nấm. Việc sử dụng vi sinh vật đối kháng có thể khống chế được nấm bệnh *A. niger*. Chủng nấm HL6 được chúng tôi phân lập từ đất quanh vùng rễ cây lạc trồng tại huyện Hải Lăng, tỉnh Quảng Trị, có khả năng ức chế sự phát triển của nấm bệnh *A. niger,* với hiệu suất đối kháng đạt 77,41% trong điều kiện *in vitro*. Kết quả định danh phân tử dựa trên so sánh trình tự nucleotide vùng ITS1-4 chỉ ra chủng nấm HL6 thuộc loài *Trichoderma asperellum.* Kết quả thử nghiệm đã cho thấy chủng *T. asperellum* HL6 không ảnh hưởng đến tỉ lệ nảy mầm của hạt lạc. Trong thử nghiệm ở điều kiện nhà lưới, việc bổ sung nấm *T. asperellum* HL6 đã làm giảm mạnh tỉ lệ cây lạc bị bệnh héo rũ gốc mốc đen, hiệu lực phòng trừ bệnh đạt được 99,55%. Kết quả nghiên cứu này là tiền đề cho nghiên cứu thử nghiệm trên đồng ruộng để đánh giá hiệu quả phòng chống bệnh héo rũ gốc mốc đen của chủng nấm *T. asperellum* HL6 nhằm tiến tới sản xuất chế phẩm sinh học phòng trừ nấm bệnh *A. niger*.

Keywords: cây lạc, héo rũ gốc mốc đen, *Trichoderma asperellum, Aspegillus niger*, kiểm soát sinh học

Evaluation of *Trichoderma asperellum* HL6 against *Aspergillus niger* Van Tieghem causing collar rot in groundnut

Nguyen Xuan Hieu[1, 2], Nguyen Đuc Huy[1], Ton Đuc Hong[3], Nguyen Tien Long[1], Thai Thi Huyen[4], Nguyen Thi Thu Thuy[4] *

[1] Institute of Biotechnology, Hue University, Nguyen Đinh Tu St., Hue, Vietnam
[2] Cua Viet Lower & Upper Secondary School, Cua Viet Town, Gio Linh, Quang Tri, Vietnam
[3] Hue University, 3 Le Loi, Hue, Vietnam
[4] University of Agriculture and Forestry, Hue University, 102 Phung Hung St., Hue, Vietnam

* Correspondence to Nguyen Thi Thu Thuy <nttthuy.huaf@hueuni.edu.vn>
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Abstract. Collar rot disease, caused by the fungus *Aspergillus niger* Van Tieghem, significantly impacts peanut production. The long-term survival of fungal pathogens in soil can decrease the effectiveness of chemical fungicides. The use of antagonistic microorganisms can help control the fungal pathogen *A. niger*. A fungus strain HL6 which has been isoolated from the soil around the root zone of peanut plants grown in Hai Lang district, Quang Tri province, exhibited the inhibitory against *A. niger* of 77.41% efficiency under *in vitro* conditions. Molecular identification revealed that the strain HL6 belonged to the *Trichoderma asperellum* species based on nucleotide sequence comparison of the ITS1-4 region. The test results showed that the *T. asperellum* HL6 did not affect the germination rate of the peanut seeds. In net-house experiments, the addition of *T. asperellum* HL6 significantly reduced the occurrence of collar rot disease in peanut plants, with a disease prevention effectiveness reaching 99.55%. These results propose the field trials to evaluate the effectiveness of the *T. asperellum* HL6 in preventing collar rot diseases for production of bio-inoculants against *A. niger*.

Keywords: groundnut, collar rot, *Trichoderma asperellum*, *Aspegillus niger,* biological control

1 Đặt vấn đề

Lạc (*Arachis hypogaea* L.) còn gọi là đậu phộng, là cây họ đậu hàng năm được trồng ở hơn 80 quốc gia ở vùng nhiệt đới, cận nhiệt đới và ôn đới ẩm [1]. Hạt lạc chứa nhiều chất dinh dưỡng như protein, carbohydrate, canxi và khoáng chất, là nguồn thực phẩm cho con người, nguyên liệu cho công nghiệp và thức ăn chăn nuôi [2]. Về mặt kinh tế, lạc thuộc nhóm cây đứng đầu danh sách cây lấy dầu trên thế giới [1].

Việc trồng lạc bị ảnh hưởng bởi nhiều loại bệnh như bệnh đốm lá, héo rũ gốc mốc đen, héo rũ gốc mốc trắng, lở cổ rễ, thối thân và quả … [3]. Trong số các bệnh ở lạc, héo rũ gốc mốc đen do nấm *Aspegillus niger* gây ra là một loại bệnh xuất hiện phổ biến, có sức tàn phá nghiêm trọng, gây thiệt hại lớn về năng suất lạc [1]. Nấm *A. niger* xâm nhập, tấn công làm thối hạt, nấm xâm nhập làm tổn thương trầm trọng phần rễ và gốc thân. Cây con và cây trưởng thành bị nhiễm bệnh, vết bệnh phát triển trên thân ngay dưới bề mặt đất và sau đó lan lên dọc theo cành. Điều này dẫn đến héo vĩnh viễn các cành của toàn bộ cây, vùng cổ rễ bị thối, phủ lớp nấm màu đen

[4]. Thiệt hại năng suất hàng năm trên thế giới do bệnh héo rũ gốc mốc đen gây ra ước tính khoảng 10% có nơi lên đến 40% ở nhiều quốc gia như Ấn Độ, Ethiopia [5, 6]. Ở Việt Nam, bệnh héo rũ gốc mốc đen dao động từ 9 đến 30%, hầu hết các giống lạc đều mẫn cảm với bệnh này [7].

Hiện nay, sử dụng thuốc hóa học để phòng trừ bệnh cho cây lạc vẫn được áp dụng rộng rãi. Tuy nhiên, phương pháp này không chỉ tốn kém mà còn gây ô nhiễm môi trường và không đem lại hiệu quả như mong đợi. Bên cạnh đó, việc lạm dụng thuốc hóa học trong thời gian dài có thể dẫn đến các đột biến gen mà cuối cùng được cho là tạo ra các kiểu gen kháng thuốc diệt nấm [8]. Vì vậy, việc sử dụng đối kháng sinh học để kiểm soát *A. niger* gây hại cây lạc được xem là giải pháp thân thiện với môi trường. Các loài *Trichoderma* đã được sử dụng rộng rãi trong nông nghiệp nhờ thể hiện những tiềm năng nổi trội như tiết ra enzyme phân giải màng tế bào của nấm [6, 9]; tiết ra các loại kháng sinh gây độc mạnh cho nấm; tạo ra các chất chuyển hóa thứ cấp có hoạt tính sinh học có thể kích thích sinh trưởng, tăng cường khả năng chống chịu stress, hấp thu tích cực các chất dinh dưỡng và bảo vệ cây trồng; cạnh tranh dinh dưỡng, không gian sống với mầm bệnh [6, 10]. Trần Thị Thu Hà và Phạm Thanh Hòa đã báo cáo về khả năng ức chế sự phát triển nấm bệnh *Sclerotium rolfsii* của *Trichoderma* trong điều kiện *in vitro* [11]. Nguyễn Đình Thi và cs. đã báo cáo về khả năng kiểm soát bệnh hại và giúp lạc sinh trưởng tốt khi sử dụng kết hợp *Trichoderma* và *Pseudomonas* trên đồng ruộng [12]. Tuy nhiên, không phải các chủng *Trichoderma* đều hoạt động tương đồng đối với một chủng nấm bệnh cụ thể [13]. Do đó, việc tiếp tục nghiên cứu tìm ra chủng nấm *Trichoderma* bản địa nhằm kiểm soát nấm bệnh *A. niger* trên cây lạc là hết sức cần thiết.

2 Vật liệu và phương pháp

2.1 Vật liệu

Nguồn nấm bệnh: Chủng nấm bệnh *A. niger* QT1 được cung cấp bởi Phòng thí nghiệm Công nghệ Enzyme và Protein, Viện Công nghệ Sinh học, Đại học Huế. Đây là chủng nấm bệnh được phân lập từ cây lạc bị bệnh héo rũ gốc mốc đen ở huyện Triệu Phong, tỉnh Quảng Trị. Chủng nấm QT1 này đã được định danh dựa vào đặc điểm hình thái, quy tắc Koch và phương pháp sinh học phân tử [7].

2.2 Phương pháp nghiên cứu

Phân lập, tuyển chọn nấm *Trichoderma* có khả năng đối kháng nấm *A. niger*

Phân lập nấm Trichoderma

Sáu mẫu đất (300 g/mẫu) được thu thập ở độ sâu 15–20 cm, xung quanh gốc các cây lạc khoẻ, ở các khu vực chuyên canh trồng lạc của tỉnh Quảng Trị và tỉnh Thừa Thiên Huế (Bảng 1). Mẫu đất được đựng trong các túi nilon, ghi nhãn rồi chuyển về phòng thí nghiệm, bảo quản ở nhiệt độ 4 °C cho tới khi sử dụng. Một gram của mỗi mẫu đất được hòa tan trong 99 mL nước cất vô trùng, khuấy đều trong 10 phút ở nhiệt độ phòng. Tiếp tục pha loãng mẫu đến các nồng độ 10^{-3}, 10^{-4}, 10^{-5} và 0,5 mL mỗi nồng độ được trải đều lên bề mặt môi trường phân lập *Trichoderma*,

bổ sung propamocarb. Các đĩa được nuôi ở 28 ± 2 °C trong 96 giờ cho đến khi nhìn thấy được các khuẩn lạc nấm xuất hiện. Chọn các khuẩn lạc nấm khác nhau, có hình thái đặc trưng của *Trichoderma* cấy chuyền sang đĩa môi trường PDA (20 g cao khoai tây và 20g dextrose, pH 7,0) bằng phương pháp cấy đơn bào tử để thu các chủng nấm thuần khiết [14]. Các chủng nấm *Trichoderma* được lưu giữ và bảo quản trên môi trường PDA, ở 4 °C.

Tuyển chọn chủng nấm Trichoderma có khả năng đối kháng

Tuyển chọn chủng nấm *Trichoderma* được thực hiện theo phương pháp của Watanabe [15]. Ở đĩa thí nghiệm, hai khoanh nấm *Trichoderma* và *A. niger* đều có đường kính 4 mm được cấy đối xứng nhau qua tâm của đĩa Petri chứa môi trường PDA, mỗi bên cách tâm 30 mm. Ở đĩa đối chứng, nấm *A. niger* được cấy tương tự như đĩa thí nghiệm, nhưng không cấy nấm *Trichoderma*. Tất cả các đĩa nấm được nuôi ở 28 ± 2 °C, sau 6 ngày nuôi cấy, phân tích sự đối kháng của mỗi chủng nấm *Trichoderma* đối với *A. niger.* Chỉ số đối kháng (H) theo tỷ lệ phần trăm ức chế sinh trưởng của *A. niger* được tính theo công thức sau:

$$H\ (\%) = \frac{D - d}{D} \times 100 \quad (1)$$

trong đó, D là đường kính tản nấm *A. niger* trên đĩa đối chứng (mm) và d là đường kính tản nấm *A. niger* trên đĩa thí nghiệm (mm). Thí nghiệm được lặp lại ba lần**.**

Định danh chủng nấm *Trichoderma* đối kháng mạnh

Nhận dạng hình thái

Các chủng nấm phân lập được nhận dạng sơ bộ dựa trên các đặc trưng hình thái của nấm *Trichoderma* như màu sắc, sự phát triển sợi nấm và kết cấu tản nấm, sự phân nhánh của bào tử, kích thước và hình dạng bào tử [16].

Định danh phân tử

Nuôi cấy chủng nấm *Trichoderma* được tuyển chọn trong môi trường PDA. Sợi nấm ở giai đoạn phát triển mạnh được chuyển sang môi trường PDB (Potato Dextrose Broth), nuôi lắc ở 28 ± 2 °C với tốc độ 180 vòng/phút trong 2 ngày để sản xuất sinh khối. DNA được tách chiết bằng kit TopPURE ® Plant DNA Extraction Kit (ABT) (Công ty TNHH Giải pháp Y Sinh ABT, Việt Nam). Vùng Internal transcribed spacer (ITS) của nấm được khuếch đại với cặp primer ITS1 (5′-TCC GTA GGT GAA CCT GCGG-3′) và ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) [17]. Quá trình khuếch đại phản ứng chuỗi polymerase (PCR) được thực hiện trong máy gia nhiệt SimpliAmp™ Thermal Cycler (Applied Biosystems, Thermo Fisher Scientific Inc., USA) với tổng thể tích phản ứng là 50 µL chứa 25 µL Go Taq Green 2X Master Mix (Promega, USA), 5 µL (50 ng) mẫu DNA, 5 µL mỗi mồi (10 pmol) và 15 µL H_2O. Chu trình khuếch đại như sau: 95 °C trong 10 phút; 30 chu kỳ ở 95 °C trong 50 giây, 53 °C trong 50 giây và 72 °C trong 1 phút; 72 °C trong 10 phút. Sản phẩm PCR được điện di trên gel agarose 1,0 % và được nhuộm bằng Safe-View™ Classic Nucleic Acid Stain (Applied Biological Materials Inc., Canada). Hình ảnh điện di được ghi nhận bằng hệ thống Ultra Slim LED Illuminator. Sản phẩm được tinh sạch và tiến hành

giải trình tự tại công ty First BASE (Malaysia). Các trình tự thô được xử lý bằng phần mềm BioEdit v. 7 [18] và so sánh với các trình tự tương ứng của các chủng đã được đăng ký trên GenBank bằng công cụ BLASTn trên National Center for Biotechnology Information - NCBI (www.ncbi.nlm.nih.gov).

Phân tích phát sinh loài

Cây phát sinh loài được xây dựng bằng 31 trình tự tham chiếu vùng ITS được thu thập và tải xuống từ NCBI của các loài thuộc chi *Trichoderma* (Bảng 2) cùng với trình tự ITS của chủng HL6. Hai chủng *Protocrea farinosa* CBS 121551 (chủng TYPE, NR_119700) và *Protocrea pallida* CBS 29978 (chủng TYPE, NR_111329) được sử dụng làm nhóm ngoài (outgroup). Các trình tự này được căn chỉnh bằng ClustalW [19]. Sau đó, cây phát sinh loài được dựng bằng phần mềm trực tuyến IQ-TREE v. 2.1.3 bằng phương pháp Maximum Likelihood với 1000 lần ultrafast bootstrap [20]. ModelFinder được sử dụng để xác định mô hình thuật toán tối ưu cho phân tích phát sinh loài [21]. Kết quả được hiển thị bằng phần mềm Interactive Tree of Life (iTOL) v. 5 và chỉnh sửa thủ công bằng Adobe Illustrator CC 2021 (Adobe Systems, CA, USA) [22].

Ảnh hưởng của chủng nấm *Trichoderma* HL6 đến sự nảy mầm của hạt lạc

Chủng nấm *Trichoderma* HL6 được nuôi cấy trên môi trường PDA trong 5 ngày ở 28 ± 2 °C**.** Dịch bào tử nấm được xác định nồng độ bằng buồng đếm Neubauer, sau đó pha loãng bằng nước cất vô trùng đến nồng độ 10^8 bào tử/mL để sử dụng cho thí nghiệm [23]. Thí nghiệm được tiến hành trên giống lạc L14. Chọn hạt lạc giống đồng đều, không bị sâu bệnh. Ở công thức thí nghiệm, lấy ngẫu nhiên 100 hạt lạc giống ngâm trong 500 mL nước cất có bổ sung 30 mL dung dịch huyền phù bào tử HL6 nồng độ $1,5 \times 10^8$ bào tử/mL trong thời gian 3 h. Ở công thức đối chứng, hạt lạc được ngâm trong 500 mL nước cất. Hạt lạc ở công thức thí nghiệm và đối chứng sau đó được vớt ra, để ráo nước và ủ riêng trong các khay ở điều kiện ở nhiệt độ phòng, theo dõi sự nảy mầm của hạt sau 48 h. Thí nghiệm được lặp lại 3 lần. Xác định tỉ lệ nảy mầm của hạt lạc theo công thức:

$$\text{Tỉ lệ nảy mầm (\%)} = \frac{\text{Số hạt lạc nảy mầm}}{\text{Tổng hạt lạc}} \times 100 \quad (1)$$

Thử nghiệm khả năng kiểm soát nấm bệnh *A. niger* của chủng nấm *Trichoderma* HL6 trong điều kiện nhà lưới

Thí nghiệm được bố trí theo khối hoàn toàn ngẫu nhiên với 3 công thức, 3 lần lặp lại, mỗi lần lặp lại được thực hiện trên 1 chậu nhựa (kích thước 35 × 30 × 30 cm) chứa 5 kg đất hữu cơ trồng cây đã được khử trùng, mỗi chậu được gieo 5 hạt lạc, các công thức gồm:

Sử dụng 15g hạt giống lạc ngâm trong 2 mL dung dịch nấm *Trichoderma* HL6 (mật độ $1,5 \times 10^8$ bào tử/mL) trong thời gian 3 giờ, sau đó gieo hạt vào chậu thí nghiệm. Hai tuần sau gieo (cây lạc có 2–4 lá), tưới 20 mL dung dịch nấm *Trichoderma* HL6 ở mật độ $1,5 \times 10^8$ bào tử/mL cho một chậu thí nghiệm vào gốc cây lạc và vùng đất xung quanh gốc theo từng công thức tương ứng. Sau 1 ngày, tưới 20 mL dung dịch bào tử nấm/chậu với mật độ 1×10^6 bào tử/mL *A. Niger* QT1 vào gốc lạc và vùng đất xung quanh gốc. Công thức 1 và 2 được sử dụng với nước cất. Sau

Bảng 1. Công thức thí nghiệm

Công thức thí nghiệm	Cách xử lý
Công thức 1 (CT1)	Cây không bị nhiễm *A. niger*, hạt lạc và cây lạc được xử lý bằng nước cất.
Công thức 2 (CT2)	Cây bị nhiễm *A. niger*, hạt lạc và cây lạc được xử lý bằng chủng nấm *Trichoderma* HL6.
Công thức 3 (CT3 – Đối chứng)	Cây bị nhiễm *A. niger*, hạt lạc và cây lạc được xử lý bằng nước cất.

đó, đặt các chậu thí nghiệm trong nhà lưới và theo dõi tỷ lệ cây chết trong mỗi công thức.

Chỉ tiêu theo dõi: Theo dõi tỷ lệ cây chết do nhiễm bệnh (%) tại các thời điểm 7, 14, 21, 28 và 45 ngày sau nhiễm nấm bệnh (SNNB) [24].

2.1 Xử lý số liệu

Số liệu được xử lý bằng phân tích phương sai One-way ANOVA (Ducan's test) bằng phần mềm SPPSS v. 26. Sự sai khác có ý nghĩa thống kê giữa các giá trị trung bình được xác định với $p < 0,05$.

3 Kết quả và thảo luận

3.1 Phân lập chủng nấm *Trichoderma* đối kháng nấm bệnh *A. niger*

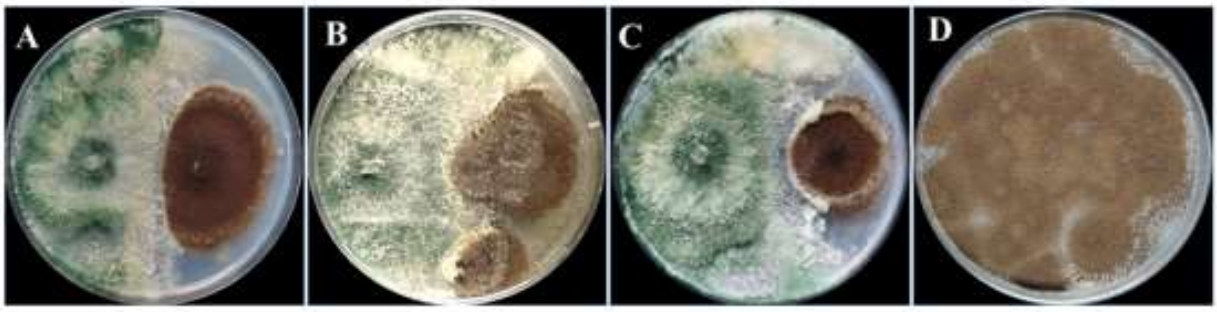
Từ các mẫu đất vùng rễ cây lạc khoẻ thu thập ở tỉnh Quảng Trị và tỉnh Thừa Thiên Huế, chúng tôi đã phân lập được 06 chủng nấm có hoạt tính đối kháng với chủng nấm bệnh *A. niger* QT1. Hiệu suất đối kháng với nấm bệnh của 6 chủng nấm này đạt từ 37,04 đến 77,41% (Bảng 2). Trong đó, chủng HL6 có hiệu suất đối kháng cao nhất, đường kính vòng ức chế sinh trưởng nấm bệnh đạt 69,67 mm, hiệu suất đối kháng đạt 77,41% và khác biệt có ý nghĩa thống kê so với các chủng còn lại. Vì vậy, chủng HL6 được lựa chọn để thực hiện các nghiên cứu tiếp theo (Hình 1).

Màu sắc sợi nấm của loài nấm *A. niger* rất phức tạp, trên các môi trường nuôi cấy khác nhau, ở các điều kiện nhân nuôi khác nhau và thậm chí là các chủng nấm khác nhau màu sắc sợi

Bảng 2. Hiệu suất đối kháng của các chủng nấm đối với nấm bệnh *A. niger* QT1

Ký hiệu chủng	Đường kính vòng ức chế (D-d, mm)	Hiệu suất đối kháng (%)	Địa điểm thu mẫu
VL1	33,33 ± 3,06 ^{f}	37,04 ± 3,40 ^{d}	Vĩnh Linh, Quảng Trị
Đak3	42,00 ± 2,00 ^{e}	46,67 ± 2,23 ^{c}	Đakrong, Quảng Trị
HL6	69,67 ± 1,15 ^{a}	77,41 ± 1,28 ^{a}	Hải Lăng, Quảng Trị
PhĐ3	43,00 ± 2,65 ^{e}	47,78 ± 2,94 ^{c}	Phong Điền, Thừa Thiên Huế
TPH4	45,33 ± 2,08 ^{e}	50,37 ± 2,31 ^{c}	Thành phố Huế, Thừa Thiên Huế
PhV1	55,33 ± 0,58 ^{bc}	61,48 ± 0,64 ^{b}	Phú Vang, Thừa Thiên Huế

Ghi chú: Các chữ cái khác nhau trên cùng một cột chỉ sự sai khác trung bình mẫu có ý nghĩa thống kê với $p < 0,05$ (Ducan's test). M ± SD: Trung bình mẫu (Mean) ± độ lệch chuẩn.



Hình 1. Khả năng đối kháng của một số chủng nấm đối kháng với nấm bệnh *A. niger* QT1 sau 6 ngày nuôi cấy trên môi trường PDA, (**A**) chủng TPH4, (**B**) chủng PhV1, (**C**) chủng HL6 và (**D**) đĩa đối chứng.

nấm có thể khác nhau. Một số nghiên cứu cho thấy màu sắc tản nấm trên môi trường nuôi cấy có màu đen [25, 26], một số nghiên cứu khác lại chỉ ra tản nấm *A. niger* có màu nâu nhạt đến nâu đậm [27–29]. Về mặt di truyền màu sắc bào tử nấm *A. niger* được quy định bởi nhiều gen khác nhau trong chu trình sinh tổng hợp polyketide. Dựa vào mức độ biểu hiện khác nhau của các gen trong chu trình này mà màu sắc của *A. niger* có thể thay đổi từ vàng nhạt, xanh oliu, nâu, đen và thậm chí không màu (màu trắng). Sự thay đổi màu sắc xảy ra khi một hoặc nhiều gen trong chu trình sinh tổng hợp polyketide không hoạt động dẫn đến sự gián đoán của chu trình tạo sắc tố ở bào tử nấm [30]. Trong nghiên cứu này, màu sắc của nấm *A. niger* có màu nâu đậm, điều này thể hiện sự đa dạng về màu sắc của loài nấm này trên môi trường nuôi cấy.

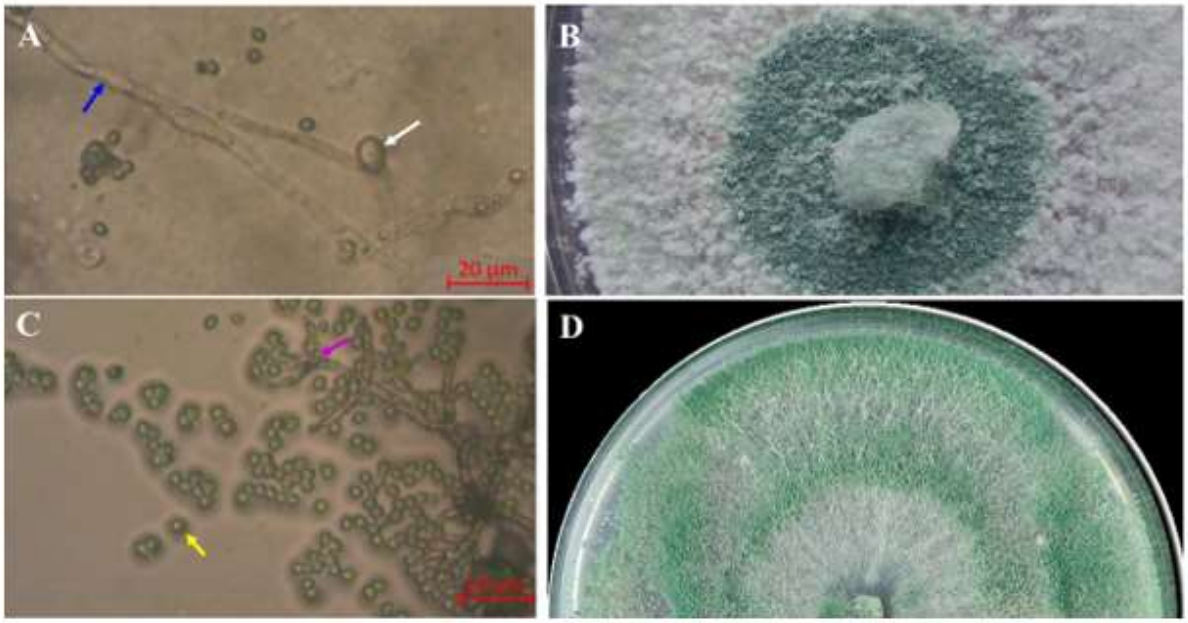
Các loài nấm thuộc chi *Trichoderma* là một trong những tác nhân kiểm soát sinh học được nghiên cứu và ứng dụng rộng rãi để phòng chống các bệnh nấm, vi khuẩn, tuyến trùng ở thực vật [31]. Trước đây, đã có các nghiên cứu xác nhận về khả năng của các loài *T. viride, T. harzianum, T. hamanatum, T. ressei và T. koningii* trong kiểm soát sinh học chống lại nấm *S. rolfsii, A. niger và A. flavus* gây bệnh ở cây lạc [32]. Kết quả khảo sát của Trần Thị Thu Hà và Phạm Thanh Hòa về khả năng đối kháng của 40 chủng nấm *Trichoderma* phân lập từ các mẫu đất ở hai tỉnh Quảng Trị, Thừa Thiên Huế cho thấy có 11 chủng đối kháng cao với nấm bệnh *S. rolfsii* với hiệu suất ức chế sợi nấm đạt được từ 61,39 đến 88,06% [11]. Gần đây, các báo cáo của Alwadai và cs., Raja và cs. cũng xác nhận rằng 3 loài *T. harzianum, T. brevicompactum* và *T. velutinum* có khả năng đối kháng với các nấm bệnh *Fusarium oxysporum, Alternaria alternata* và *Exserohilum rostratum, …* [33, 34].

3.2 Định danh chủng nấm HL6

Trên môi trường PDA, sợi nấm của chủng HL6 khi mới xuất hiện có màu trắng, lúc hình thành bào tử có màu xanh lục hoặc xanh lục nhạt. Tản nấm sinh trưởng nhanh, lúc đầu có màu trắng, hơi xốp; sau 2 ngày tản nấm chuyển thành màu xanh lục, sau 6 ngày đường kính tản nấm đạt tới 90 mm và hình thành nhiều bào tử. Bào tử có dạng hình cầu, hình trứng, bề mặt nhẵn, thể bình có dạng hình trụ (Hình 2).

Kết quả điện di sản phẩm PCR ghi nhận xuất hiện băng ở vị trí khoảng 600 bp so với thang chuẩn. Quá trình định danh phân tử được tiến hành bằng cách sử dụng trình tự sau căn chỉnh của chủng HL6 so sánh với các chuỗi trình tự tham chiếu trong cơ sở dữ liệu nucleotide NCBI

bằng công cụ BLASTn. Kết quả BLASTn ghi nhận rằng chủng nấm HL6 có mức độ tương đồng cao (100%) với trình tự tham chiếu vùng ITS của chủng *T. asperellum* CBS 433.97 (chủng TYPE, MH021852) (Bảng 3).



Hình 2. Đặc điểm của chủng nấm HL6, quan sát dưới kính hiển vi Olympus BX51 (độ phóng đại 40X) (A) sợi nấm; (C) bào tử; (B và D) đặc điểm tản nấm HL6 trên môi trường PDA.

Bảng 3. Thông tin các chủng tham chiếu sử được sử dụng trong phân tích phát sinh loài

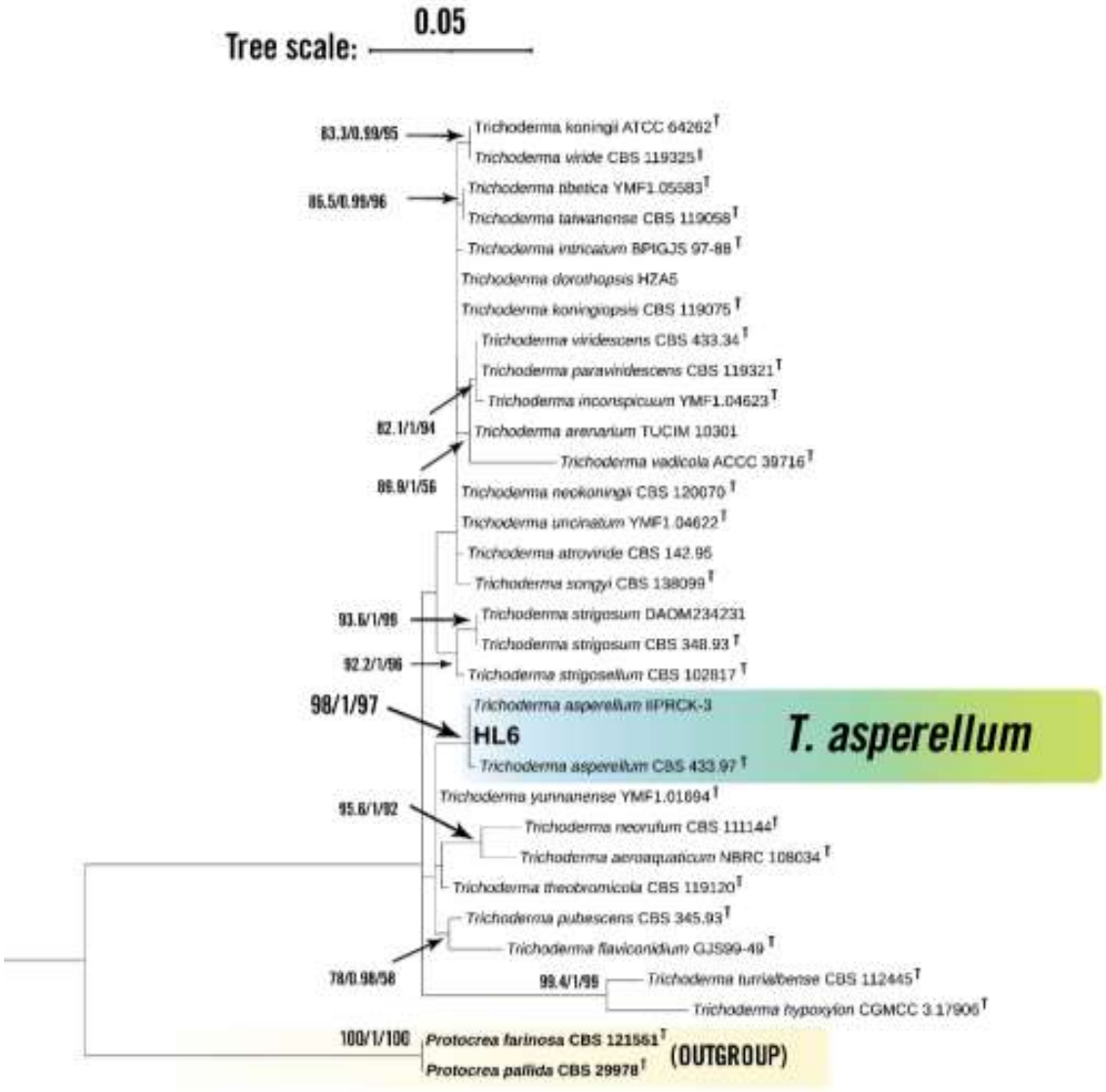
Tên loài tham chiếu	Chủng	Mã truy cập Genbank	Mức độ tương đồng (%)	Ghi chú
Trichoderma asperellum	CBS 433.97	NR_130668	100	TYPE
Trichoderma pubescens	CBS 345.93	MH862413	98,49	TYPE
Trichoderma strigosum	DAOM 234231	EU280114	97,48	
Trichoderma koningii	CBS 345.93	NR_138456	97,44	TYPE
Trichoderma dorothopsis	HZA5	MH624140	97,32	
Trichoderma neorufum	CBS 111144	NR_077132	96,90	TYPE
Trichoderma uncinatum	YMF 1.04622	MK795994	97,09	TYPE
Trichoderma inconspicuum	YMF 1.04623	MK795993	96,98	TYPE
Trichoderma turrialbense	CBS 112445	NR_138448	90,51	TYPE
Trichoderma atroviride	CBS 142.95	AF456917	97,09	
Trichoderma flaviconidium	GJS 99-49	DQ023301	97,66	TYPE
Trichoderma strigosum	CBS 348.93	AY387661	97,50	TYPE

Tên loài tham chiếu	Chủng	Mã truy cập Genbank	Mức độ tương đồng (%)	Ghi chú
Trichoderma arenarium	TUC IM10301	MT217123	97,44	
Trichoderma viridescens	CBS 433.34	AY380905	97,10	TYPE
Trichoderma yunnanense	YMF1.01694	AY941823	99,47	TYPE
Trichoderma theobromicola	CBS 119120	MH863052	98,68	TYPE
Trichoderma asperellum	IIPRCK-3	MK841004	99,66	
Trichoderma hypoxylon	CGMCC 3.17906	NR_153269	90,28	TYPE
Trichoderma vadicola	ACCC 39716	NR_184886	95,55	TYPE
Trichoderma aeroaquaticum	NBRC 108034	NR_134333	97,04	TYPE
Trichoderma paraviridescens	CBS 119321	NR_134367	96,79	TYPE
Trichoderma viride	CBS 119325	NR_138441	97,02	TYPE
Trichoderma intricatum	BPI GJS 97-88	NR_134343	97,10	TYPE
Trichoderma tibetica	YMF 1.05583	NR_176705	97,25	TYPE
Trichoderma neokoningii	CBS 120070	NR_138446	97,58	TYPE
Trichoderma strigosellum	CBS 102817	NR_134437	97,11	TYPE
Trichoderma songyi	CBS 138099	NR_159053	97,24	TYPE
Trichoderma taiwanense	CBS 119058	NR_144874	97,49	TYPE
Trichoderma koningiopsis	CBS 119075	NR_131281	97,49	TYPE
Protocrea farinosa	CBS 121551	NR_119700	83,48	TYPE
Protocrea pallida	CBS 299,78	NR_111329	83,68	TYPE

Ghi chú: TYPE là chủng chuẩn tham chiếu.

Kết quả này cũng được xác nhận bằng cách sử dụng phương pháp cây phả hệ - phân tích phát sinh loài. Mô hình tối ưu: JC được lựa chọn theo Bayesian information criterion (BIC) để phân tích phát sinh loài. Cụ thể, chủng HL6 được tái cấu trúc (reconstruction) trong cụm đơn ngành (monophyletic clade) với sự có mặt của các chủng tham chiếu như *T. asperellum* CBS 433.97 (chủng TYPE) và *T. asperellum* PRCK-3 (MK841004) với tham số hỗ trợ cao (SH-aLRT)/ Bayesian posterior probability (BPP)/Ultrafast bootstrap (UFB): 98/1/97 (Hình 3). Dựa trên các kết quả trên, cho thấy chủng HL6 được xác định thuộc loài *T. asperellum*.

Tại mỗi nút, các giá trị biểu thị các giá trị tham số hỗ trợ theo thứ tự lần lượt là SH-aLRT/ xác suất hậu nghiệm Bayesian (BPP)/ultrabootstrap (UFB) ML. Các chủng TYPE tham chiếu đại diện được ký hiệu bằng "T". Thanh tỷ lệ biểu thị tỷ lệ thay thế nucleotide trên mỗi vị trí. Hai chủng Protocrea farinosa CBS 121551 và Protocrea pallida CBS 29978 được sử dụng làm nhóm ngoài (outgroup).



Hình 3. Cây phát sinh chủng loài dựa trên trình tự ITS của chủng nấm HL6.

3.3 Ảnh hưởng của chủng *T. asperellum* HL6 đến sự nảy mầm của hạt lạc

Tỷ lệ nảy mầm của hạt lạc giống đạt 99,33 % đối với công thức thí nghiệm và đạt 98,67% đối với công thức đối chứng nhưng không khác biệt ở mức ý nghĩa thống kê giữa hai công thức (Bảng 4, Hình 4).

Như vậy, có thể khẳng định chủng *T. asperellum* HL6 không làm giảm tỉ lệ nảy mầm của hạt lạc. Kết quả nghiên cứu này cũng phù hợp với nghiên cứu của Ayyandurai và cs., cho biết chủng *T. longibrachiatum* SP-20 đã không ảnh hưởng đến sự nảy mầm của hạt lạc với tỷ lệ nảy mầm đạt 93,33% [35].

Bảng 4. Tỷ lệ nảy mầm của hạt lạc khi được xử lý với chủng nấm *T. asperellum* HL6

Công thức	Tỉ lệ hạt nảy mầm (%)
Xử lý với nấm *T. asperellum* HL6	$99,33^{a} \pm 0,58$
Đối chứng	$98,67^{a} \pm 1,16$

Ghi chú: Các chữ cái khác nhau trên cùng một cột chỉ sự sai khác trung bình mẫu có ý nghĩa thống kê với $p < 0,05$ (Ducan's test). M ± SD: Trung bình mẫu (Mean) ± độ lệch chuẩn (Standard deviation).



Hình 4. Tỷ lệ nảy mầm của hạt lạc, (A) xử lý bằng dung dịch bào tử nấm *T. asperellum* HL6, (B) xử lý bằng nước cất.

3.4 Khả năng hạn chế bệnh héo rũ gốc mốc đen trên cây lạc của chủng nấm *T. asperellum* HL6 trong điều kiện nhà lưới

Chủng nấm *T. asperellum* HL6 thể hiện khả năng hạn chế bệnh héo rũ gốc mốc đen trên cây lạc khi thử nghiệm ở điều kiện nhà lưới (Bảng 5).

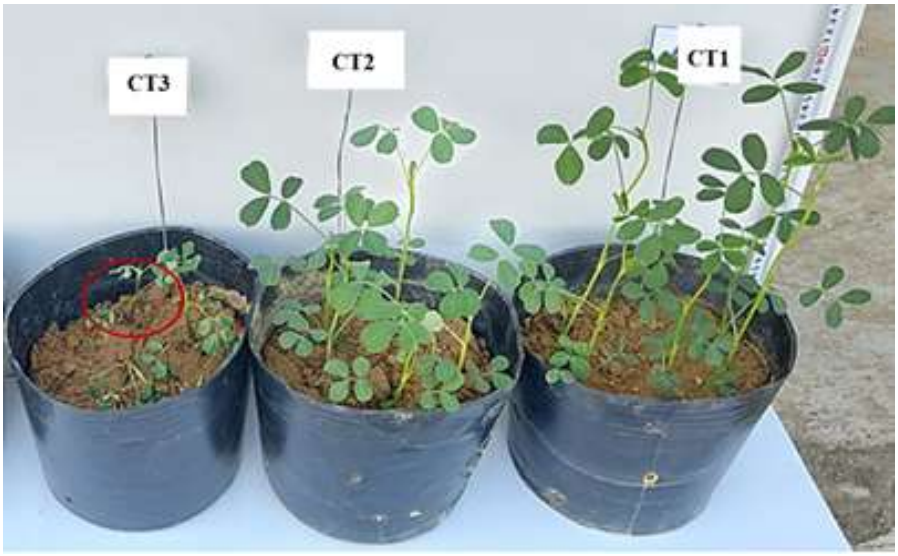
Bảy ngày sau nhiễm nấm *A. niger* QT1, ở CT3 có tỉ lệ cây chết lên đến 60%, khác biệt rõ so với các công thức còn lại, tiếp đến CT2 (*T. asperellum* HL6) tỉ lệ cây bị chết là 2,2%. Như vậy sử dụng dung dịch bào tử nấm *T. asperellum* HL6 có hiệu quả phòng trừ bệnh héo rũ gốc mốc đen ở mức sai khác có ý nghĩa thống kê.

Bảng 5. Hiệu quả phòng trừ sinh học của chủng nấm *T. asperellum* HL6 đối với bệnh héo rũ gốc mốc đen trên cây lạc trong điều kiện nhà lưới

Công thức	Tỉ lệ (%) cây lạc bị chết do héo rũ gốc mốc đen tại các thời điểm điều tra					Hiệu quả phòng chống bệnh 45 NSN (%)
	7 NSN	14 NSN	21 NSN	28 NSN	45 NSN	
CT1	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	100
CT2	$2,2 \pm 3,8^{b}$	$4,5 \pm 3,8^{b}$	$4,4 \pm 3,8^{b}$	$4,4 \pm 3,8^{b}$	$4,4 \pm 3,8^{b}$	95,5
CT3 (Đ/c)	$60,0 \pm 6,6^{a}$	$100 \pm 0,0^{a}$	$100 \pm 0,0^{a}$	$100 \pm 0,0^{a}$	$100 \pm 0,0^{a}$	0,0

Ghi chú: Các chữ cái khác nhau trên cùng một cột chỉ sự sai khác trung bình mẫu có ý nghĩa thống kê với $p < 0,05$ (Ducan's test). M ± SD: Trung bình mẫu (Mean) ± độ lệch chuẩn (Standard deviation). NSN: Ngày sau nhiễm.

Mười bốn ngày sau nhiễm nấm bệnh, tỷ lệ cây chết cao nhất lên đến 100% được ghi nhận ở CT3, tuy nhiên ở CT2 có tỷ lệ cây chết thấp hơn (4,4%). Ở các kỳ điều tra tiếp theo, tỉ lệ cây chết của các công thức không thay đổi. Hiệu quả phòng chống bệnh héo rũ gốc mốc đen đạt 95,5% sau 45 ngày lây nhiễm. Như vậy, kết quả khảo sát cho thấy công thức xử lý bằng chủng nấm *T. asperellum* HL6 thể hiện khả năng kiểm soát tốt bệnh héo rũ gốc mốc đen hại lạc và duy trì hiệu quả ổn định trong điều kiện nhà lưới (Hình 5).



Hình 5. Khả năng kiểm soát bệnh héo rũ gốc mốc đen trên cây lạc của chủng nấm *T. asperellum* HL6 trong điều kiện nhà lưới 14 ngày sau nhiễm nấm *(Khoanh tròn là cây bị héo vì nhiễm nấm, cổ rễ bị thối, phủ lớp nấm màu đen)*

4 Kết luận và đề nghị

4.1 Kết luận

Trong điều kiện *in vitro*, chủng HL6 có khả năng đối kháng mạnh với nấm bệnh *A. niger* QT1, đường kính vùng ức chế sinh trưởng nấm bệnh đạt 69,67 mm, hiệu suất đối kháng đạt 77,41%. Phân tích trình tự ITS và so sánh trình tự trên NCBI bằng công cụ BLAST cho thấy chủng HL6 thuộc loài *T. asperellum*. Chủng nấm *T. asperellum* HL6 không ảnh hưởng đến khả năng nảy mầm của hạt lạc. Thử nghiệm lây nhiễm nhân tạo trong điều kiện nhà lưới, cho thấy chủng nấm *T. asperellum* HL6 thể hiện khả năng kiểm soát tốt bệnh héo rũ gốc mốc đen trên cây lạc do nấm *A. niger* gây ra khi xử lí hạt giống trước khi gieo và tưới chế phẩm vào thời điểm trước khi nhiễm nấm bệnh 1 ngày với hiệu quả kiểm soát bệnh đạt 95,55%.

4.2 Đề nghị

Tiếp tục thử nghiệm chế phẩm nấm *T. asperellum* HL6 trên đồng ruộng để đánh giá hiệu quả phòng trừ sinh học của chủng nấm này được chính xác hơn.

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