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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).

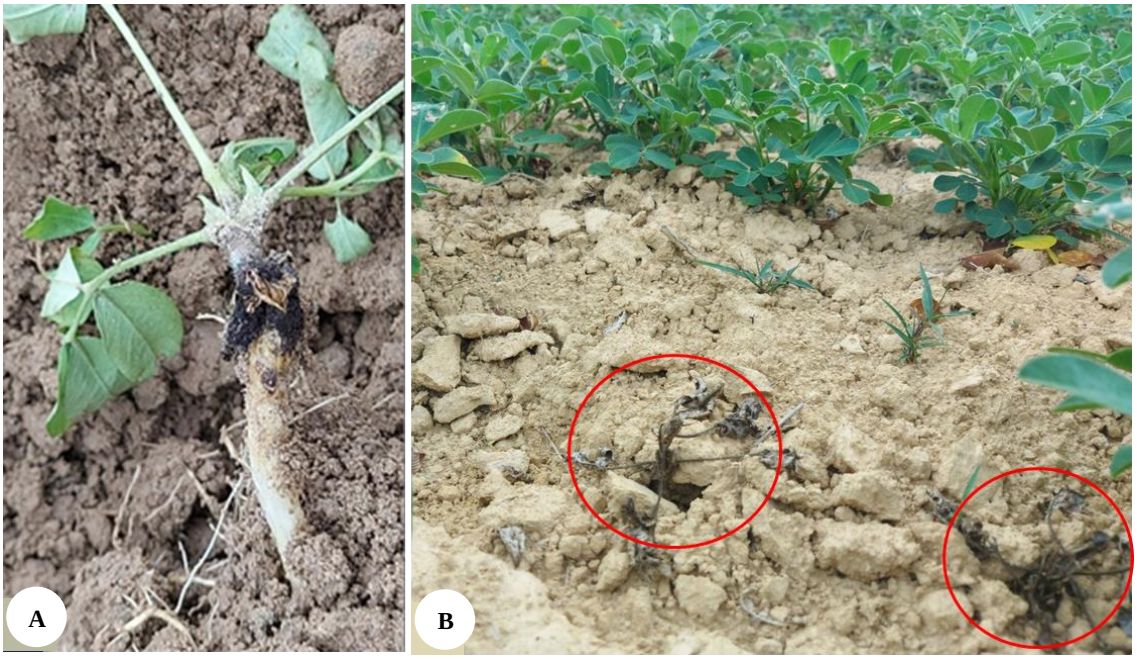

Figure 1. Collar rot symptoms observed on L14 cultivar groundnut on the field. A. A close-up image of plant exhibiting rot symptom on collar, B. Plant exhibiting rot symptoms on collar in Quang Tri province (Red circle indicates diseased plant with collar rot disease of groundnut)

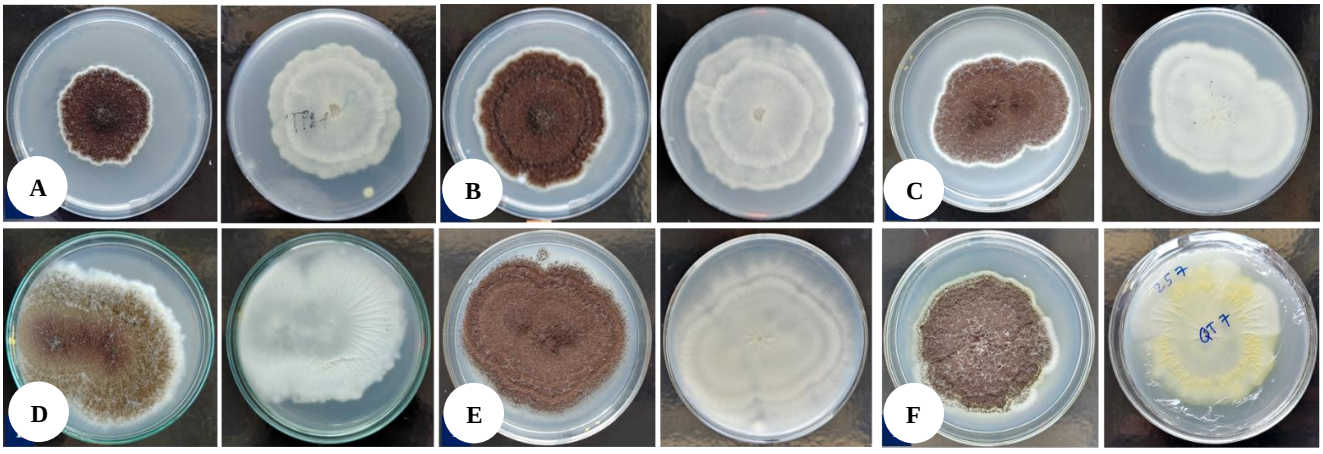

Figure 2. Cultural characteristics of *Aspergillus niger* isolates: (A-F) Colonies on PDA medium

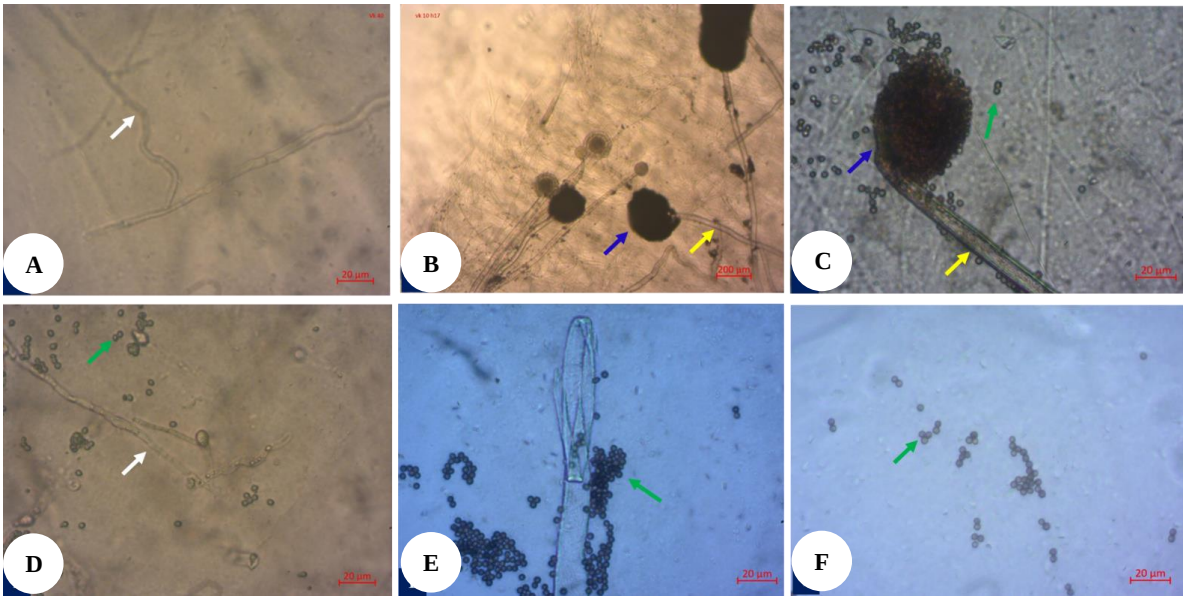

Figure 3. Morphological characteristics of *Aspergillus niger* isolates A-F: Microscopic observations (x 40 magnification). White arrows indicate fungal hyphae, blue arrows indicate vesicle, yellow arrows indicate conidiophores, green arrows indicate conidia, and red bars indicate size references (scalebar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

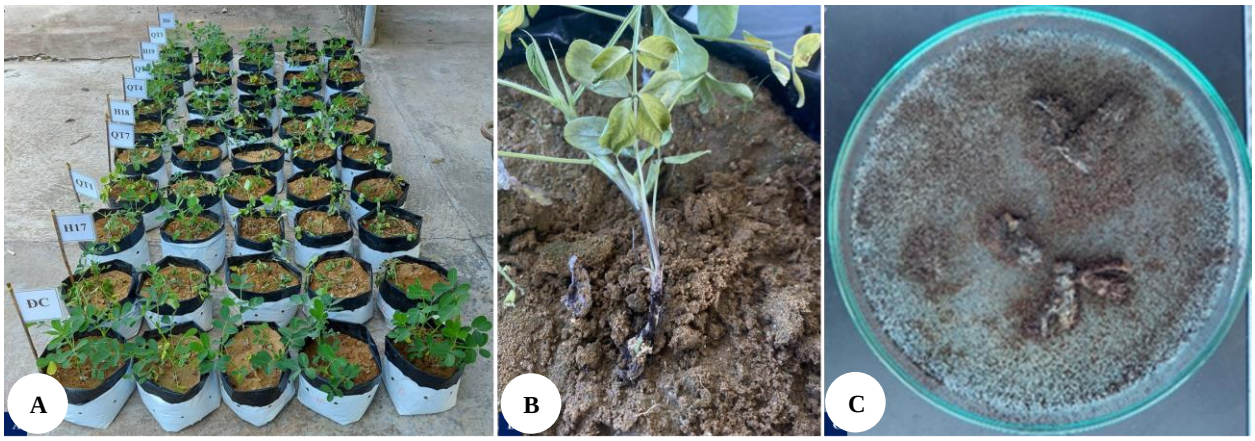

Figure 4. Pathogenicity test on healthy groundnut plants. A. Inoculation of nine fungal isolates on healthy plants (10 days after inoculation), B. Plant exhibiting rot symptom on collar after fungal inoculation, C. Re-isolation of *A. niger* from infected plants

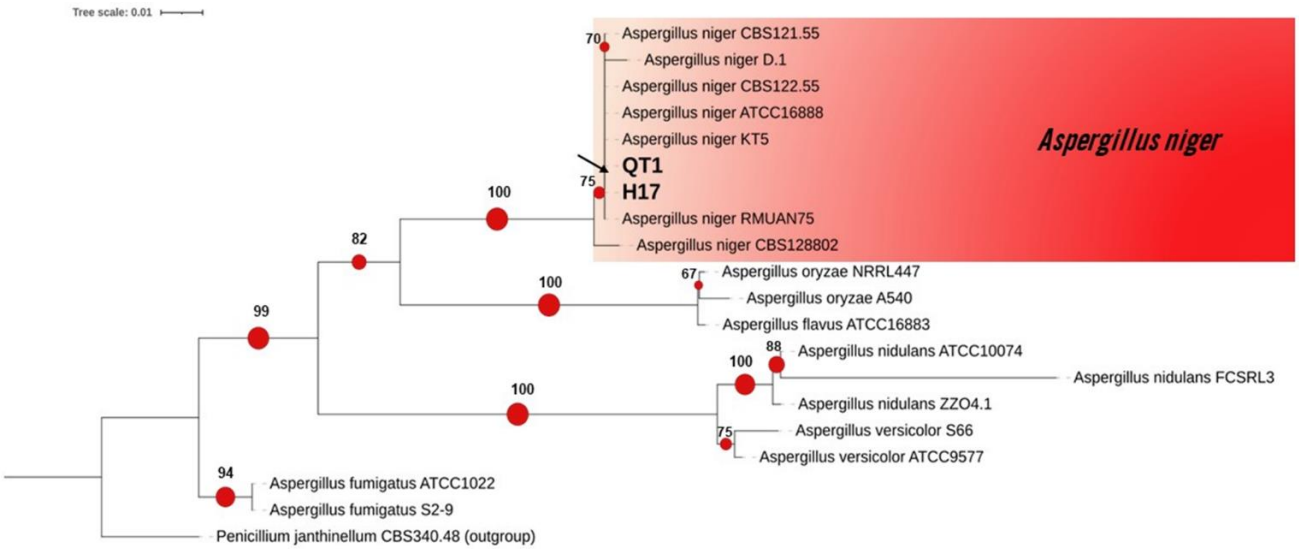

Figure 5. Phylogenetic tree based on ITS sequences of two *Aspergillus niger* isolates (QT1 and H17)

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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