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Investigation of fumonisin production and plant cell wall degrading enzyme activity of *Fusarium verticillioides* causing pink ear rot in Maize (*Zea mays* L.)

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Abstract Maize (*Zea mays* L.) cultivation is challenging due to pink ear rot caused by *Fusarium verticillioides*, which not only compromises maize yield and quality but also produces fumonisin mycotoxins, posing serious risks to human health. This study aimed to islating fungal pathogens and investigating possible machenisms regarding the fungal pathogenicity. Our reults showed that a total of 14 fungal strains were isolated from infected maizes. These strains exhibited high percentage of the pathogenicity up to 90% inhibiting maize seed germination and carried fumonisin-related genes. Additionally, 13 out of 14 fungal strains showed significant plant cell wall–degrading enzyme activities including amylase, cellulase, pectinase and protease, which likely facilitated fungal invasion and tissue breakdown in maize. Among them, the DN8 fungal strain was the most deleterious pathogen and was identified as *F. verticillioides* through sequencing of the Internal Transcribed Spacer (ITS) region. Overall, these findings showed that the fungal isolates caused the pink ear rot and detrimentally infected maize seeds by reducing the germination rate, accumulating fumonisins, and exhibiting aggressive activities, and also emphasized the need for effective management of *F. verticillioides* in maize cultivation.

Keywords: *Fusarium verticillioides,* Fumonisin, Pink ear rot, Plant cell wall-degrading enzyme

Introduction

Maize (*Zea mays* L.) is the third staple cereal crop after wheat and rice, and serving as a primary source of human food and livestock feed (Abbassian, 2006; Omotayo *et al.,* 2022). Maize is rich in carbohydrates, sugars and contains a significant amount of vitamin sources (Swapna *et al.*, 2020). In addition, Maize is considered as a functional food due to its pigments and bioactive metabolites with antioxidant properties that help reduce the risk of diseases such as cancer, cardiovascular disorders, and vision impairment (Kean *et al.*, 2008).

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Maize cultivation faces significant challenges from various effects including environmental condition, varieties and crop management systems. Among them, plant disease cause by pathogens including fungal infections pose the greatest threat to maize cultivation (Chaubey *et al.*, 2015). Fungal plant pathogens can infect any stages of the plant growth and development however the maize infected by fungal pathogen reduces yield and its quality. Some deleterious fungal genera can produce mycotoxins, which have detrimental effects on human and animal health (Surendra *et al.*, 2011; Salau *et al.*, 2017).

Mycotoxins represent a major threat to maize, with the most common types being aflatoxins, fumonisins, trichothecenes, and zearalenone, commonly produced by *Aspergillus, Fusarium*, and *Penicillium* (Pitt, 2014). Among them, *Fusarium verticillioides* has been reported as the most prevalent pathogenic species infecting maize (Cao *et al.*, 2014; Deepa *et al.*, 2016), especially a pink ear rot disease (Bottalico and Perrone, 2002). It not only reduces maize yield and quality (Einloft *et al.*, 2021) but also produces various mycotoxins, especially fumonisins B1 with high concentrations (Sherif *et al.*, 2023). Some fungal pathogens in *Fusuarium* genera such as *F. nygamai*, *F. acutatum*, *F. begoniae*, *F. brevicatenulatum*, *F. phyllophilum* và *F. napiforme* can produced fumonisin with many concentrations (Rheeder *et al.*, 2002). According to Kamle *et al.* (2019), up to 25% of annual crop production is contaminated with mycotoxins at harvest, including fumonisin, resulting in significant economic losses in agriculture and a decline in product quality.

To successfully infect and cause the disease, these fungal pathogens must breakdown the plant cell wall (Hématy *et al.*, 2009; Underwood, 2012), which is highly heterogeneous, consisting of polysaccharides, proteins, and aromatic polymers (Rose *et al.*, 2004; Cosgrove, 2005). Several studies showed that *Fusarium* spp. can produce essential enzymes to damage plant tissues such as cellulase (Sharafaddin *et al.*, 2019; Perincherry *et al.*, 2021), amylase (Gao *et al.*, 2017; Sharafaddin *et al.*, 2019), pectinase (Sharafaddin *et al.*, 2019; Perincherry *et al.*, 2021). The study by Roncero *et al.* (2000) showed that the plant pathogenic fungus *F. oxysporum* can produced plant cell wall-degrading enyzmes such as cellulases, xylanases, pectinases, and proteases. In addition, the saprophytic pathogenic fungus *Alternaria alternata* also produces plant cell wall–degrading enzymes, which have been shown to play an important role in enabling the pathogen to achieve full virulence (Ma *et al*., 2019). Ramzi *et al.* (2019) reported that the pathogenicity of the saprophytic fungus *Ganoderma boninense* in oil palm is associated with plant cell wall-degrading enzymes produced during the saprophytic and necrotrophic stages of host infection.

In the southern of Vietnam, the high humidity in rain season elevated temperatures and create a suitable condition for the growth of *F. verticillioides*.

Besides that, poor post-harvest handling and storage conditions increase the risk of contamination by this pathogen (Tran *et al.*, 2021). Recently, some extensive researchs has focused on the ability of fungi to produce degrading enzymes, particularly fungi linked to maize. However, the production of extracellular enzymes by *F. verticillioides* remains insufficiently studied, despite its critical role in host infection and crop damage. This study aimed to isolating fungal pathogens and investigating possible machenisms regarding the pathogen's enzymatic activities, contributing to the understanding of effective management strategies for pink ear rot in maize.

Materials and methods

Isolation of fungal pathogen

Sixteen diseased maize samples were collected from Dong Nai province and individually stored in sterile nylon bags under a cool storage condition for laboratory analyses (Figure 1A). In the laboratory, the affected kernels from the disease maize (Figure 1B) were collected and placed on the PDA medium supplemented amoxicillin (50µg/ml) and 5 kernels per petri dish. All petridishes were incubated at 28°C in dark condition for 3-5 days (Degani *et al.*, 2021). When the mycelium appeared, a mycelial agar block (ø= 6mm) from the actively growing edge was cut and transferred into fresh PDA plates to purify the fungal isolate. The mycelium of fungal isolates was purified for three times until the homoginy of fungal mycelium was appeared by checking the mecelium under light microsope (Ma *et al.,* 2024). The pure fungal isolates were characterized their morphologies including color of mycelial mat, mycelium, micro-conidia, macro-conidia, phialides. In addition, the all-fungal isolates were also recorded their growth rate by measuring the colony diameter of mycelial growth every 24 hours for 7 days and results reported as the average diameter in millimeters ± standard deviation (LeClaire and Fortwendel, 2015).

Figure 1. Maize samples were collected and pink ear rot symptoms on maize
Note: A) Infected maize samples collected in Dong Nai, Vietnam; B) Infected maize kernels were used to isolate the pathogen.

Table 1. Coordinates of the sampling sites

No.	Sampling site	Coordinate
1	Hamlet 1, Suoi Nho Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°02'31.75"N 107°16'10.62"E
2	Hamlet 2, Suoi Nho Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°02'00.78"N 107°16'22.93"E
3	Hamlet 3, Suoi Nho Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°02'23.90"N 107°17'35.59"E
4	Hamlet 4, Suoi Nho Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°03'20.79"N 107°16'52.68"E
5	Hamlet 5, Suoi Nho Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°04'42.67"N 107°16'18.19"E
6	Hamlet 6, Suoi Nho Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°00'51.02"N 107°17'48.07"E
7	Cho Hamlet, Suoi Nho Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°02'40.09"N 107°16'57.37"E
8	Bau Coi Hamlet, Xuan Bac Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°01'02.14"N 107°16'53.57"E
9	Hamlet 1, Xuan Bac Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°00'23.21"N 107°19'19.05"E
10	Hamlet 2A, Xuan Bac Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°01'56.24"N 107°19'12.81"E
11	Hamlet 3B, Xuan Bac Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°01'47.02"N 107°18'45.07"E
12	Hamlet 4, Xuan Bac Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°03'10.57"N 107°16'45.52"E
13	Hamlet 5, Xuan Bac Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°02'12.4"N 107°17'18.0"E
14	Hamlet 6, Xuan Bac Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°00'16.09"N 107°17'27.93"E
15	Hamlet 7, Xuan Bac Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°05'05.58"N 107°17'32.51"E
16	Hamlet 8, Xuan Bac Commune, Dinh Quan District, Dong Nai Province, Vietnam	11°01'05.13"N 107°20'08.00"E

Detection of fumonisin-producing F. verticillioides by PCR technique

The 7-day-old mycelium of 14 fungal isolates was collected for DNA extraction using the method described by Al-Samarrai and Schmid (2000). Then, the PCR reaction to determine fumonisin-producing *F. verticillioides* was performed using the primer pair VERTF-1 (5'-GCGGGAATTCAAAAGTGGCC-3') and VERTF-2 (5'-GAGGGCGCGAAACGGATCGG-3'), which was designed based on the intergenic spacer (IGS), following the procedure of Patinõ *et al*. (2004). The PCR products were loaded into a 2% agarose gel containing SafeView (ABM, Canada) and a PCR product of Mili-Q water was used as the negative control.

After that, an electrophores process, the DNA bands were visualized using the Gel Doc XR+ System (Bio-Rad, USA). The appearance of the band was about 400bp represented for the positive results in the PCR assay with primers VERTF-1/VERTF-2. The fungal isolates carrying this gene were chosen for the collected spores.

Pathogenicity test

Fungal isolate preparation: All fungal islates were cultured on PDA medium for 7 days. When the spore appeared on the mycelial surface, 10 mL of sterile distilled water was added onto the surface of fungal mycelium and collected the spore following Bhunjun *et al.* (2021) method and was adjusted to 10^5 spores/ml using a hemocytometer (Neubauer Corporation, Germany). The supension of fungal spores was used for maize seed inoculation.

Maize seed preparation: Maize seed used in this experiment was hybrid variety (Southern Seed Corporation, Vietnam). They were surface sterilized following the protocol of Gai *et al.* (2017) and then allowed to dry under the laminar flow cabinet for 30 minutes.

Maize seed inoculation: An experiment was conducted based on the method of Covarelli *et al.* (2012), the seeds were submersed either in 200 mL of the fungal spore suspension for 60 minutes, or in sterile distilled water serving as a negative control. Each fungal isolate was inoculated with 20 seeds and three replicates each. The seeds were then placed on sterile petri dishes contained a moist sterilized paper towel (New Toyo Pulppy Co., Ltd.) and incubated at 30^{o}C in a dark condition for 8 days. On the third day of the incubation period, the number of germinated seeds were counted to calculate the percentage of the seed germination. At the eighth day of the incubation, the healthy seedlings were collected and measrured the length of hypocotyl and root. The vigor index (VI) was calculated according to Randhawa *et al.* (1985):

The percentage of seed germination (%G) = (number of germinated seeds/ the total of seeds) × 100 (1)

The vigor index (VI) = VI = %G × (HL + RL) (2)

Where VI: Vigor index; %G: % germinated; HL: hypocotyl length; RL: root length.

Qualitative tests of plant cell-wall degrading enzymatic activity

Fungal spore preparation: Fungal spore suspensions were prepared similarly to the pathogenicity test according to Bhunjun *et al*. (2021).

Agar well diffusion preparation: The agar well diffusion method described by Oetari *et al*. (2018) was used to evaluate extracellular enzyme activities of fungal isolates. A medium of Czapek-Dox Agar - CDA (30g/L D-glucose, 2 g/L $NaNO_3$, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L KCl, 0.01 g/L $FeSO_4.7H_2O$ and 20 g/L agar) was used as the basal medium supplemented with different substrates depending on the targeted enzyme activity. The CDA medium was amended with 1% starch for amylolytic activity (Zhang and Chen, 2023), with 1% carboxymethyl cellulose (CMC) for cellulolytic activity, and with 1% pectin for pectinolytic activity (Adeleke *et al*., 2012). The skim milk agar – SMA (1 g/L D-glucose, 28 g/L skim milk powder, 5 g/L casein enzyme hydrolysis, 2.5 g/L yeast extract and 20 g/L agar) was used for proteolytic activity (Karimi *et al.,* 2019).

Enzyme activity test: For each plate, four wells were created on the agar plate. Three wells were loaded with 15 µl of fungal spore suspension (10^5 spores/ml), while the fourth well served as a control containing sterile distilled water. All plates were incubated at 30ºC for 5 days in dark condition.

Qualitative assessment of enzyme activity: Amylolytic and cellulolytic activities was assessed by staining plates with 1% Lugol's iodine to visualize the clear zones formed around each well (Adeleke *et al.,* 2012; Zhang and Chen, 2023). Pectinase was indicated by the formation of clear orange zones around the wells after staining plates with 0.3% Congo Red (Haile and Tafesse, 2022; Kolhe et al., 2024) and proteolytic activity was evaluated based on clear zones formed around the wells on SMA (Karimi *et al.*, 2019). Measurements of colony diameter and clear zones were performed. The following formula was used to determine the enzymatic index (EI): R/r, where R was the diameter of the clear zone, and r was the diameter of the colony (Islam *et al.*, 2022).

Identification of the most virulent fungal isolate

Based on the enzyme activity assay, the fungal strain exhibiting the highest pathogenic potential was selected for molecular identification using sequencing of the Internal Transcribed Spacer (ITS) region. The primer pair of ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5' TCCTCCGCTTATTGATATGC-3') was used for the PCR reaction following the protocol of Fujita *et al*. (2001). The identification process was conducted by Loci Institute of Molecular Biology (Ho Chi Minh City, Vietnam). The obtained sequence was then used BLAST tool on the NCBI database to compare it with existing sequences. The phylogenetic tree was constructed on MEGA (version 12) using selected fungal strain sequence and reference sequences retrieved from NCBI including *Fusarium verticillioides* (PV242103.1), *Fusarium verticillioides*

(PV082123.1), *Fusarium ipomoeae* (NR_154596.1), *Fusarium equiseti* (ON417701.1), *Fusarium oxysporum* (MZ496570.1), *Fusarium solani* (NR_163531.1) and *Fusarium falciforme* (NR_164424.1) to determine the phylogenetic relationships among species.

Data analyses

All the experiments were performed in triplicate and the results expressed as the mean value. Differences in data were determined by one-way analysis of variance (ANOVA) followed by the Tukey test ($P<0.05$). Statistical analysis was performed by the Minitab Statistical Software 22 (version 22, LLC).

Results

The isolation and morphological characterization of fungal pathogens

There are 14 fungal strains isolated from sixteen diseased maize samples collected at Dong Nai Province. Their mycelial morphologies exhibited different pigments like orange, pinkish orange and purple. The aerial mycelia of these isolates were cottony mycelium sparse (Figure 1A, Table 2). Their hyphae were hyaline, branched, and septate (Figure 1B). Monophialides were present, and four isolates, including DN3, DN8, DN10, and DN11, exhibited V-shaped structures resembling rabbit ears (Figure 1C).

The microconidia of the fungal strains were cylindrical or oval, hyaline, and non-septate (Figure 1D). However, the microconidia of strains DN5, DN9, DN12, and DN13 were elliptical, with their sizes ranging from 4.0-17.8×1.3-6.0 µm (Table 3). In addition, results from the microscopic observation showed that the microconidia of the DN10 strain had 1-2 septa. Besides, the macroconidia of fungal strains were straight, long, or slightly curved, with a tapered end (14.28%), foot-shaped end (21.43%) and rounded end (64.29%), and had 2-5 septa, measuring 11.5-51.5×2.3-6.1 µm (Figure 1E, Table 3). The strains of DN2, DN3, DN6 and DN11 showed their macroconidia with a thin wall, 2-5 septa and measured 11.5–51.5×2.3–6.1 µm. The macroconidia of strains DN5, DN6, and DN8 were falcate, tapered at both ends, and septate, with dimensions ranging from 4.0–17.8×1.3–6 µm (Table 3).

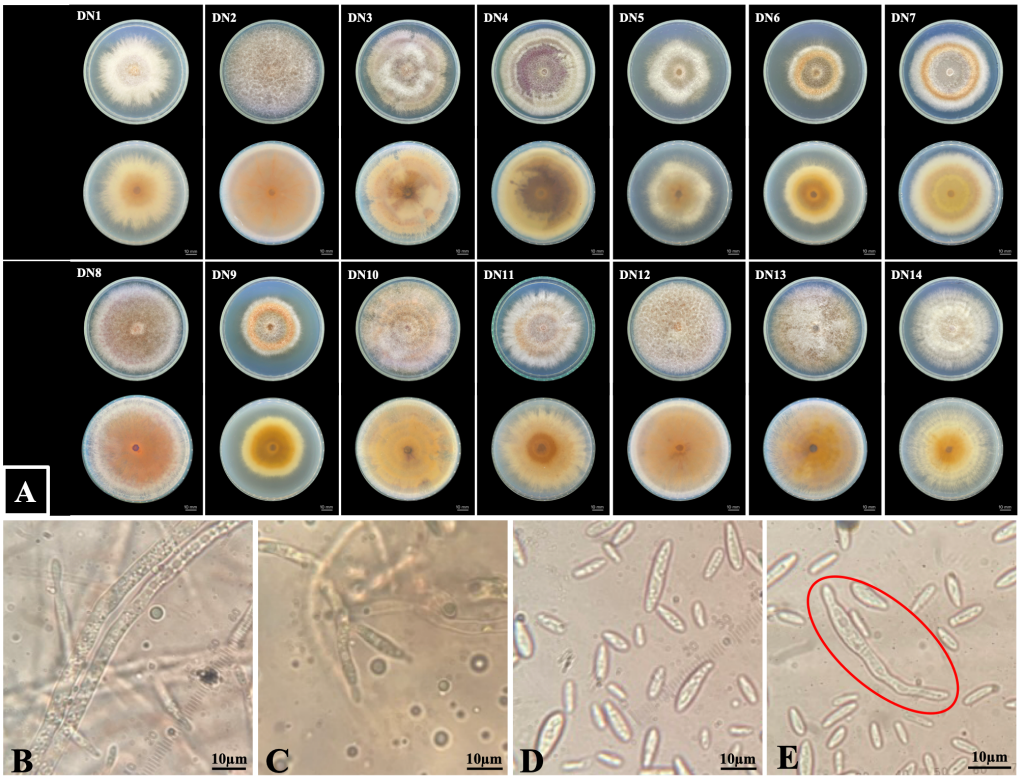

Figure 2. Macroscopic view on PDA medium and microscopic view taken under light microscope with ×100 magnetic of different fungal strains isolated from infected maize: A) Colonies of 14 fungal strains on PDA medium; B) A mycelium of fungus; C) Phialides; D) Microconidia and; E) Macroconidia.

Table 2. Macroscopic characterization of 14 fungal colonies isolated from infected maize

Fungal strain	Colony shape	Margin	Color	Texture
DN1	Circular	Serrate	White with light orange center	Cottony
DN2	Circular	Entire	Pinkish orange	Cottony
DN3	Circular	Serrate	Orangish purple	Cottony
DN4	Circular	Entire	Purple	Cottony
DN5	Circular	Undulate	White with light orange center	Cottony
DN6	Circular	Serrate	White with orange concentric rings	Cottony
DN7	Circular	Entire	White with orange concentric rings	Cottony
DN8	Circular	Entire	Pinkish orange	Cottony
DN9	Circular	Entire	White with orange concentric rings	Cottony
DN10	Circular	Entire	Pinkish orange	Cottony
DN11	Circular	Serrate	White with orange center	Cottony
DN12	Circular	Entire	Pinkish orange	Cottony
DN13	Circular	Entire	Pinkish orange	Cottony
DN14	Circular	Entire	White with orange center	Cottony

Table 3. Microscopic characterization of 14 fungal strains isolated from infected maize

Fungal strain	Description			Conidial size (length×width)	
	Hyphae	Phialides	Conidia	Macroconidia	Microconidia
DN1	Septate	Mono phialides	Cylindrical/oval, non-septate microconidia Slightly curved/straight, foot-shaped end, 3-4 septate macroconidia	23.5-40.0×3.3-5.1	6.1-15×1.9-4.3
DN2	Septate	Mono phialides	Cylindrical/oval, non-septate microconidia Slightly curved/straight, rounded end, 3-5 septate macroconidia	11.5-20.5×3.5-5.0	5.2-15.8×1.8-4.7
DN3	Septate	Mono and V-shaped phialides	Cylindrical/oval, non-septate microconidia Slightly curved/straight, rounded end, 3-5 septate macroconidia	12.8-23.0×3.2-5.1	6.2-17.8×2.2-5.8
DN4	Septate	Mono phialides	Cylindrical/oval, non-septate microconidia Curved, tapered end, 2-4 septate macroconidia	15.5-22.0×3.5-4.3	4.8-13.3×1.3-4.0
DN5	Septate	Mono phialides	Cylindrical/oval, non-septate microconidia Curved/straight, rounded end, 2-3 septate macroconidia	15.1-26.3×3.1-4.1	4.0-10.0×1.8-3.5
DN6	Septate	Mono phialides	Cylindrical/oval, non-septate microconidia Slightly curved/straight, rounded end, 3-5 septate macroconidia	20.5-51.5×3.5-5.0	6.2-16.2×1.8-6.0
DN7	Septate	Mono phialides	Cylindrical/oval, non-septate microconidia Slightly curved/straight, rounded end, 3-4 septate macroconidia	14.5-27.8×2.3-6.1	6.0-13.2×2.0-4.1
DN8	Septate	Mono and V-shaped phialides	Cylindrical/oval, non-septate microconidia Slightly curved/straight, foot-shaped end, 3-5 septate macroconidia	24.0-67.1×4.5-5.2	5.1-18.1×2.0-5.1
DN9	Septate	Mono phialides	Cylindrical/oval, non-septate microconidia	16.5-22.1×3.8-4.2	6.3-11.2×2.5-4.0

Fungal strain	Description			Conidial size (length×width)	
	Hyphae	Phialides	Conidia	Macroconidia	Microconidia
			Slightly curved/straight, rounded end, 2-3 septate macroconidia		
DN10	Septate	Mono and V-shaped phialides	Cylindrical, non-septate microconidia Slightly curved/straight, rounded end, 3-5 septate macroconidia	19.2-23.1×3.5-4.1	6.1-14.4×2.5-3.5
DN11	Septate	Mono and V-shaped phialides	Cylindrical/oval, non-septate microconidia Slightly curved/straight, slender, foot-shaped end, 3-5 septate macroconidia	20.1-27.2×3.1-5.1	5.2-13.5×1.5-4.1
DN12	Septate	Mono phialides	Cylindrical/oval, non-septate microconidia Slightly curved/straight, rounded end, 3-5 septate macroconidia	20.2-36.1×3.2-5.5	7.3-14.2×2.1-4.0
DN13	Septate	Mono phialides	Cylindrical/oval, non-septate microconidia Slightly curved/straight, rounded end, 2-3 septate macroconidia	22.5-32.3×4.0-5.5	5.8-17.5×2.0-5.3
DN14	Septate	Mono phialides	Cylindrical, non-septate microconidia Slightly curved/straight, tapered end, 2-4 septate macroconidia	14.0-24.0×3.0-4.3	4.7-14.0×1.5-3.0

The growth rate of fungal strains

Results of the growth rate indicated that all fungal strains exhibited their vigorus growth (Figure 2). The isolates with the highest growth rates were DN2, DN8, and DN12 and gained 12.86 mm/day ($P<0.05$). Mycelia of these strains completely covered the petri dish at 7 days of the incubation. In contrast, the DN4 and DN9 strains exhibited the slowest growth rate with 9.14 mm/day and 9.52 mm/day, respectively ($P<0.05$).

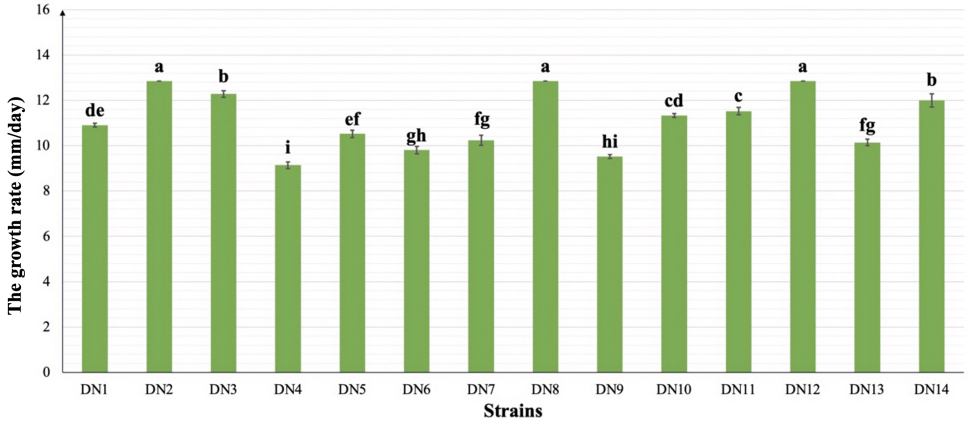

Figure 3. The growth rate of fungal isolates after 7 days of incubation
Note: Data are presented as mean ± standard deviation (SD); different letters above the column denote significant differences at ($P<0.05$) using Tukey's test at a 95% confidence level.

Detection of fumonisin-producing F. verticillioides

The electrophoresis results clearly demonstrated that the PCR reaction of 14 fungal strains applied the primer pair successfully and amplified a unique 400 bp sequence of the IGS region. These PCR products exhibited a single, distinct, and bright band on the agarose gel, and no amplification products were detected with the control (Figure 4). This result provided strong evidence that 14 fungal strains were *F. verticillioides* strains carrying the specific gene region responsible for fumonisin production.

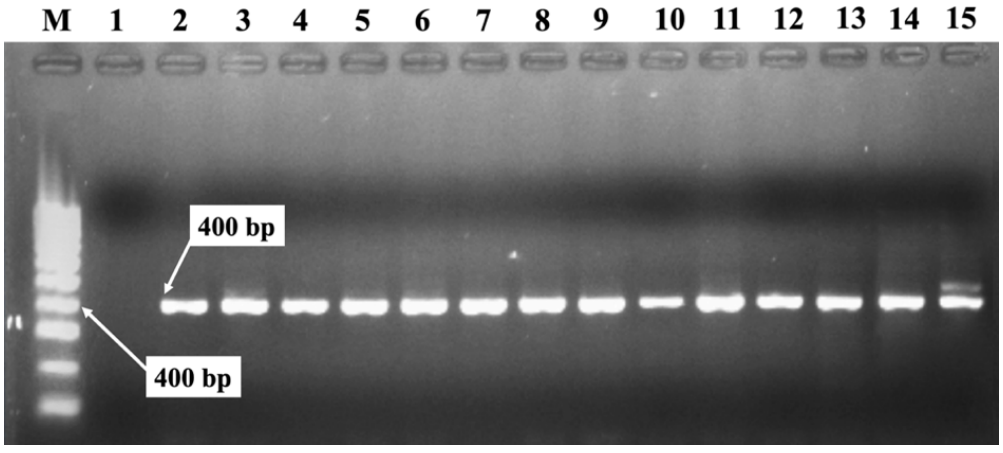

Figure 4. Electrophoresis results with Lane M: HyperLadder™ 100 bp standard from Bioline, England; Lane 1: negative control; Lane 2-15: Fungal strains of DN1 to DN14

Pathogenicity test

After an 8-day incubation period, 14 fungal strains significantly inhibited maize seed germination compared to the control. The germination rate of fungal strains ranged from 1.67-23.33%. Among them, the DN8 strain exhibited the strongest inhibition on the seed germination with 1.67%. Four strains of DN1, DN5, DN11 and DN14 also significantly reduced the seed germination rate to less than 10% (Table 4).

The vigor index of the maize seedling showed that the control treatment had the highest vigor index of 16.32 ($P<0.05$). All seedlings of the treatment inoculated with the DN8 strain were died after 8 days of incubation, resulting in a vigor index of 0.00. The strains of DN1, DN2, DN4, DN5, DN9, DN11, DN12 and DN14 had the vigor indices from 0.26 to 0.89.

In addition, the hypocotyl and root length are considered as important indicators reflecting the viability of seedlings under fungal infection. These results showed that all the fungal strains infected and reduced both hypocotyl and root length, especially root length. The seedlings inoculated with the DN12 and DN4 strain exhibited low hypocotyl length, respectively 0.96 cm and 1.35 cm, which indicated their significant impact on the seedling's growth from the early growth stage. Notably, seedlings incubated with the DN1, DN3, DN4, DN5, DN9, DN11, DN12 and DN14 strain exhibited the lowest root length ranging from 0.90 – 2 cm ($P<0.05$).

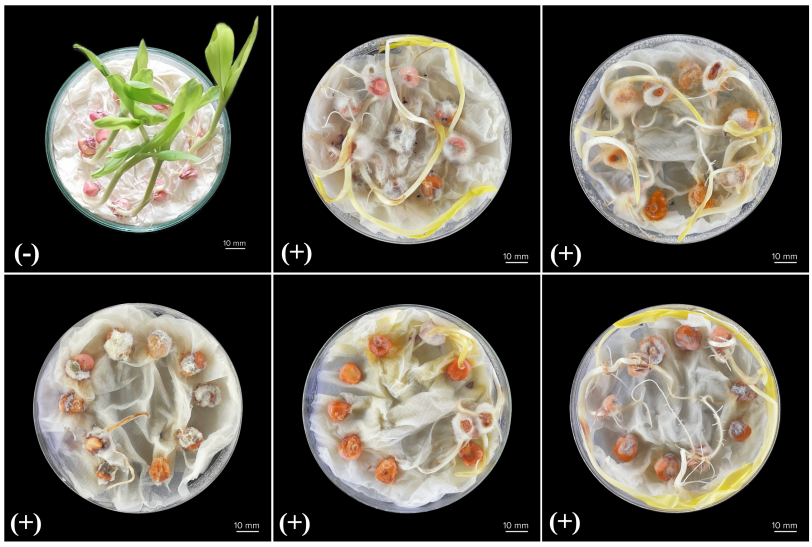

Figure 5. Infection and pathogenicity of fungal strains on maize seeds after 8 days of inoculation: (-) seeds treated with sterile distilled water; (+) seeds treated with the fungal spore suspension

Table 4. Effects of fungal strains on the germination rate, seedling mortality rate, hypocotyl length, root length, and vigor index of maize seeds

Fungal strain	Germination rate (%)[1]	Hypocotyl length (cm)[1]	Root length (cm)[1]	Vigor Index (x100)[1]
Control	100^{a}	6.55^{bc}	9.77^{a}	16.32^{a}
DN1	6.67^{gh}	2.47^{def}	0.90^{fg}	0.26^{f}
DN2	10.00^{efg}	4.55^{cd}	2.00^{ef}	0.66^{ef}
DN3	16.67^{bcde}	8.34^{b}	1.93^{ef}	1.72^{bcd}
DN4	23.33^{b}	1.35^{fg}	1.06^{fg}	0.56^{ef}
DN5	8.33^{fgh}	2.23^{efg}	1.28^{fg}	0.30^{f}
DN6	21.67^{bc}	6.92^{b}	4.35^{cd}	2.44^{b}
DN7	15.00^{cdef}	6.23^{bc}	3.16^{de}	1.41^{cde}
DN8	1.67^{h}	0.00^{g}	0.00^{g}	0.00^{f}
DN9	15.00^{cdef}	4.42^{cde}	1.51^{f}	0.89^{def}
DN10	16.67^{bcde}	8.32^{b}	7.36^{b}	2.63^{b}
DN11	8.33^{fgh}	1.47^{fg}	1.57^{f}	0.28^{f}
DN12	18.33^{bcd}	0.96^{fg}	1.32^{fg}	0.41^{f}
DN13	11.67^{defg}	12.33^{a}	5.82^{c}	2.12^{bcd}
DN14	6.67^{gh}	2.92^{def}	1.98^{ef}	0.32^{f}
P-value	**[2]	**	**	**
CV (%)	13.24	16.36	17.06	14.88

[1]/ Within the same column, values followed by different letters indicate statistically significant differences using the Tukey test at 5% level.
[2]/ P<0.05

Qualitative tests of plant cell-wall degrading enzyme

After 5 days of the incubation, all fungal strains exhibited the ability to produce clearing zones on specific medium. Results from the CDA medium Among 14 fungal strains, the DN8 strain showed the highest enzymatic indices of amylase, cellulase and pectinase with values of 0.644, 1.019 and 0.829, respectively (P<0.05). On SMA medium, the strains of DN8 and DN4 exhibited the highest degradation zone values with the values of 0.761 and 0.797, respectively (P<0.05). Overall, the DN8 strain showed the highest enzyme activities in all tests and it also showed the strongest virulence in the germination

test. Therefore, this fungal strain was selected to sequence the ITS gene region to identify the species.

Identification of the most virulent fungal isolate

Results frem the identification of the ITS sequencing showed that the DN8 strain sequence exhibited 100% similarity with *Fusarium verticillioides* PV242103.1 in the NCBI database. The phylogenetic analysis also revealed that the DN8 strain clustered within a small clade alongside *Fusarium verticillioides* (PV242103.1) and *Fusarium verticillioides* (PV082123.1), with a bootstrap value of 100, indicating their close evolutionary relationship compared to other species (Figure 7).

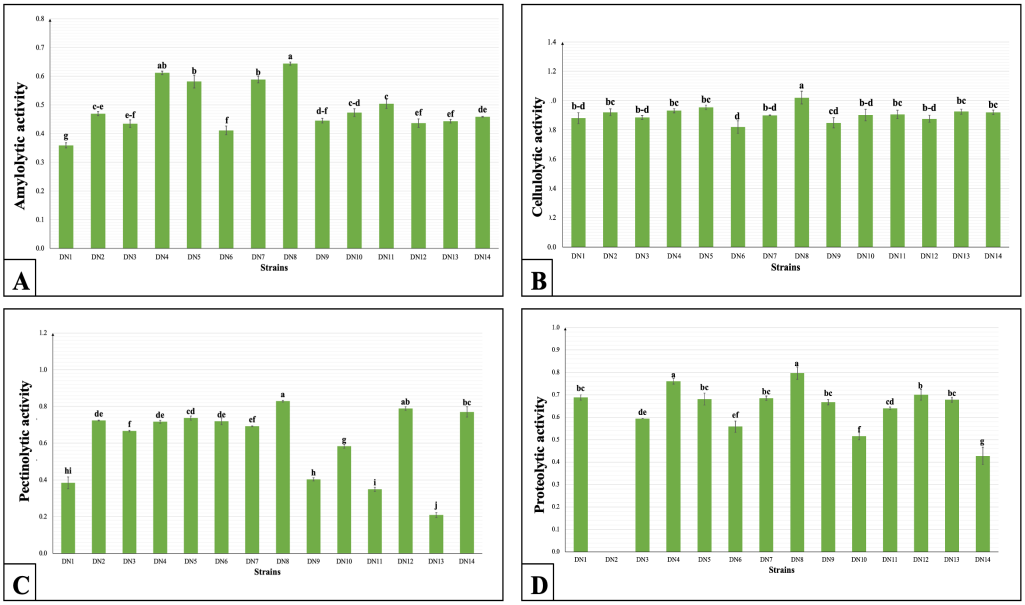

Figure 6. Screening enzymatic activities of the fungal isolates
Note: A) amylase, B) cellulase, C) pectinase, D) protease. Data are presented as mean (n=3) ± standard deviation (SD). Different letters above the column denote significant differences ($P<0.05$) using Tukey's test at a 95% confidence level.

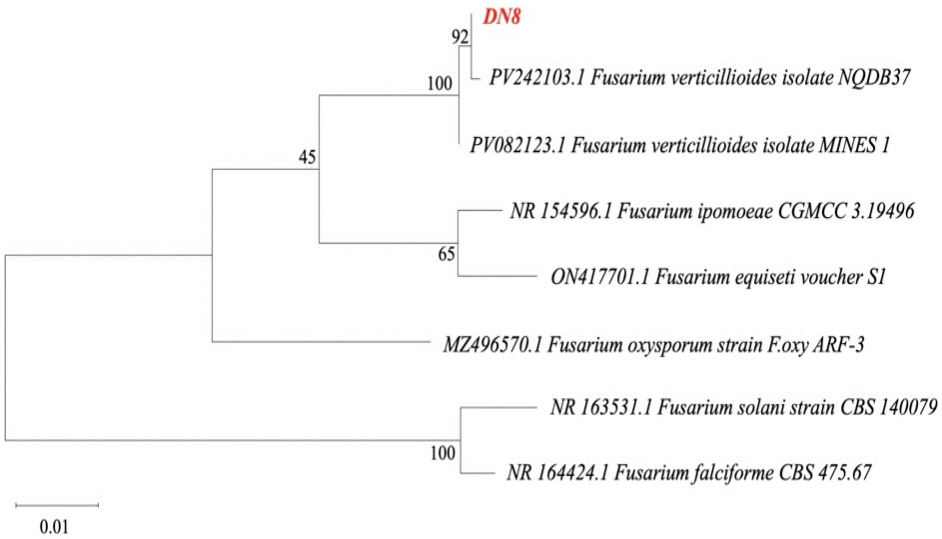

Figure 7. A phylogenetic tree based on the ITS sequence of the DN8 strain conducted using the neighbor-joining method in MEGA 12. Numbers on branchces indicate bootstrap support based on 1000 replicates

Discussion

Based on our results, the morphology of 14 fungal isolates was consistent with previous studies on *F. verticillioides* (Chandra *et al.*, 2008; Kaur *et al.*, 2016; Gagkaeva and Yli-Mattila, 2020). Colonies of *Fusarium verticillioides* isolated from the corn exhibited light pink to pale purple colors along with fine white to off-white aerial hyphae. In line with these findings, Harleen *et al.* (2016) also observed the pigmentation of *F. verticillioides* colonies ranging from purple to dark purple, with some isolates displaying light pink to pale purple shades. An exhibition of V-shaped structures of phialides also aligns with the description that Macial *et al*. (2016) reported about *F. verticillioides*.

As a part of the microbial community in the soil, *F. verticillioides* can infect maize seeds and cause the growth of the seedlings damaged within 72 hours of the infection and destroy the crop entirely (Omotayo and Babalola, 2023). When the seeds were contaminated by pathogenic fungi, these pathogens progressively branched, broke down and penetrated protoplasmic cells as the seed coat deteriorated (Mehrotra and Aggarwal, 2003). The infection and virulence of the fungal pathogenic strains significantly affected on maize seed germination and the level of seedling vigor index. The lower germination rates, the more severe the damage. Stagnati *et al.* (2020) also reported that the inculation of *F. verticillioides* on maize seed caused reduced 70-90% of the seed

germination. In this study, the DN8 strain exhibited the strongest virulence when it significantly reduced the germination rate, and the seeds which formed seedlings could not survive afterwards. Not only the DN8 strain, the strains of DN4, DN5, DN11 and DN12 also showed serious damage to seedlings, causing the root length and hypocotyls to be significantly lower than those of the control. In contrast, the DN13 strain had less effect on root and hypocotyl length than that of the other strains; however, this fungal strain still infected and significantly inhibited seed germination rate. This can be highlighted that the toxicity of these fungal strain not only impaired seed germination and but also inhibited seedling growth, in turn seriously impacting yield and quality of maize (Báez-Astorga *et al*., 2025), especially the DN8 strain.

In addition, when *Fusarium* infected plants, the secondary metabolites released from them such as trichothecenes, deoxynivalenol and especially the fumonisins B1, B2, and B3, were reported to rapidly induce seedling wilting, stem softening, and leaf necrosis (Marasas *et al.*, 1995; Mehrotra and Aggarwal, 2003). This may explain a reason for 14 fungal strains, particularly strain DN8, were able to infect maize seeds and seedlings.

This study also illustrated the ability of these fungal strains to release plant cell wall-degrading enzymes, including amylase, cellulase, pectinase, and protease. These enzymatic activities help degraded the protective cuticle and plant cell wall, thereby facilitating tissue penetration and nutrient acquisition. These enzymes may also cause servered effect in later stages of the infection, when the fungi invaded the physical barriers like cellulose microfibrils and lignin (Gibson *et al.*, 2011). Among 14 strains, the DN8 strain showed the highest enzymatic indices in all tests. This result was clarified the mechanism of infection of the DN8 strain and emphasize that the DN8 strain is a dangerous pathogen that needs to be strictly managed.

In conclusion, this study successfully isolated 16 fungal pathogens and provided evidences that 14 out of 16 pathogenic fungal strains exhibited key characteristics of *F. verticillioides* carrying the gene responsible for fumonisin production using the primers VERTF-1 and VERTF-2 detection. These fungal strains inhibited maize seed germination and seedling growth. Among the strains studied, the DN8 strain carried genes responsible for fumonisin production, damaged seed germination severely, and produced all extracellular enzymes related to cell wal degradation capacity. This study's results showed that *Fusarium verticillioides* DN8 not only caused serverely pink ear rot in maize but also reproduced fumonisin, which potentially affects maize yield, quality, and even consumer health.

Conflict of interest

The authors declare no conflict of interest.

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