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Discovery of Ganoderma Species Associated with Dieback of the *Delonix regia* Trees

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ABSTRACT

Background: *Delonix regia* is a flowering tree of the Fabaceae family, widely grown as an ornamental plant in urban areas and considered a symbol of summer in Vietnamese culture. From January 2023 to November 2024, more than 100 cases of *Delonix regia* trees dying from a fungus growing at the base or roots of the trees were recorded in Hue city, Vietnam. The study determines the causal organisms for management purposes urban green trees.

Methods: Fruit body samples collected from the wild were analysed morphologically following the method of Trinh Tam Kiet. Microscopic structures of fungal hyphae, spores and hymenium holes were observed and photographed using an Olympus BX51 optical microscope. Sample were sterilized with alcohol 70% to isolate fungal hyphae using the tissue culture to create pure cultures for DNA extraction and studies on the growth characteristics of this fungus. The mycelium was used for DNA extraction according to the method. The PCR reaction was performed using the ITS1 and ITS4 primer pairs (ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′; ITS4: 5′TCCTCCGCTTAT TGATAT GC-3′), following the method described. The PCR products were analysed on agarose gel and then subsequently sequenced and were compared against gene sequences in the GenBank database using the BLAST tool. Mushrooms cellulase activities analysis by colorimetric method using dinitrosalicylic acid (DNS).

Result: Research about the morphology, observe the basidiospores by microscopy and analysed the phylogeny based on ITS sequences and identified the fungus causing *Delonix regia* tree death disease distributed in Hue city as *Ganoderma multipileum*. The cellulase activity of *Ganoderma multipileum* mycelium reached a maximum value of 0,185 IU/mL after 5 days of culture at 35°C.

Key words: *Delonix regia, Ganoderma multipileum,* ITS gene, Morphology.

INTRODUCTION

Urban green trees offers numerous health benefits, including regulating microclimates, providing shade, blocking wind, reducing rainwater runoff, lowering noise pollution and sequestering carbon. The *Delonix regia*, a flowering plant from the Fabaceae family, is widely cultivated as an ornamental tree along streets, in parks, schools and office premises. In Vietnamese culture, it symbolizes summer, with parts of the tree being used in traditional medicine such as: the bark has the effect of reducing fever, lowering blood pressure, reducing joint pain and swelling. While the leaves can cure belching, heartburn and constipation (Do Tat Loi, 2004).

Nowadays, protecting urban trees is essential as most of them are woody. Their damage due to insect and wood-decaying fungal attacks can pose risks to humans (Huynh, 2024). Therefore, identifying pathogenic threats to these trees is crucial for effective management.

In this study, a Ganoderma species causing the dieback of *Delonix regia* was discovered at the base of these trees in Hue city, Thua Thien Hue province, Vietnam. The morphological characteristics and molecular sequence data of the species are detailed below.

MATERIALS AND METHODS

Ganoderma samples were randomly observed and collected from different *Delonix regia* trees aged over 20 years from January 2023 to November 2024 in Hue City (Fig 1). The experiments were conducted from March 2023 to September 2025 at the Department of Biology, Faculty of Science, Hue University, Vietnam.

Strain isolation

Samples were brought to the laboratory and sterilized with alcohol 70% to isolate fungal hyphae using the tissue culture method of Nguyen (2003). Under sterile conditions, fungal fruiting body samples were cut into tissue fragments measuring 0.5×0.5×0.1 cm. These were then placed in sterilized potato dextrose agar (PDA) petri dishes to create pure cultures for DNA extraction and studies on the growth characteristics of this fungus.

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Morphological characterization

Fruit body samples collected from the wild were analysed morphologically following the method of Kiet (2011). Microscopic structures of fungal hyphae, spores, etc were observed and photographed using an Olympus BX51 optical microscope.

Molecular identification

The mycelium was used for DNA extraction according to the method of Gardes and Bruns (1993): 50 mg of mycelium was crushed for 5 minutes, 500 µL of solution Lysis buffer, vortexed and left at room temperature for 10 minutes, then centrifuged and the supernatant was collected. The DNA was precipitated with alcohol 96% and washed twice with 70% alcohol. The DNA was then vacuum dried for 10 minutes at 45°C and dissolved in 100 µL TE 0.1X. Finally, the DNA quality was checked through spectrophotometry and electrophoresis on 0.8% agarose gel. Qualified products were stored at -20°C for the next steps.

The PCR reaction was performed using the ITS1 and ITS4 primer pairs (ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′; ITS4: 5′TCCTCCGCTTAT TGATAT GC-3′), following the method described by White *et al.* (1990) and Ramesh (2022). The PCR products were analysed on agarose gel and then subsequently sequenced. The obtained sequences were compared against gene sequences in the GenBank database using the BLAST tool (Basic Local Alignment search tool).

The sequences were aligned using ClustalW software. A phylogenetic tree illustrating the genetic relationships between the studied sample and other *Ganoderma* species listed in GenBank was constructed using MEGA 11 software. The tree was based on the Maximum Parsimony method with a bootstrap confidence level of 100% (Altschul *et al.*, 1990).

Isolation medium

PDA (Potato dextrose agar) includes 200 g potato extract, 20 g dextrose, 20g agar, distilled water to 1000 mL (Kartik *et al.*, 2022).

Level 1 propagation medium

- **PGA (Potato glucose agar):** 200 g potato extract; 20 g glucose; 20 g agar; distilled water to 1000 mL.
- **Raper:** 2 g peptone; 2 g yeast extract; 0,5 g $MgSO_4.7H_2O$; 0,46 g KH_2PO_4; 1 g K_2HPO_4; 20 g glucose; 20 g agar; distilled water to 1000 mL.
- **Peptone:** 20 g cornstarch; 20 g glucose; 1 g KH_2PO_4; 0,5 g $MgSO_4.7H_2O$; 1 g peptone; 20 g agar; distilled water to 1000 mL (Nguyen, 2003).

Mycelium culture medium for cellulase enzyme collection

- **Rice bran:** 50 g.
- **40 mL mineral solution including:** 3 g $NaNO_3$; 0,5 g $MgSO_4$. 7 H_2O; 0,5 g KCl; 1 g KH_2PO_4; 0,1 g $FeSO_4.7H_2O$; 10 g CMC, 1000 mL H_2O, pH= 6-6.5.

Mix well and make cotton plug. Sterilize at 1 atm, 30 minutes.

Determining cellulase activity

The reaction mixture including 1 mL enzyme extract; 0,5 mL phosphate buffer and 1 mL 1% CMC was mixed well and incubated for 30 minutes at 25°C. The reaction was terminated by adding 1 mL DNS reagent. The colour was created by boiling the mixture for 5 minutes. Optical density was measured at 575 nm against a blank (Miller, 1959).

RESULTS AND DISCUSSION

Morphological characteristics

The fruit body consists of a stipe, cap and hymenophore with diverse shapes and structures. The mushroom stalk is cylindrical, single or branched, often attached to one side of the mushroom cap, the outside is covered with a hard, shiny brown tissue layer (Fig 2B). The mushroom cap is typically fan-shaped, semicircular, kidney-shaped, multi-layered. The upper surface of the cap often features concentric rings, ripples, or folds and has a shiny reddish-brown colour. The margin is usually light yellow, transitioning to reddish-brown towards the centre (Fig 2A).

The lower surface of the mushroom cap is often white or light gray (Fig 2B), covered with many light brown polygonal hymenium holes (Fig 2C). Young fruiting bodies develop from the surface of old ones, forming overlapping layers.

When cutting across the mushroom cap (Fig 2D), it is possible to distinguish from top to bottom: the cap crust, which is typically very hard, shiny and reddish-brown; the context tissue beneath the crust, consisting of spongy,

Fig 1: Observed and collected *Ganoderma* mushroom samples.

lightweight cells that are very tightly bound and the hymenophore layer under the context tissue, made up of closely arranged cylindrical tubes containing basidiospores.

Basidiospores when observed under high magnification (10×100) appear pale yellow, measuring 9-12 µm and are typically short-ovoid or elliptical in shape. The spore wall has two layers: an outer layer that is smooth and transparent and an inner layer that is pale yellow to light brown with evenly distributed protrusions. The spores do not contain starch (Fig 2E). The hyphae observed at magnification (10×40) are thin, branched, non-septate, usually colourless (Fig 2F), which are vegetative hyphae that often appear in large quantities in the substrate or when cultivated to harvest fruiting bodies.

Based on the morphological characteristics of the fruiting body and basidiospores of the fungus being studied, compared to the descriptions provided by Kiet (2011), this sample belongs to a species within the *Ganoderma* genus. However, to confirm the precise identification, genetic sequencing was performed using the PCR method.

Results of ITS gene region analysis

To obtain more accurate identification results, we purified the strain on PDA medium to collect fungal hyphae for rRNA analysis using molecular biology methods. The ITS gene region sequence was determined and after removing primer sequences and noise signal regions, we obtained the nucleotide sequence shown in Fig 3.

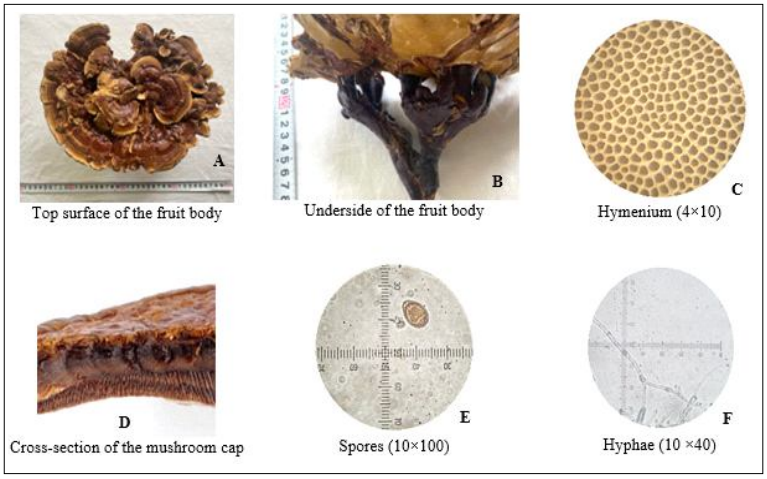

Fig 2: Morphological characteristics of mushrooms.

Score	Expect	Identities	Gaps	Strand	Frame
1120 bits(606)	0.0()	606/606(100%)	0/606(0%)	Plus/Plus	

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Query  1    GATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCATGTGCACGCCCTGC  60
Sbjct  12   GATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCATGTGCACGCCCTGC  71
Query  61   TCATCCACTCTACACCTGTGCACTTACTGTGGGCTTCAGATCGTAAAACGGGTCCCTTTA  120
Sbjct  72   TCATCCACTCTACACCTGTGCACTTACTGTGGGCTTCAGATCGTAAAACGGGTCCCTTTA  131
Query  121  CCGGGCTTGCGGAGCGTGTCTGTGCCTGCGTTTATCACAAACTCTATAAAGTATCAGAAT  180
Sbjct  132  CCGGGCTTGCGGAGCGTGTCTGTGCCTGCGTTTATCACAAACTCTATAAAGTATCAGAAT  191
Query  181  GTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGC  240
Sbjct  192  GTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGC  251
Query  241  ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA  300
Sbjct  252  ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA  311
Query  301  TCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTG  360
Sbjct  312  TCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTG  371
Query  361  TCATGAAATCTTCAACCTGCAAGCTTTTGTGGTTTGTAGGCTTGGACTTGGAGGCTTGTC  420
Sbjct  372  TCATGAAATCTTCAACCTGCAAGCTTTTGTGGTTTGTAGGCTTGGACTTGGAGGCTTGTC  431
Query  421  GGCCGTTGTTGGTCGGCTCCTCTTAAATGCATTAGCTTGGTTCCTTGCGGATCGGCTCTC  480
Sbjct  432  GGCCGTTGTTGGTCGGCTCCTCTTAAATGCATTAGCTTGGTTCCTTGCGGATCGGCTCTC  491
Query  481  AGTGTGATAATGTCTACGCTGCGACCGTGAAGCGTTTGGCGAGCTTCTAACCGTCTCAGT  540
Sbjct  492  AGTGTGATAATGTCTACGCTGCGACCGTGAAGCGTTTGGCGAGCTTCTAACCGTCTCAGT  551
Query  541  TGGAGACAACTTTATGACCTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGC  600
Sbjct  552  TGGAGACAACTTTATGACCTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGC  611
Query  601  ATATCA  606
Sbjct  612  ATATCA  617
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Fig 3: Sequencing results of the ITS gene region.

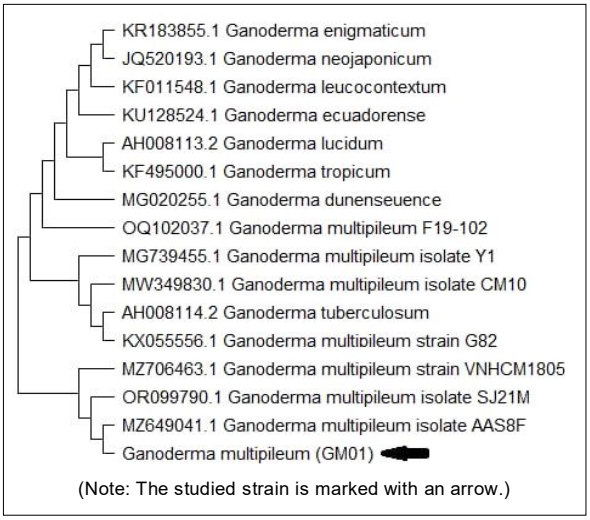
The results of ITS region sequencing were compared with the database on NCBI GenBank (Table 1). The 606 bps rRNA gene segment of the ITS region of the fungus had a similarity rate of 100% with the species *Ganoderma multipileum* (Acession number: MZ649091.1). Comparing the results of morphological description according to Kiet (2011), Dong (2009), Nguyen *et al.* (2023) and combining the analysis of rRNA in the ITS region, we found that this fungus sample is the species *Ganoderma multipileum* Ding Hou (1950).

The phylogenetic tree was built based on the ITS sequence of the studied strain and closely related strains of the species *Ganoderma multipileum* shown in Fig 4.

Growth pattern of *G. multipileum* in different growth media

The results of isolation on PDA medium showed that the fast growing with dense and uniform mycelium. When cultivating mycelium on three types of media surveyed for level I propagation including PGA, Pepton and Raper, it was shown that the Rapper medium had a statistically significant difference in the growth process of mycelium compared to the Pepton and PGA medium (Table 2 and Fig 5).

The survey results of the primary mycelium culture medium of *Ganoderma multipileum* showed that Raper medium is the most suitable because this medium has all the necessary nutrients for mycelium growth, a large mycelium system, grows quickly and branches evenly after 5 days of subculturing. Compared with the research results of Ho (2017) when using Raper medium to cultivate *Ganoderma applanatum* mycelium collected in Tinh Bien area, An Giang province, with a mycelium speed diameter of 4,45 cm after 8 days of culture, our survey results are higher, especially in terms of mycelium growth time, which is much shorter. Based on the survey of the characteristics of mycelium and the diameter of mycelium growing on 3 primary propagation media, we chose Raper medium to propagate *Ganoderma multipelium*.



(Note: The studied strain is marked with an arrow.)

Fig 4: Phylogenetic tree showing the genetic relationships of *Ganoderma multipileum* (GM01).

According to Ryu and Mandels (1980): the cellulase system consists of three soluble extracellular enzymes, namely 1,4-β-endoglucanase, 1,4-β-exoglucanase and β-glucosidase (β-Dglucoside glucohydrolases or cellobiase), which hydrolyse cellulose into glucose. Pilotti *et al.* (2004) showed that Ganoderma species grow as a facultative parasite, which can live as a saprophyte on decaying tree stumps and roots by decomposing lignin as well as cellulos. We believe that this *Ganoderma multipileum* species is capable of producing cellulase enzymes that hydrolyse cellulose, a major component of wood and plants, converting it into simple sugars for the fungus to absorb as a source of energy and nutrients. This shows the potential for the application of this fungus in the treatment of agricultural waste to create valuable products. The results of cultivating mycelium at 30°C on agar medium supplemented with 1% CMC after 5 days showed that mycelium grew a lot, with a mycelium diameter of 4.3±0.11 cm. After staining with Lugol's reagent, the ability to decompose CMC (carboxyl methyl cellulose) was the part that did not absorb the dye with a diameter of 5.1±0.13 cm (Fig 6). This shows that *Ganoderma multipileum* has a high ability to produce cellulase enzyme.

To evaluate the cellulase activity of *Ganoderma multipileum,* we cultured mycelium on rice bran medium supplemented with mineral solution and 1% CMC, the culture process was conducted at the following temperatures: 25°C, 30°C, 35°C and 40°C. After 5 days, the cellulase enzyme was separated by 0.5% NaCl solution at a ratio of 1 g of culture medium to 4 mL of 0,5% NaCl,

Table 1: Similarity of collected mushroom sequences with *Ganoderma multipileum* on NCBI.

Description	Max score	Total score	Query cover	E. value	Per. ident	Accession
Ganoderma multipileum isolate AAS8F_ITS1F internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene	1083	1083	100%	0.0	100%	MZ649041.1
Ganoderma multipileum isolate SJ21M small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	1083	1083	100%	0.0	100%	OR099790.1

Table 2: Results of the first-level breeding environment survey.

Environment	Mycelial spread after 3 days (cm)	Mycelial spread after 5 days (cm)	Mycelial characteristics
PGA	2.82[c]	4.51[c]	Mycelial growth is thick, white, fluffy, uniform, slow growing
Pepton	3.86[b]	5.14[b]	Mycelial growth is abundant, white, sparse, unevenly developed
Raper	4.12[a]	6.28[a]	Mycelial growth is abundant, white, fluffy, fast growing, evenly branched

Note: Mean values in the same column followed by different letters indicate statistically significant differences (P<0.05).

Fig 5: Mycelium growth on different nutrient media after 5 days.

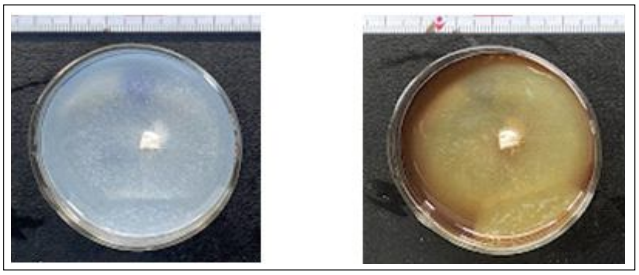


Fig 6: Cellulase production ability of *Ganoderma multipileum* on 1% CMC medium.

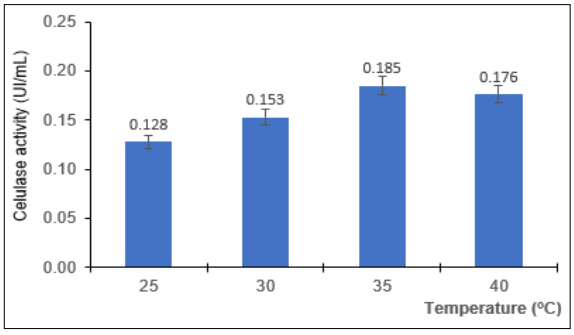


Fig 7: Cellulose activity of mycelium at different culture temperatures.

Fig 8: Life forms of *Ganoderma multipileum* on *Delonix regia* trees.

cold centrifugation to collect the extract and determine the enzyme activity by spectrophotometry with DNS reagent.

The results shown in the figure show that: the cellulase enzyme of *Ganoderma multipileum* is highly active at 30-40°C with a maximum activity of 0,185 UI/mL when cultured at 35°C, then tends to decrease due to the inhibition of cellulase catalytic activity at higher temperatures (Fig 7).

The research results of Nyi and Ginayanti (2017) on the cellulase enzyme activity of *Ganoderma applanatum* and *Ganoderma tropicum* in Indonesia showed that the cellulase of *Ganoderma applanatum* reached 0,184 UI/mL, which was stronger than *Ganoderma tropicum* (0,112 UI/mL) at 45°C. Compared with this result, the cellulase activity of *Ganoderma multipileum* is equivalent to that of *Ganoderma applanatum*.

Characteristics of *Ganoderma multipileum* causing dieback in *Delonix regia*

In nature, *Ganoderma multipileum* often parasitizes or acts as a saprophyte on the trunk or roots of living *Delonix regia* trees (Fig 8A) or as a saprophyte on the stumps of dead flamboyant trees (Fig 8B).

In nature, *Ganoderma multipileum* produces basidiospores that are dispersed through the air and soil. Under favourable conditions (temperature and humidity) and when the *Delonix regia* tree is damaged at its roots or trunk, these spores can penetrate the tree, germinate and develop into vegetative hyphae (Fig 9C). These hyphae branch out to form a network. On the bark of the trunk, the vegetative hyphae grow vigorously, branching and forming light grey patches on the inner surface of the bark (Fig 9A). During their growth, the vegetative hyphae penetrate deeply into the bark, extracting water and nutrients, disrupting the connections between cells in the bark's parenchyma and the cambium (a meristematic cell layer separating the bark

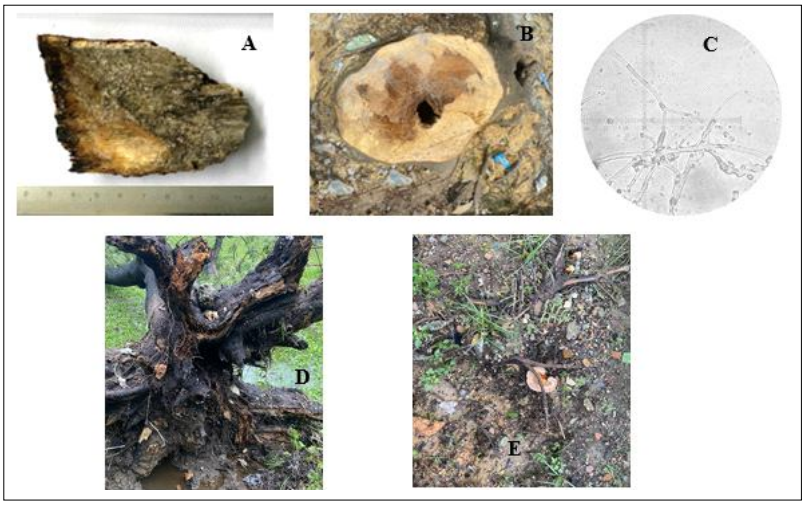


Fig 9: Pathogenic phenomena of *Ganoderma multipileum* in *Delonix regia* trees.

and wood). They also destroy the vascular structures within the bark's phloem. These disruptions to the bark's structure severely impact the tree's ability to transport organic substances. Observations in nature show that as the fruit bodies of *Ganoderma multipileum* grow and develop, the bark begins to peel away from the wood in large patches, causing the trunk to dry out (Fig 8, 9). This results in wilting and yellowing leaves due to nutrient deprivation. Prolonged bark peeling can eventually lead to the death of the *Delonix regia* tree.

In the wood of the roots and trunk, after *Ganoderma multipileum* spores penetrate and develop into vegetative hyphae, they branch into fine networks. These networks disrupt the connections between the cells in the wood parenchyma and the xylem structures, creating hollow spaces within the trunk and roots, leading to necrosis (Fig 9B). This significantly impairs water and mineral transport, as well as the tree's mechanical support capabilities, which can result in breakage or collapse (Fig 9D).

When a tree collapses, *Ganoderma multipileum* spores remain viable in the environment. Under favourable conditions, they can form new fruit bodies on soil containing remnants of the host tree's root system (Fig 9E). The study of Taslim *et al.,* (2025) to assess how effective the use of organic and liquid fertilizers derived from palm oil mill waste as an alternative to improve soil quality and control Ganoderma. The results showed that using organic fertilizer obtained from palm oil mill waste significantly improved soil quality. Compared with conventional methods, this treatment also succeeded in reducing the level of Ganoderma attacks by 40% and increasing production yields by 15%.

CONCLUSION

The results of morphological analysis, basidiospore evaluation under microscope and phylogenetic analysis based on ITS sequence have identified the pathogenic fungus causing the dieback of *Delonix regia* trees in Hue city as *Ganoderma multipileum*. Raper medium for the best level I propagation of *Ganoderma multipileum* had a uniform and fast mycelial spreading speed of 6.28 cm after 5 days of culture compared to other media. The cellulase activity of *Ganoderma multipileum* reached a maximum value of 0.185 IU/mL after 5 days of culture at 35°C. The result of this study clearly showed a connection between the presence of *Ganoderma multipileum* and the observed disease symptoms in *Delonix regia*.

Conflict of interest

The authors declare no conflict of interest.

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