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Extracellular enzymatic activity of endophytic fungi isolated from spines of rattan palm (*Calamus castaneus* Griff.)

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ABSTRACT

Aims: *Calamus castaneus* is a non-climbing rattan plant widely distributed in tropical rainforests. The sharp spines of rattan palm harbour endophytic fungi, which may produce extracellular enzymes that contribute to various functions without harming the host plant. This study was aimed to evaluate the ability of fungal endophytes isolated from the *C. castaneus* spines to produce extracellular enzymes, including protease, pectinase, amylase, lipase and cellulase.
Methodology and results: Thirty-four (34) endophytic fungal isolates were tested for their ability to produce extracellular enzymes using the agar plate method. Enzyme activity was measured using the enzyme index (EI) by measuring the halo (clear zone) on the agar medium. The EI value indicates the strength of the enzyme produced by the endophytes. Results demonstrated that all thirty-four fungal endophytes could produce at least one extracellular enzyme. *Xylaria cubensis* BR90 showed the highest protease activity of 5.73 EI. *Muyocopron laterale* (SM60) showed the highest pectinase activity of 2.74 EI. For lipase and cellulase activities, *Cyphellophora guyanensis* (BR71) produced 2.26 EI while *Acremonium hennebertii* (BR70) produced 1.97 EI, respectively.
Conclusion, significance and impact of study: Endophytic fungi from spines of *C. castaneus* were able to produce cellulase, pectinase, lipase, protease and amylase. The extracellular enzymes degraded different substrates, suggesting different types of interaction of the fungal endophytes with the host plant.

Keywords: Calamus castaneus, endophytic fungi, extracellular enzymes, spines

INTRODUCTION

Calamus castaneus, one of the most common non-climbing rattan palm plants in Malaysian forests, is covered in hard, yellow-based spines, while the surface of the middle part of its upper leaves is covered in softer bristle spines (Dransfield, 1979). The spines on the stem function as defence structures against herbivores and mammals from grazing and climbing plants (Dransfield, 1979; Liu *et al.*, 2020).

The spines of *C. castaneus* harbour endophytic fungi (Azuddin *et al.*, 2021), which form a mutualistic association with the plant host. Endophytic fungi reside in healthy plant hosts for at least part of their life cycle without causing apparent disease symptoms (Petrini, 1991; Schulz *et al*., 1993). Most endophytic fungal genera identified from the spines of *C. castaneus* are common genera reported to produce extracellular enzymes (Azuddin *et al.*, 2021) such as cellulase, pectinase, lipase, protease, and amylase, which are produced by fungal cells and secreted outside for various functions (Archer and Wood, 1995; Choi *et al*., 2005).

The extracellular enzymes produced by fungi play an essential role in defence mechanisms against pathogen invasion, hydrolysis of food substances and obtaining nutrients from the host. Extracellular enzymes also play a role in pathogenicity by degrading plant cell walls and facilitating the penetration of endophytes into the host plant (Sunitha *et al.*, 2013; Desire *et al.*, 2014).

Cellulase and amylase produced by endophytes could be an indication that the endophytes can turn into saprophytes, whereas the production of pectinase is an indication that the endophyte could be an opportunistic or latent pathogen. Lipase is secreted mainly for nutrient acquisition, whereas proteases associated with mycoparasitism suggest that endophytes are potential biocontrol agents (Benítez *et al.*, 2004; Choi *et al.*, 2005; Feng *et al.*, 2005).

Endophytic fungi from the spines of *C. calamus* might also produce extracellular enzymes with the same functional roles. The present study was conducted to determine the ability of endophytic fungi isolated from the spines of *C. castaneus* to produce extracellular enzymes such as cellulase, pectinase, lipase, proteinase and

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amylase. The extracellular enzymes produced by fungal endophytes recovered spines of *C. castaneus* can be used as preliminary information on their functional roles.

MATERIALS AND METHODS

Fungal isolates

The information on spine sample collection, isolation and identification of endophytic fungi isolated from the spines of *C. castaneus* are described in Azuddin *et al.* (2021). Thirty-four isolates of endophytic fungi recovered from the spines were chosen to determine their ability to produce extracellular enzymes, including cellulase, pectinase, lipase, proteinase, and amylase. The fungal endophytes were selected to represent each genus (Table 1) of the endophytic fungi.

Agar plate preparation

The agar plate method was used for the detection and production of extracellular enzymes, in which each medium contained specific enzyme substrates. The extracellular enzymes were qualitatively measured on the specific agar plates. A mycelial plug (5 mm diameter) was inoculated at the centre of each specific agar plate with three replicates and the experiment was repeated twice. The inoculated agar plates were incubated at room temperature (25 ± 1 °C) for 5-10 days, depending on the medium used. The uninoculated plates served as the controls.

To detect protease production, Nutrient Agar (NA) (Himedia®, India) was added to 8% gelatin, whereas for amylase, 0.2% soluble starch was added to the agar medium. The gelatin solutions were sterilised separately and added to 100 mL of NA medium. Two (2) g of soluble starch were added in 1 L of NA and sterilised. The inoculated agar plates were incubated for 7 days. Saturated ammonium sulphate in an aqueous solution was coated onto the plates after 7 days of incubation. The formation of a clear, opaque zone indicates proteolytic production (Hankin and Anagnostakis, 1975). A yellow-clear zone was formed after the plates were coated with 1% iodine in 2% potassium iodide for amylase production.

Pectinase production was detected using yeast extract agar containing pectin and mineral salt solution (Hankin and Anagnostakis, 1975). Ten (10) g of pectin (citrus), 2 g of yeast extract and 25 g of straw agar were mixed in 1 L of mineral salt solution. The salt solutions contained $(NH_4)_2SO_4$ (2 g), KH_2PO_4 (4 g), Na_2HPO_4 (6 g); $FeSO_4 \cdot 7H_2O$ (0.2 g), $CaCl_2$ (1 mg), H_3BO_3 (10 µg), $MnSO_4$ (10 µg), $ZnSO_4$ (70 µg), $CuSO_4$ (50 µg) and MoO_3 (10 µg) in 1 L of distilled water. After 5 days, the inoculated plates were overlaid with a 1% aqueous solution of hexadecyltrimethylammonium bromide (cetyltrimethylammonium bromide). Pectinase production occurred via the formation of a clear zone around the colony.

Table 1: Endophytic fungal isolates used in extracellular enzyme production.

Endophytic fungi	Isolate code
Neopestalotiopsis saprophytica	BP1
Neopestalotiopsis formicarum	BP2
Colletotrichum horii	BP3
Colletotrichum fructicola	BP5
Colletotrichum endophytica	BP9
Colletotrichum endophytica	BP10
Colletotrichum endophytica	SM31
Colletotrichum horii	BP13
Colletotrichum boninense	SM21
Colletotrichum cliviae	SM25
Xylaria cubensis	SM22
Xylaria cubensis	BR90
Phyllosticta carochlae	SM27
Diaporthe arengae	SM28
Diaporthe cf. *heveae*	SM36
Diaporthe hongkongensis	SM42
Diaporthe arecae	SM45
Diaporthe tectonae	SM62
Diaporthe cf. *nobilis*	BR67
Arthrinium urticae	SM47
Curvularia lunata	SM54
Muyocopron laterale	SM60
Fusarium lateritium	BR66
Fusarium decemcellulare	BR72
Fusarium solani	BR92
Fusarium oxysporum	BR86
Acrocalymma fici	BR68
Acremonium hennebertii	BR70
Cyphellophora guyanensis	BR71
Penicillium indicum	BR91
Penicillium oxalicum	BR102
Trichoderma harzianum	BR94
Trichoderma koningiopsis	BR96
Endomelanconiopsis endophytica	BR98

For lipase detection, peptone agar, supplemented with Tween 20, was used. The peptone agar consisted of 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2 \cdot 2H_2O$ and 25 g straw agar in 1 L distilled water. Tween 20 (10 mL) was sterilised separately and added to 1 L of peptone agar before pouring into Petri dishes. The inoculated plates were incubated for 7 days and precipitation of calcium salts around the fungal colony indicated lipase production (Hankin and Anagnostakis, 1975).

Potato Dextrose Agar (PDA) (Himedia® India) supplemented with 0.5% carboxymethylcellulase (Lynd *et al.*, 2003) was used to detect cellulase. Five (5) g of carboxymethylcellulase were added to 1 L of PDA and sterilised at 121 °C for 20 min. The inoculated plates were incubated for 5 days. After incubation, the plates were overlaid with 0.2% aqueous Congo red solution in 1 M Tris-HCl (pH 7.5) for 20 min and rinsed several times with 1 M NaCl (Hankin and Anagnostakis, 1975). The diameter of the clear zone was then measured.

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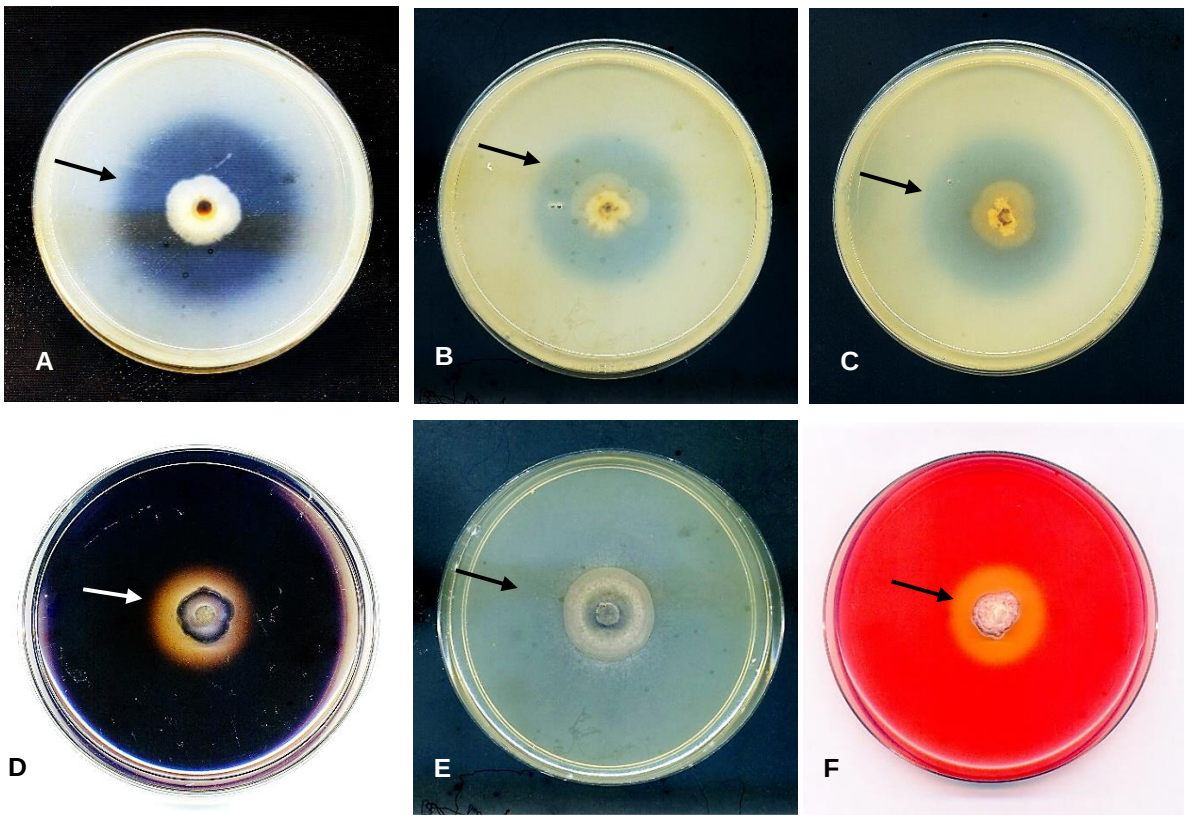

Figure 1: Clear zone around the colony (see area shown by arrows) indicated positive extracellular enzymes activity. (A) Protease; (B) Pectinase; (C) Pectinase; (D) Amylase; (E) Lipase; (F) Cellulase.

Table 2: Extracellular enzymatic reaction category based on enzyme activity index*.

Enzyme activity index (EI)	Reaction category
Higher than or equal to 2 ($x \geq 2$)	Strong reaction
Less than 2, but more than 1 ($1< x < 2$)	Medium reaction
Equal or less than 1 ($x \leq 1$)	Weak reaction
No zone observed (0)	No formation of a clear zone

*Adapted from Choi *et al.* (2005).

Enzyme activity index

The enzyme activity index (EI) was used to screen and calculate the strength of the extracellular enzymes produced by endophytes. After 5-10 days of incubation, the growth of fungal colonies and halo formation (clear zone) around the colonies were measured and recorded. EI was calculated using the following formula (Choi *et al.*, 2005):

Enzyme activity index (EI)
= Diameter of zone (cm)/Diameter of fungal growth (cm)

From the EI value, the enzymatic reactions of the isolates were classified based on the reaction category shown in Table 2.

Enzyme activity data were analysed using ANOVA and significant differences among the isolates were analysed using Tukey's test (p<0.05) using SPSS Statistics for Windows, version 26 (Armonk, NY, USA, IBM Corp.).

RESULTS

All 34 endophytic fungal isolates produced at least one extracellular enzyme. None of the isolates produced all the extracellular enzymes evaluated (Table 3).

Protease

Thirty-two isolates exhibited clear, opaque zones around colonies that were positive for protease production (Figure 1A). Thirteen isolates were categorised as having strong reactions with EI ranging from 2.02-5.73, with *X. cubensis* (BR90) producing the highest EI (5.73) and being significantly different from the other isolates.

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Table 3: Enzyme index (EI) of extracellular enzymes produced by fungal endophytes from *C. castaneus* and classification of reaction category.

Isolates	Enzymes / EI*				
	Protease	Pectinase	Amylase	Lipase	Cellulase
Control	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
C. fructicola (BP5)	0.00^{a}	0.00^{a}	0.00^{a}	1.23 ± 0.02^{hij} (Medium)	0.00^{a}
F. decemcellulare (BR72)	0.00^{a}	0.00^{a}	1.18 ± 0.09^{b} (Medium)	1.04 ± 0.02^{b} (Medium)	0.00^{a}
T. harzianum (BR94)	1.102 ± 0.04^{ab} (Medium)	0.00^{a}	0.00^{a}	1.18 ± 0.01^{efghi} (Medium)	0.00^{a}
C. horii (BP13)	1.02 ± 0.04^{ab} (Medium)	1.03 ± 0.02^{b} (Medium)	0.00^{a}	1.66 ± 0.01^{pq} (Medium)	0.00^{a}
P. oxalicum (BR102)	1.05 ± 0.02^{abc} (Medium)	1.16 ± 0.01^{b} (Medium)	0.00^{a}	1.06 ± 0.01^{bc} (Medium)	0.00^{a}
F. solani (BR92)	1.08 ± 0.01^{abc} (Medium)	1.11 ± 0.04^{b} (Medium)	2.00 ± 2.60^{e} (Strong)	1.12 ± 0.02^{cde} (Medium)	0.00^{a}
F. oxysporum (BR86)	1.17 ± 0.01^{bc} (Medium)	1.15 ± 0.00^{b} (Medium)	2.72 ± 0.18^{f} (Strong)	1.11 ± 0.01^{cd} (Medium)	0.00^{a}
P. indicum (BR91)	1.18 ± 0.10^{bc} (Medium)	0.00^{a}	0.00^{a}	1.54 ± 0.16^{n} (Medium)	0.00^{a}
D. tectonae (SM62)	1.24 ± 0.07^{bc} (Medium)	0.00^{a}	0.00^{a}	1.16 ± 0.02^{def} (Medium)	1.32 ± 0.10^{bc} (Medium)
C. cliviae (SM25)	1.26 ± 0.04^{bc} (Medium)	1.06 ± 0.02^{b} (Medium)	0.00^{a}	1.55 ± 0.01^{n} (Medium)	0.00^{a}
C. horii (BP3)	1.26 ± 0.04^{bc} (Medium)	1.08 ± 0.00^{b} (Medium)	0.00^{a}	1.87 ± 0.02^{s} (Medium)	0.00^{a}
T. koningiopsis (BR96)	1.27 ± 0.04^{bc} (Medium)	0.00^{a}	0.00^{a}	1.41 ± 0.03^{lm} (Medium)	0.00^{a}
N. saprophytica (BP1)	1.42 ± 0.04^{bc} (Medium)	0.00^{a}	1.64 ± 0.05^{cd} (Medium)	1.62 ± 0.02^{op} (Medium)	0.00^{a}
C. endophytica (BP10)	1.46 ± 0.03^{bcd} (Medium)	1.03 ± 0.01^{b} (Medium)	0.00^{a}	1.58 ± 0.03^{no} (Medium)	0.00^{a}
C. lunata (SM54)	1.46 ± 0.02^{bcd} (Medium)	0.00^{a}	0.00^{a}	1.19 ± 0.01^{fghij} (Medium)	0.00^{a}
D. cf. *heveae* (SM36)	1.54 ± 0.06^{bcde} (Medium)	0.00^{a}	0.00^{a}	1.17 ± 0.02^{defg} (Medium)	0.00^{a}
F. lateritium (BR66)	1.56 ± 0.03^{bcde} (Medium)	0.00^{a}	0.00^{a}	1.24 ± 0.02^{ij} (Medium)	0.00^{a}
C. endophytica (BP9)	1.60 ± 0.03^{bcde} (Medium)	1.04 ± 0.02^{b} (Medium)	0.00^{a}	1.22 ± 0.03^{fghij} (Medium)	0.00^{a}
C. endophytica (SM31)	1.61 ± 0.02^{bcde} (Medium)	1.03 ± 0.01^{b} (Medium)	0.00^{a}	1.58 ± 0.03^{no} (Medium)	0.00^{a}
C. boninense (SM21)	1.66 ± 0.06^{bcde} (Medium)	1.07 ± 0.01^{b} (Medium)	0.00^{a}	1.17 ± 0.03^{efgh} (Medium)	0.00^{a}
N. formicarum (BP2)	1.94 ± 0.06^{bcdef} (Medium)	0.00^{a}	1.78 ± 0.08^{de} (Medium)	1.71 ± 0.02^{q} (Medium)	0.00^{a}
P. carochlae (SM27)	2.02 ± 0.10^{bcdef} (Strong)	0.00^{a}	2.71 ± 0.34^{f} (Strong)	1.60 ± 0.01^{no} (Medium)	0.00^{a}
A. fici (BR68)	2.10 ± 0.09^{bcdefg} (Strong)	1.84 ± 0.17^{c} (Medium)	0.00^{a}	1.24 ± 0.01^{ij} (Medium)	0.00^{a}
D. arecae (SM45)	2.18 ± 0.03^{cdefg} (Strong)	1.06 ± 0.02^{b} (Medium)	0.00^{a}	1.40 ± 0.02^{l} (Medium)	0.00^{a}
D. cf. *nobilis* (BR67)	2.19 ± 0.14^{cdefg} (Strong)	1.04 ± 0.02^{b} (Medium)	2.06 ± 0.17^{e} (Strong)	1.40 ± 0.01^{l} (Medium)	1.24 ± 0.01^{b} (Medium)
D. hongkongensis (SM42)	2.60 ± 0.12^{defgh} (Strong)	1.34 ± 0. 16^{b} (Medium)	0.00^{a}	1.54 ± 0.03^{n} (Medium)	0.37 ± 0.65^{a} (Weak)
D. arengae (SM28)	2.66 ± 0.05^{efgh} (Strong)	1.09 ± 0.04^{b} (Medium)	1.27 ± 0.10^{bc} (Medium)	1.78 ± 0.03^{r} (Medium)	1.27 ± 0.02^{b} (Medium)
X. cubensis (SM22)	2.83 ± 0.15^{fgh} (Strong)	1.09 ± 0.02^{b} (Medium)	1.61 ± 0.34^{cd} (Medium)	1.45 ± 0.02^{lm} (Medium)	1.18 ± 0.01^{b} (Medium)
C. guyanensis (BR71)	3.24 ± 0.10^{ghi} (Strong)	0.00^{a}	0.00^{a}	2.26 ± 0.01^{t} (Strong)	1.28 ± 0.05^{b} (Medium)
A. urticae (SM47)	3.25 ± 0.20^{ghi} (Strong)	2.42 ± 0.06^{d} (Strong)	0.00^{a}	1.62 ± 0.02^{op} (Medium)	1.71 ± 0.06^{cd} (Medium)
M. laterale (SM60)	3.58 ± 1.08^{hi} (Strong)	2.74 ± 0.12^{d} (Strong)	0.00^{a}	1.32 ± 0.01^{k} (Medium)	1.43 ± 0.03^{bc} (Medium)
E. endophytica (BR98)	4.17 ± 1.59^{i} (Strong)	0.00^{a}	0.00^{a}	1.46 ± 0.01^{lm} (Medium)	0.00^{a}
A. hennebertii (BR70)	4.34 ± 0.22^{i} (Strong)	0.00^{a}	1.51 ± 0.05^{bcd} (Medium)	1.23 ± 0.03^{ghij} (Medium)	1.97 ± 0.38^{d} (Medium)
X. cubensis (BR90)	5.73 ± 0.64^{j} (Strong)	1.13 ± 0.04^{b} (Medium)	0.00^{a}	1.43 ± 0.02^{lm} (Medium)	1.04 ± 0.02^{b} (Medium)

*EI, (Diameter of colony + Halo zone)/Diameter of fungal growth; Mean of EI ± SD (n=6) followed by the same letter are not significantly different (p<0.05) according to Tukey's test.

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Nineteen isolates were classified as medium reactions (EI=1.02-1.94). *Trichoderma harzianum* (BR94) and *C. horii* (BP13) produced the lowest protease activity (EI=1.02).

Pectinase

Nineteen endophytes produced visible clear zones around colonies that were positive for pectinase production (Figure 1B and 1C). The EI values ranged from 1.03-2.74 (Table 3). *Mycoleptodiscus indicus* (SM60) produced the highest pectinase activity with an EI of 2.74, followed by *Art. urticae* (SM47), with an EI of 2.42; both were classified as strong reactions and were not significantly different. Seventeen endophytes were classified as having medium reactions, with EI values ranging from 1.03-1.84.

Amylase

Ten endophytes exhibited a visible yellow clear zone around colonies that were positive for amylase production (Figure 1D). The EI values ranged from 1.18-2.72 (Table 3). Four endophytes were categorised as strong reactions with *F. oxysporum* (BR86), which produced the highest amylase activity with an EI of 2.72, followed by *P. carochlae* (EI=2.71), with no significant difference between the two EI. The other six endophytes were classified as medium reactions, with EI values of 1.18-1.78.

Lipase

All 34 endophytes exhibited visible precipitation around colonies and were positive for lipase production (Figure 1E). The EI values produced by the endophytes ranged from 1.04-2.26 (Table 3). *Cyphellophora guyanensis* (BR71) produced the highest lipase activity, with an EI of 2.26 that was significantly different from that of other endophytes. *Fusarium decemcellulare* (BR72) produced the least lipase, with the lowest EI value (1.04). The other endophytes were classified as having medium reactions, with EI values ranging from 1.04-1.87.

Cellulase

Only 10 endophytes produced visible yellow, clear zones around colonies that were positive for cellulase production (Figure 1F). The EI value produced by the isolates ranged from 0.37 to 1.97 (Table 3). Nine endophytes produced a medium reaction, with EI ranging from 1.04-1.97 with no significant difference. *Diaporthe hongkongensis* (SM42) produced the least cellulase enzyme, had the lowest EI value (0.37) and was classified as a weak reaction.

DISCUSSION

The results of the present study indicate that the endophytic fungal isolates tested showed different strengths of extracellular enzyme activity. Cellulase, pectinase, lipase, protease, and amylase tested in this study are cell wall-degrading enzymes that utilise different substrates. According to Carroll and Petrini (1983), fungal endophytes exhibit different functional roles depending on the types of extracellular enzymes produced.

Thirty-two fungal endophytes produced proteases with medium-to-strong enzymatic reactions. Microorganisms produce proteases, which are commonly related to mycoparasitism, as proteases hydrolyse protein-peptide bonds in fungal pathogen cells, which eventually degrade the cell wall. This allows endophytes to penetrate pathogenic tissues (Haggag *et al.*, 2006). Endophytic *X. cubensis,* in this study, produced the most potent protease activity. However, based on a study by Azuddin *et al.* (2021), *X. cubensis* exhibited weak or no antagonistic effects against several tested plant-pathogenic fungi. In contrast, *Xylaria* showed antagonistic effects against bacteria, in which *X. psidii* produced high protease activity, indicating antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* (Indarmawan *et al.*, 2016). In the present study, *Endomelenoconiopsis endophytica* was among the endophytes that produced strong protease activity, suggesting the potential of the endophyte as a biocontrol agent. In an antagonistic study by Azuddin *et al.* (2021), *Endomelenoconiopsis endophytica* moderately inhibited most tested fungal pathogens.

Several studies have reported endophyte-producing proteases as potential biocontrol agents. Endophytic *C. gloeosporioides* has the potential to be developed as a biocontrol agent because it produces high protease activity and inhibits *Pestalotiopsis theae*, a pathogen of foliar grey blight on tea plants (*Camellia sinensis*) (Rabha *et al.*, 2014). Endophytic *Trichoderma* sp. from date palm (*Phoenix dactylifera*) produced high protease activity and exhibited strong antagonistic effects towards *F. oxysporum*, a pathogen associated with diseased olive plants (Abdennabi *et al.*, 2017).

Nineteen fungal endophytes produced pectinase with medium-to-strong enzymatic reactions. The ability of fungal endophytes to produce pectinase indicates that endophytes are likely to be latent pathogens. In a study by Azuddin *et al.* (2021), endophytic *Colletotrichum* spp. (*C. boninense*, *C. fructicola*), *Diaporthe* spp. (*D. hongkongenesis*, *D. arengae* and *D.* cf. *nibilis*) and two *Fusarium* spp. (*F. solani* and *F. oxysporum*) were pathogenic to *C. castaneus* and *bertam* leaves, as well as to chili and banana fruits. These fungal isolates also produced pectinase, suggesting that endophytes could be latent pathogens. In a study by Sunitha *et al.* (2013), endophytic *C. gloeosporioides*, *F. solani*, *Xylaria* sp. and *F. oxysporum* from four medicinal plants (*Alpinia calcarata*, *Bixa orellana*, *Calophyllum inophyllum* and *Catharanthus roseus*) were among the endophytes that produced pectinase and implied as potential latent pathogens. Uzma *et al.* (2016) reported that endophytic *Penicillium* sp. from *Hedychium coronarium* and endophytic *Colletotrichum* sp. and *Phomopsis* sp. from medicinal plants (*Piper longum*) produced elevated levels of pectinase, suggesting that fungal endophytes can

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become pathogens. Fungal endophytes residing in plant hosts may switch to pathogens when an imbalance antagonism occurs between the plant defence and the virulence of the fungal endophytes. Imbalanced antagonism may also occur in senescent tissues. Fungal endophytes that transform into pathogens produce pectinase to degrade pectin contained in the middle layer of the plant cell wall and then begin to sporulate, which leads to disease development (Schulz and Boyle, 2005; Sunitha *et al.*, 2013).

Ten fungal endophytes from the genera *Fusarium*, *Neopestalatiopsis*, *Pestalatiopsis*, *Diaporthe*, *Acremonium* and *Xylaria* produced amylase with medium to strong enzymatic reactions. These results suggest that these fungal endophytes can act as decomposers and at the beginning of decomposition, fungi produce high levels of amylase (80-100%) compared to other enzymes (Kjøller and Struwe, 2002).

According to Sun *et al.* (2011), endophytic fungal genera, including *Xylaria*, *Phomopsis* and *Fusarium* were among the endophytes that produced amylase, which is involved in the decomposition of leaves of woody trees (*Acer truncatum*). Sun *et al.* (2011) suggested that fungal endophytes that produce amylase are likely to be decomposers, as endophytes can degrade starch as a food source. Fungal endophytes that produce amylase can degrade starch or carbohydrates stored in plant tissues as nutrient sources, as amylase hydrolyses starch into sugar as a source of nutrients and energy (Buléon *et al.*, 1998). Starch stored in plant tissues is also available during senescence. Fungal endophytes are the first colonisers of the host; they secrete amylase to degrade starch, and the products are consumed by the endophytes soon after the host dies (Choi *et al.*, 2005).

Lipase was produced by all 34 fungal endophytes with medium-to-strong enzymatic activity. Choi *et al*. (2005) reported comparable results in which all endophytes recovered from *Brucea javanica*, namely, *Colletotrichum* sp., *Fusarium* sp., *Phomopsis* sp. and *Xylaria* sp. produced lipase. These results indicated that in addition to cellulase and pectinase, all endophytes from *C. castaneus* were able to produce lipases. The ability of fungal endophytes to produce lipase may indicate that the endophytes used lipase to acquire nutrients from the host. Studies have shown that fungal endophytes produce lipases to obtain energy from lipid degradation. Lipase production by endophytic fungi has been reported in endophytes isolated from medicinal plants (Bezerra *et al.*, 2012; Toghueo *et al.*, 2017).

Ten isolates produced cellulase, ranging from weak to medium enzymatic reactions. The fungal endophytes producing cellulase in this study were also likely to be pathogens and saprophytes. Chen *et al.* (2018) reported that the endophytic *Diaporthe* is a latent pathogen that produces cellulase. Endophytic *Phomopsis longanae* (synonymous with *Diaporthe*) from healthy fruits of longan (*Dimocarpus longan*) was a latent pathogen, as the fungus was able to infect longan fruits and a high level of cellulase was detected during disease development. Cellulases are cell wall-degrading enzymes that influence the process of endophytes becoming saprobes or latent pathogens by hydrolysing the cellulose in the plant host cell wall to absorb nutrients for growth and survival (Promputtha *et al.*, 2007; 2010). After the colonisation of the plant host, the fungal endophytes become dormant or latent and reside in the middle lamellae. Tissue senescence or stress conditions modify the host tissues, trigger the endophytes to grow and sporulate, and switch from endophytes to saprophytes or latent pathogens (Promputtha *et al.*, 2007; 2010).

Endophytic *X. cubensis* and *Cyp. guyanensis* are likely to become saprophytes, whereas *Acr. hennerbertii*, *Art. urticae*, *Myc. indicus*, *D. tectonae*, *D. arengae*, *D*. cf. *nobilis* and *D. hongkongensis* are latent pathogens. Several studies have indicated that endophytes that produce cellulase are involved in cellulose decomposition. For example, *Cyp. guyanensis* has been reported from rotting wood (Feng *et al.*, 2014) and *X. cubensis* has been isolated from decaying decorticated dicotyledonous wood (Lee *et al.*, 2002).

In the present study, the agar plate method was applied, which is a qualitative method to detect the production of extracellular enzymes by the endophytic fungi, which is the first step to determine the ability of the endophytes to produce extracellular enzymes. The activity of the extracellular enzymes using quantitative methods such as fluorometric or colorimetric microplate as well as spectrophotometer would give more information on their functional roles during the interaction with the host plant. Other extracellular enzymes such as laccase, asparaginase, xylanase and chitinase should also be tested to obtain more information on their functional roles and their beneficial effect on the host plant.

Extracellular enzymes secreted by endophytic fungi degrade complex materials into smaller compounds that are easier to assimilate. According to Traving *et al.* (2015), microbial extracellular enzymes are preferred by various industries due to their stability, availability, and cost effectiveness. Thus, several extracellular enzymes produced by endophytic fungi, including protease, pectinase, amylase, lipase and cellulase, have the potential to be developed as useful products in several industrial applications.

Cellulase degrades cellulose and related polysaccharides and, therefore, is commonly applied in textile, pulp, and paper industries (Yadav *et al.*, 2017). Cellulases have also been used in the food industry and its preservation (Ejaz *et al.*, 2021). Pectinase degrades pectin and it is widely used to decompose agricultural and industrial waste (Garg *et al.*, 2016) and in textile manufacturing (Pusic *et al.*, 2015). Pectinase has major roles in food industries, including extractions of fruit juice and vegetable oil, production of jam and jellies as well as fermentation and concentration of coffee, tea and cocoa (Barman *et al*., 2015; Kubra *et al*., 2018). Proteases hydrolyse protein into simple constituents such as amino acids and proteases secreted by fungi are preferred due to their stability (Mefteh *et al*., 2019). Fungal proteases are among the important enzymes in many industrial

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applications, including food, detergents and leather processing (Razzaq *et al.*, 2019). Lipase catalyzes the hydrolysis of fats into fatty acids and glycerol (Melani *et al.*, 2020) and is important in the manufacturing of cleaning products such as detergents, dry cleaning solvents and skin cleansers (Alabdalall *et al.*, 2021). Amylases convert starch into glucose units and are among the important enzymes in the manufacturing of food, paper, textile and detergent (de Souza and de Oliveira Magalhães, 2010). Fungal amylases are known to be thermostable (Toghueo *et al.*, 2017) and are potentially valuable for pharmaceutical and fine-chemical industries (de Souza and de Oliveira Magalhães, 2010).

CONCLUSION

The fungal endophytes from the spines of *C. castaneus* were able to produce proteases, pectinases, amylases, lipases and cellulases, depending on the species. The enzymes utilised different substrates, suggesting functional roles of the fungal endophytes in the host plant. The fungal endophytes may play a role as decomposers, pathogens and potential biocontrol agents.

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