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Characterization and Pathogenicity Evaluation of *Ceratocystis* sp. Isolated from Various Hosts on *Acacia crassicarpa* Seedlings

Karakterisasi dan Evaluasi Patogenisitas Isolat Ceratocystis *sp. dari Berbagai Inang pada Semai* Acacia crassicarpa

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Ceratocystis sp.,
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ABSTRACT

Acacia crassicarpa is widely grown in forest plantations with *Acacia mangium* and *Eucalyptus* spp. *Ceratocystis* sp. is identified as a significant pathogen, causing substantial damage to *A. mangium* plantations as well as infecting *A. crassicarpa*, *Eucalyptus* spp., and several fruit trees such as *Lansium* spp., which led to yield losses. Research reported that isolates of *Ceratocystis* derived from various hosts have varying pathogenicity. Therefore, this research aimed to characterize the morphological properties and evaluate pathogenicity levels of eight *Ceratocystis* isolates (AC1, AC2, AM1, AM2, AM3, AM4, EP1, and LA1) on *A. crassicarpa* seedlings. The investigation occurred in the shade house and at the Faculty of Forestry UGM, Forest Health and Protection Laboratory in Yogyakarta. Four-month-old *A. crassicarpa* seedlings were artificially inoculated with *Ceratocystis* isolates from *A. mangium*, *A. crassicarpa*, *Eucalyptus* spp., and *Lansium* spp. hosts. The experiment employed a completely randomized design with four replicates. The results showed that the characteristics of the isolates varied, but the differences in perithecium size were statistically insignificant. EP1 had a lighter color (greyish olive) than the other isolates. It was the most virulent and had a high potential for use in screening the resistance of *A. crassicarpa* clones against *Ceratocystis* sp. in the future.

KATA KUNCI
Ceratocystis *sp.*,
Acacia crassicarpa,
inoculate *buatan,*
virulen

INTISARI

Acacia crassicarpa *merupakan spesies yang dikembangkan di hutan tanaman selain jenis* Acacia mangium *dan* Eucalyptus *spp.* Ceratocystis *sp. telah dilaporkan menginfeksi* A. mangium, A. crassicarpa, Eucalyptus *spp., dan tanaman buah seperti* Lansium *spp. sehingga menyebabkan penurunan produktivitas. Isolat* Ceratocystis *sp. dari berbagai inang telah dilaporkan memiliki patogenisitas yang bervariasi. Penelitian ini bertujuan untuk mendeskripsikan morfologi dan mengevaluasi patogenisitas delapan isolat* Ceratocystis *sp. (AC1, AC2, AM1, AM2, AM3, AM4, EP1, dan LA1) pada semai* A. crassicarpa. *Penelitian dilakukan di* shade house *dan Fakultas Kehutanan UGM, pada Laboratorium Perlindungan dan Kesehatan Hutan, di Yogyakarta. Semai* A. crassicarpa *umur empat bulan diinokulasi menggunakan metode inokulasi buatan dengan delapan isolat yang berasal dari* A. mangium, A. crassicarpa, Eucalyptus *spp., dan* Lansium *spp. Penelitian ini menggunakan desain uji Random Acak Lengkap (RAL) dengan empat ulangan. Karakteristik delapan isolat* Ceratocystis *sp. bervariasi, tetapi berdasarkan ukuran atribut, perithecium tidak signifikan. EP1 memiliki warna koloni yang lebih terang (*greyish olive*) dibandingkan dengan isolat lain dan EP1 merupakan isolat yang paling virulen serta berpotensi digunakan untuk skrining klon* A. crassicarpa *yang tahan terhadap* Ceratocystis *sp. di masa mendatang.*

Introduction

Between 1990 and 2020, the global area covered by planted forests expanded by 123 million hectares, reaching 294 million hectares (FAO & UNEP 2020). In Indonesia, plantation forests increased from 20.67 million hectares in 2017 to 34.44 million hectares in 2020, leading to an 8.44 million m^3 rise in wood production, from 37.79 million m^3 to 46.23 million m^3 over three years (Direktorat Jenderal Pengelolaan Hutan Lestari 2021). Among the species flourishing in the plantation forest is *Acacia crassicarpa* A. Cunn. ex Benth. This exotic species is native to northern Australia, southwestern Papua New Guinea, and the Papua Province of Indonesia (Nirsatmanto & Sunarti 2019). It is particularly more adaptive to growing on peatlands, showing higher survival and faster growth than other acacia species. The wood properties of the species meet the requirement for pulp and paper raw materials (Martins et al. 2020). Despite these benefits, *A. crassicarpa* plantations have faced significant threats from infections caused by *Ceratocystis* sp. (Wingfield et al. 2023).

Research reported that *Ceratocystis* sp. causes widespread infection in *A. mangium* (Tarigan et al. 2011), *A. crassicarpa* (Wingfield et al. 2023), *Eucalyptus* spp. (Nasution et al. 2019), and other fruit trees such as *Lansium* spp. (Muslim et al. 2022), leading to yield losses. Some of the disease symptoms caused by the fungus include wilting and cancer. It is important to know that the fungus initiates infection through plant wounds Acacia decurrens (Rahayu et al. 2015). Confirmed hosts of *Ceratocystis* sp. are *Platanus* sp. (EFSA Panel on Plant Health (PLH) et al. 2016), *Mangifera indica* L. (Arriel et al. 2016), *Gmelina arborea* Roxb. (Méndez-Álvarez et al. 2021), *Theobroma cacao* L. (Kamgan et al. 2008), *Citrus* sp. (Ploetz et al. 2013), *Coffea arabica* L. (Almeida et al. 2024), *Hevea brasiliensis* (Valdetaro et al. 2015), *Spathodea* sp. (Johnson et al. 2005), and *Ficus carica* L. (Tsopelas et al. 2021).

The genus *Ceratocystis* is currently used broadly to describe a diverse group of morphologically and ecologically similar fungi (Muslim et al. 2022). However, the influence of morphology on the virulence remains unclear, specifically on *A. crassicarpa*. *Ceratocystis* has currently been restricted to species that are phylogenetically closely related to *C. fimbriata* (Lee et al. 2016). The isolates from various hosts have been reported to have varying pathogenicity (Lee et al. 2016; Valdetaro et al. 2019; Muslim et al. 2022). According to Muslim et al. (2022), *C. fimbriata* isolates from *L. domesticum* are pathogenic on *L. domesticum* and *A. mangium*, moderate on *A. crassicarpa*, *E. urophylla*, and *Melaleuca cajuputi*, while being non-pathogenic on *Dyera costulata*, *H. brasiliensis*, and *Alstonia scholaris*. This research focused on the virulence of various *Ceratocystis* isolates against *A. crassicarpa* seedlings. The objective was to describe the morphological properties and evaluate the virulence of eight isolates on *A. crassicarpa* seedlings.

Methods

Location and Material

The morphological characterization and pathogenicity testing of *Ceratocystis* sp. on *A. crassicarpa* seedlings was conducted between July and October 2022. The research location was the shade house and Forest Health and Protection Laboratory, Faculty of Forestry, Universitas Gadjah Mada (UGM), Yogyakarta. Eight *Ceratocystis* sp. collection isolates maintained in the laboratory were utilized for morphological characterization and pathogenicity evaluation, as detailed in Table 1. Pathogenicity test was conducted on four-month-old *A. crassicarpa* seedlings, which measured $\pm$ 35 cm in height and had semi-woody stems. These seedlings were grown from seeds collected from the progeny test (F1-21104) provided by APP (Asia Pulp and Paper) in Riau Province, Sumatra.

Isolate Characterization

Eight *Ceratocystis* isolates (Table 1) were characterized morphologically. The isolates were cultured on Potato Dextrose Agar (PDA) medium, and the colony growth rates were examined. Colony area measurements were taken every two days for 30 days to determine growth rates. The isolates were evaluated for their perithecium attributes and colony color. The observed characteristics were a) the length and width of the perithecium, b) the shape and length of the ostiolar hyphae, c) the length and width of the ascospores, and d) the length of the conidia. These features were assessed using an Olympus CX33

Table 1. List of *Ceratocystis* isolates based on host and origin used in this research

No	Host	Origin	Isolate Code	Collection
1	*A. crassicarpa*	Sumatra	AC1	UGM 2022
2	*A. crassicarpa*	Kalimantan	AC2	Suheri 2022
3	*A. mangium*	Java	AM1	UGM 2019
4	*A. mangium*	Kalimantan	AM2	UGM 2018
5	*A. mangium*	Kalimantan	AM3	UGM 2018
6	*A. mangium*	Kalimantan	AM4	UGM 2018
7	*Eucalyptus* sp.	Sumatra	EP1	UGM 2019
8	*Lansium* sp.	Sumatra	LA1	UGM 2019

microscope. Colony coloration was determined using Ridgway's (1912) color standard estimates.

Pathogenicity Test

This research used a completely randomized design with eight isolates of *Ceratocystis* sp., as presented in Table 1, and four replications. Artificial inoculation was performed following a modified Brawner et al. (2022) method, with an inoculation point height of ± 8 cm. The inoculum utilized was prepared from a 14-day-old culture, and removed with a 5 mm cork borer. The inoculum was affixed to wounds on the seedling stems, secured, and protected with masking tape to ensure proper placement. The inoculated seedlings were maintained in a 40% shaded environment at room temperature (± 25°C). Seedlings received 30 cc of sterile water in the morning and evening, and 5 mL of sterile water was sprayed daily using a sprayer to maintain moist conditions at the inoculation point (Brawner et al. 2020).

Assessment of Pathogenicity Test

Observations were made every seven days from the 1st to 35th days after inoculation (DAI). Parameters recorded included the outer lesion length (OLL), the percentage of seedling mortality, and the mortality day. The internal lesion length (ILL) was measured for seedlings that died. The virulence level of the isolate was determined based on the length of the outer and inner lesions and the time required for seedling death (Brawner et al. 2022). The percentage of mortality caused by artificial inoculation with each isolate was calculated using the following formula.

$$\text{Percentage of Mortality} = \frac{\text{number of dead seedling}}{\text{Total seedling}} \times 100\%$$

For selecting virulent isolates, the highest total score of the *in vivo* (the longest outer lesion, the longest inner lesion, and the highest and fastest seedling mortality) and *in vitro* (the widest colony growth) items were considered.

Data Analysis

The morphological characteristics of *Ceratocystis* isolates were analyzed qualitatively and descriptively. The size of the perithecium attributes was measured using Miconos Opti Lab Viewer 4 Software. Microsoft Excel 2013 Software was adopted to analyze the average colony growth of *Ceratocystis* isolates, the average length of the outer lesion, the average length of the inner lesion, and the percentage of seedling mortality in the pathogenicity test. Analysis of variance for the size of the perithecium attributes was performed using RStudio software version 4.3.0.

Results and Discussions

Characterization of *Ceratocystis* sp.

The morphological characteristics of eight *Ceratocystis* isolates showed similarities in colony color, shape, size, and perithecium features, as presented in Table 2. Visual assessments of the isolate colony colors of the isolates present the same hue (deep greyish olive), except for EP1, which originated from *E. pellita* (Figure 1). All *Ceratocystis* isolates produced globose ascomata bases in the form of ascus that generated hat-shaped ascospores and cylindrical conidia. The elongated neck connecting the ascomata with the ostiolar hole produced the ascospores. The shape of the ostiolar hyphae showed variations, such as a divergent tip pattern (isolates AC1 and AC2) and straight edges (isolates AM1, AM2, AM3, AM4, EP1, and LA1). Furthermore, the size of perithecium attributes, namely the length and width of the perithecium, the length of the ostiole hyphae, the length and width of the ascospores, and the size of the conidia, was not significantly different (p=0.05) (Table 2). Figures 2 to 9 show the morphological characteristics of *Ceratocystis* isolates.

Table 2. Summary of morphological characters of eight isolates of *Ceratocystis* in pathogenicity tests

Isolat Code	Colony Colors*)	Perithecium		Ostiolar Hyphae		Ascospores		Conidia's size (µm²)
		Length (µm)	Wide (µm)	Shape	Length (µm)	Length (µm)	Wide (µm)	
AC1	Deep greyish olive	203,5±42,5	203,1±12,2	Divergent	73,6±4,1	4,9±0,7	3,4±0,4	83,0±23,1
AC2	Deep greyish olive	193,1±10,5	180,0±14,8	Divergent	53,9±3,0	4,9±0,3	3,6±0,5	83,3±20,9
AM1	Deep greyish olive	207,5±44,6	250,7±8,2	Straight	54,6±2,7	4,9±0,5	3,5±0,6	110,1±50,4
AM2	Deep greyish olive	310,3±86,2	301,9±62,4	Straight	63,4±14,7	5,4±0,4	4,3±0,4	125,2±20,6
AM3	Deep greyish olive	265,3±19,9	245,5±25,5	Straight	74,4±6,3	5,2±0,5	4,1±0,7	141,4±32,7
AM4	Deep greyish olive	252,0±18,0	251,3±22,9	Straight	54,5±3,3	5,3±0,6	3,6±0,7	108,7±32,4
EP1	Greyish olive	169,0±29,9	174,7±6,9	Straight	66,9±4,5	5,0±0,7	3,7±0,5	67,0±21,6
LA1	Deep greyish olive	219,5±70,3	201,4±32,6	Straight	56,6±1,5	5,0±0,8	3,7±1,1	83,1±14,4

*) Colony colors with Ridgway's notation (1912)

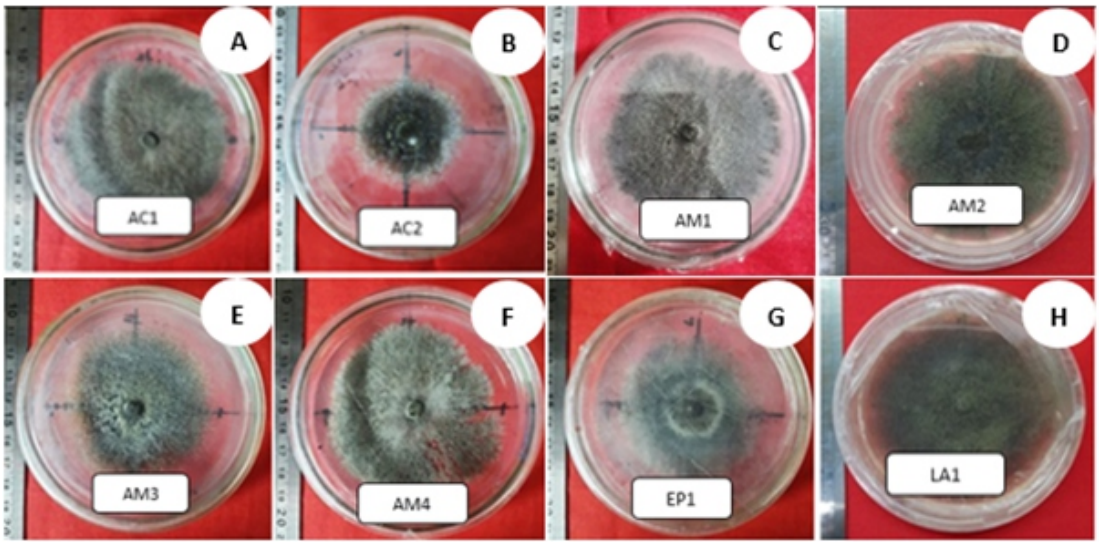

Figure 1. The isolates features of *Ceratocystis* spp.: A. AC1, B. AC2, C. AM1, D. AM2, E. AM3, F. AM4, G. EP1, H. LA1

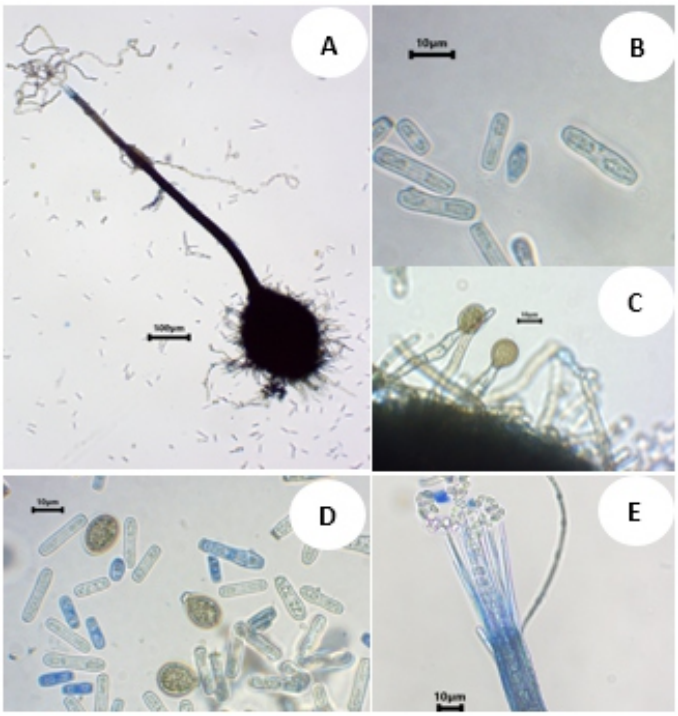

Figure 2. Microscopic characteristic of isolate AC1: A. The perithecium, B. Cylindrical conidia, C. Terminal thick walled of aleurioconidium, D. Aleurioconidium and Cylindrical conidia, E. Divergent ostiolar hyphae with hat-shaped ascospores at the top

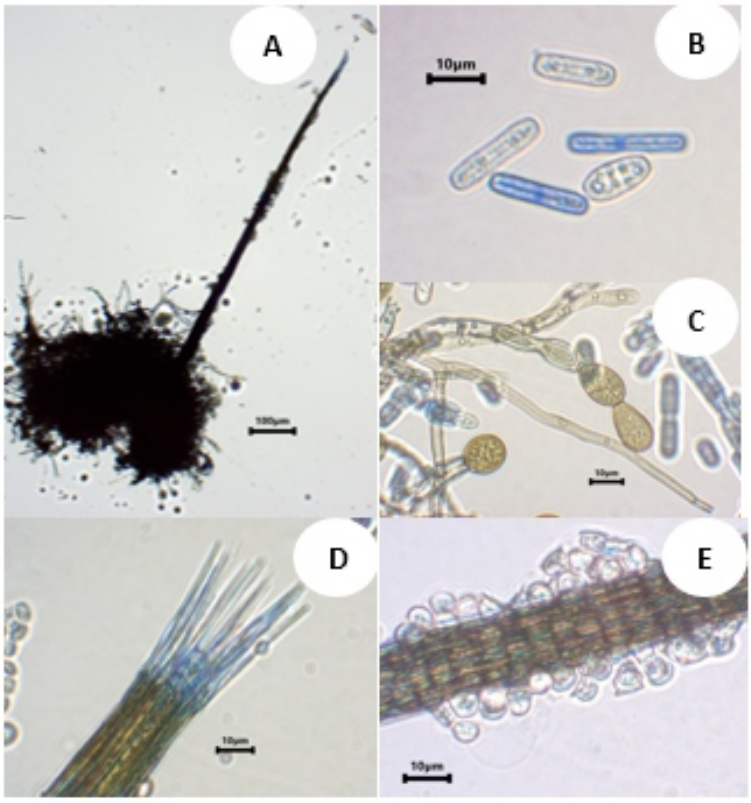

Figure 3. Microscopic characteristic of isolate AC2: A. The perithecium, B. Cylindrical conidia, C. Terminal thick walled of aleurioconidium, D. Divergent ostiolar hyphae, E. hat-shaped ascospore morphology

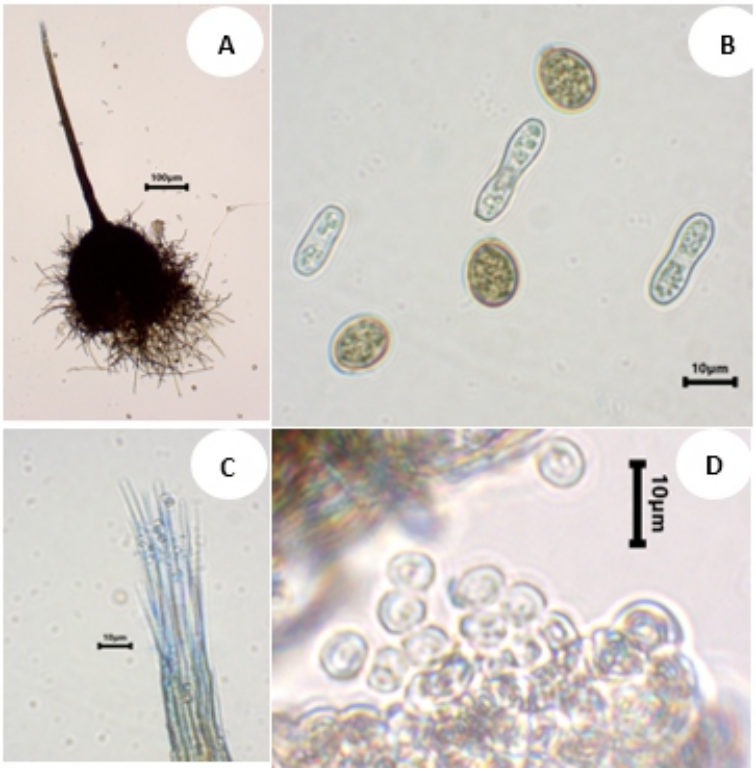

Figure 4. Microscopic characteristic of isolate AM1: A. The perithecium, B. Cylindrical conidia, C. Straight ostiolar hyphae, D. Hat-shaped ascospore morphology

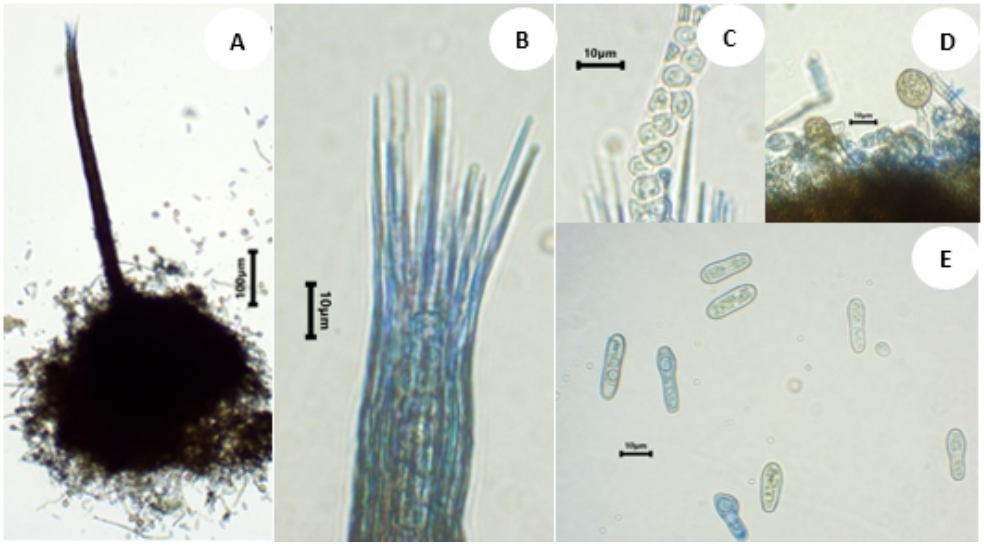

Figure 5. Microscopic characteristic of isolate AM2: A. The perithecium, B. Cylindrical conidia, C. Straight ostiolar hyphae, D. Hat-shaped ascospore morphology

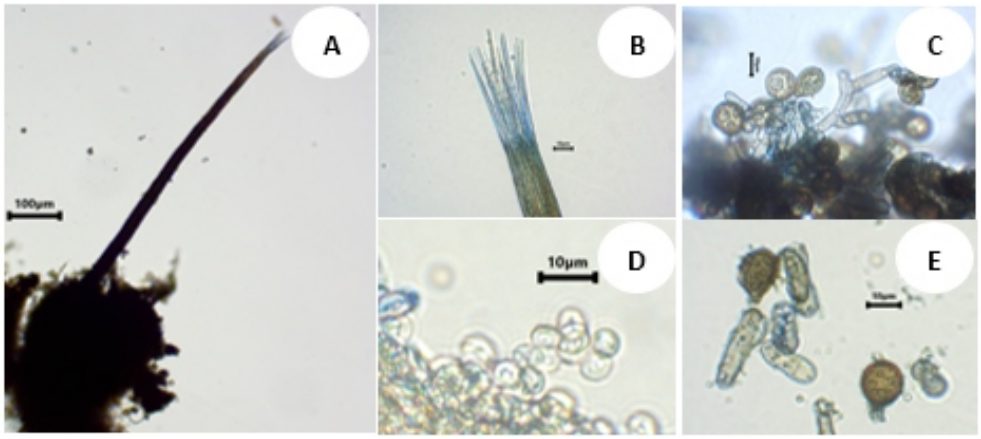

Figure 6. Microscopic characteristic of isolate AM3: A. The perithecium, B. Straight ostiolar hyphae, C. Terminal thick-walled aleurioconidium, D. Hat-shaped ascospore, E. Aleurioconidium, and cylindrical conidia

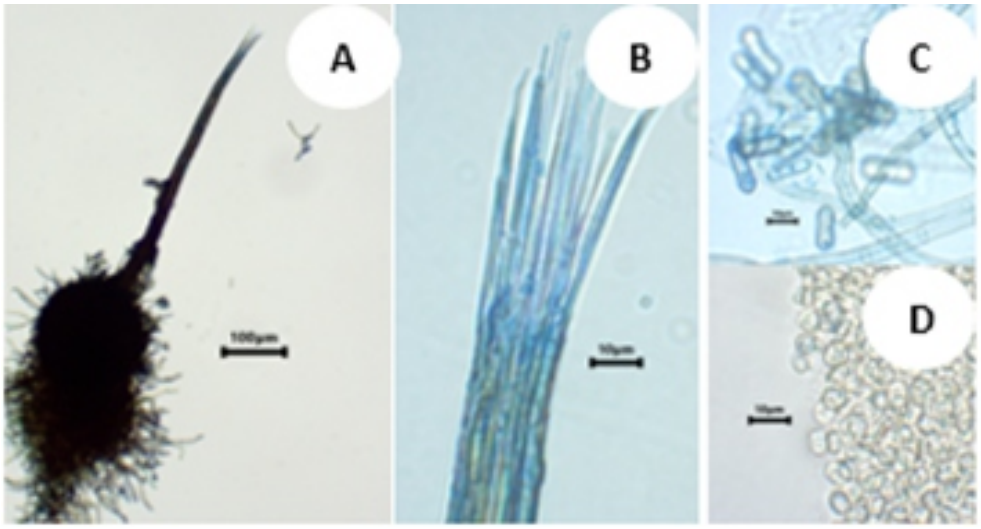

Figure 7. Microscopic characteristic of isolate AM4: A. The perithecium, B. Straight ostiolar hyphae, C. Cylindrical conidia, D. Hat-shaped ascospore morphology

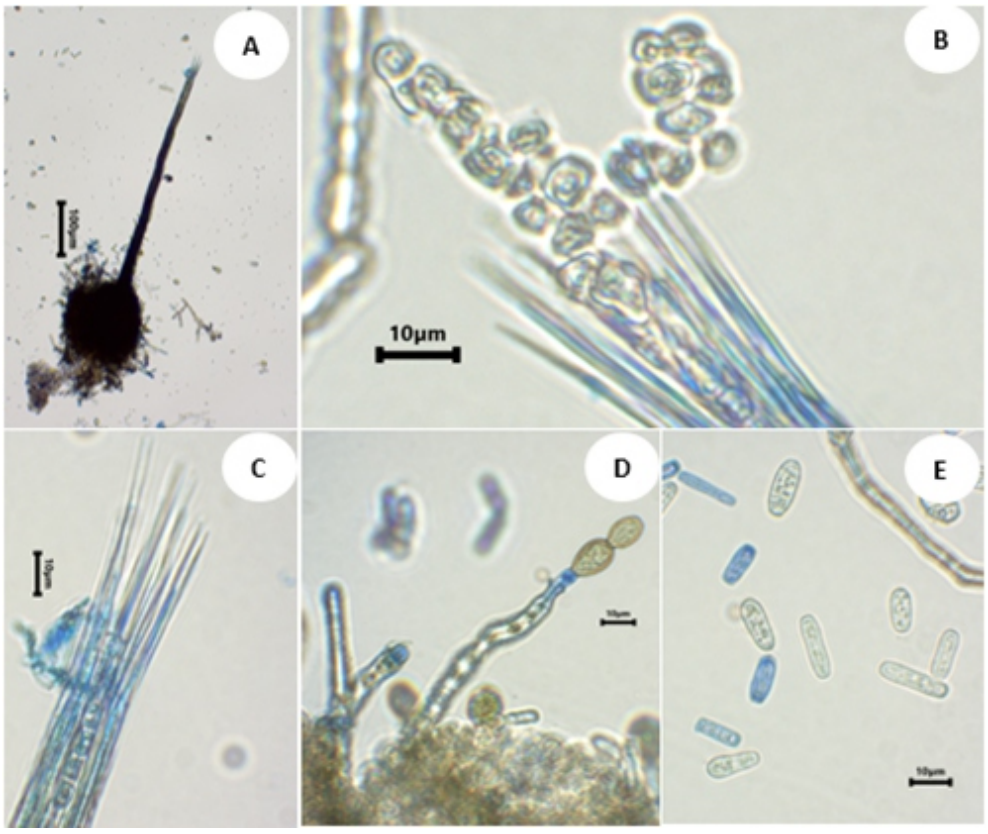

Figure 8. Microscopic characteristic of isolate EP1: A. The perithecium, B. hat-shaped ascospore release from ostiole, C. Straight ostiolar hyphae, D. Terminal thick-walled aleurioconidium. E. Cylindrical conidia

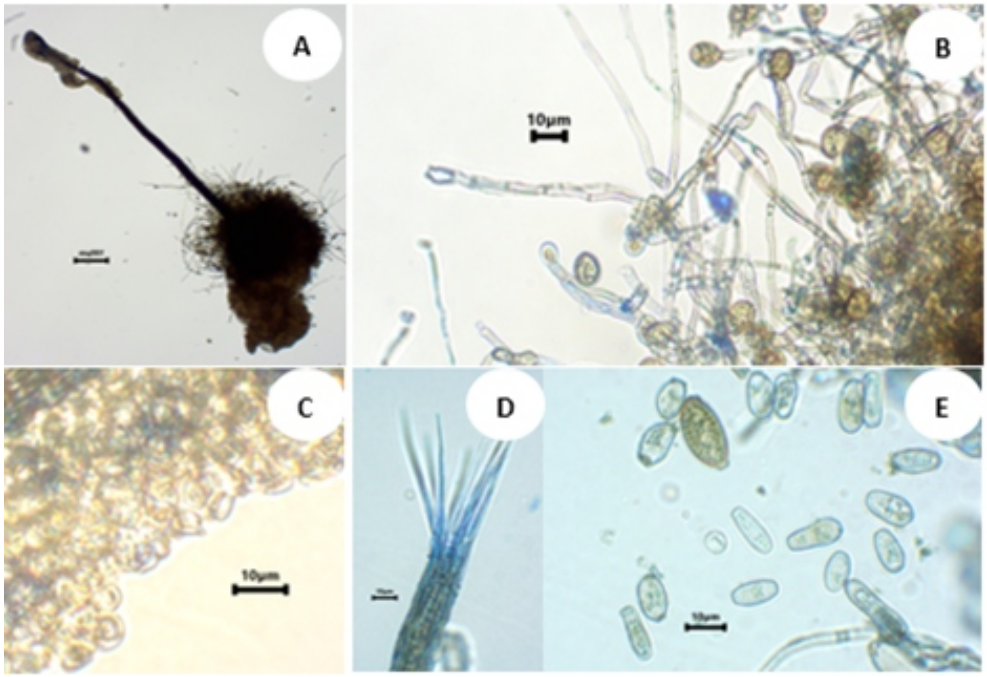

Figure 9. Microscopic characteristic of isolate LA1: A. The perithecium, B. terminal thick-walled aleurioconidium, C. Hat-shaped ascospore, D. Straight ostiolar hyphae, E. Aleurioconidium, and cylindrical conidia

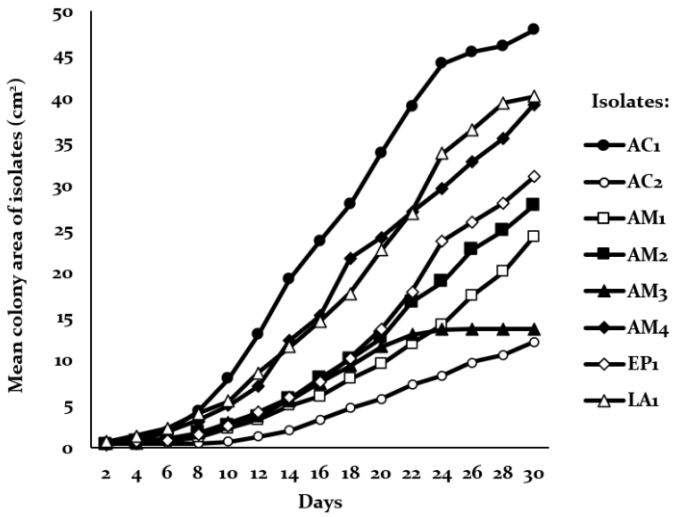

Figure 10. The average colony area of eight isolates of *Ceratocystis* sp. on PDA media for 30 days

Growth of *Ceratocystis* Isolates

The growth trends of eight *Ceratocystis* isolates cultured on PDA medium showed significant differences over 30 days, as presented in Figure 10. From day 10 to day 30, the AC1 isolate derived from *A. crassicarpa* Sumatra had the fastest growth and the biggest colony area (47.8 cm^2). Meanwhile, AC2 from *A. crassicarpa* Kalimantan host grew slowly and had the smallest colony area until the 30^{th} day of observation (12.0 cm^2). Understanding the variability of isolates was important for identifying the most virulent for resistance screening. According to Harrington et al. (2011), due to the long history of *Ceratocystis* sp., particularly *C. fimbriata* in many parts of the world, isolates from the same host had variable virulence. In this research, isolates AC1 and AC2, despite being sourced from *A. crassicarpa*, differed in morphological characteristics and colony growth on the PDA medium. The results suggested potential differences in their virulence. Therefore, the identification using DNA markers needed to be conducted.

Pathogenicity Test

Ceratocystis sp. was known to cause wilt disease, primarily through the infection of plant tissue following the entry of fungal pores into natural or artificial wounds. Spores inside the tissue develop into hyphae that invade and infect the host. Symptoms due to attacks by *Ceratocystis* sp. ranged from the appearance of outer and internal lesions on the stem to plant death. As detailed in Figure 11A, outer lesions signified hyphae's invasion into the epidermis and phloem tissue. Meanwhile, the appearance of internal lesions, as presented in Figure 11B, showed the penetration into xylem tissue, which affected the water and nutrient transfer process. This penetration led to tissue degradation and, eventually, plant death, as detailed in Figure 11C. Pathogen-produced enzymes that harm host tissue and degrade the transport network (Hubert 1931). Specifically, *C. fimbriata* enzymes included β-xylosidase and xylanase, which destroyed hemicellulose-containing plant cell walls, according to Martins et al. (2018). Those processes caused plant wilting and eventually death.

Based on the artificial inoculation result, all isolates produced outer lesions on *A. crassicarpa* seedlings in 14 days, except AM3 and AM4, as shown in Figure 12A. Isolates AC1, AC2, EP1, and LA1 generated the highest lesion lengths at 35 DAI. Furthermore, AC1 and EP1 also produced the longest inner lesion, which ranged from 29.2 cm to 31.6 cm at 35 DAI (Figure 12B). Only EP1 caused 100% seedling mortality in this research, while AC1, AC2, AM2, and LA1 produced 50% to 75% mortality, as shown in Figure 12C. The tested isolates were able to induce outer or inner lesions but varied substantially in aggressiveness to *A. crassicarpa*. It was found that two isolates from *A. crassicarpa,* one from *Eucalyptus* spp., and one from *Lansium* sp., were particularly aggressive, but strong evidence of specialization to the host was not observed. The EP1 originated from *Eucalyptus* spp. host in Riau, Sumatra was the most virulent to *A. crassicarpa*. It could kill 100% of seedlings at 35% DAI.

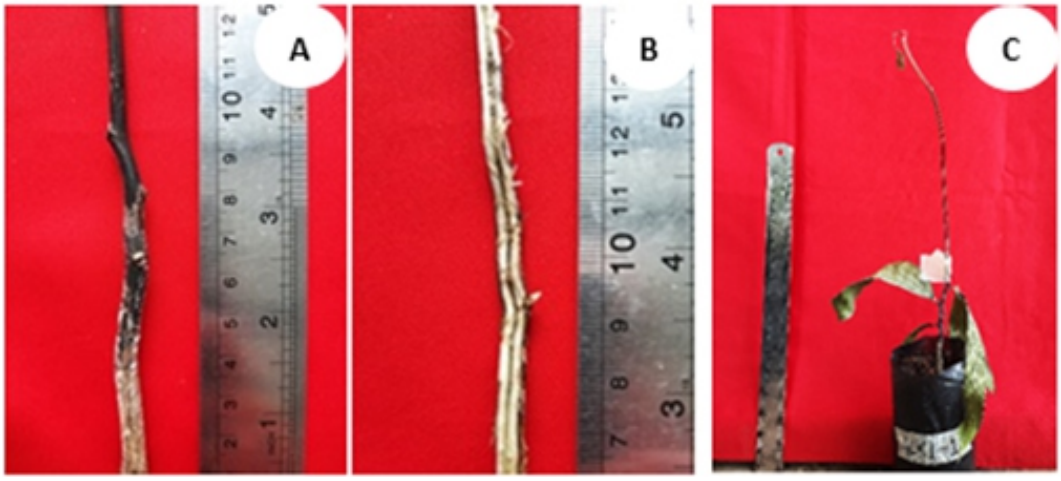

Figure 11. *Ceratocystis* sp. infection signs on *A. crassicarpa* seedlings artificially inoculated with isolates 35 DAI (days after inoculation): A. Outer lesion symptoms; B. Internal lesion symptoms; and C. Seedling death symptoms

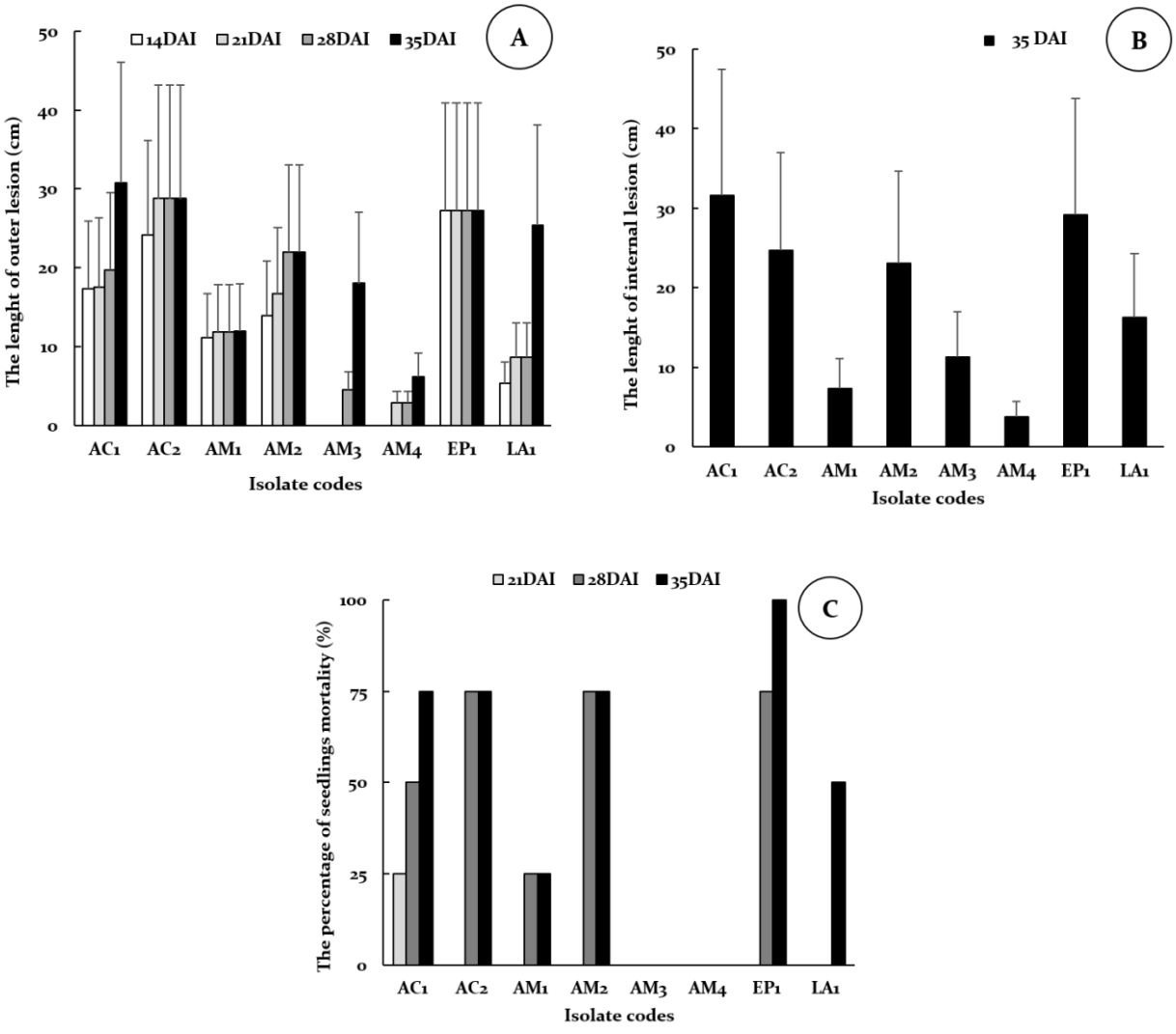

Figure 12. Histogram of pathogenicity test results for eight *Ceratocystis* sp. isolates: A. Length of the outer lesion; B. Length of the internal lesion; and C. Percentage of seedlings mortality of *A. crassicarpa* seedlings inoculated with *Ceratocystis* sp. The vertical line on the bar is the standard deviation.

Oliveira et al. (2016) reported a weak correlation between the origin of *Ceratocystis* sp. isolates and their aggressiveness toward mango in Brazil. Valdetaro et al. (2015) discovered that isolates from *Hevea brasiliensis* caused significant xylem discoloration and high mortality in *Crotalaria juncea* with less impact on *H. brasiliensis*. Rossetto and Ribeiro (1990) emphasized planting resistant genotypes as a key control strategy for *Ceratocystis* wilt. Breeding programs for disease resistance require a deep understanding of factors influencing variability in pathogen virulence and aggressiveness (McDonald & Linde 2002). The most aggressive isolate (EP1) was recommended for future artificial inoculation trials to support resistance screening to *Ceratocystis* sp.

Conclusion

In conclusion, this research confirmed that small variations existed among eight isolates of *Ceratocystis* sp. obtained from *A. mangium*, *A. crassicarpa*, *Eucalyptus* spp., and *Lansium* sp. based on perithecium size, morphology, and colony growth on PDA. Pathogenicity tests on *A. crassicarpa* seedlings showed that the isolate from *Eucalyptus* spp. (EP1) was the most virulent, followed by isolates from *A. crassicarpa* (AC1 and AC2), *A. mangium* (AM2), and *Lansium* sp. (LA1), which caused 100%, 75%, and 50%, respectively, at 35 days after inoculation (DAI). In contrast, AM3 and AM4 from the *A. mangium* host were not pathogenic to *A. crassicarpa*. This research recommended using EP1 for resistance screening of *A. crassicarpa* genotypes.

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