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Morphological and Molecular Characteristics of Endophytic Fungi in Sugarcane as Antagonists of the Pathogen *Fusarium sacchari*

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Fusarium sacchari is a pathogenic fungus that causes the pokkah boeng disease in sugarcane plants. The symptoms of pokkah boeng disease are systemic, spreading throughout all parts of the plant. The control measures implemented so far still rely on synthetic pesticides but have not yielded optimal results and are not environmentally friendly. One of the environmentally friendly control methods is the use of endophytic fungi. Endophytic fungi live within plant tissues without causing disease symptoms. This research aims to discover endophytic fungi derived from sugarcane and evaluate their ability to suppress the growth of the pathogen *F. sacchari*. The research began with the isolation of sugarcane tissue, followed by the morphological and molecular characterization of endophytic fungi and testing their inhibitory capacity against the pathogen *F. sacchari*. Morphological identification was based on colony and microscopic characteristics, while molecular identification was conducted using PCR and sequencing, with similarities based on GenBank. Inhibition tests were conducted through dual culture and volatile compound testing, as well as enzyme production. The research results obtained 38 endophytic fungal isolates from sugarcane plants that have the ability to inhibit the pathogen *F. sacchari*. Five of these isolates showed the highest inhibition against *F. sacchari* and produced cellulase, protease, and chitinase enzymes. Molecular identification results indicated that these five-sugarcane endophytic fungal isolates are *Daldinia eschscholdzii*, *Hypoxylon pulicicidum*, *Trichoderma virens*, *Trichoderma harzianum*, and *Diaporthe phaseolorum*. The endophytic fungus *T. harzianum* provides the highest inhibition rate of 83.33% in dual culture and 55.5% inhibition through the production of volatile compounds.
Keywords: Endophytic fungi, inhibitory power, *Fusarium sacchari*, morphological characteristics, molecular characteristics, pokkah boeng disease, sugarcane plants.

INTRODUCTION

The plant microecosystem is highly diverse, generally consisting of fungi and bacteria that colonize plants and subsequently affect plant growth and development (Fisher *et al*., 1992). The interactions between fungi and plants are also varied, one of which is a mutualistic interaction, including endophytic fungi. The diversity of endophytic fungi colonizing plants is distributed across all tissues, including roots, stems, leaves, and fruits (Viogenta *et al*., 2020). Endophytic fungi are fungi that live within plant tissues without causing disease symptoms (Strobel, 2018). The interaction between plants and endophytic fungi can benefit plants by assisting in nutrient absorption and protecting against biotic and abiotic stresses (Munif, 2003).

Sugarcane (*Saccharum officinarum* L.) is a plantation crop that serves as the main raw material for sugar production and is a strategic plantation commodity in the Indonesian economy (Subiyakto *et al*., 2016). The area of sugarcane plantations in Indonesia from 2018 to 2022 has increased, but sugar production still fluctuates. The sugarcane plantation area in Indonesia in 2022 was 0.49 million hectares, and White Crystal Sugar (WCS) production reached 2.4 million tons. Fluctuating sugar production has implications for government programs to increase sugar imports. The volume of sugar imports in Indonesia experienced a deficit of

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5,605.53 thousand tons in 2022 (BPS, 2023). Many factors affected the decrease in sugarcane productivity, including: insufficient fertilizer use (Diana *et al*.,2016), lack of sugarcane varieties resistant to diseases (Risdianti *et al*., 2017), and disturbances by plant diseases (Subiyakto *et al*., 2016).

The pokkah boeng disease caused by *Fusarium sacchari* is very detrimental to sugarcane plants, as it can attack the plants from the young stage to the mature stage. The symptoms of pokkah boeng begin with chlorosis, which develops to the leaf sheath (the base of the leaf), followed by bud rot, curled leaves, necrosis; and the cutting phase where the sugarcane bark appears as if it has been cut with a knife (Subiyakto *et al*., 2016). Losses due to pokkah boeng disease in sugarcane plants in Indonesia were estimated to reach 0.6 - 1.2 trillion rupiah annually (Subiyakto *et al*., 2016). Various efforts to control pokkah boeng disease have been made, but the results were still varied and not optimal. Therefore, a more effective alternative control based on endophytic fungal biological agents is needed.

Biological disease control using endophytic fungi is a control method that uses fungi to inhibit pathogen growth through various mechanisms, namely producing mycotoxins, extracellular enzymes, parasitism, antibiosis, nutrient competition, and induction of resistance (Pratiwi *et al*. 2013; Jamwal and Gandhi 2019). Biological control can occur naturally or through manipulation of the environment, host, or antagonistic agents with mass introduction of one or more types of antagonists. Endophytic microbes can be isolated from all parts of the plant, including roots, stems, or leaves, and can also come from plants that are not host plants of a pathogen (Fouda *et al.*, 2015). Endophytic fungi *Penicillium chrysogenum* and *Alternaria alternata* stimulate corn plant growth by producing IAA and can control *Candida albicans* disease by producing extracellular enzymes (Fouda *et al*., 2015). Endophytic fungi *Penicillium* sp. and *Gliocladium* sp. can improve tomato plant growth and control Fusarium wilt disease (Asniah *et al.*, 2020).

Endophytic fungi have diverse morphological characteristics, ranging from colony color and shape, septate to non-septate hyphae, conidia or not forming conidia on certain media. Endophytic fungi isolated from various banana plant tissues can control the pathogen *Fusarium oxysporum* (Suciatmih *et al.*, 2014). The high diversity of endophytic fungal characters makes identification difficult up to the species level. Molecularly, endophytic fungi have high diversity, with various species that can be found even in a single host plant. Molecular analyses such as DNA sequencing have been widely used to study endophytic fungal communities and identify endophytic fungal species (Manias *et al*., 2019). A deep understanding of the morphological and molecular characteristics of endophytic fungi can provide new insights regarding their roles and potential uses in health, agriculture, and the environment. The aim of this research is to obtain and identify endophytic fungi from sugarcane plants, both morphologically and molecularly, that have the potential to inhibit the pathogen *F. sacchari* in vitro.

MATERIALS AND METHODS

***Materials*:** Materials used in the research include: diseased sugarcane plant samples showing pokkah boeng symptoms, healthy sugarcane plant samples, potato dextrose agar (PDA) medium, potato dextrose broth (PDB) medium, 70% ethanol, spirtus, distilled water, sodium hypochlorite (NaOCl), czapek dox agar (CDA), malt extract agar (MEA), malt extract broth (MEB), mercury(II) chloride ($HgCl_2$), carboxymethyl cellulose (CMC), yeast extract, iodine, dimethyl ether ($(CH_3)_2OH$), dimethyl sulfoxide (DMSO), colloidal chitin, ethyl acetate, tissue paper, cotton, and filter paper. Equipment used: laminar air flow cabinet, autoclave, bunsen burner, forceps, scissors, cutter, machete, petri dishes (9 cm diameter), erlenmeyer flasks, beakers, hotplate, oven, inoculation loop, test tubes, binocular microscope, camera, and writing materials.

***Sampling of Healthy and Diseased Plants*:** The sampling location for healthy and diseased plants was in the sugarcane plantation of PT. Jonlin, Bombana Regency, Southeast Sulawesi. Healthy plant examples that show better growth were used as sources of endophytic fungi, while examples of diseased plants with pokkah boeng symptoms were used as sources of F. sacchari isolates. A total of 5 healthy plant samples were taken, distributed among the population of sugarcane plants where there were plants with pokkah boeng symptoms. The roots and stems of the diseased plants were then brought to the laboratory for isolation.

***Isolation of Endophytic Fungi and Pathogens*:** The isolation methods for endophytic fungi and pathogens are the same, with the difference being in the plant samples used. For the isolation of endophytic fungi, all tissues from healthy plants were used, while for the isolation of pathogenic fungi, the plant parts used were the stem sections showing Pokkah boeng symptoms. The isolation procedure followed the method described by Asniah *et al*. (2023).

Identification was carried out based on the morphological characteristics of the colonies, specifically the color and shape of the colonies, observed under a binocular microscope using standard manual identification keys (Moubasher & Moustafa, 1970).

***Testing for Inhibition Activity through Dual Culture*:** The in vitro inhibition test of endophytic fungal isolates against pathogens was conducted through a dual culture test (antagonistic test) to determine the ability of endophytic fungal isolates to inhibit the pathogen *F. sacchari*. The incubated pathogen isolate was then transferred to the same PDA medium as the endophytic fungus, placed 3 cm from the edge of the dish, while the pathogen isolate piece was placed

3 cm from the edge of the dish on the front side of the endophytic fungus. Endophytic fungal isolates showing an inhibition percentage against the pathogen ≥ 50% on the 4th day became candidate endophytic fungal isolates for testing the inhibitory power of volatile compounds. Observations of inhibitory power were made at 2, 4, and 6 days after inoculation of the endophytic fungi. The percentage of inhibition was calculated using the formula Supriati *et al.*, (2010) as follows: P = (R1 - R2) / R1 x 100%, where: P = inhibition percentage; R1 = radius of the pathogen colony towards the edge of the Petri dish; R2 = radius of the pathogen colony towards the endophytic fungal culture.

***Morphological and Physiological Characterization*:** Identification was carried out based on morphological characteristics observed under a binocular microscope using standard manual identification keys according to Moubasher & Moustafa (1970) for fungi with fruiting bodies and spores, and sterile hyphal fungi. Physiological characterization was done by testing endophytic fungi for extracellular enzyme production. The production and activity test of extracellular enzymes, namely cellulase, protease, and chitinase, followed the method of Sunitha *et al*. (2013) as modified by Susilowati *et al*. (2020).

***Inhibition Test of Volatile Compounds*:** Endophytic fungal isolates with high inhibitory power were further tested to observe the effect of volatile compounds produced against pathogenic fungi. The test was conducted using the vapor method. Each piece of pure culture of antagonistic and pathogenic fungi was taken from pure cultures on PDA media using a cork borer (0.8 cm) and then placed in the center of separate Petri dishes containing PDA media. The bases of both dishes were then sealed using plastic wrap. Subsequently, the two Petri dishes were inverted facing each other. The pathogenic fungus was placed on top and the endophytic fungus at the bottom, and they were incubated at 27°C until the pathogenic fungus in the control was full. Observations were made by measuring the diameter of the pathogenic fungal colony every 24 hours until the pathogenic fungal culture was six days old or had filled the Petri dish (Liswarni *et al*., 2018). The results of the inhibition study were analyzed using descriptive analysis with the Microsoft Office Excel 365 software.

***Molecular Characterization of Potential Endophytic Fungi*:** Endophytic fungi identified by PCR technique and analyzed for genetic diversity were those showing ≥ 30% inhibition against pathogens on day 4 through dual culture testing or volatile compound testing. Isolation of genomic DNA of endophytic fungi used the modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Muzuni *et al.*, 2014). PCR components for fungi used the universal primer pair ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (Sandy *et al.*, 2015). DNA amplification was then performed following the method of Sandy *et al.* (2015). DNA fragment samples from PCR amplification were sequenced to see the DNA arrangement of each population. Sequencing was carried out using an ABI-Prism 377 sequencing machine. Sequencing was performed with 1 DNA sample combined with forward primer and reverse primer. Sequencing was done using the Sanger method, using a terminator dye in the form of fluorescent rhodamine dye (PRISM reaction dyedoaxy terminator cycle sequencing kit). After obtaining the sequencing results, the sequences were then aligned using the NCBI BLAST program. The sequencing results were used to determine their similarity by comparing them with sequences in GeneBank using the BLAST (Basic Local Alignment Search Tools) program provided by NCBI (National Center for Biotechnology Information) through www.ncbi.nlm.nih.gov/blast. The Bioedit application with the ClustalW Alignment program was used to analyze the alignment of sample rDNA fragments and analyze restriction enzyme sites, MEGA version 5.0 application was used for the construction of phylogenetic relationships, and the Phydit application was used to determine similarity values. After this series of procedures was carried out, the organism analyzed could be determined its species (Haidin, 2017).

RESULT

***Morphological Characteristics of Endophytic Fungi from Sugarcane Plants*:** The exploration of endophytic fungi from healthy sugarcane plants successfully isolated 38 fungal isolates, consisting of 15 isolates (39.4%) from leaf tissue, 11 isolates (29%) from stems, and 12 isolates (31.6%) from sugarcane root tissue. The morphology of most endophytic fungal isolates has been characterized based on colony growth on PDA medium and microscopically observed under a binocular microscope. The morphology and microscopic features of these 38 endophytic fungal isolates are presented in Table 1.

Table 1 shows that based on the colony growth of endophytic fungi on PDA media, there is variation in the upper surface color, lower surface color, texture, edge, elevation, and colony shape. The highest percentage of endophytic fungal colony texture, 53% (20 out of 38 isolates), is Velvety; the highest percentage of endophytic fungal colony edge, 74% (28 out of 38 isolates), is entire; the highest percentage of endophytic fungal colony elevation, 37% (14 out of 38 isolates), is flat; and the highest percentage of endophytic fungal colony shape, 68% (26 out of 38 isolates), is zonate. The microscopic characteristics of endophytic fungi from sugarcane, related to the presence and type of conidia, shape of conidia or spores, hyphae, and specific features, are presented in Table 2.

Table 2 shows the highest number of endophytic fungal isolates in root tissue, with 12 isolates, followed by 11 isolates in stem tissue, and 15 isolates in leaf tissue. Among the identified endophytic fungal isolates, 50% (19 out of 38 isolates) were found to have asexual spores using a binocular

Table 1. Macroscopic Characteristics of endophytic fungi isolated from *S. officinarum*.

Isolates	Plant tissue	Surface color	Reverse color	Texture	Margin	Elevation	Pattern
CEA13	Root	White cycle	White	Velvety	Entire	Umbonate	Flowery
CEA14.Bl	Root	Purplish White	Purplish White	Velvety	Entire	Umbonate	Zonate
CEA11	Root	White	White	Cottony	Lobate	Umbonate	Zonate
CEA04	Root	Yellowish White	Yellow	Velvety	Entire	Raised	Radiate
CEA03	Root	Grey around	Black	Granular	Entire	Umbonate	Zonate
CEA01	Root	Black	Dark	Velvety	Entire	Raised	Radiate
CEA12	Root	Yellowish Green	Yellowish	Granular	Lobate	Raised	Zonate
CEA09	Root	Greenish white	Green	Velvety	Entire	Raised	Radiate
CEA10	Root	Green	Green	Velvety	Entire	Raised	Radiate
CEA05	Root	Greenish grey	Grey	Granular	Entire	Convex	Zonate
CEA06	Root	Grey	Grey	Velvety	Entire	Flat	Radiate
CEA08	Root	White	White	Cottony	Entire	Umbonate	Zonate
CEB.Bl.06	Stem	White	White	Granular	filamentous	Umbonate	Zonate
CEB01	Stem	Greenish grey	Grey	Granular	Entire	Convex	Zonate
CEB.Bl.07	Stem	Kuning keabuan	Grey	wrinkled	Lobate	Flat	Zonate
CEB.ASA	Stem	Grey	Dark	Velvety	Lobate	Convex	Zonate
CEB.ASA2	Stem	Grey	Grey	Velvety	Lobate	Flat	Zonate
CEB05	Stem	Greyish white	White	Granular	Entire	Flat	Flowery
CEB03	Stem	Greyish white	White	Cottony	Lobate	Flat	Zonate
CEB04	Stem	White	White	Velvety	Lobate	Convex	Zonate
CEB21	Stem	Grey	Black	Velvety	Entire	Flat	Radiate
CEB22	Stem	White	White	Cottony	Entire	Flat	Flowery
CEB24	Stem	Greyish white	White	Cottony	Entire	Flat	Radiate
CED.Bl.e	Leaf	Dark	Black	Velvety	Entire	Flat	Zonate
CED.Bl.d	Leaf	Grey	Dark	Velvety	Lobate	Flat	Zonate
CED13	Leaf	Greenish grey	Grey	Granular	Entire	Convex	Zonate
CED.Bl.f	Leaf	White	White	Cottony	Entire	Umbonate	Zonate
CED01	Leaf	White	White	Cottony	Entire	Flat	Zonate
CED07	Leaf	Putih krem	Krem	Cottony	Entire	Convex	Zonate
CED11	Leaf	Putih krem	Krem	Cottony	Entire	Convex	Zonate
CED.Bl.a	Leaf	Dark grey	Dark	Cottony	Entire	Convex	Zonate
CED08	Leaf	Blackish grey	Dark	Velvety	Entire	Convex	Radiate
CED12	Leaf	Greenish grey	Grey	Velvety	Entire	Flat	Zonate
CED02	Leaf	Dark grey	Dark	Velvety	Entire	Flat	Zonate
CED.Bl.c	Leaf	Dark	Black	Velvety	Entire	Convex	Zonate
CED05	Leaf	Reddish grey	Red	Velvety	Entire	Convex	Radiate
CED03	Leaf	Dark grey	Black	Velvety	Entire	Convex	Zonate
CED04	Leaf	Grey	Dark	Velvety	Lobate	Flat	Zonate

microscope, while the remaining 19 isolates were not identified.

Percentage of Inhibition of Endophytic Fungal Isolates Against Pathogens: The percentage of inhibition of endophytic fungi against the pokkah boeng disease pathogen was assessed through dual culture and volatile compound tests. The testing media used was PDA media with a petri dish diameter of 9 cm. The isolated endophytic fungal isolates were tested for inhibitory activity against the pathogen *F. sacchari* using the dual culture method. The average percentage of inhibition of endophytic fungal isolates against the pokahbung disease pathogen is presented in Table 3.

Table 3 shows that endophytic fungal isolates can inhibit the growth of the test pathogen fungi within a range of 35% to 83.33% at 6 DAI. Endophytic fungal isolates CEA09 and CEA10 demonstrate the highest inhibition percentage, each at 83.33%, and are known microscopically as the genus Trichoderma.

Inhibition percentage of the pathogen in the volatile compound test. This test was conducted by covering both dishes with endophytic fungi and pathogens. The average inhibition percentage of endophytic fungal isolates against the pokkah boeng disease pathogen is presented in Figure 1.

Table 2. Microscopic Characteristics of endophytic fungi isolated from *S. officinarum*

Isolate code	Shape of spores	Hypha	Specific characteristics
CEA13	Round	Septate	Short conidiophores, crescent-shaped conidia, microconidia, macroconidia, short conidiophores
CEA14.Bl	Round	Septate	Short conidiophores, crescent-shaped conidia, microconidia, macroconidia, short conidiophores
CEA11	Round	Septate	Short conidiophores, crescent-shaped conidia, microconidia, macroconidia, short conidiophores
CEA04	Round	Septate	Short conidiophores, crescent-shaped conidia, microconidia, macroconidia, short conidiophores
CEA03	Round	Septate	Konidiofor panjang, tegak, berdinding tebal, memiliki vesikel, konidia bersusun dengan menempel pada vesikel.
CEA01	Round	Septate	Long, upright conidiophores with thick walls, having vesicles, conidia arranged in clusters attached to the vesicle.
CEA12	Round	Septate	Long, erect conidiophores with thick walls, possessing vesicles, conidia arranged in a row attached to the vesicle
CEA09	Round	Septate	Spherical conidia with single cells, growing at the tip of the phialid on the conidiophore, which branches out
CEA10	Round	Septate	Round conidia with single cells, growing at the tip of the phialid on the conidiophore, which branches out
CEB.Bl.06	Round	Septate	The conidia are single-celled, rectangular or somewhat elongated in shape, with the conidiophore resembling a twig
CEB01	Round	Septate	Long, erect conidiophores with thick walls, having vesicles, conidia arranged in rows attached to the vesicle.
CEB21	Round	Septate	The conidiophores are upright and branched, hyphae are septate, short phialids, and the conidia are ellipsoidal in shape
CED.Bl.e	Round	Septate	The dark-colored upright conidiophores, conidia with 3-5 cells with one of them curved, and the conidia are formed at the tip of the conidiophore
CED.Bl.d	Round	Septate	Upright conidiophores, branching to form a complex structure, with conidia forming in chains at the tips of the conidiophore branches
CED13	Round	Septate	Upright conidiophores, branching to form a complex structure, with conidia forming in chains at the tips of the conidiophore branches
CED.Bl.f	Round	Septate	Short conidiophores, crescent-shaped conidia, microconidia, macroconidia
CED01	Round	Septate	Short conidiophores, crescent-shaped conidia, microconidia, macroconidia
CED07	Round	Septate	Short conidiophores, crescent-shaped conidia, microconidia, macroconidia
CED11	Round	Septate	Short conidiophores, crescent-shaped conidia, microconidia, macroconidia
CED.Bl.a	-	Septate	Septate mycelium
CED08	-	Septate	Septate mycelium
CED12	-	Septate	Septate mycelium
CED02	-	Septate	Septate mycelium
CED.Bl.c	-	Septate	Septate mycelium
CED05	-	Septate	Septate mycelium
CED03	-	Septate	Septate mycelium
CED04	-	Septate	Septate mycelium
CEA05	-	Septate	Septate mycelium
CEA06	-	Septate	Septate mycelium
CEA08	-	Septate	Septate mycelium
CEB.Bl.07	-	Septate	Septate mycelium
CEB.ASA	-	Septate	Septate mycelium
CEB.ASA2	-	Septate	Septate mycelium
CEB22	-	Septate	Septate mycelium
CEB24	-	Septate	Septate mycelium
CEB05	-	Septate	Septate mycelium
CEB03	-	Septate	Septate mycelium
CEB04	-	Septate	Septate mycelium

Note: - = No conidia were found on the PDA medium

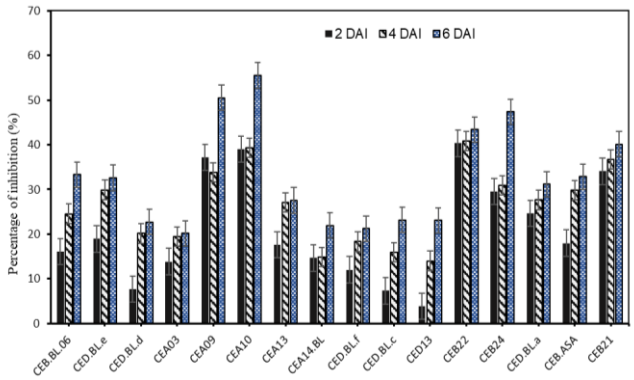

Figure 1. Inhibition activity of endophytic fungi from sugarcane against the pathogen *F. sacchari* by producing volatile compounds observed at 2, 4, and 6 DAI.

Figure 1. shows the percentage of inhibition from volatile compounds produced by endophytic fungi ranging from 20.17% to 55.50% observed at 6 DAI. The highest inhibition percentage from volatile compound testing is isolated CEA10 at 55.50%, while the lowest is isolated CEA03 at 20.17%.

***Characteristics of Extracellular Enzymes of Endophytic Fungi*:** The biochemical characteristics of endophytic fungi also indicate that some isolates produce extracellular enzymes including cellulase, protease, and chitinase. These biochemical characteristics were assessed using solid media suspected of secondary metabolites from the endophytic fungi successfully released in that medium. The presence of extracellular enzyme activity from an endophytic fungus was demonstrated by the formation of clear zones in the specific enzyme test medium. These clear zones indicated the presence of enzyme activity degrading the medium containing

the enzyme substrate. The enzymatic activity of the sixteen endophytic fungal isolates tested can be seen in Figure 2.
Picture 2 shows that 16 endophytic fungal isolates produce protease enzymes, 11 isolates produce cellulase enzyme, and 6 isolates produce chitinase enzyme. Isolates CEA09, CEA10, CED13, CEB22, CEB24, and CEB21 produces protease, cellulase, and chitinase enzymes. Among these five endophytic fungal isolates, 3 isolates do not produce spores as a reproductive tool and 2 isolates produce conidia and are microscopically identified as Trichoderma genus.

Table 3. Percentage of inhibition (%) of endophytic fungi against the pathogen *F. sacchari* using the dual culture method.

Isolate code	Percentage of Inhibition in Dual Culture Test (%) on the... day after inoculation (average ± standard deviation)		
	2	4	6
CEB.BL.06	30.00±4.71	54.36±0.27	65.00±2.36
CED.BL.e	16.67±14.1	45.65±9.22	58.33±7.07
CED.BL.d	17.42±1.07	56.32±5.21	68.33±2.36
CEA03	36.41±13.8	59.78±13.8	70.00±9.43
CEA01	29.17±5.89	65.82±3.09	73.33±0.00
CEB01	16.08±9.89	37.73±10.9	46.43±0.00
CEA09	77.75±1.17	80.00±0.00	83.33±0.00
CEA10	77.75±1.17	80.00±0.00	83.33±0.00
CEA13	20.00±0.00	46.43±5.05	50.00±0.00
CEA14.BL	10.51±3.99	41.34±6.36	58.33±2.36
CED.BL.f	16.67±4.71	41.79±14.4	58.33±2.36
CEA11	10.99±4.66	46.74±4.61	51.79±2.53
CED01	9.73±2.88	51.00±1.41	46.41±0.36
CED07	8.01±0.45	41.74±2.46	57.78±3.14
CEA04	21.43±10.1	52.17±6.15	56.00±5.66
CED11	3.57±5.05	31.61±20.1	53.70±2.62
CEA12	7.69±10.9	30.52±17.5	41.67±2.36
CEB.BL.07	6.90±0.34	39.88±2.53	55.00±2.36
CED.BL.c	3.13±4.42	29.89±6.92	50.00±0.00
CED13	22.71±5.60	46.54±7.97	60.00±4.71
CEB22	51.60±14.1	67.22±2.84	74.48±3.20
CEB24	53.94±0.86	59.17±1.18	74.42±5.43
CED.BL.a	7.42±0.39	46.74±4.61	58.33±2.36
CEB.ASA	9.55±0.64	55.84±1.84	68.33±2.36
CEB.ASA 2	25.63±7.95	48.91±1.54	61.67±2.36
CED08	47.76±8.61	60.26±9.07	70.71±1.01
CED12	13.33±0.00	44.00±0.00	51.67±2.36
CED02	6.90±0.34	45.76±9.37	56.67±4.71
CEB21	51.67±2.36	71.96±1.59	77.14±4.04
CEA06	30.95±3.37	65.33±1.89	66.67±0.00
CEA08	8.33±0.00	49.00±1.41	49.00±1.41
CEB05	11.54±16.3	41.74±2.46	47.00±4.24
CED05	20.51±18.1	21.29±0.64	35.00±2.36
CED03	8.33±0.00	53.26±4.61	51.67±2.36
CED04	25.63±7.95	49.81±3.10	50.86±1.22
CEB03	16.67±0.00	45.08±1.52	51.67±2.36
CEB04	16.08±9.89	48.91±1.54	53.33±0.00
CEA05	23.21±2.53	55.43±7.69	61.67±2.36

Note: ± = standard deviation. data on isolates from sugarcane leaves have been published Asniah *et al.* (2023)

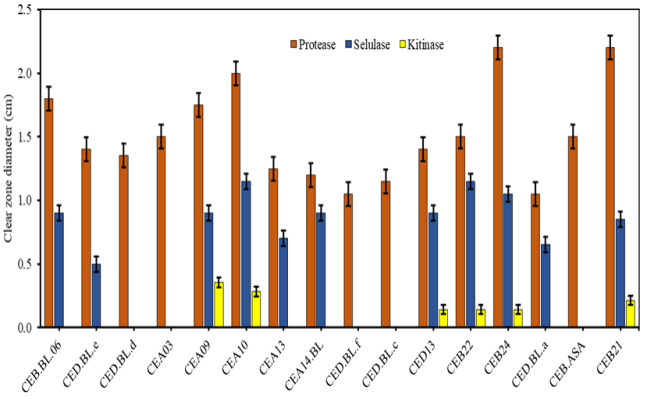

Figure 2. Histogram of the clear zone diameter activity of endophytic fungal isolates from sugarcane producing protease, cellulase, and chitinase enzymes at 6 HSI on PDA medium.

***Selection of Endophytic Fungal Identifications*:** Based on the inhibition percentage test results from dual culture and volatile compound tests, and the ability to produce extracellular enzymes, 5 potential endophytic fungal isolates have been selected for further in vivo testing or in sugarcane plants in a greenhouse. These five isolates were CEA09, CEA10, CEB21, CEB22, and CEB24. These potential isolates will undergo confirmation through molecular identification. The molecular identification results confirmed that CEA09 and CEA10 were *Trichoderma virens* and *Trichoderma harzianum*, CEB21 was *Daldinia eschscholzii*, CEB22 was *Diaporthe phaseolorum*, and CEB24 was *Hypoxylon pulicicidum*. The species identification results of these 5 isolates through molecular methods are presented in Table 4.
Table 4 indicates that the five endophytic fungal isolates from sugarcane are classified within the Phylum Ascomycota, class Sordariomycetes, Orders Hypocreales, Xylariales, and Diaporthales. The identification percentage based on the Blast gene is above 99%. The sequencing results. reference identity percentage in GenBank. and the phylogenetic tree for the selected five isolates are presented in Figures 3, 4, 5, 6, and 7.
Picture 3 indicates that isolate CEA09 based on morphological characteristics, pertains to the genus Trichoderma. Molecularly identified as *Trichoderma virens* species with a similarity percentage of 99.01% with accession number MK791709.1.
Figure 4 indicates that isolate CEA10 based on morphological characteristics, pertains to the genus Trichoderma. Molecularly identified as *Trichoderma harzianum* species with a similarity percentage of 99.68% with accession number MK209008.1.

Table 4. The BLAST analysis results of endophytic fungal isolates from sugarcane plants at PT Jonlin Plantation.

Isolate code	Phylum	Kelas	Ordo	Species	Accession	% Similarity
CEA09	Ascomycota	Sordariomycetes	Hypocreales	*Trichoderma virens*	MK791709.1	99.01%
CEA10	Ascomycota	Sordariomycetes	Hypocreales	*Trichoderma harzianum*	MK209008.1	99.68%
CEB21	Ascomycota	Sordariomycetes	Xylariales	*Daldinia eschscholtzii*	MH712260.1	99.08%
CEB22	Ascomycota	Sordariomycetes	Diaporthales	*Diaporthe phaseolorum*	MT043777.1	99.48%
CEB24	Ascomycota	*Sordariomycetes*	Xylariales	*Hypoxylon policicidum*	ON715774.1	99.17%

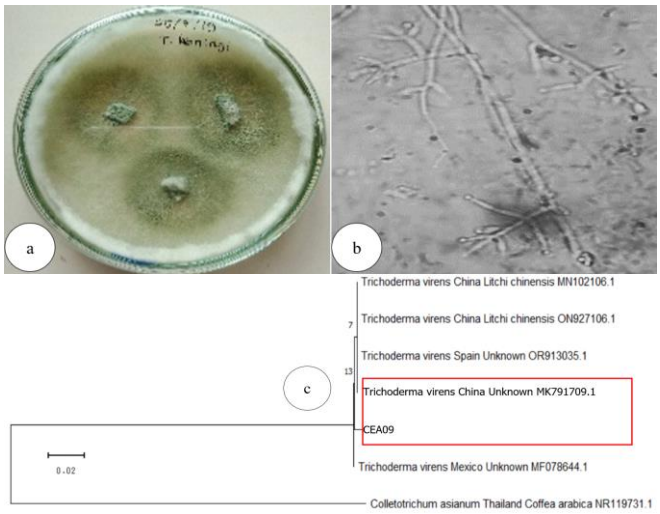

Figure 3. Identification of CEA09 isolates a) colonies on PDA media. b) microscopic magnification 400 times and c) phylogenetic tree.

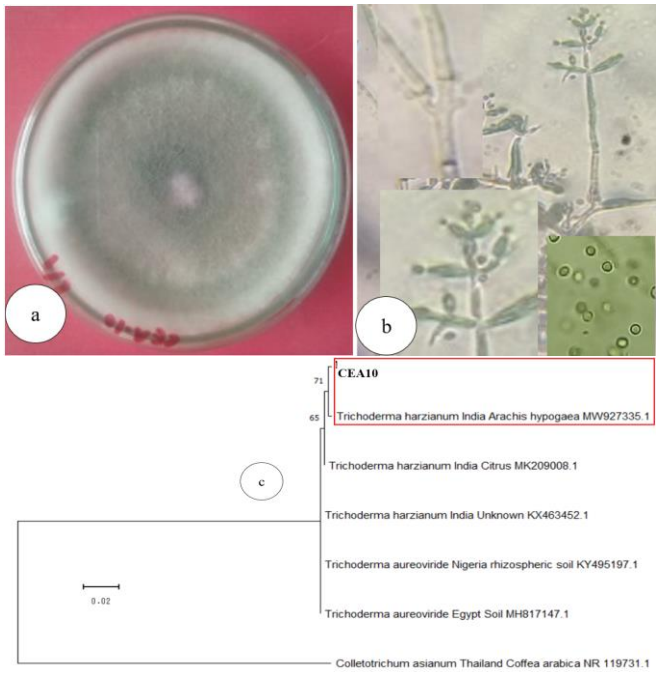

Figure 4. Identification of CEA10 isolates a) colonies on PDA media. b) microscopic magnification 400 times and c) phylogenetic tree.

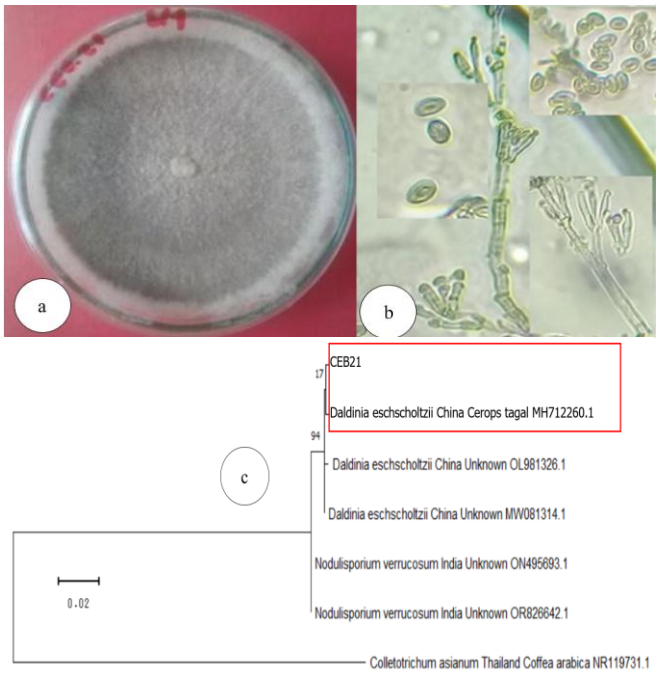

Figure 5. Identification of CEB21 isolates a) colonies on PDA media. b) microscopic magnification 400 times and c) phylogenetic tree.

Figure 5 indicates that isolated CEB21 based on morphological characteristics, possesses elongated conidia with branched conidiophores. Molecularly identified as *Daldinia eschscholtzii* species with a similarity percentage of 99.08% with accession number MH712260.1.

Figure 6 indicates that isolate CEB22, based on morphological characteristics, does not produce conidia or only produces mycelium, with white mycelium. Molecularly identified as *Diaporthe phaseolorum* species with a similarity percentage of 99.48% with accession number MT043777.1.

Figure 7 indicates that isolate CEB24, based on morphological characteristics, does not produce conidia or only produces grayish-white mycelium. Molecularly identified as *Hypoxylon pulicicidum* species with a similarity percentage of 99.17% with accession number MT043777.1.

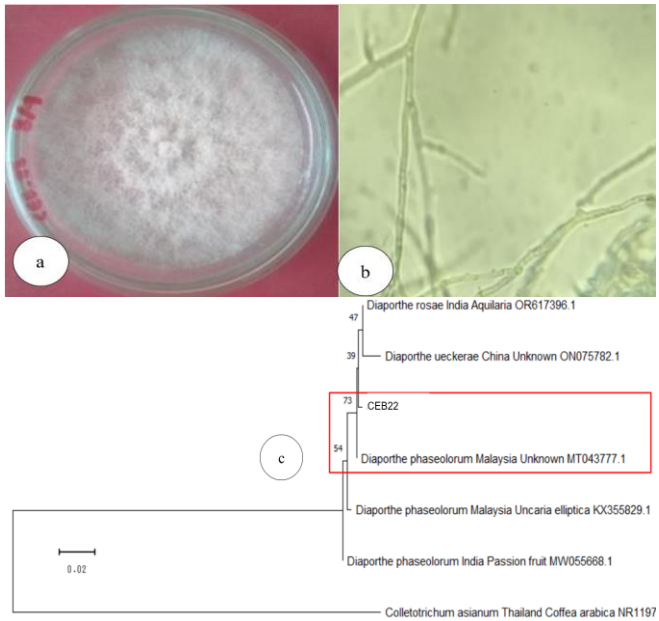

Figure 6. Identification of CEB22 isolate a) colonies on PDA media. b) microscopic magnification 400 times and c) phylogenetic tree.

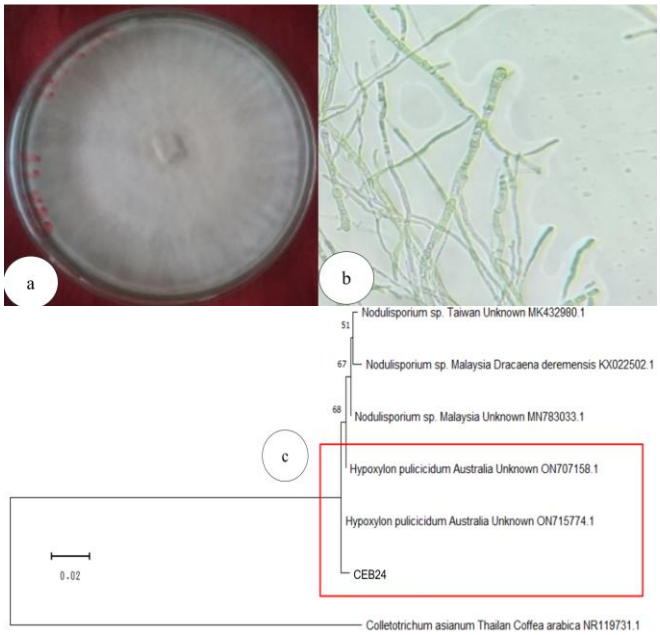

Figure 7. Identification of CEB24 isolate a) colonies on PDA media. b) microscopic magnification 400 times and c) phylogenetic tree.

DISCUSSION

Endophytic fungi have become an increasingly important focus of research in the fields of microbiology and plant biotechnology today. Endophytic fungi are fungi that live within plant tissues without showing symptoms of disease (Schulz & Boyle, 2005; Selim *et al*., 2012; Soesanto and Mugiastuti, 2023), and have great potential in enhancing plant growth and resistance to various biotic and abiotic stresses. Endophytic fungi represent the largest group of organisms on Earth that have not been extensively explored and characterized. Out of more than 1.5 million plant species, only about 5% have been successfully isolated as endophytic microorganisms (Strobel, 2018).

This study successfully isolated 38 endophytic fungal isolates from the root, stem, and leaf tissues of healthy sugarcane plants. Based on morphological identification, endophytic fungi from sugarcane plants have different colors and colony shapes. Some isolated endophytic fungal isolates did not produce reproductive structures in the form of conidia and could not yet be identified. Species-level identification was only performed on endophytic fungal isolates that showed potential as plant growth promoters and antagonistic agents against the pathogen *F. sacchari*. which causes pokkah boeng disease in sugarcane plants at PT. Jonlin Sugarcane Plantation (unpublished). Different host plants and/or different plant tissues produce different endophytic fungi in terms of both species and numbers (Yuanwar & Ainy, 2019). This is an implication of the adaptation mechanism of endophytic microbes to the specific microecology and physiological conditions of each host plant (Wahyudi, 2001).

The exploration of endophytic fungi from various plant species has become an interesting topic for researchers in recent years. Understanding the interaction between endophytic fungi and host plants to maximize their potential in sustainable agriculture is crucial (Porras-Alfaro & Bayman, 2011). Several researchers have successfully isolated endophytic fungi from various host plants, including sugarcane plants in Nepal (Jamwal & Gandhi. 2019), tomato plants (Asniah *et al.,* 2014), bamboo roots (Asniah *et al.,* 2013), cacao (Assad *et al.,* 2017; Taufik *et al.,* 2019; Taufik *et al.,* 2021), pearl grass (Viogenta *et al.,* 2020), and *Garcinia porrecta* and *Garcinia forbesii* plants (Radji *et al.,* 2009).

Based on the research results, endophytic fungal isolates CEA09 and CEA10 showed the highest inhibitory activity against *F. sacchari* compared to other isolates with 83.33% inhibition. Endophytic fungi have been widely reported as biological agents with high antagonistic mechanisms against pathogens, including the ability to compete for space and nutrients, produce toxins, produce antibiotics, enzymes that can degrade pathogenic fungal cell walls, hypersensitivity, and induction of plant resistance (Suswanto *et al*., 2018). Endophytic fungi *Trichoderma* sp. from tomato plants were able to inhibit the pathogen *Colletotrichum* sp. with an inhibition percentage of 74.14% (Asniah *et al*., 2014). According to Liswarni *et al*. (2018), each fungus has different growth rates and abilities to compete for nutrients from the growth medium. The antagonistic mechanism occurs due to competition between two types of fungi grown side by side

due to the same needs of each fungus. namely the need for growing space and nutrients from the growth media used (Melysa *et al*., 2013). Isolates CEA09 and CEA10, which have the highest percentage of inhibition against the pathogenic fungus *F. sacchari* at 83.33% with strong inhibitory activity are suspected to have high nutrient and space competition rates as well as other antagonistic mechanisms, namely antibiosis and mycoparasitism. Manurung *et al.* (2014) stated that inhibition of pathogenic fungal growth can occur through mechanisms of space competition, mycoparasitism, and antibiosis.

Based on the percentage of inhibition from the dual culture method, where isolates with inhibition percentages above 50% were further tested for volatile compound inhibition and extracellular enzyme activity. Screening results found 16 isolates that were tested for volatile compound inhibition and extracellular enzyme production. The percentage of volatile compound inhibition from endophytic fungal isolates against the pathogen *F. sacchari* ranged from 21-56%. It is suspected that the endophytic fungal isolates from sugarcane can produce volatile compounds on PDA medium. *Trichoderma* spp. microbes have antagonistic mechanisms including space and nutrient competition, antibiosis, and cell wall degradation through enzyme production, and produce both non-volatile and volatile metabolite compounds (Adhikari, 2023; Petrisor *et al.*, 2017). Volatile compounds inhibit mycelium and reduce sclerotia production in the pathogens *Sclerotinia sclerotiorum* and *S. rolfsii* (Amin *et al.*, 2010; Nagamani *et al*., 2017). Endophytic fungi *T. virens* and *T. harzianum* produce volatile metabolite compounds that inhibit the growth of *Colletotrichum capsici* mycelium in culture media and reduce disease incidence in fruit (Yan & Anh, 2018). Endophytic fungi *Penicillium commune, P. canescens*, and *Alternaria alternata* isolated from olive trees produce secondary metabolites of volatile compounds as antimicrobials that inhibit the growth of Gram-positive bacteria, Gram-negative bacteria, and yeast (Nagamani *et al*., 2017).

Endophytic fungi from sugarcane are also capable of producing extracellular enzymes on specific media for each enzyme: 16 isolates produced protease enzymes, 11 isolates produced cellulase enzymes, and 6 isolates produced chitinase enzymes. The isolates that produced all three enzymes were CEA09, CEA10, CEB21, CEB22, CEB24, and CED13. One of the antagonistic mechanisms of endophytic fungi in protecting plants is cell wall degrading enzymes such as chitinase, protease, and cellulase, as well as antibiotic secondary metabolites to control pathogen growth. The diameter of the clear zone formed indicates the strength of each fungus in producing enzymes. The genus Trichoderma produces protease enzymes to degrade pathogen cell walls (Nagamani *et al.*, 2017).

Potential endophytic fungi can be identified by their ability to produce extracellular enzymes in liquid media (Sunitha *et al.*, 2013). Endophytic fungi directly contribute by producing hydrolytic enzymes such as β-glucanase, chitinase and protease as well as antibiotics that suppress pathogens (Mamat *et al*., 2018). The differences in clear zone diameters indicate that there are differences in the extracellular enzyme activity capabilities produced by each CE isolate. The higher the clear zone diameter, the higher the activity of producing extracellular enzymes, which also increases the ability to degrade pathogenic fungal cell walls. Most enzymes degrade pathogen cell walls because most pathogen cell walls contain β-glucan and chitin compounds (Deng & Cao, 2017). The endophytic fungal isolate *Penicillium oxalicum* T3.3 produces chitinase, β-glucanase, protease, cellulase enzymes that can control the pathogen *Colletotrichum gloesporioides*, the cause of anthracnose disease in dragon fruit plants (Andhikawati *et al*., 2014; Mamat *et al*., 2018). Endophytic fungi *Fusarium solani* and *Talaromyces* sp. produce secondary metabolites in the form of cellulase enzymes with high biomass, thus being able to control Fusarium pathogens (Seethikal *et al*., 2015). Some endophytic fungi can trigger systemic defense responses in host plants, enhancing the plant's ability to fight pathogen attacks. This may involve increased production of defense enzymes such as peroxidase and chitinase. or accumulation of defense compounds such as salicylic acid and phytoalexins (Hardoim *et al.*, 2015).

Based on the results of molecular identification of 5 selected endophytic fungal isolates from sugarcane, the species identified were *Trichoderma virens, T. harzianum, Daldinia eschscholtzii, Hypoxylon pulicicidum,* and *Diaporthe phaseolorum*. The endophytic fungi *T. virens* and *T. harzianum* were found in the roots, while *Daldinia eschscholtzii, H. pulicicidum*, and *Diaporthe phaseolorum* were found in the stems of sugarcane plants. Stone *et al*. (2004) state that endophytic fungi from the genera Daldinia, Hypoxylon, and Trichoderma are commonly found in shoots, stems, and leaves, both in annual and perennial plants. Four endophytic fungal isolates of the genus Diaporthe isolated from the medicinal plant *Catharanthus roseus* growing in botanical gardens in China are endophytic fungi that microscopically do not form conidia and are able to inhibit *Aspergillus* spp. pathogens by producing antibiotics from the volatile compound group (Yan *et al.*, 2018). The endophytic fungus *Daldinia eschscholtzii* MFLUCC19-0493 isolated from ginger plants (*Zingiber officinale*) and Stemona tuberosa roots inhibits the growth of pathogens *Colletotrichum acutatum* and *Sclerotium rolfsii* by producing 60 metabolite compounds, including elemicin (24%), Benzaldehyde dimethyl acetal (8%), Ethyl sorbate (7%), Methyl geranate (6%), Trans-sabinene hydrate (5%) and 3.5-dimethyl-4-heptanone (5%) found most abundantly. Daldinia concentrica produces 27 volatile organic compounds (VOCs) including 3-methyl-1-butanol, (2-methyl-1-butanol), 4-heptanone, isoamyl acetate and trans-2-octenal which are antimicrobial

in controlling anthracnose that attacks strawberry fruit (Khruengsai *et al.*, 2021).

Based on the research findings, the five endophytic fungi identified from sugarcane plants exhibited inhibitory mechanisms against the pathogen *F. sacchari* in vitro, namely through competition for space and nutrients, enzymatic production, volatile compounds, and the production of secondary metabolites (unpublished data). This research is still at the laboratory scale, so further studies at the greenhouse and field scales are essential. The results of this study have the potential to identify promising candidates for the development of biocontrol agents. Endophytic fungal isolates that show strong and consistent antagonistic activity against *F. sacchari* can be targets for subsequent studies, including the development of effective endophytic fungal formulations, optimization of application methods, and environmental safety evaluations, which are important steps in transforming laboratory findings into biocontrol products that can be used in the field. Identification and characterization of antifungal compounds also become the next research focus. not only important for understanding antagonistic mechanisms but also potentially paving the way for the development of new natural fungicides.

***Conclusions*:** Endophytic fungi from sugarcane plants were successfully isolated, yielding 38 isolates that demonstrated inhibitory capabilities against the pathogen *F. sacchari* in dual culture, with inhibition percentages ranging from 35% to 83.33%. Among these, 16 isolates produced protease enzymes, 11 isolates produced cellulase enzymes, and 6 isolates produced chitinase enzymes. Five isolates showed the highest inhibitory ability against the pathogen *F. sacchari* and produced cellulase, protease, and chitinase enzymes. Molecular identification of these five-sugarcane endophytic fungal isolates, which have potential as controllers of pokkah boeng disease, revealed them to be *Daldinia eschscholdzii, Hypoxylon pulicicidum, Trichoderma virens, Trichoderma harzianum*, and *Diaporthe phaseolorum.*

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