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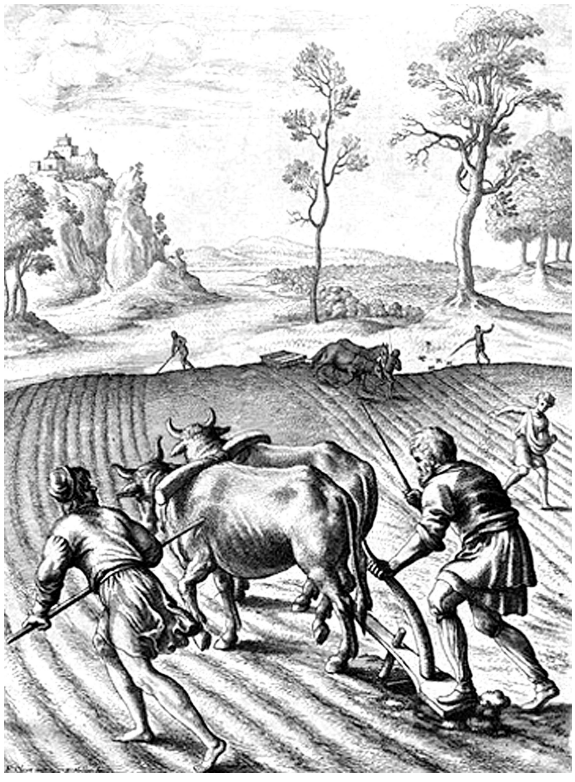

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Research Article
Lasiodiplodia theobromae Fungus Causing Stem Canker Disease on Rubber Tree (*Hevea brasiliensis*) in Indonesia

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Abstract

Background and Objectives: Stem canker diseases of rubber tree (*Hevea brasiliensis*) is a new disease and as so far not reported yet in Indonesia. Now-a-days the occurrence of this disease is increasing by time. The objectives of this study were: (1) To determine the causative agent of stem canker and (2) To characterize the morphology and molecular properties of the pathogen. **Materials and Methods:** The study was conducted at Sembawa Research Laboratory, Rubber Research Center, South Sumatera and mycology Laboratory, IPB Plant Protection Department, from January, 2014-January, 2016. This research was conducted in three stages, namely, 1: Morphological identification based on the form of colonies and conidia as a reference used Barnet and Hunter identification keys and confirmed with previous studies, 2: Molecular identification using ITS 4 and ITS5 primers and sequence analysis using the BLAST program at www.ncbi.nlm.nih .gov website and 3: Pathogenicity test using Koch's postulate. **Results:** The result showed that morphology observation and molecular identification indicated that pathogen causing stem canker disease on rubber was *Lasiodiplodia theobromae*. The result of pathogenicity test showed that there was similarity of symptoms that arise between artificial inoculation and natural symptoms in the field. Another similarity that was also seen was the appearance of the fruiting body on the infected part. **Conclusion:** *Lasiodiplodia theobromae* was a pathogen causing stem canker disease on rubber tree, proven by morphology, molecular identification and Koch's postulates.

Key words: Molecular, morphology, postulate Koch's, rubber tree, stem canker

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Various diseases have been caused low rubber production in Indonesia. Diseases attack on rubber trees can inhibit the growth, damaging the internal organs, decrease rubber yield, or even cause plant death. Among of rubber diseases, stem canker increasingly tends to be a serious problem. It was first seen as small lesions such as scabies, which then merges into larger lesions on the rubber bark surface. As the disease develops, it forms brown necrosis in the cambium and spreads continuously to plant canopy. Severe infections can cause latex bleeding, bark crack and decay (gummosis). This disease, allegedly caused by *Fusarium* sp., is found in almost of rubber plantation in south Sumatra and south Kalimantan, which attack 12 clones with various intensity[1]. Pha *et al*.[2] showed epidemics with similar symptoms in Vietnamese traditional rubber planting since 1998. Such disease was thought to be caused by *Lasiodiplodia theobromae*.

Lasiodiplodia theobromae has been known as a fungus with a wide host range, estimated more than 280 plant species[3,4] and with varied pathological effects on its hosts[5]. In our preliminary observations, the fungus was constantly found inmost of infected plant samples and showing the symptoms as reported by Budiman and Suryaningtyas[1]. This fungus is well known as an opportunistic pathogen, saprophytes on decaying plant materials and potentially being endophyte[6,7]. Many of this fungus have been reported as the causal agent of canker and dieback diseases on various hosts.

In Indonesia, there has been no information about this fungus as the disease agent in rubber and whether *L. theobromae* from various other hosts can infect rubber plants. The accurate identification of plant disease causal agent and better understanding of its bioecology are essential to develop proper management of the disease. At the same time, research with different themes has also been carried out to find out the host range of these pathogens (*L. theobromae*) and the host's cross-pathogenicity[8]. Whereas, in this study will be discussed about the symptoms of stem canker on each stage of rubber plants, in the nursery, immature and mature plant and Koch postulates to prove that this disease is really caused by *L. theobromae* not by *Fusarium* sp. This study was performed to identify and characterize, by means of morphological properties, molecular and pathogenicity studies, the causal agent of rubber stem canker in Indonesia.

MATERIALS AND METHODS

Field surveys, disease symptom and fungal isolation: Field surveys were conducted at Experimental Farm of Sembawa Research Center and Rubber Estate of PT Perkebunan Nusantara VII, Padang Pelawi Business Unit, Bengkulu, Musilandas Business Unit, Palembang and Buyut Tulung Business Unit, Lampung and this research was conducted start January, 2014-January, 2016. Various types of symptoms related to the disease such as branches/twigs death, stem bleeding and decay were observed and collected for isolation. Infected plants with various symptoms were collected from rubber clones of BPM 24, RRIM 921 and IRR 112 at Experimental Farm of Sembawa Research Center. The samples were also collected from other rubber plantations, namely PT. Perkebunan Nusantara VII, Padang Pelawi Business Unit Bengkulu (PB 260 and IRR 112 rubber clones) and Tulung Buyut Business Unit Lampung (PB 260 clone). Since the preliminary observation. Fungi was suspected as the causal agent, then proper medium for this organism will be used in the isolation steps. The isolation method is a modification of the method of plant pathogen isolation Mehrotra and Aggarwal[9]. Plant parts with specific symptomatic diseases were cleaned with running water to remove any debris or attached soil, then cut on the margin between necrotic and apparently healthy tissue into 1-3 cm. The tissue pieces were then surface disinfected by soaking into 3% clorox solution for 3 min and then rinsed with sterilized water for three times. After air drying in laminar air flow, the tissue was cut again into sections with a size of 0.5 cm, then planted in potato dextrose agar (PDA) medium and incubated at room temperature for 7 days until fungal colonies were observed. Fungal colonies, suspected as *Lasiodiplodia* were separated based on colony morphology after 7 days. Fungal mycelium from these isolates transferred to fresh PDA plate and incubated at room temperature and hyphal tips from 2 days old colonies were aseptically placed on PDA Petri plates using dissecting microscope to obtain pure cultures. The same isolates determined as pathogen from five different hosts were also isolated using the same method.

Pathogens identification on morphological characters basis: Three isolates collected from infected rubber represent various symptom types and 5 isolates from other hosts were used for morphological characterization. All isolates first were identified based on colony and conidial morphology. Identification Method Based on Morphological Characteristics using the Tuite method[10]. Identification was done using the identification key according to Barnett and Hunter[11] for identification to genus and Dugan[12] for identification to the species. Confirmation of fungi was conducted by comparing to the previous study[13-15]. Isolates were examined daily for pycnidia and conidia formation. Conidial morphology, e.g., cell wall, shape, color and septal presence or absence was recorded using compound microscope.

Molecular identification: Identification of fungal isolates was conducted by molecular analysis based on Internal Transcribed Spacer (ITS) ribosomal DNA of fungi. The PCR amplification was using ITS 4 primary: 5'-TCC TCC GCT TAT TGA TAT GC -3 'and ITS 5 primary: 5'-GGA AGT AGT AAC AAA AAG G-3'[16,17] with DNA fragment 400-600 bp. The DNA extraction procedure was refer to Doyle[18] based CTAB. Reaction DNA amplification was performed using the PCR machine (MJ Research type PCT-100) with PCR conditions as follows: one cycle of 3 min at 94°C followed by 45 cycles of 1 min at 94°C (denaturation), 1 min at 37°C (annealing), 2 min at 72°C (extension). The entire DNA amplification product is equipped with an extension for 1 min at 72°C. After amplification, the gel will be reanalysis for electrophoresis. The RDNA sequence analysis was performed in one reaction and the primers used are forward primer which is also used in the amplification process. Sequence of gene fragment sequences were then uploaded to the Gene Bank through the BLAST program at www.ncbi.nlm.nih.gov website. Other analysis is determine the similarity of fungal origin rDNA gene fragment of rubber with other fungi through phylogenetic. Phylogeny tree was made based on data that processed using Mega program.

Pathogenicity test: Three isolates from three rubber plant that show diseased symptom were used for pathogenicity test by accomplishing Koch's postulates. Pathogenicity test using the Situmorang methods[19]. Inoculation for pathogenicity test was performed in two ways, namely with and without wounding. Seedlings of rubber clone PB 260 were used for the pathogenicity test. Inoculation was carried out on the rubber seedling branches with green brownish color. Inoculation without wounding was performed by sticking pieces of culture inoculums, diameter 0.5 cm on the bark surface of the rubber seedlings. Meanwhile, inoculation with wounding was performed by inserting the same inoculum under the seedling branch's barks. As a control treatment, on the part of plants either with or without wounding were only inoculated with a 0.5 cm in diameter PDA medium plug. Inserted agar plug with and without inoculum was wrapped with grafting plastic to avoid drying during infection process. All the inoculated seedlings were then covered with plastic bag to keep the relative humidity suitable for infection process. Daily observation was conducted after 2 days from inoculation until the symptom appeared. To ensure that the same fungus associated with symptomatic inoculated seedlings, reisolation was performed with the same method as in isolation step. In addition, microscopically observation was also conducted to determine the fungus growth.

RESULTS

Observation disease symptoms in the field: Field observations both in the nursery and mature tree area found several variations of symptoms of stem canker disease. A large number of scabs that appears on the plant begun with the formation of small brown scabs with sized about 0.3 mm to 1 cm on the stems and branches of plants. The scab emerged on the surface of the plants caused coarse texture. On the heavy attacks branches, the leaves turned yellow and dry up with the time. If the attacks continued, stem became die (dieback). In addition to the symptoms of branch death, also found the appearance of brown spots that eventually resulted in the stem drying.

Field observations on the immature (1 year old) plants (TBM) showed the death of the green buds on the stems of plants and when the stems were split then it could be seen brown part in the xylem (Fig. 1). Based on observations in the field with the appearance of symptoms, the possibility of this disease was caused by *Fusarium* sp. associated with other pathogens, but based on microscopic observation of fruiting bodies and spores of the pathogen, the possibility of disease-causing pathogens of stem rot rubber disease was not *Fusarium* sp. and it was possibly caused by other pathogens.

Symptoms of the immature plant at age 2-4 years old were scabs and coarse skin. Scabs were constantly developing and spreading causing decayed of xylem tissue. Furthermore, if the condition was developed up to this stage, the plant would be dry and leaves were falling and the death symptoms of branches occurred (Fig. 2).

Besides attacking immature trees, a pathogen of stem rot also attacked the mature plants where the tapping panel was attacked. The results of field observations revealed that the tapping panel of mature plants are attacked by pathogens stem rot and caused damage, bursting and decaying on tapping panel, so the renewed bark could not be tapped again.

Morphological and molecular identification: After observation in the field, then diseased plant samples were observed under a stereo microscope. Symptoms observed were scabies from these seedlings. From the observation, it was seen a set of pathogens fruiting bodies were round protruding covered mycelia as cotton. If the fruit body was broken, it could be seen pathogen spores with oval-shaped, one septa and brown (Fig. 3).

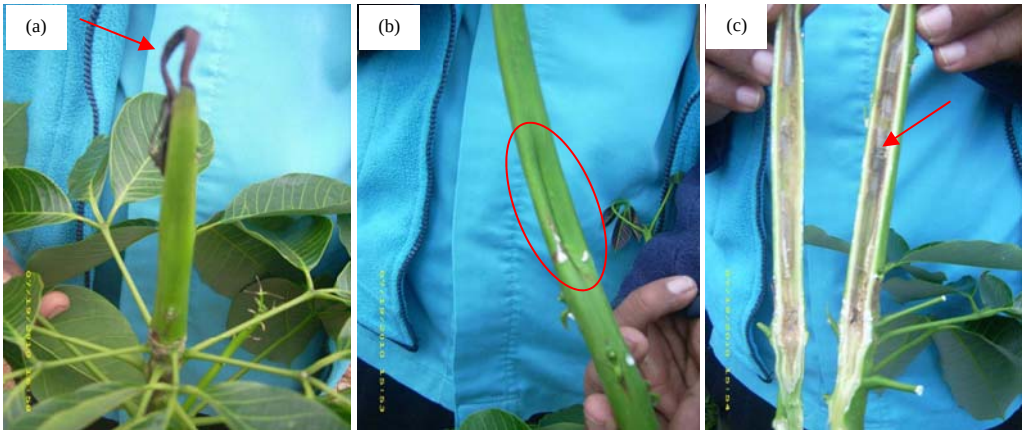

Fig. 1(a-c): Symptoms of stem decay rubber disease on 1 year old immature plant (a) Shoot dieback, (b) Curly up symptom and (c) Brown part in the xylem

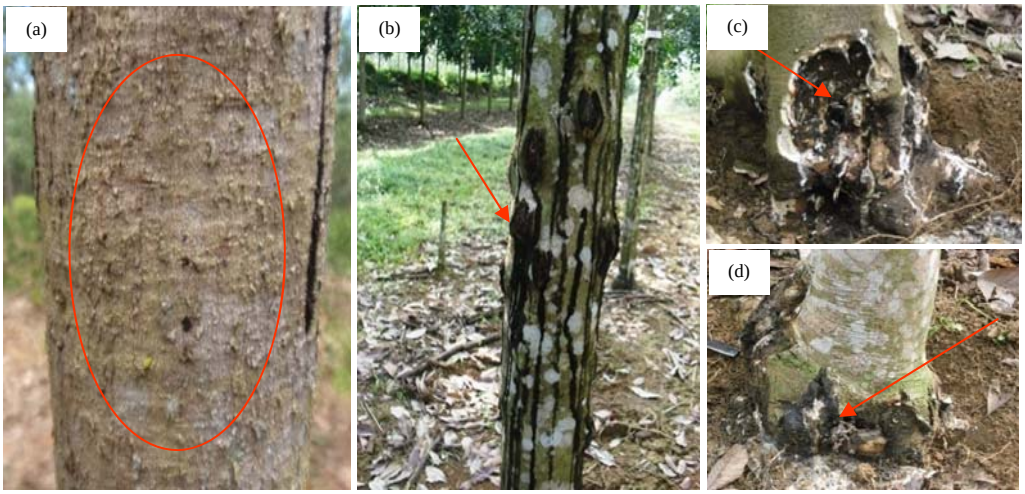

Fig. 2(a-d): Symptoms of decaying stems on immature aged 2-4 years (a) Spreading many scab, (b) Lesions were burst release black latex (bleeding) and (c, d) Weathered and bleeding attacks the roots

Morphological observation indicated that pathogens causing stem canker disease on rubber were *Lasiodiplodia theobromae*. The colony has a blackish-gray color with a growth rate of 1,3 cm/day. Immature conidia was unicellular and a septate formed after 25 days of culture with the size of 16-20×6-9 µm. Mature conidia formed after 35 days of culture with size of 16-20×6-9 µm, oval-shaped and had a septate and slightly brown in color. Pycnidia formed after 20 days of culture. Initially, conidia was unicellular, hyaline, granulose, sub-ovoid until ellipsoid, thick-walled and septae.

Identification of *L. theobromae* isolates was conducted molecularly based on Internal Transcribed Spacer (ITS) ribosomal DNA of the fungi. DNA band with 500 bp was successfully amplified from *L. theobromae* isolates from rubber using ITS 4 and ITS 5 primers (Fig. 4). Based on the results of BLAST, it was obtained that isolates show 98% homology with *Lasiodiplodia theobromae* strain CMM4499. This indicated that the fungus causing stem canker disease on rubber is *B. theobromae* or *Lasiodiplodia theobromae*.

Pathogenicity test: Isolates originating from three types of symptoms were necrosis (isolate A), bleeding (isolate B) and decay (isolate C). All these isolates cause the same symptoms, with different incubation periods. Symptoms begin with the appearance of spots on the skin after 10-13 days incubation period and developed into a scab. The symptoms continue to develop and spread causing rotten of the xylem tissue, plant drying, leaves fall and dead twigs symptoms appear. Symptoms of death twigs will appear at 25 days after inoculation (Table 1, 2).

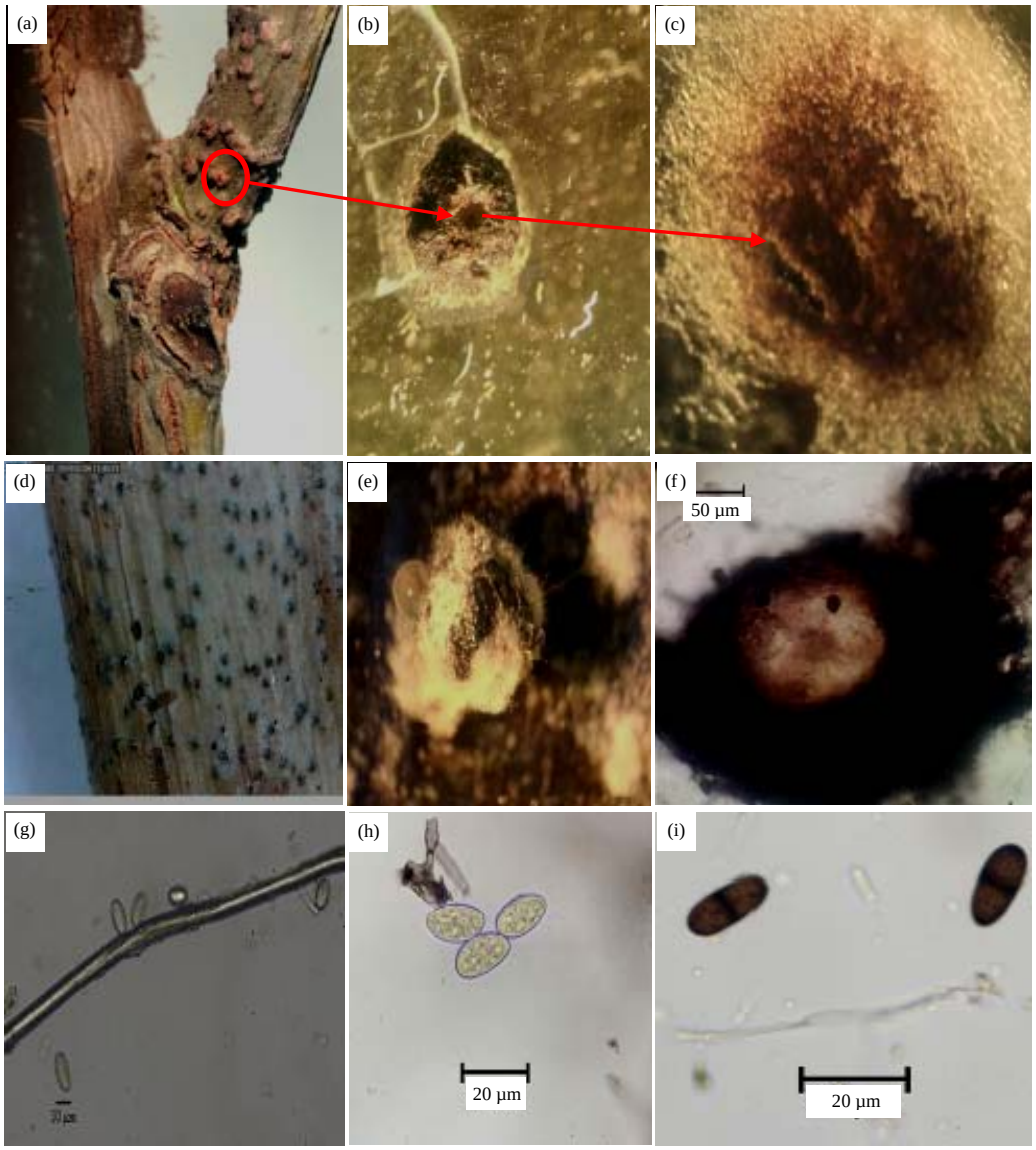

Fig. 3(a-i): Pycnidia dan conidia *Lasiodiplodia theobromae* (a) Pycnidia in symptomatic plants of stem canker, (b) Pycnidia with 4×10 magnification, (c, d) Pycnidia with 10×10 magnification, (e) Pycnidia has broken and release conidia, (f) Conidia that comes out of rom pycnidia, E, (g, h) Immature conidia and (i) Mature conidia

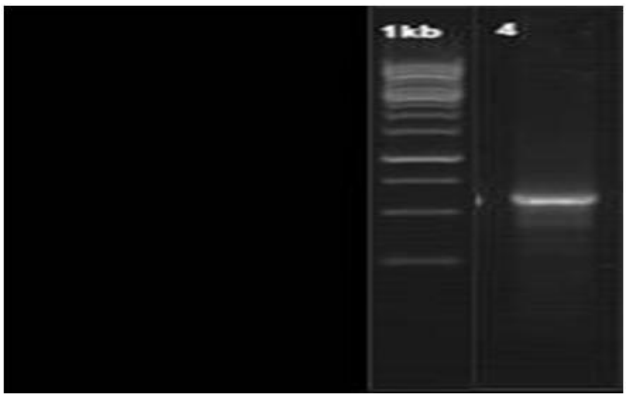

Fig. 4: Amplification of DNA bands *L. theobromae* of rubber using ITS4 and ITS 5 primary on agarose gel

Table 1: Incubation period of each isolates inoculated on rubber seedlings

	Stages of Koch's postulates			Incubation period (day)	
Isolate	Isolation	Inoculation	Reisolation	With wound	Without wound
A	+	+	+	10	13
B	+	+	+	10	12
C	+	+	+	11	12

Table 2: Time needed for each symptom appears

Kind of symptoms	Day after inoculation
Spot	10-13
Scab	11-20
Skin and leave drying	17-25
Decay ed xylem tissue	22-28
Branch dry and die	25-35

DISCUSSION

The canker pathogen was tested more quickly enter the injured plant tissue without having to apply mechanical stress and other entry processes and directly penetrate and infected. The initial symptoms that appeared in each isolate were brown spots and necrosis. Symptoms continue to develop into a scabs and continue to develop and expand causing fouling of the pith tissue and sometimes out of the gum, leaves withering and symptoms of dead branches.

Symptom development as obtained in this study was also widely reported by several other researchers on various types of plants and generally triggered by the presence of mechanical injury[20-24]. Attack of canker pathogens on durian results in canker symptoms and results in scars such as reddish brown color (gummosis). If the bark is peeled it looks blackish brown lines along the cortical tissue. Symptoms in the root of black rot, especially in young root. As a result of attacks on the roots and stems, resulting in yellowing leaves then fall. In severe attacks can result in death and canopy decline[25].

Observation of morphology (shape of conidia, konidiogen cells and growth of structures mycelial) indicated that disease-causing pathogen stem canker disease was *Lasiodiplodia theobromae*. Identification was done by using the identification key, according to Dugan[12]. *Lasiodiplodia theobromae* (Pat.) is the synonym of *Botryodiplodia theobromae* (Pat.) Griff. and Maubl which has an asexually stage from the genus *Botryosphaeria rhodina* (Berk. and MA Curtis) ARX[20]. *Lasiodiplodia theobromae* is a form of *Botryosphaeria rhodina* Anamorphic (Berkeley and Curtis) von Arx and a fungus that has a class Deuteromycetes[26].

Fungus *L. theobromae* has a wide host range, other than to attack citrus plants, cacao, rubber, mangosteen and bananas, the fungus can also attack crops of mango, pineapple, avocado, melon, coconut, eggplant, peppers, peanuts, corn, sugarcane and tobacco[27] Punithalingam[28] stated that morphological, *L. theobromae* fungus was characterized by mycelial growth like smooth hair or cotton yarn and aerial mycelium was abundant. Early colony had sepia color and turned into grey and then black. Pycnidia was clustered, often aggregate, stromatic, ostiolate and it had width up to 5 mm.

The color and shape of *L. theobromae* pycnidia. Pycnidia was a fruiting body shaped like a pumpkin in which there conidiophores and producing conidia. In general pycnidia to dark brown and covered with mycelia. Pycnidia will burst and release young conidia. According Masilamani and Muthumar[29], on natural conditions mature pycnidia will produce conidia mature that come out through the hole ostiole on pycnidia and then spread. Furthermore, mature conidia will be formed after the age of colony 32 days. Pycnidia formed after the age of colony 20 days and usually clustered like cotton on PDA media, which when be pressed release some water. According to Punithalingam[28], pycnidia simple, clustered, sometime aggregate, stromatik, ostiole, width up to 5 mm.

Young conidia formed after the age of colony 25 days with the size of 16-20 µm×9-12 Lm. Young conidia unicellular and aseptate, mature conidia sized 16-22×9-13 µm oval-shaped, dark in color and has a septa and slightly brown color. According to Watanabe[30], conidia *L. theobromae* scattered singly, hyaline, elliptic or cylindrical and generally mature conidia consists of two cells (insulated one).

This research indicated that *L. theobromae* was the only pathogen that associated with stem canker disease on rubber tree confirmed by similarity index with BLAST-N program that found the fungus 98% identical with *L. theobromae*. In Indonesia, there has been no information yet about this fungus as the disease agent in rubber tree and so this is the first report that found *L. theobromae* as the causal agent of Hevea stem canker disease.

CONCLUSION

Morphological observation indicated that pathogens causing stem canker disease on rubber were *Lasiodiplodia theobromae*. Molecular identification of *L. theobromae* isolates was successfully amplified *L. theobromae* isolates from rubber trees and based on the BLAST-N results showed that the fungus 98% homolog with *Lasiodiplodia theobromae* strain CMM4499. Pathogenicity studies by the Koch postulate test in Hevea seedlings showed the same symptoms with infected plant in the field. This test indicated that *L. theobromae* was the causal agent of rubber tree stem canker disease.

SIGNIFICANCE STATEMENT

This study discovered the pathogenic fungus that cause stem canker disease on rubber tree, namely *L. theobromae*, where a many researchers formerly thought this disease caused by *Fusarium* species. So this study will give a better understanding of *L. theobromae* fungus bioecology that are essential to develop proper management of stem canker disease on rubber tree.

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