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Identification of cassava black stem and root rot agents in Thailand

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ABSTRACT

Cassava (*Manihot esculenta* Crantz) black stem and root rot (BSRR) caused by complex fungi is one of the most serious fungal disease of cassava in Thailand. The objective of this study was to identify the causal agent of BSRR disease of cassava in Nakhon Ratchasima province, Thailand. From June 2015 to May 2018, the outbreak areas in three districts of Nakhon Ratchasima province were surveyed for cassava plants with the characteristic symptoms. From 1800 diseased samples, 139 fungal pathogens were isolated and separated by morphological traits. Among them, 33 fungal isolates were subsequently tested for pathogenicity on detached stems and roots of CMR 43-08-89, a susceptible cassava cultivar, and proved to be pathogenic with different levels of aggressiveness. Next, PCR amplification of DNA of eight pathogenic isolates with high aggressiveness using two sets of universal primers ITS1/ITS4 and Ef1-688F/Ef1-1251R, revealed that the TEF1-α gene region could be used for the identification and classification at the species level. The analysis results fit well with that of the morphological studies on growth and colonial characteristics of the eight isolates. Based on the GenBank database, they were identified as *Lasiodiplodia theobromae*, *L. euphorbiaceicola* and *Neoscytalidium dimidiatum*.

Key words: Cassava disease, *Lasiodiplodia* spp., *Manihot esculenta*, *Neoscytalidium dimidiatum*.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most important crops of Thailand. It is categorized as an agro-based industrial crop with well-growing industries and markets in and outside the country. Thailand has been the world-leading exporter of cassava products with a production volume of 31 million tons in 2018-2019 (Arunmas, 2019). Unlike in Africa or South America, in which cassava product is mainly consumed as a staple food; in Thailand, it has been mainly used as raw materials in industries for the production of tapioca starch, modified starch, ethanol, and animal feed. As such, it has been treated as a cash crop benefiting millions of subsistence Thai farmers. Cassava has changed its roles to a profitable crop on account of the classification of many process industries in which cassava can be used as industrial basic materials. To encourage food and energy industries, an escalation of cassava production by using good agricultural practices has been promoted in Thailand (Sangpueak et al., 2018). Since then, its cultivation has drastically changed in terms of planting acreage and practices to meet the continuously increasing demand. As a result, the outbreak and severity of pests and diseases have increased since 2010 (Sangpueak et al., 2018).

Cassava plants could be attacked by more than 34 kinds of pathogens, causing various degrees of losses (Sangpueak et al., 2018; Maruthi et al., 2019). In South America, Africa, and India, black stem and root rot (BSRR) has been considered one of the important diseases on the account that it can cause as much as

80%-100% loss to the cassava crops. The fungi *Lasiodiplodia theobromae* and *Neoscytalidium dimidiatum* have been identified to be the causal agents of the disease (Nyaka et al., 2015; Boas et al., 2017; Bodah, 2017). In addition, there have been reports in Brazil and neighboring countries that it was caused by the *L. euphorbiaceicola*, *L. pseudotheobromae*, *L. hormozganensis*, *L. parva*, *L. brasiliensis*, *L. caatinguensis*, *L. iranensis*, *L. laeliocattleyae*, *N. hyalina* and *Macrophomina pseudophaseolina* (Machado et al., 2014a; Brito et al., 2019; 2020). The disease is widespread during the hot and wet seasons. Usually, the diseased plant bark becomes darkened and has a split that extends upward on the stem. Moreover, Botryosphaeriaceae species are complex with various economic crops hosts, including citrus, coconut, cactus prickly pear, coconut palms, tea (Phillips et al., 2013; Rosado et al., 2016; Feijo et al., 2019; Santos et al., 2020; Rathnayaka et al., 2021).

Although *L. theobromae* and *N. dimidiatum* are the two fungi commonly reported as the causal agents, their identification has been mainly based on their morphology, which could be inadequate to characterize fungus at a species level. Presently, studying its morphological and molecular characteristics together has been generally accepted as a standard method for the identification of species and taxonomy of pathogens (Machado et al., 2014a; 2014b). The internal transcribed spacer (ITS) region has been used as a standard barcode for detecting many fungal pathogens (Schoch et al., 2012). However, the imprecision of some species could not be distinguished when only the ITS region was used (Ismail et al., 2012; Marques et al., 2013). Therefore, some additional sequences of the translation elongation factor EF-1 alpha (TEF1-α) gene have to be used along with the ITS for confirmation of the Botryosphaeriaceae species (Marques et al., 2013; Rosado et al., 2016; Brito et al., 2020; Santos et al., 2020). Even though BSRR disease was found in Thailand many years ago, its causal agent has not been precisely elucidated. In this study, identification of the causal fungi of BSRR was performed using a combination of morphological and molecular studies.

MATERIALS AND METHODS

Diseased sample collection and isolation of the pathogens

Field surveys were carried out from June 2015 to May 2018 in cassava (*Manihot esculenta* Crantz) plantations of Nakhon Ratchasima province, Thailand, to collect 1800 black stem and root rot (BSRR) diseased samples with collar rot, stem rot, leaf fall, wilt, and root rot symptoms. Typical symptoms of BSRR disease were vascular discoloration, offensive odor, blackening, decaying of the stem, and rotting of roots. Small-sized black pycnidia and dieback symptoms were also observed on the diseased cassava stems and stalks.

To isolate the causal pathogen, the diseased samples of cassava were cleaned by washing with running tap water. Subsequently, the diseased portions were cut into small pieces, about 2 mm in size and surface-sterilized with 1% sodium hypochlorite (NaOCl) for 2-3 min. They were further washed with sterile distilled water at least three times before being dried between sterilized filter papers. The cassava samples were then placed on water agar (WA) medium and incubated at room temperature for 48 h. After that, the growing hyphal tips were transferred onto potato dextrose agar (PDA).

To induce spore formation, fresh cultures were transferred onto sterilized toothpicks soaked in potato dextrose broth (PDB) in Petri dishes and incubated at 25 °C with a 12:12 h light/dark cycle for 3 wk. Subsequently, single spore-isolates were taken from each of the pycnidium formed on the toothpicks. The pure isolates were cultured on PDA and stored at 4 °C for further experiments (modified Phillips et al., 2013; Machado et al., 2014a; 2014b).

Morphological characterization

Each pure fungal isolate was transferred onto PDA, and a double-sterilized toothpick was placed adjacent to it, after that the agar plate was incubated at 25 °C with a regime of 12:12 h photoperiod for up to 3 wk to stimulate the formation of pycnidia. All relevant morphological characters of the isolates were studied using a light microscope (slightly modified from Machado et al., 2014a; 2014b).

Pathogenicity tests

Each fungal isolate was grown on PDA (7 d, room temperature) and used for inoculation of pathogenicity test.

Stem inoculation. The stem inoculation method was adapted from Trakunyingcharoen et al. (2013) and Chen et al. (2016). Stalks of healthy cassava plants, susceptible variety (CMR 43-08-89), were cut into 10 cm portions and surface-sterilized with 10% NaOCl for 1 min. Next, the portions of cassava stalk were washed out with sterilized water three times and blotted dried with sterile paper towel. To inoculate the fungus, a 6-mm-

diameter hole was cut into the bark using a sterile cork borer. Then, a piece of PDA slice with 7-d-old fungal culture was inserted into the hole and sealed with a strip of parafilm. The inoculated cassava portions were finally kept in a moist chamber for observation. After 3 wk, the parafilm was removed from the bark and the fungus was re-isolated and rechecked (Trakunyingcharoen et al., 2013; 2015).

Root inoculation. The inoculation was done similarly to that of the stem inoculation but the storage root portion was used instead. The inoculated roots were kept in moist chambers for 1 wk for observation (Ismail et al., 2012; Li et al., 2015). The pathogens from infection lesions were re-isolated in culture media and rechecked.

Disease severity scores of each isolate were evaluated during the observation using the scale described by Trakunyingcharoen et al. (2013) and Chen et al. (2016).

Fungal DNA extraction, sequencing, and phylogenetic studies

One gram of pathogenic mycelia was frozen and ground in liquid nitrogen, then transferred into a 1.5 mL microcentrifuge tube, then 500 µL extraction buffer (0.2 M Tris-HCl, 0.25 M NaCl, 25 mM EDTA, and 2% sodium dodecyl sulfate, pH 8.5) was added. The tube was incubated at 65 °C for 30 min and then centrifuged at 14000 rpm, for 10 min. The supernatant was transferred into a new tube, and 200 µL chloroform:isoamyl alcohol (24:1 v/v) was added. The mixture was vortexed for 30 s and then centrifuged at 14000 rpm for 10 min. Subsequently, the supernatant was transferred to a new tube, and 2 volumes of ice-cold absolute ethanol were added. The mixture was carefully mixed by vortexing for 30 s, centrifuged at 10000 rpm for 5 min, then, the supernatant was discarded, and the DNA pellet was washed with 70% ethanol (400 µL). The washed pellet was dried under a vacuum condition for 30 min and resuspended in 50 µL TE buffer (0.1 mM EDTA and 10 mM Tris-HCl, pH 8.5) by gentle agitation to dissolve the DNA and stored frozen (-20 °C) until further use (Sangpueak et al., 2018).

The PCR amplification of the fungal DNA was done using primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3')/ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Fernández-Herrera et al., 2017), and Ef1-688F (5'-CGG TCA CTT GAT CTA CAA GTG C-3')/Ef1-1251R (5'-CCT CGA ACT CAC CAG TAC CG-3') (Wang and Song, 2021). The 25 µL reaction mix was prepared as follow: Taq DNA polymerase (0.2 µL, Invitrogen by Gibthai Company, Thailand), 5 µL 10X buffer, 0.5 µL 50 mM $MgCl_2$, 0.75 µL 10 mM dNTP, 0.5 µL 10 µM primers, 1 µL genomic DNA and adjusted to the final volume with sterile deionized water. The PCR amplification was carried out using a thermal cycler (PCR MJ Mini, BIO-RAD, Hercules, California, USA). Following an initial denaturation at 95 °C for 1 min, the DNA templates were amplified for 35 cycles. Each cycle consisted of a denaturation step at 95 °C for 1 min, an annealing step at 55 °C for 1 min (for TEF1-α) or 52 °C for 1 min (for ITS), and an extension step at 72 °C for 2 min. A final extension step (72 °C for 10 min) was included at the end of the cycle. Amplification products were separated on 1% (w/v) agarose gels in Tris-acetate-EDTA (TAE) buffer. Before electrophoresis, gels were stained with 6X DNA loading buffer (Novel Juice, GeneDireX, Taiwan, China), and the DNA bands were visualized with UV light (UVP GelDoc-It2 Imager, Analytik Jena, Jena, Canada). The instrument was also used for data and image capturing (Sompong et al., 2012; Sangpueak et al., 2018).

Purified PCR products were twice sequenced by the Pacific Scientific (Jakarta Barat, Indonesia), and analyzed using the Chromas Lite software (Technelysium Pty Ltd., South Brisbane, Queensland, Australia) to determine the consensus sequence. Nucleotide sequence alignment was performed with the BLAST-N program and algorithm MEGABLAST, based on NCBI gene bank data (National Center for Biotechnology Information, Bethesda, Maryland, USA; http://www.ncbi.nlm.nih.gov) (Phillips et al., 2013).

Table 1. Black stem and root rot disease incidence in cassava plants surveyed in Nakhon Ratchasima province, Thailand during June 2015 to May 2018.

Location	Surveyed cassava fields	Inspected cassava samples	Disease incidence
	Nr	Nr	%
Khon Buri	6	600	34
Soeng Sang	9	900	46
Nong Bun Mak	3	300	28
Total	18	1800	-

The nucleotide sequences of *Lasiodiplodia* and *Neoscytalidium* obtained from the Genbank database were analyzed using the Clustal W program to arrange relationships (multiple sequence aliment) for neighbor-joining (NJ) Unweighted pairs group methods with arithmetic mean (UPGMA) and Maximum likelihood (ML) by MEGA 6 (Tamura et al., 2013; De Silva et al., 2019). Bootstrap values were obtained from 1000 NJ bootstrap replicates. Maximum-parsimony analysis was performed using the heuristic search option with 1000 random taxon additions and tree bisection and reconnection (TBR) as the branch-swapping algorithm to prove the best and most accurate. Then analyzed together with the morphology and biomolecular characterization and phylogenetic analysis of pathogens to confirm that cause root rot of cassava.

RESULTS

Sample collection and fungal isolation

A total of 18 cassava fields in three districts of Nakhon Ratchasima province had been surveyed, including Soeng Sang, Khon Buri, and Nong Bun Mak. From 1800 cassava samples inspected, disease incidences among districts were varied, with Soeng Sang being the highest at 46%, while those of Khon Buri and Nong Bun Mak were at 34% and 28%, respectively (Table 1). The BSRR symptoms could be found on all three parts of the cassava plants; but most frequently on the stems and roots, starting from 1-mo old plants until harvest. On the stems, the wounds were initially light brown and turned to black necrotic lesions with numerous small-sized black pycnidia. The infected stems normally withered or die-back, resulting in plant death. Similar wounds were also observed on the stalks. The withering or dying plants, when uprooted, showed rotten roots with an offensive odor and blackening of the woody part. Both affected roots and stems displayed internal discoloration and dark-browning of vascular regions when cut open (Figure 1).

Morphological studies

In total, 139 isolates of fungal pathogens were obtained from the cassava samples collected in three districts of Nakhon Ratchasima. Among them, 33 isolates were selected from morphological identification and locations, 50 single-spore representatives were chosen for this study. From their growth habits and morphological characters, the isolates were identified as *Lasiodiplodia* and *Neoscytalidium* in the Botryosphaeriaceae family. Morphological characterizations of the isolates are as follows:

Figure 1. Symptoms of black stem and root in cassava found during the surveys: Wilted plants (A-C), root rot (D-G), black pycnidia protruding from the affected bark (H-I), and black stem rot (J).

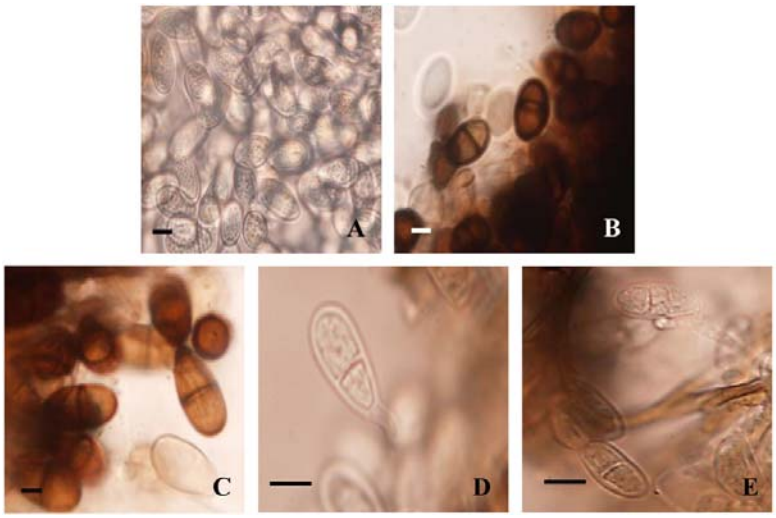

Figure 2. Colonies and conidia of *Lasiodiplodia* spp.: Colonies on toothpick culture (A, B), conidiogenous cells (C), paraphyses (D), non-septate immature conidia (E). Scale bar = 10 µm.

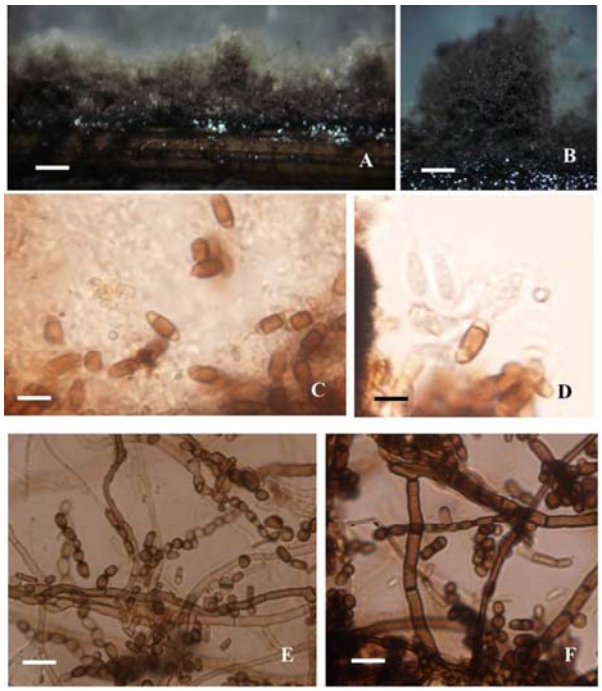

Figure 3. Colonies and conidia of Neoscytalidium sp.: Colonies on toothpick culture (A, B), three celled conidia (C, D), chained arthrospores (E, F). Scale bar = 5 µm.

Group 1, *Lasiodiplodia* spp.: Twenty-eight isolates were classified into this group. Their colonies were dark grayish, green-olive to greyish black on PDA medium. Immature conidia were clear, oval-shaped, and one-celled. Mature conidia were striated, dark brown, oval-shaped and one septum or aseptate (Figure 2). Based on conidia size, the 28 isolates were subdivided into three subgroups; large (L), medium (M) and small (S). The L group consisted of three isolates L1STRM, L5KBSH1, and L28SBB, having average conidial sizes at 19.50-32.80 × 12.40-19.70 µm. The M group, contained 13 isolates; L4STR1, L7STRW, L9STR2, L12SHRD,

L14LSR, L16BLSR, L18KBSH3, L19KBSH2, L21SRE, L23STWH, L24HRWH, L25SRC, and L26HRWB) having 19.00-30.10 × 10.10-19.32 µm conidial sizes. The S group contained 12 isolates, namely L2SHSR1, L3BSR, L6HRD, L8HDBB, L10BBLB, L11HSR2, L13SRTF, L15HDBB, L17STRB, L22HRW, L20FHTB1 and L27SRBT having 12.50-26.30 × 9.51-15.00 µm conidial sizes (Table 2).

Group 2, *Neoscytalidium* sp.: five isolates including N1SRTTC, N2SHCA, N3DTD2, N4BBPM and N5STRST were classified into this group. Their colonies were black, growing rapidly on PDA medium. The conidia were dark brown, smooth, thick-walled, 1-2 and occasionally 3 celled and 10.15-16.25 × 3.63-6.25 µm in sizes. Similar arthroconidia were also found formed in chains (Figure 3) and averaged 6.14-14.36 × 2.96-5.12 µm in size (Table 3). Thirty-three isolates representing 139 specimens of the fungal pathogen were chosen to do the pathogenicity test.

Pathogenicity test

By inoculating the 33 isolates to detached stems and roots of CMR 43-08-89, all of them could infect the plants but showed different degrees of aggressiveness (Table 4, Figures 4A-4C and Figures 5A-5C).

Table 2. Conidial size, paraphyses size and septation of *Lasiodiplodia* groups isolated from cassava plants with black stem and root rot disease in Nakhon Ratchasima, Thailand. [1]Average of 50 conidia for each isolate on water agar medium.

Species/Isolate	Conidial size[1]	Paraphyses size	Paraphyses septation
	µm	µm	
L group			
L1STRM	22.87-30.00 × 13.00-19.35	-	-
L5KBSH1	19.50-32.80 × 12.40-19.70	-	-
L28SBB	22.12-30.00 × 12.50-19.35	-	-
M group			
L4STR1	20.00-26.30 × 10.10-17.20	60 × 3-4	Aseptate
L7STRW	22.12-30.10 × 12.50-16.52	-	-
L9STR2	20.00-26.30 × 10.10-17.20	-	-
L12SHRD	20.00-24.00 × 12.00-14.00	74 × 3-4	Aseptate
L14LSR	20.10-24.50 × 10.10-14.00	-	-
L16BLSR	24.00-28.00 × 10.36-13.24	-	-
L18KBSH3	24.00-28.00 × 10.10-17.20	50 × 3-4	Aseptate
L19KBSH2	19.00-23.50 × 11.00-12.50	-	-
L21SRE	24.00-28.00 × 10.10-17.20	-	-
L23STWH	20.00-26.30 × 10.10-17.20	-	-
L24HRWH	20.00-26.30 × 10.10-13.24	-	-
L25SRC	24.00-28.00 × 10.30-12.60	-	-
L26HRWB	24.00-28.00 × 12.50-19.32	-	-
S group			
L6HRD	17.00-23.00 × 10.00-13.00	60 × 3-4	Aseptate
L8HDBB	17.54-23.35 × 10.36-14.45	56 × 3-4	Aseptate
L11HSR2	12.50-22.50 × 10.00-15.00	46 × 3-4	Aseptate
L13SRTF	16.00-23.00 × 9.50-12.50	59 × 3-4	Aseptate
L20FHTB1	17.50-24.10 × 10.10-12.50	56 × 3-4	Aseptate
L27SRBT	20.00-26.00 × 10.00-13.00	63 × 3-4	Aseptate
L2SHSR1	17.54-23.00 × 10.36-13.24	-	-
L3BSR	16.50-20.50 × 10.30-12.60	-	-
L10BBLB	16.50-22.10 × 10.30-14.50	-	-
L15HDBB	19.80-22.00 × 12.10-14.00	-	-
L17STRB	17.50-24.10 × 10.10-12.50	-	-
L22HRW	18.20-21.50 × 10.00-14.50	-	-

Table 3. Conidial and arthroconidia sizes of *Neoscytalidium* spp. isolated from cassava plants with black stem and root rot disease in Nakhon Ratchasima, Thailand. [1]Average of 50 conidia for each isolate on water agar medium.

Isolate	Conidial size[1]	Arthroconidia size
	µm	µm
N1SRTTC	11.25-16.25 × 5.00-6.25	8.65-14.36 × 2.96-5.12
N2SHCA	11.25-16.25 × 3.63-4.88	6.25-13.50 × 2.96-5.12
N3DTD2	10.00-15.00 × 4.00-5.00	6.00-14.00 × 3.00-5.00
N4BBPM	10.25-16.25 × 3.63-6.25	6.25-12.36 × 2.96-5.12
N5STRST	11.25-16.25 × 3.63-6.25	7.50-12.36 × 2.96-5.12

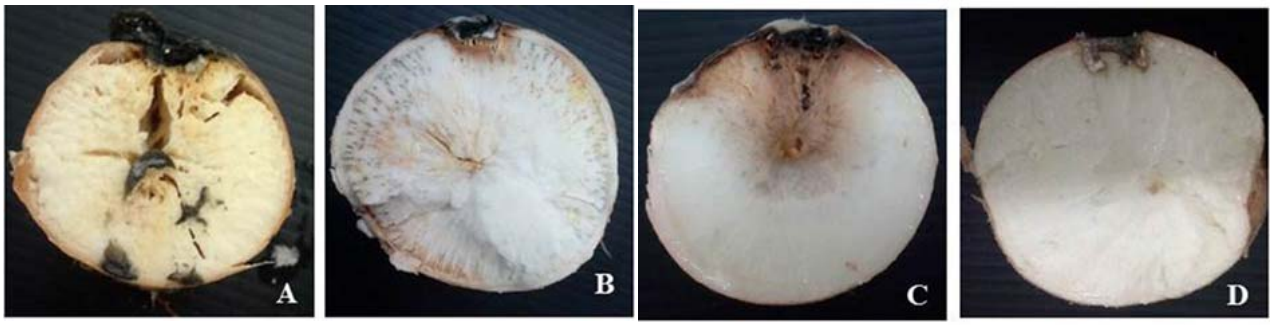

Figure 4. Symptoms developed on cassava roots at 7 d after being inoculated with L11HSR2 (A), L6HRD (B), L18KBSH3 (C), and uninoculated control (D).

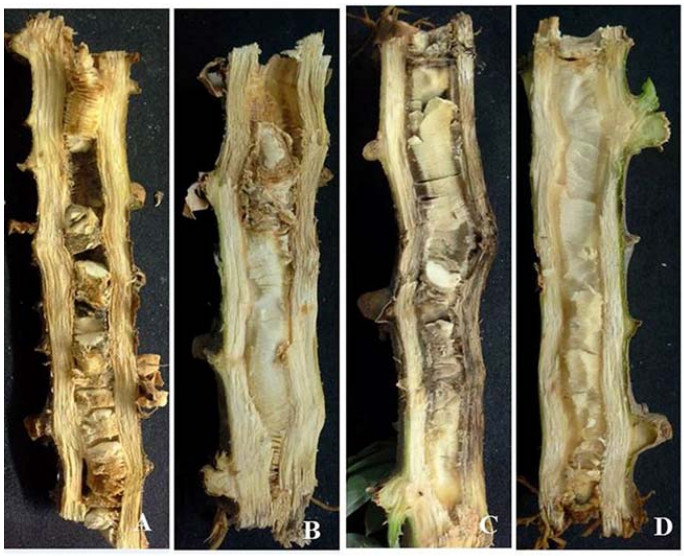

Figure 5. Symptoms developed on cassava stems at 15 d after being inoculated with L11HSR2 (A), L6HRD (B), L18KBSH3 (C), and uninoculated control (D).

Root inoculation: All inoculated roots showed symptoms of fungal infection occurring externally on the root bark after one week (Figures 4A-4C). Observed symptoms included necrotic bark lesions around the inoculation points and the root bark was enveloped by a brown to black or greyish mycelia. Such symptoms were not observed on the control of uninoculated roots (Figure 4D).

Stem inoculation: Forty-five days after the inoculation, all isolates could infect the stems exhibiting internal discoloration and developing large lesions. Some parts of the stems turned into brown to black tissues (Figures 5A-5C). No disease symptoms were observed in the control of uninoculated stems (Figure 5D). Based on the results of the pathogenicity test, eight representative isolates were chosen to do on the following studies.

Table 4. Pathogenicity and aggressiveness test of 33 isolates on cassava CMR 43-08-89. [1]Standard error ± S.E. different characters mean statistical difference at a probability level of 0.05 by comparing Duncan's new multiple range test (DMRT) mean. [2]Disease scores 1-5 as follows: 1 = non-symptomatic, 2 = symptom less than 25% of root/stem, 3 = symptom 25%-50% of root/stem, 4 = symptom 50%-75% of root/stem, 5 = symptom more than 75% of root/stem. [3]**Significantly different at the 0.01 level; ns: nonsignificant.

	Root inoculation		Stem inoculation	
Pathogens isolates	Wound size[1]	Disease scores[2]	Wound size[1]	Disease scores[2]
	mm		mm	
L1STRM	27.50 ± 0.90d-g	3	24.00 ± 2.27c-g	2
L2SHSR1	28.00 ± 1.06c-g	3	29.00 ± 6.75b-e	2
L3BSR	29.25 ± 3.13c-g	3	37.60 ± 1.92ab	3
L4STR1	23.75 ± 2.72e-h	3	34.50 ± 3.75ab	3
L5KBSH1	21.13 ± 2.50f-h	3	11.00 ± 2.35h-j	2
L6HRD	34.25 ± 2.81b-e	4	15.63 ± 1.57g-j	2
L7STRW	23.88 ± 0.67e-h	3	14.38 ± 2.19g-j	2
L8HDBB	30.38 ± 3.01b-f	3	20.00 ± 5.40e-i	2
L9STR2	27.00 ± 2.62d-g	3	30.25 ± 4.27a-e	2
L10BBLB	26.25 ± 1.29d-h	3	22.75 ± 1.31d-g	2
L11HSR2	46.63 ± 2.09a	5	41.25 ± 1.25a	3
L12SHRD	40.08 ± 4.04a-c	4	37.50 ± 6.61ab	3
L13SRTF	35.25 ± 4.75a-e	4	27.75 ± 1.25b-e	2
L14LSR	17.00 ± 2.47g-i	1	33.50 ± 2.90a-d	3
L15HDBB	17.50 ± 1.25g-i	1	30.75 ± 8.10a-e	2
L16BLSR	26.25 ± 4.80d-h	3	8.75 ± 2.14j	2
L17STRB	35.00 ± 4.68a-e	4	16.75 ± 1.18f-j	2
L18KBSH3	26.25 ± 4.80d-h	3	9.50 ± 2.02ij	2
L19KBSH2	35.00 ± 4.68a-e	4	10.50 ± 1.66h-j	2
L20FHTB1	42.38 ± 1.32ab	4	15.00 ± 2.04g-j	2
L21SRE	31.60 ± 3.93b-f	3	33.75 ± 3.75a-d	3
L22HRW	38.13 ± 1.04a-d	4	20.50 ± 1.55e-i	2
L23STWH	13.88 ± 1.94hi	2	16.25 ± 0.52f-j	2
L24HRWH	28.50 ± 6.23c-g	3	0.63 ± 2.13e-h	2
L25SRC	31.50 ± 2.02b-f	3	8.75 ± 0.52j	2
L26HRWB	22.50 ± 6.73e-h	3	7.00 ± 0.46j	2
L27SRBT	40.25 ± 5.09a-c	4	33.75 ± 3.15a-d	3
L28SBB	33.50 ± 2.02b-f	4	10.63 ± 1.49h-j	2
N1SRTTC	26.50 ± 1.95d-g	3	36.00 ± 4.20ab	3
N2SHCA	26.30 ± 1.67d-h	3	26.50 ± 3.30b-f	2
N3DTD2	22.75 ± 0.41e-h	3	36.00 ± 4.85ab	3
N4BBPM	25.50 ± 1.89d-h	3	36.75 ± 2.69ab	3
N5STRST	24.15 ± 0.31e-h	3	30.00 ± 3.11b-e	2
Control	6.50 ± 0.56i	1	6.03 ± 0.02j	1
F-test[3]	**		**	
CV, %	25.87		28.33	

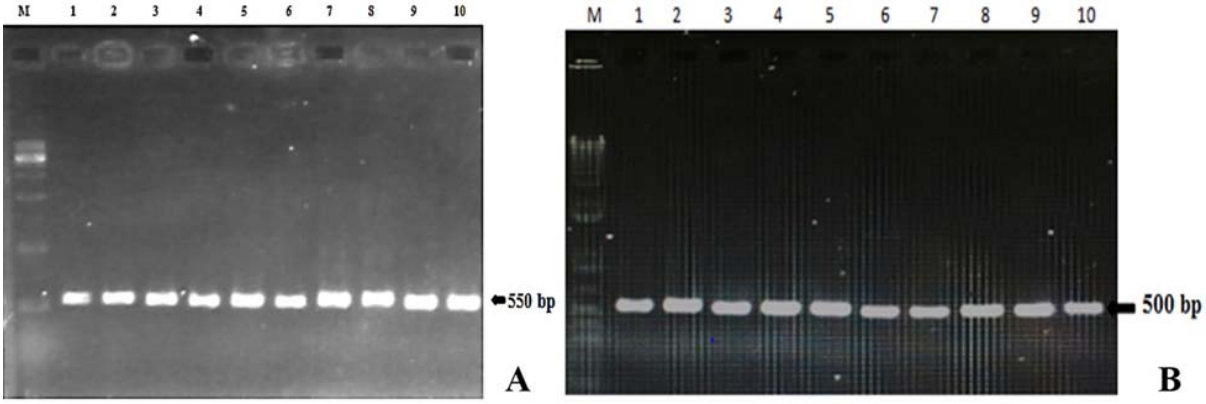

Figure 6. PCR products of the extracted DNA from fungi causing black stem and root rot of cassava in Nakhon Ratchasima amplified with ITS1/ITS4 primers (A), Ef1-688F/Ef1-1251R primers (B). Lane M: Marker 1 kb DNA ladder; lane 1: L6HRD; lane 2: L11HSR2; lane 3: L12SHRD lane 4: L13SRTF; lane 5: L18KBSH; lane 6: L20FHTB1; lane 7: L22HRW; lane 8: L27SRBT; lane 9: N1SRTTC; lane 10: N3DTD2.

Table 5. Similarity of DNA sequences of the *Lasiodiplodia* and *Neoscytalidium* isolates causing black stem and root rot disease in Nakhon Ratchasima (NR), Thailand amplified by ITS primers, compared to those of the references in the GenBank.

NR isolate nr	Morphology identifications	Reference GenBank	Reference species	Sequence similarity
		Accession Nr		%
L6HRD	*Lasiodiplodia* sp.	MK530047	*L. theobromae*	96
L11HSR2	*Lasiodiplodia* sp.	KJ596529	*L. theobromae*	97
L12HSRD	*Lasiodiplodia* sp.	KJ596529	*L. theobromae*	97
L13SRTF	*Lasiodiplodia* sp.	MT075444	*L. theobromae*	95
L18KBSH3	*Lasiodiplodia* sp.	MT497430	*L. theobromae*	96
L20FHTB1	*Lasiodiplodia* sp.	MT472040	*L. theobromae*	99
L27SRBT	*Lasiodiplodia* sp.	LC275062	*L. theobromae*	99
N3DTD2	*Neoscytalidium* sp.	MT075446	*L. theobromae*	96

Table 6. Similarity of DNA sequences of the *Lasiodiplodia* and *Neoscytalidium* isolates causing black stem and root rot disease in Nakhon Ratchasima (NR), Thailand amplified by TEF1-α primers, compared to those of the references in the GenBank.

NR isolate nr	Morphology identifications	Reference GenBank	Reference species	Sequence similarity
		Accession nr		%
L6HRD	*Lasiodiplodia* sp.	KJ417861	*L. euphorbiaceicola*	96
L11HSR2	*Lasiodiplodia* sp.	MK495418	*L. euphorbiaceicola*	96
L12HSRD	*Lasiodiplodia* sp.	KJ417861	*L. euphorbiaceicola*	97
L13SRTF	*Lasiodiplodia* sp.	MK495416	*L. euphorbiaceicola*	96
L18KBSH3	*Lasiodiplodia* sp.	KU507444	*L. theobromae*	97
L20FHTB1	*Lasiodiplodia* sp.	JX464021	*L. theobromae*	96
L27SRBT	*Lasiodiplodia* sp.	MK495396	*L. euphorbiaceicola*	95
N3DTD2	*Neoscytalidium* sp.	MK495384	*N. dimidiatum*	95

PCR amplification and DNA sequencing

When extracted DNA of eight representative isolates of the stem and root rot fungi (L6HRD, L11HSR2, L12HSRD, L13SRTF, L18KBSH3, L20FHTB1, L27SRBT and N3DTD2) were amplified using the combination of ITS1/ITS4 primers, 550 bp fragments of the ITS region were obtained from all of them (Figure 6A). But when the combination of Ef1-688F/Ef1-1251R primers was used, 500 bp fragments of the TEF1-α region were obtained instead (Figure 6B). After the 550 bp fragments (ITS) had been subsequently sequenced and further analyzed, the results showed that all of them had 95%-99% similarity to that of *L. theobromae* in the GenBank (Table 5).

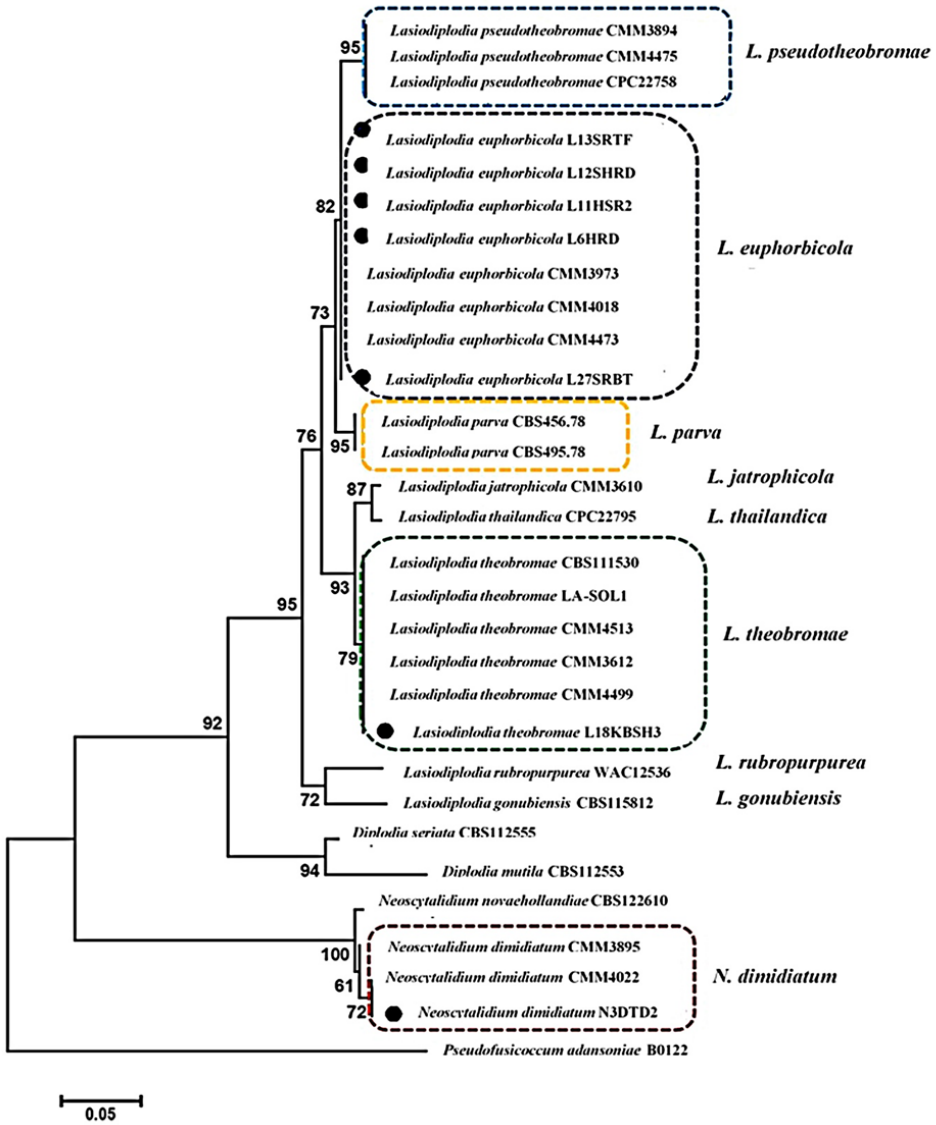

Figure 7. A neighbor-joining tree derived from the combined sequences of TEF1-α of Botryosphaeriaceae represent bootstrap values (%) from 1000 replicates. A phylogenetic tree was conducted using MEGA 6.0 with kimura-two parameter model.

Similar sequencing and analysis had also been performed on the 500 bp fragments (TEF1-α) of the eight isolates and it was found that those of L18KBSH (accession numbers MT472034) and L20FHTB1 (accession numbers MT472039) showed 96-97% similarity to that of *L. theobromae* accession numbers KU507444 and JX464021 in the GenBank, respectively. Sequences of the five isolates including L6HRD (accession numbers

MT472028), L11HSR2 (accession numbers MT472030), L12SHRD (accession numbers MT472032), L13SRTF (accession numbers MT472030), and L27SRBT (accession numbers MT472040) had 95%-96% likeness to that of *L. euphorbiaceicola*, while that of N3DTD2 (accession numbers MT472042) shared 95% similarity to that of *N. dimidiatum* accession number MK495384 in the GenBank (Tables 5 and 6).

Phylogenetic analysis using the TEF1-α align sequence data set of the eight isolates delineated them into three distinct species of Botryosphaeriaceae: two species of *Lasiodiplodia* and one species of *Neoscytalidium*. The three species of *L. euphorbiaceicola*, *L. theobromae*, and *N. dimidiatum* were grouped in separate clades (Figure 7).

DISCUSSION

Results of our research demonstrate that the main causal agents of the BSRR disease of cassava in Thailand are fungi in the Botryosphaeriaceae family, but using their morphological characteristics alone cannot elucidate their identity at the species level. The colony and conidial characteristics of the 33 isolates indicated that they belong to either *Lasiodiplodia* or *Neoscytalidium* genus but their species determination based on such characters is still doubtful. This assumption can be seen when the conidial size was used for the identification that the *Lasiodiplodia* having large conidial sizes (L group 19.50-32.80 × 12.40-19.70 µm) could be identified as *L. theobromae* (21-31 × 13-15.5 µm), or *L. citricola* (22-27 × 12-17 µm) or *L. crassispora* (27-30 × 14-17 µm) (Marques et al., 2013). Similar uncertainty is also applied to those with small-sized conidia (S group 12.50-26.30 × 9.51-15.00 µm) which could be identified as *L. euphorbiaceicola* (15-23 × 9-12 µm) or *L. pava* (16-23.5 × 10.5-13 µm) (Machado et al., 2014a; Brito et al., 2020). Therefore, additional criteria are needed for the precise identification of these pathogens. Comparing the ITS sequences of the representative fungal isolates with those of GenBank, all eight isolates were identified as *L. theobromae*, including N3DTD2 that had been identified as *Neoscytalidium* based on ITS unique production of arthroconidia and conidial characters. This result indicates that the additional criterion of ITS homology that has been used by other researchers is inadequate for the identification of fungi in this group (Munirah et al., 2017). However, when the homology of the TEF1-α region was used for the comparison, the eight representative isolates could be distinctively separated into three species: *L. theobromae*, *L. euphorbiaceicola*, and *N. dimidiatum*. This study has been the first report of finding *N. dimidiatum* and *L. euphorbiaceicola* infecting cassava in Thailand.

Our findings show that the TEF1-α sequence is a more reliable criterion for the identification of *Lasiodiplodia* species, is consistent with the study of Berraf-Tebbal et al. (2020). These authors reported that the TEF1-α sequences are better than the ITS for the determination and phylogenetic analysis of the *Lasiodiplodia* species. In the study of Rosado et al. (2016), TEF-1-α was major mostly used to generate and analyze *Lasiodiplodia* species. Combination of ITS and β-tubulin was just used to support phylogenetic TEF1-α. For the disease symptomatology, we found that the symptoms could be found in all parts of cassava plants but most frequently on the stem and root. In terms of the disease etiology, the finding of this research is similar to that of De Silva et al. (2019). These authors reported that the major pathogens of cassava root rot disease in sub-Saharan Africa were identified as *L. theobromae*, *Nattrassia mangiferae* (synonyms: *Scytalidium hyalinum* or *Neoscytalidium hyalinum*/*N. dimidiatum*) and *Fusarium* spp. Additional major causal agents found in their study were *L. theobromae*, *Macrophomina phaseolina*, *Fusarium* spp. and *Pythium* sp. Some of other results showed that only *L. theobromae* was extremely aggressive when inoculated into cassava stem cuttings (Vilas Boas et al., 2017). In addition, Motokura et al. (2014) identified the causal agent of stem rot as *L. parva* in Japan. Moreover, Machado et al. (2014b) found that the causal agents of cassava black root rot in Brazil are *L. euphorbiaceicola*, *L. pseudotheobromae*, and *N. dimidiatum*. In Thailand, Buensanteai and Athinuwat (2012) initially reported the causal agent of severe stem rot as *L. theobromae*.

CONCLUSIONS

From the results of this research, it can be concluded that the black stem and root rot (BSRR) disease in Thailand is caused by more than one fungal species. *Lasiodiplodia theobromae*, *Neoscytalidium dimidiatum*, and *L. euphorbiaceicola* have been proved to cause symptoms similar to those found in the field. At present, there is not yet a recommendation for the controlling of this disease in Thailand. Therefore, the knowledge generated by this research should be of assistance for the development of an effective control measure in the future.

Author contributions
Methodology: S.D., C.S. Software: C.S. Validation: T.L.T., N.B. Formal analysis: S.D., N.H.H. Investigation: N.H.H. Resources: C.S., N.K.P. Data curation: N.K.P. Writing-original draft: R.S. Writing-review & editing: R.S., P.P. Visualization: T.L.T. Supervision: P.P. Project administration: N.B. All co-authors reviewed the final version and approved the manuscript before submission.

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