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MOLECULAR IDENTIFICATION OF FUNGI ASSOCIATED WITH PLANT ROT DISEASE OF SOURSOP (*Annona muricata*) IN SERI MENANTI, NEGERI SEMBILAN

Nurul Syafika Muslimin[1]*, Farida Zuraina Mohd Yusof[1,2], Nur Qursyna Boll Kassim[3], Khalilah Abdul Khalil[1]

[1]*School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA, 40450 UiTM, Shah Alam, Selangor, Malaysia.*
[2]*Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi MARA (UiTM) Puncak Alam, Malaysia*
[3]*Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, UiTM Jasin, 77300 Merlimau, Melaka, Malaysia*

*Corresponding Author: pyka07.ns@gmail.com

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Abstract

Soursop (*Annona muricata*) is renowned for its ethnomedicinal uses, leading to the increasing demand for this fruit. Soursop rot disease has become one of the major constraints in soursop fruit production in Peninsular Malaysia, especially for the smaller commercial producer, much likely due to the indefinite identity of pathogens associated with the disease. As such, this study was designed as a preliminary identification of the fungal species that contribute towards soursop rot diseases in Negeri Sembilan, Malaysia using the ITS primer. The infected soursop leaves and soil samples were collected from a farm located in Seri Menanti, Negeri Sembilan, Malaysia. Cultivation was done on Potato Dextrose Agar (PDA) medium. Twenty-five (25) isolates were collected, and six (6) different species of fungi were discovered through molecular identification. They were suggested to have different roles and contributions in various types of crops worldwide, namely the *Gongronella butleri, Fusarium beomiforme, Lasiodiplodia theobromae, Hypocreals sp., Neopestalotiopsis sp.* and *Colletotrichum siamense.*

INTRODUCTION

Annona muricata, commonly known as soursop, is a member of the Annonaceae family. It is a small tropical tree that can be found and widely cultivated in tropical countries. Recent studies have shown the important roles of this underutilised fruit, where it has extensive ethnomedicinal uses for the treatment of asthma, cough, and fever as well as skin remedies [1,30]. The benefits of this fruit for medication purposes were spread widely throughout the world and the fruit was classified as a traditional medicine against an array of human ailments and diseases [30]. As a result, the fruit has a high demand in Malaysia, as stated by the soursop farmer in New Straits Times on April 2, 2016.

Like all plant trees, soursop is also susceptible to being attacked by pests and disease. The most common plant diseases of soursop are root rot, pink disease, and anthracnose. Many cases of soursop plant disease have been reported around the world. In Mexico, the *Colletotrichium gloeosporoides* and *Rhizopus stolonifer* were associated with the pre-harvesting deterioration of soursop fruit in Colombian plantations [33]. Escobar and Sanchez [10] pointed out that *Phytophthora* species and *Rhizoctonia* species were among the fungal pathogens that attack the roots of *Annona muricata* causing root rot diseases. Research had shown that four fungal isolates: *Fusarium spp, Botryodiplodia theobroma, Aspergillus niger,* and *Rhizopus stolonifera* were associated with the pre-harvest deterioration of soursop [32]. The findings added that *B. theobromae* was the most prevalent of the pathogens, followed by *Fusarium spp,* while *R. stolonifer* was found associated with only 7% of the fruits used in the study [32].

In Peninsular Malaysia, the rot disease has been resulting an income loss as well as damage to soursop production every year. The impact would be double, particularly on the smaller farmers. Claimed to have financial support given by the government, the disease problems are still unstoppable due to the lack of studies done on soursop disease in those

areas. The majority of studies were mostly focused on their nutrient content and utilisation in medical aspects. The field visit to the soursop farm reported that the rot disease can destroy more than 500 trees per year. This case alone covers only a single farm in Negeri Sembilan. Despite the case, proper identification of fungi associated with the disease is yet to be done. To address this situation, this study aimed as a preliminary identification of the contribution fungi on soursop rot disease in Peninsular Malaysia, thus was conducted on a soursop farm located in Seri Menanti, Negeri Sembilan, Malaysia to identify the fungi associated with soursop plant rot disease. The experimental works were constructed with three consecutive steps: (1) fungal isolation, (2) morphology identification, and (3) molecular identification. The study also give insight on their reported roles and contributions to the various types of crops globally.

MATERIALS AND METHODS

Fungal Isolation

The visit to the soursop farm at Seri Menanti in Negeri Sembilan of Peninsular Malaysia (2.69496 North, 102.19487 South) was conducted in November 2019. The farm was chosen as the representative for small commercial farming that has contribution to the production of various soursop-based products in the local market. This area is classified as a tropical rainforest climate with a significant amount of rainfall during the year even for the driest month with an accumulative temperature of 26°C, ranging between 2000-2500 mm of annual rainfall [8]. The soursop plants with external symptoms such as discolouration of the stems and bark, leaf browning and wilting were observed, as these properties were indicators of plant rot infection (Figure 1). The soil and leaves from the infected soursop trees were collected. About twenty samples of soil and infected leaves were transported with sterile sealed bags and brought to the Cell Biology Laboratory, Faculty of Applied Sciences, Universiti Teknologi MARA where the cultivation of fungi was carried out using potato dextrose agar (PDA) medium.

The soil sample was undergone a serial dilution plate method in which the protocols were optimized and modified from Warcup [49]. About 1 gram of soil sample was dissolved in 10 ml of sterile distilled water to make a soil suspension. An aliquot of 1 ml from the suspension then was transferred to another tube with 9 ml of sterile water (10^{-1} dilution). Next, 10^{-2} dilution was made by transferring 1 ml from 10^{-1} dilution to another tube also with 9 ml sterile water. Thus, 10^{-3} dilution, 10^{-4} dilution, and 10^{-5} dilution were made as much as required. From the dilution of choice in this case the last three dilutions in which 1 ml of each of the dilutions was plated on PDA plates. The plates were then incubated at 30°C for 5-7 days or till the single spore appeared. The single spores were picked aseptically and transferred into new PDA plates to get pure culture.

Meanwhile, the method for fungal isolation from the infected soursop leaves was followed and modified based on the Quintana-Obregón protocol [41]. The 70% alcohol wipes were used to get rid of debris from the leaf surface before small pieces of the fungal colony were picked from the infected area using sterile forceps and transferred to several PDA plates. The agar plates then were incubated at 30°C for 5-7 days. After the incubation, the single spores were picked aseptically and transferred into new PDA plates to get pure culture. All those experimental steps were undertaken in the presence of a fire torch to assure an aseptic condition throughout the isolation and culturing process.

Figure 1. The image of the infected soursop root and leaves. The root and leaves show symptoms of rotting: black spots on the end bark, brown spot, and lesion on leaves as well as bruised root. These samples were taken from soursop farm in Sri Menanti, Negeri Sembilan

Morphological Observation

Two approaches were done in the morphological identification. First is the macroscopic approach. Each of the isolates was macroscopically identified by their colony characterisation on the PDA plates. The characteristics that were focused on are the colour of the isolate elevation, texture, type of spore production and the presence of hyphae. The second is the microscopic approach. The isolates were microscopically identified with the agar block method. About 2mm x 2mm mycelium agar block of a 7-day-old fungal isolate was cut using sterile blade and put on the glass slide and covered with a coverslip before it was put inside a sterile empty plate. The plates then were half-filled with sterile distilled water to provide humidity to the culture. The set-up apparatus was placed at room temperature for 2-3 days until a small portion of the fungal spore grows surrounding the agar block. The glass slide with the mycelium block was then observed under a light microscope with the power of 400x magnification.

DNA Extraction

The DNA was extracted using Vivantis© GF-1 Plant Extraction Kit. The fungal colony was transformed into a powder form using liquid nitrogen. The colony powder was then proceeded for DNA extraction where the technique was optimised based on the instructions given by the manufacturer's protocol. About 0.5-2g colony powder was mixed with 300µl buffer PL and 20µl Proteinase K then vortex eventually. The mixture was incubated at 65℃ for 120 minutes. After that, the mixture was centrifuged for at least 5 minutes at 10,000 rpm speed to get the supernatant, which was then transferred to a new 1.5 ml tube. Next, 640µl of buffer PB was added to the supernatant and the tubes were inverted a few times to get an even mixture. The mixture was then again incubated at 65℃ for 10 minutes. After the incubation, 200µl of absolute ethanol was added and mixed quickly by inverting it a few times to prevent clumping of extracted DNA to form. A maximum of 650µl of the mixture was then transferred to a filter column tube that was provided by the kit. The tube was centrifuged at 10,000 rpm for 1 minute. This step is repeated for the remaining solution. The filter column was then washed using a 650 µl diluted wash buffer. The tube was centrifuged again for 1 minute at 10,000 rpm. The centrifuge step was duplicated to get rid of all the wash buffers from the filter column. Lastly, the upper part of the filter column was transferred to a new 1.5ml tube and preheated 60µl elution buffer was pipetted in at the centre of the filter column, to make sure that the filter was covered evenly before the tube incubated at room temperature for 60 minutes. The tube is then centrifuged, and the extracted DNA can be stored at -20℃ for long-term storage. The DNA extracted size was inspected using the gel electrophoresis technique which was then observed visually under a UV transilluminator.

PCR Amplification

The DNA extracted was amplified using 18s rRNA universal primer pair, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') as forward primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as reverse primer [44]. The reaction mixture is a mixture of 1X buffer, 2.0 mM MgCl, 0.2 nM dNTP, 0.1 µM Forward primer, 0.1 µM Reverse primer, 1.25 U *Taq* polymerase and 2 µl DNA template. The final volume of each reaction mixture was made up to 50 µl with sterile distilled water. The PCR profiles are consisting of initial denaturation at 94ºC for 1 minute followed by 30 cycles of DNA denaturation at 94ºC for 30 seconds, DNA annealing at 49ºC for 45 seconds, DNA extension at 72ºC for 3 minutes and finally, another DNA extension step at 72ºC for 7 minutes. The final products were stored at -20ºC for future use. The expected size of PCR products from this work is 400-800 base pairs.

BLAST

The amplified gene was sequenced by a commercial company, Apical Scientific Sdn. Bhd., Selangor, Malaysia. The sequenced DNA provided by the company was then aligned with NCBI GenBank by using BLAST software (http://blast.ncbi.nlm.nih.gov).

RESULTS AND DISCUSSION

The study found there are six different fungal species were identified isolated from the infected soursop trees in Sri Menanti farm, Negeri Sembilan, namely, *Gongronella butleri, Fusarium beomiforme, Lasiodiplodia theobromae, Hypocreales sp., Neopestalotiopsis sp.,* and *Colletotrichum siamense* (Table 1). In order to determine their correlation of species, the phylogenetic tree of these fungi was constructed using ClustalW multiple alignment with 1000 bootstraps replications. The bootstrap value represents the confidence level of their relationship where the values are acceptable with high confidence if values recorded are more than 85%, meanwhile less than 50% of bootstrap value indicates a low-level of confidence [21]. The tree demonstrated three class of fungi consist of *Sordariomycetes*, *Dothideomycetes,* and *Mucoromycetes* (Figure 2). In class *Sordariomycetes*, isolate OP784569 *F. oxysporum* formed monophyletic group with OP784571 *Hypocreales sp.* with 50% bootstrap value and these isolates were formed polyphyletic group with another isolate OP784573 *C. siamense* with bootstrap value of 73%. Moreover, these groups then create paraphyletic with OP784572 *Neopestalotiopsis sp.* with high level confidence of 97% bootstrap value. These groups forming indicate that four fungal species share a few of similar genetic characteristics with each other. Meanwhile, the other two isolates identified as OP784570 *L. theobromae* and OP784568 *G. butleri* show no correlation and presented as outgroup from other isolates.

Each of the identified species possessed distinct morphological characteristics (Figure 3 and Figure 4). ***Gongronella butleri*:** The mycelium sporulates in yellowish colour which has white mycelia edges. The structure was velvety, rough surface, and thick. The sporulation is dense. The reverse was pigmented to yellowish white on the edge and dark yellow at the centre. The colony was buff but flat and encrusted when reattached from the agar medium making it hard for the harvest process. Under the microscope, the conidiophores were erect, arising mainly from aerial hyphae, consisting of verticillate branches with whorls of two to four phialides. Conidia, which were observed in long divergent chains, were ellipsoidal to fusiform, apiculate, and rough-walled.

Table 1. The fungal species were identified using NCBI BLAST software. The gene sequence for each fungal species has been registered under GenBank with provided accession number

Sample ID	Fungal Species	GenBank Accession Number
P1	*Gongronella butleri*	OP784568
N3	*Fusarium beomiforme*	OP784569
LN7	*Lasiodiplodia theobromae*	OP784570
DN1	*Hypocreales sp.*	OP784571
DN4	*Neopestalotiopsis sp.*	OP784572
DN5	*Colletotrichum siamense*	OP784573

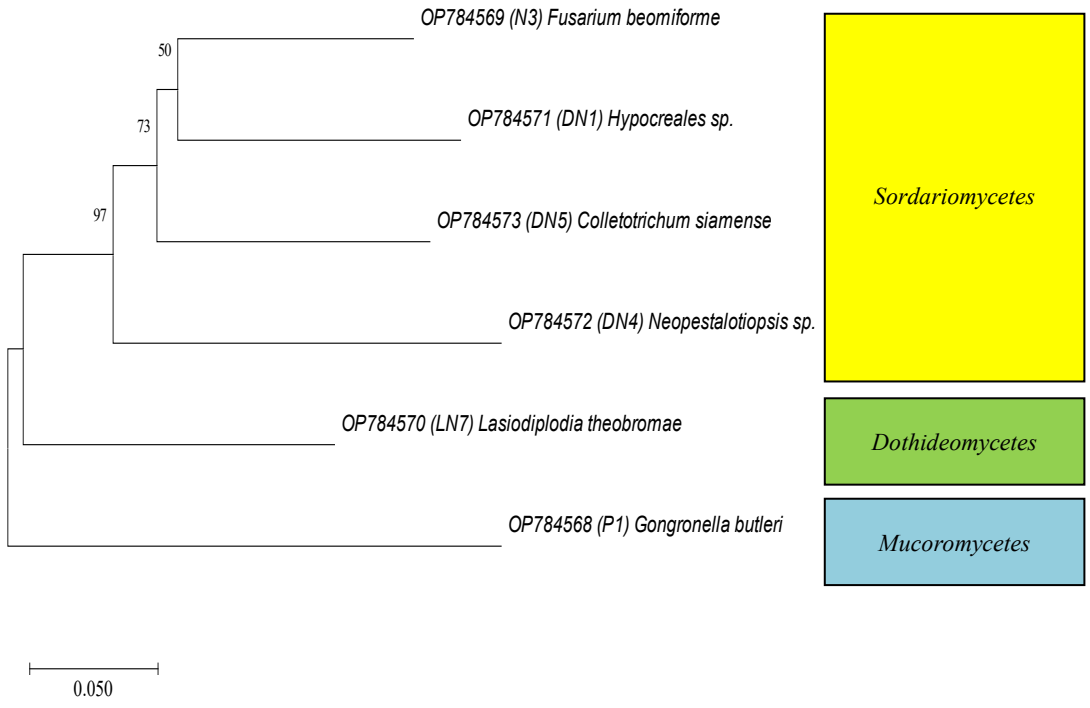

Figure 2. The phylogenetic tree of the fungal isolates constructed using ClustalW multiple alignment with 1000 bootstraps replications. The bootstrap value was acceptable with high confidence if value recorded more than 85% and less than 50% of bootstrap value indicates a low-level confidence

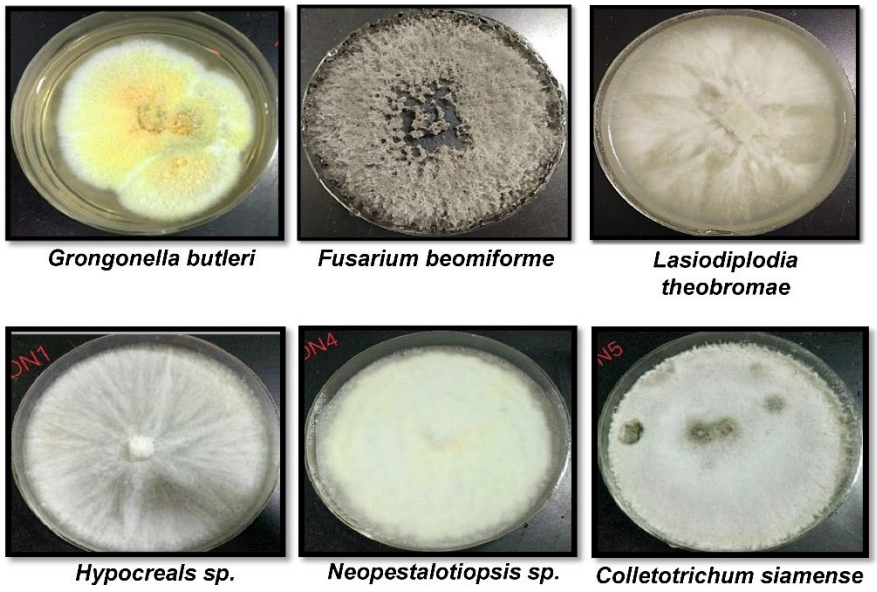

Figure 3. The macroscopic observation of *G. butleri, F. oxysporum, L. theobromae, Hypocreales sp., Neopestalotiopsis sp.,* and *C. siamense* on PDA agar plates (90 x 15 mm) after incubated at 30℃ for 5-7 days. The figure shows distinctive morphological characteristics based on their colour, texture, and elevation of mycelium

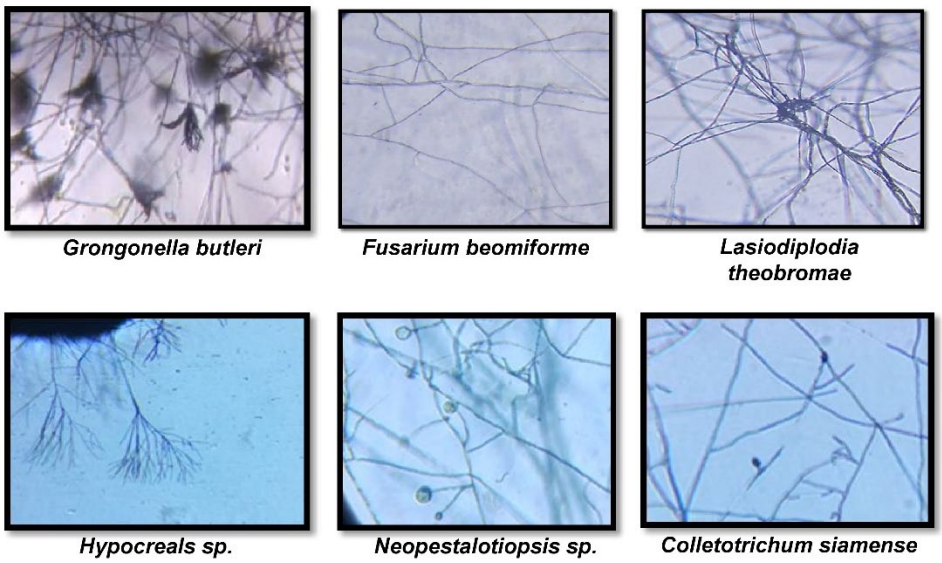

Figure 4. The microscopic observation of *G. butleri, F. oxysporum, L. theobromae, Hypocreales sp., Neopestalotiopsis sp.,* and *C. siamense* under light microscope at 400x magnification using agar block technique

***Fusarium beomiforme*:** The mycelium appeared black with dark greyish long mycelia. The structure is cotton-like and thick. The sporulation was on the whole plate and was abundant. The reverse was black on the edge as well as the centre. The colony appeared buff and smooth which easily reattached from the agar medium making it easier for high mass harvest. Meanwhile, under the microscope, a few septate hyphae appeared with no conidiophore visualized. Usually, this species has slightly bent and pointed at the apex's microconidia which exist in high quantities, unicellular, elongated drop-like usually with 5-12 × 1.5-2.5µm in size [11].

***Lasiodiplodia theobromae*:** The mycelium appeared milky white. The structure is cotton-like and thin. The

sporulation is on the whole plate with a star-like shape, and it is abundant and appeared like a shrub. The reverse was white with light yellow colour. The colony appeared flat and encrusted when reattached from the agar making it difficult during the harvest process. Under the microscope, the fungal compact with septate hyphae with no order. The visual was not captured the conidiophore. However, the metulae appeared in a group and was very short. The species appeared with no phialides. The conidia were globose and attached to the metulae in groups and usually 21-31 × 13-15µm in size [3].

***Hypocreales sp.*:** The mycelium appeared white in colour overall. The structure is shrub-like and pointy. The sporulation is the whole plate but not abundance. The reverse was also white with a dense white at the edge. The colony appeared thin, flat, and encrusted when reattached from the agar medium. Under the microscope, bunch of aerial hyphae are visualized but with a blunt end. The short, rounded protrusions hyphae resemble an aborted clamp connection. The narrow hyphae forming loose knots form caused by the less volume of the hyphae presence. The presence of spore or conidia failed to be visualized which usually appeared hyaline, smooth, and slightly curved.

***Neopestalotiopsis sp.*:** The mycelium appeared milky white, especially at the centre. The structure is cotton-like, soft surface and appeared dry. The sporulation was dense and covered the whole plate. The reverse was also white on the edge as well as the centre with a slightly different range of colour density may be due to the medium colour intervention. The colony was buff and soft when reattached from the agar. Under the microscope, the sporangiophore produced long branches with loose knots. The sporangium appeared hyaline, smooth-walled, and as one-celled spores in globose shape. The matured sporangium appeared in a variety of sizes.

***Colletotrichum siamense*:** The mycelium appeared white in colour and has milky white edge. The structure is shrub-like at the edge and velvety at the centre with less dense mycelium. It has rough surface and appears wet. The sporulation was on the whole plate and was abundant. The reverse was light white on the edge as well as the centre. The colony was buff, soft, and easily reattached from the agar medium. Under the microscope, the sporangiophore produced loose knot branches that entirely attached to small sporangium. The hyphae appeared not as abundant as other species of fungi. The sporangium appeared in a globose shape. Some of the hyphae branches appeared with a blunt end where the short, rounded protrusions of hyphae resemble aborted clamp connections.

These fungal species were found to be demonstrated various roles in diverse crop plantations. Some were reported as the causal agents for various plant rot diseases whilst the symbiosis of some others was found to have the capability as a Bio-control agent (BCA) and form a mutualistic relationship with the plant, increasing the improvement of the plant growth. Their contribution to multiple crops over the globe is shown in Table 2. *Gongronella sp.* are saprophytes found in the soil. It is among the most commonly occurring and economically important fungi used in Chitosan production, the most promising renewable polymeric materials for extensive application as an antifungal agent in industrials and agricultural fields [7]. Their capacity to inhibit the mycelial growth of other fungi such as *Fusarium sp.* has been put into evidence [28].

Table 2. The reported cases regarding the roles and contributions of the identified fungi in various types of crop plantations around the world

Isolates	Role	Contributions to Plant Disease	References
Gongronella butleri	BCA	- Antifungal agent - A plant growth elicitor - Enhancer in orchid tissue culture	[7,36]
Fusarium beomiforme	Pathogenic fungi & Endophytes	- Claimed to caused mycotoxin contamination on Sorghum plant - Caused irrigation on farmland for crop production in Kenya	[23,37]
Lasiodiplodia theobromae	Pathogenic fungi	- Caused various types of rot diseases - Act as endophytic fungus on several plant including soursop - Caused pre-harvest disease of mango in Malaysia	[4,29,35,45,48]
Hypocreales sp.	BCA	- Control potato pest insects, *Bactericera cockerelli*, and zebra chip disease - Entomopathogenic fungi	[22,50]
Neopestalotiopsis sp.	Pathogenic fungi	- Causing stem canker on hibiscus flower - Grapevine trunk disease in France - Causal agent of dry flower disease of macadamia - Causal agent of guava scab in Colombia	[2,16,46]
Colletotrichum siamense	Pathogenic fungi	- Causing anthracnose of *Capsicum annuum* in Asia - Novel leaf pathogen of *Sterculia nobilis* in China	[9,51]

F. biomiforme is one of the examples of *Fusarium sp.* that suggested could associates as endophytes as well as a disease causal agent for important cash plants. The contribution of this species reportedly was still unclear and fewer reports could prove the important roles of *F. beomiforme* in crop plantation. Originally isolated from soil in Australia [23] where the species was once suspected to cause mycotoxins contamination towards sorghum, one of the important species of cereals worldwide. However, isolated from the sorghum plantation soil in Thailand, *F. beomiforme* was reported not colonized nor pathogen to sorghum [37]. In another study, the abundance of this species was reported to cause irrigation towards farmlands only while grouped with another species of *Fusarium*, resulting in a disturbance in crop production in Kenya [27]. Moreover, Laraba reported that *F. beomiforme* was associated with causing novel symptoms to novel crown rot on wheat in Algeria and further suggesting *F. beomiforme* could be a potential pathogen towards many plant crops [23].

Meanwhile, *Lasiodiplodia theobromae* is well known pathogenic fungi from the Botryosphaeriaceae family that is particularly found in tropical and subtropical regions. Similar to other species from Botryosphaeriaceae, *L. theobromae* has been contributing as a non-host pathogenic fungus, endophytes, associated with various host plants causing diverse diseases including root rot, fruit rot, dieback, and canker which are responsible for many serious important cash plant losses [29,35,45]. In the study by Wang and the members in 2005, *L. theobromae* reported acting as an endophytic fungus in several plants including *Taxus baccata, Pinus tabulae, Piper hispidum, Annona muricata* and many others [38,48]. However, there is a thin line agreement between this species' pathogenicity and endophytic towards the plant, raising its actual relationship with the host [43]. In pathogenicity case, *L. theobromae* was reported to cause fruit rot on the pre-harvest disease of mango in Malaysia and cause vascular streak dieback (VSD) – like symptoms of cacao in the Philippines, resulting in huge production losses [4,34]. Furthermore, there are about 134 chemically defined bioactive compounds that belonging to *L. theobromae* assessed could have the ability to the phytotoxic, cytotoxic, and antimicrobial activity which might directly involve host-fungus interaction [43].

Species in *Hypocreales* are known for their ability to derive nutrition from diverse nutrient sources [19]. The ancestral condition is hypothesised to have been a reliance on plant-based nutrition, either the decomposition of dead plant tissue or plant pathogens [47]. In both managed and natural ecosystems, species of *Hypocreales* play an important role in the regulation of insect populations [13,17,18]. On the other hand, *Neopestalotiopsis spp*. is a significant plant pathogenic genera with wide host ranges [26]. Several of the species have been reported to cause dark spots on the leaf in fruit crops, including sweet almond [6], strawberry [24], grapevine [15] and macadamia [40]. Generally, the pathogenic ability of the fungi depends upon their ability to produce pestalopyrones, phytotoxins, hydroxypestalopyrones as well as pestalosides [26].

Fungi from Colletotrichum sp. are mostly symbionts to plants as an endophyte (living within the plant) or act as phytopathogens [42]. Many of the species in this genus are plant pathogens, but some of the species may have a mutualistic relationship with the host. A special form of this genus, *Colletotrichum gloesporioides f. sp. manihotis* is causes an important disease of cassava in tropical Africa, which is anthracnose diseases on cassava [5,24] where the disease transmitted through breeder seeds and post-harvest debris in the field [12] and was reported to cause total crop failure [14,24]. The *Colletotrichum siamense* that was identified in this study was reported to cause anthracnose of *Capsicum annuum* in Asia [9] and was found as a novel pathogen of *Sterculia nobilis* in China [51].

The infectivity of the fungi identified in this study on several types of crops suggests they could be a potential pathogenic fungus that contributes to the soursop rot problems as well as to other crops disease problem. Their possibility of being isolated from the infected trees, and the infection symptoms shown on the infected trees itself is the possible evidence of their likelihood to demonstrate pathogenicity activity on the soursop trees. The activity could be performed by an interaction of multiple fungal species present or might be demonstrated by a sole fungal agent. However, while this information useful, these findings are preliminary and further investigation should be executed from this study for more detailed findings.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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