Int.J.Curr.Microbiol.App.Sci (2022) *11*(02): 256-263

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 11 Number 02 (2022)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2022.1102.028

The First Report: Rotten Fruit Sugar-Apple in Bali

I. Made Sudarma*, Khamdan Khalimi, Ni Wayan Suniti and Ni Nengah Darmiati

Program study Agroecotechnologi, Faculty of Agriculture Udayana University. JL. PB. Sudirman Denpasar-Bali, Indonesia

**Corresponding author*

Keywords

Sugar-apple, *Lasiodiplodia theobromae*, internal transcribe spacer (ITS)

Article Info

Received:
08 January 2022
Accepted:
05 February 2022
Available Online:
10 February 2022

A B S T R A C T

Fruit rot disease in sugar-apple plants, is the main cause of fruit damage in sugar-apple cultivation in Bali province. Symptoms of illness begin with black marks on the skin of fruit, which gradually spread throughout the fruit. The result of gene amplification of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using 1.2% agarose gel with TAE buffer 1x at 50 volts for 30 minutes. The DNA bands seen above the UV transilluminator as seen on electroferegram, produce a 600 bp DNA fragment. The cause of the illness was identified using DNA sequences and matched with GenBankie *Lasiodiplodia theobromae*, with a percentage of 100% resemblance. The phylogenetic tree of one class pathogen with *Lasiodiplodia theobromae*.

Introduction

The sugar-apple plant (*Annona squamosa* L.) beside the fruit can be eaten, is now widely used to prevent disease in the human body, such as the leaves can be used to treat as vermicide, for tumor or cancer treatment, insect bites, and other skin diseases problems (Saha, 2011). The extract of sugar-apple fruit can be used to inhibit the growth of some microbe such as *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae, Esherichia coli, Salmonella typhi, Streptococcus pyogene* and *Aspergillus niger*. The three extracts (acetone, ethanol, and aqueous) of sugar-apple, show a positive influence on all the above microorganisms (Vijayalakshmi and Nithiya, 2015). Many of the diseases that have been found to interfere with sugar-apple crops include: *Pseudocercospora* patchy diseases, rotten fruit of *Diplodia*, purple stripes (caused by *Phytophthora palmivora*), *Cylindrocladium colhounii* and *Cylindrocladium scoparium*, and X (unidentified), (Agrilink, 1998).

Spots are very dark, irregular in shape and witha distinct edge. They become hard and cracked. The external symptoms on the fruit are similar to Black Canker. However, with Diplodia rot, the internal discoloration extends well into the fruit, producing a brown, dry, corky appearance. Affected fruit usually

shrivel, dry out (mummify) and remain on the tree. Symptoms are sometimes confused with calcium deficiency which tends to occur at the basal end of the fruit (George *et al.*, 2015).

Materials and Methods

Place and time of research

The research was conducted in two places: 1) looking for sick, healthy plant specimens from cocoa planted in Bukit Jimbaran area. 2) Laboratory of Plant Disease Science and Agricultural Biotechnology Laboratory. The study was conducted from April to August 2018.

Study of Plant Disease and Determining Pathogen

Pain crops observed, the fruits are brought to the laboratory for proven cause of the illness. Pieces of sick fruits are placed in Petri dishes containing PDA media. Five pieces were made, and then placed in a Petri dish. After 3 days, the culture is refined again on a Petri dish filled with PDA. Furthermore, cultures are observed under a light microscope, and continued to sequence DNA.

Identification of the Best Endophytic Fungus with PCR and Sequencing

Detection Procedure

Detection is done through the stages of total DNA extract of fungi using DNeasy Plant Mini Kit (Qiagen/Germany).

Stages of DNA Extraction

A total of 0.1 gram samples were crushed using pistil and mortar until smooth and then fed into a 1.5 ml micro tube and added 400 µl buffer AP1 and 4 µl RNase A stock solution then crushed to homogenize the solution. Furthermore the tube containing the mixed solution was incubated for 10 minute in a water bath with temperature 65ºC, and tube is reversed every 5 minute, to mixed solution added 130 µl buffer AP2 then crushed and incubated in refrigerator for 5 minutes. After that centrifugation is done at 14,000 rpm for 5 minutes. The supernatant (upper phase) produced at this stage is then piped and inserted into the DNeasy Mini spin column (lilac color), and centrifuged at 14,000 rpm for 2 minutes, the fraction located in the lower tube (collection tube) is removed in a new tube (2 ml) without including the pellet formed, then add 1.5 AP3 / E buffer volume and mix it using a pipette (by sucking and removing the mixture using a micropipette), then pippeted 650 µl The mixture, including when there is a precipitate formed, is fed into a DNeasy mini spin column (white) and centrifuged for 1 min at 8000 rpm. The liquid in the 2 ml collection tube is removed. This stage can be repeated on the remainder of the mixture, then the collection tube is discharged with the fluid present in it.

The DNeasy mini spin column (white) was placed on a newly available micro tube, added 500 ul AW buffer and centrifuged at 8000 rpm for 1 minute. Dumped solution in the tube. Added 500 µl of the AW buffer to the DNeasy Mini Spin Column, then centrifuged for 2 minutes at 14,000 rpm. Then transferred DNeasy Mini Spin Column to a new 1.5 ml tube, added 100 ul buffer AE and inserted directly to DNeasy membrane, incubated at the room temperature for 5 minutes, then centrifuged for 1 minute at 8000 rpm, the resulting DNA can be directly used or stored at a temperature of - 20ºC until it will be used.

Furthermore, the resulting DNA is used as a template for PCR. The PCR reaction composition was: 1 µl template DNA was added to the PCR reaction mixture consisting of: 12.5 µl 2x Dream Taq Green PCR Master Mix (Thermo Scientific), each 1 µl Primary Forward and Reverse 10 mM, and water to total volume 25 µl. The primer used is the primary pair of ITS1 (5 'TCCTCCGCTTATT GATATGC 3') and ITS4 (5 'TCCGTAGGTG AACCTGCGG 3') that will amplify the internal transcribed spacer (ITS) region of ribosomal DNA

(rDNA) (White at al. PCR conditions were: 94°C for 5 minutes 1 time, followed 94°C for 1 minute, 56°C for 1 minute and 72°C for 2 minutes, repeated 35 times, last 72° C for 10 minutes.

The amplification results were electrophoresed using 1.2% agarose gel with TAE 1x buffer at 50 volt for 30 minutes. DNA bands are seen above the UV transilluminator. Produce ± 600bp DNA fragment. Furthermore, DNA fragments are sent to PT Macrogen Inc. Korea) to perform nucleotide base tracings to determine the identity of the fungus.

Results and Discussion

Disease study

Symptoms of the disease

Symptoms of the disease found in black fruit usually from the base, or certain parts of the fruit that over time causes black fruit. Fruits are infected occasionally by secondary pathogens. All fruit age levels are attacked by pathogens that cause fruit rot. The infected fruit in the plant is 60% of the total fruits present, and when it is attacked it is difficult to control or heal again (Fig. 1).

The cause of the disease

After isolation of pathogens from sick fruits and grown on PDA media, white mycelia was found from the sides of the sliced fruit skin (Fig.2A). The white mycelia was then transferred and grown into PDA media until the mycelia fills the entire Petri dish (age 7 days) (Fig.2B). After being observed under a microscope, single-sided conidia (Diplodia) was dark brown.

This pathogen is then sequenced with the help of polymerase chain reaction (PCR), to know the sequence of nitrogen base sequence from the pathogen, then with the services of DNA Bank using Bioinformatics found the intended pathogen (Fig.2C). The pathogen was called *Lasiodiplodia theobromae* with synonyms *Botryosphaeria rhodina* (Berk & Curt.) V. Arx. (telemorf), and *Botryodiplodia theobromae* Patoluillard. The identification of the fruits contains very dark patches, irregular shapes and with apparent edges.

The fruit becomes hard and crusty. The interior changes color to outer fruit, resulting in a brown, dry and crusty appearance (Agrilink, 1998).

Gray to black colonies, like cotton with abundance of air mycelium, when reversed in black. Piknidia is simple or flocking, often fused, the size can reach 5 mm. *Hyaline conidiophore*, simple, occasionally septed, rarely bifurcated cylinders, emerges from the inner layers of pycnidial lining of the pit cell. Conidial cells are hyaline, simple, cylindrical to subobpyriform, subvoid to ellipsoid-oblong, thick-walled, single-spaced conidia often extending by 20-30 x 10-15 µm (Fig. 4.2C). Paraphyses are hyaline, cylindrical, occasionally caustic to 50 µm in length.

Identification of Pathogens by PCR and Sequencing

The result of gene amplification of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using 1.2% agarose gel with TAE buffer 1x at 50 volts for 30 minutes. The DNA bands are seen above the UV transilluminator as seen on electroferegram, producing a 600 bp DNA fragment (Fig. 3).

Based on the alignment of the gene sequence of the internal transcribed spacer (ITS) region of the rebosom DNA (rDNA) with GenBank base data using BalstN, the patent isolate fungus with the DNA sequence as follows:

Sequence of the patsir fungi

TGCGGAAGGATCATTACCGAGTTTTCGAGCT
CCGGCTCGACTCTCCCACCCTTTGTGAACGT
ACCTCTGTTGCTTTGGCGGCTCCGGCCGCCA
AAGGACCTTCAAACTCCAGTCAGTAAACGC
AGACGTCTGATAAACAAGTTAATAAACTAA
AACTTTCAACAACGGATCTCTTGGTTCTGGC

ATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCACATTGCGCCCCTTGG
TATTCCGGGGGGCATGCCTGTTCGAGCGTCA
TTACAACCCTCAAGCTCTGCTTGGAATTGGG
CACCGTCCTCACTGCGGACGCGCCTCAAAG
ACCTCGGCGGTGGCTGTTCAGCCCTCAAGCG
TAGTAGAATACACCTCGCTTTGGAGCGGTTG
GCGTCGCCCGCCGGACGAACCTTCTGAACTT
TTCTCAAGGTTGACCTCGGATCAGGTAGGGA
TACCCGCTGAACTTAAGCATATCAATAAGGC
GGA

Comparison of similarity percentage of 18S rRNA gene of an isolate fungus with multiple DNA sequences in GenBank using BLAST program (Table 1).

The results of phylogeny tree analysis using Maximum Pasimony (MP) with 1000 times Bootstrap repetition showed that isolate patsir was *Lasiodiplodia theobromae*. Isolates patsir one clade with KY657465 1 *Lasiodiplodia theobromae* isolate BTMA10, KY657464 1 *Lasiodiplodia theobromae* isolate BTMA9, KY657463 1 *Lasiodiplodia theobromae* BTMA8, KY657462 1 *Lasiodiplodia theobromae* isolate BTMA6, KY657461 1 *Lasiodiplodia theobromae* BTM5, KY657459 1 *Lasiodiplodia theobromae* BTM4, KY657458 1 *Lasiodiplodia theobromae* BTM3, and KY657458 1 *Lasiodiplodia theobromae* BTM2 (Fig. 4).

*Lasiodiplodia theobromae*was reported to cause root and leaf rot of jatropha (*Jatropha curcas* L.) plants in Gujarat (Prajapati *et al.*, 2015). According to Ismail *et al.*, (2012) states that *L. theobromae* is a pathogen that can infect trees in tropical and subtropical regions. The year 2010 investigated mango plants in Egypt resulted in the isolation of 26 *Lasiodiplodia* isolates. Khanzada *et al.*, (2004) stated that mango plants in different areas were found to be symptomatic of the disease showing dry symptoms on the branches of the tip with severe exudation accompanied by yellowish brown blendok from branches and stems as well as brown vascular tissue. Symptoms like this are caused by *L. theobromae*.

Lasiodiplodia theobromae is a common pathogen in a large number of hosts in the tropics and subtropics. The isolate collections are identified as *L. theobromae* which has been studied on the basis of sequence data from the ITS region and the EF1-α gene (Alves *et al.*, 2008). This fungus secretes several types of enzymes usually including cell wall degradation and pathogenesis. Increased global temperatures can increase the fungus, such as *L. theobromae* to alter its properties. The temperature modulation expresses the enzyme, and this affects more markedly when the fungus is grown at 37 ° C than below the temperature (Felix *et al.*, 2018). Pathogens have been successfully collected from 225 *L. theobromae* isolates from 52 plants and from many parts of the world (Mehl *et al.*, 2017).

Table.1 Similarity of patsir isolate with isolate there are at GenBank

Lasiodiplodia theobromae	**Similarity percentage (%)**	***Accession Number***
***Lasiodiplodia theobromae* isolat BTMA10**	100	KY657465
***Lasiodiplodia theobromae* isolat BTMA9**	100	KY657464
***Lasiodiplodia theobromae* isolat BTMA8**	100	KY657463
***Lasiodiplodia theobromae* isolat BTMA7**	100	KY657462
***Lasiodiplodia theobromae* isolat BTMA6**	100	KY657461
***Lasiodiplodia theobromae* isolat BTMA5**	100	KY657460
***Lasiodiplodia theobromae* isolat BTMA4**	100	KY657459
***Lasiodiplodia theobromae* isolat BTMA3**	100	KY657458
***Lasiodiplodia theobromae* isolat BTMA2**	100	KY657457

Fig.1 Fruit rot symptom (A), and symptoms of severe attacks, black fruit (B) on the left and healthy to the right

Fig.2 The pathogen isolation results (A), (B) were transferred into 7 days old PDA media, and (C) Pycnidia pathogen *Lasiodiplodia theobromae*

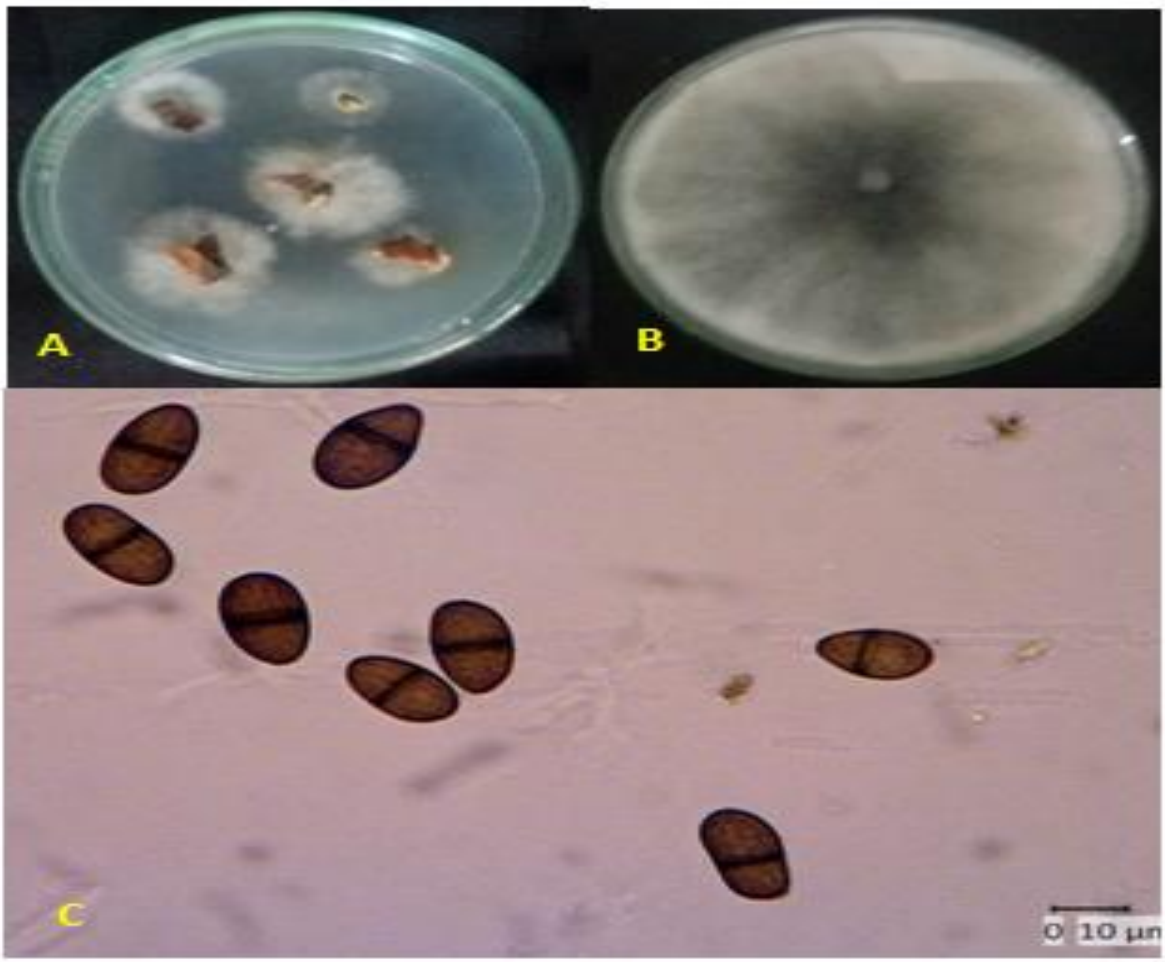

Fig.3 Pathogen sugar-apple (PatSir) Amplikon gene 18S rRNA. M. DNA Ladder 100bp; 1. Patsir isolates

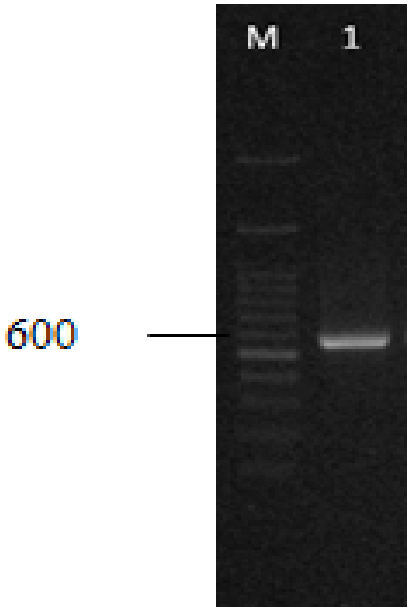

Fig.4 The phylogenetic tree is based on the MP method for the internal gene sequence of the transcribed spacer (ITS) region of the rebosom DNA (rDNA) showing the association between the patsir isolate and the GenBank database, the bootstrap value (%) is based on 1000 replications

Fig.5 The pathogenicity test by inoculation of pathogen into fruit, healthy (left) and rot (right) after inoculation by pathogen (three days after inoculation)

Pathogenicity test

The results of pathogenicity test by inoculation of pathogens into fruit, indicating that the symptoms are similar to the first symptoms seen in the tree, after inoculation of the fruit for 3 days (Fig. 5).

The pathogen of fruit rot of sugar-apple was *Lesiodiplodia theobromae.* The result of gene amplification of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using 1.2% agarose gel with TAE buffer 1x at 50 volts for 30 minutes. The DNA bands seen above the UV transilluminator as seen on electroferegram, produce a 600 bp DNA fragment. The cause of the illness was identified using DNA sequences and matched with GenBankie *Lasiodiplodia theobromae*, with a percentage of 100% resemblance. The phylogenetic tree of one class pathogen with *Lasiodiplodia theobromae*.

Acknowledgements

Authors wish to thank to the Rector of Udayana University for their assistance and the opportunity given so that research can be resolved, Dean of the Faculty of Agriculture, Udayana University, and Chairman of the Institute for Research and Community Service Udayana University, for their help and cooperation so that research can be funded to completion.

References

Agrilink. 1998. Custard apple information kit. Queensland Government. Reprint-Information Current in 1998. 1-26 h.

Alves, A., P. W. Crous, A. Correia, and A. J. L. Phillips. 2008. Morphological and molecular data reveal cryptic speciation in *Lasiodiplodia theobromae. Fungal Diversity* 28: 1-13.

Felix, C., S. Liborio, M. Nunes, R. Felix, A. S. Duarte, A. Alves, and A. C. Esteves. 2018. *Lasiodiplodia theobromae* as a producer of biotechnologically relevant enzymes. *International Journal of Molecular Science* 19(29): 1-15.

George, A., R. Broadley, D. Hutton, S. Redpath, G. Bignell, B. Nissen, D. Bruun, and G. Waite. 2015. Intergrated pest and disease management manual for custard apple. Third Edition. Queensland Departement of Agriculture and Fisheries, Marronchy Research Facility. Queensland Government.

Ismail, A. M., G. Cirvilleri, G. Polizzi, P. W. Crous, J. Z. Groenewald, and L. Lombard. 2012. *Lasiodiplodia* species associated with

dieback disease of mango (*Mangifera indica*) di Egypt. Australian Plant Pathol. 41:649-660.

Khanzada, M. A., A. M. Lodhidan S. Shahzad. 2004. Pathogenicity of *Lasiodiplodia theobromae* and *Fusarium solani* on mango. Pak.J.Bot., 36(1): 181-189.

Mehl, J., M. J. Wingfield, J. Roux, and B. Slippers. 2017. Invasive everywhere? Phylogeographic analysis of the globally distributed tree pathogen *Lasiodiplodia theobromae*. *Forests* 8(145): 1-22.

Prajapati, H. N., J. K. Patel and R.K. Patil. 2015. *Lasiodiplodia theobromae*: the causal agent of root rot and collar rot of biofuel palnt (*Jatropha curcas*) and its variability. Pl.Dis.Res. 29(2): 174-177.

Saha, R. 2011. Pharmacognosy and pharmacology of *Annona squamosa* L: A review. *Int. J. of Phar, & Life Sci*. (IJPLS) 2(10): 1183-1189.

Vijayalakshmi, R. and T. Nithiya. 2015. Antimicrobial activity of fruit extract of *Annona squamosa* L.). *World Journal of Pharmacy and Pharmaceutical Sciences* 4(5): 1257-1267.

How to cite this article:

I. Made Sudarma, Khamdan Khalimi, Ni Wayan Suniti and Ni Nengah Darmiati. 2022. The First Report: Rotten Fruit Sugar-Apple in Bali. *Int.J.Curr.Microbiol.App.Sci.* 11(02): 256-263.
doi: https://doi.org/10.20546/ijcmas.2022.1102.028