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DNA Barcoding of Cacao Pod Rot Fungi in Papua Province

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A B S T R A C T

Cocoa (Theobroma cacao) pod rot fungi have various types. In general, cause cocoa pod rot are fungi of the species Fusarium sp, Rhizoctonia sp, Phytium sp, Colletotricum sp, Amerosporium sp, Phomopsis sp and Phytopthora sp. Various species of fungi that can rot cocoa pods have high similarity in morphology characters even though the treatment for each species of fungi is different. This problem occurs in the people's nucleus cocoa plantations in the Yapsi district, Jayapura, Papua province. Samples of cacao pods infected with the fungus were taken and the isolates obtained were reinoculated into uninfected cacao pods. Isolates showing dominant infectivity were taken and identified molecularly. Identification was carried out by creating DNA barcoding by sequencing the Internal Transcribed Spacer (ITS) region of the 18SrDNA marker gene from the fungal isolates SP4DYAPSI-1, SP4 DYAPSI-2, SP3DYAPSI-1 and SP6DAUN3. Sequencing data in the form of isolate fungi DNA barcodes were analyzed with the ClustalX program analyses. The resulting phylogenetic tree construction shows that the four isolates are related to the fungus species Fusarium solani with a nucleotide similarity index above 95%. This shows that the cause of damage to cocoa pods in the Yapsi area, Jayapura, Papua, Indonesia is the fungus Fusarium solani.

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INTRODUCTION

Cocoa fruit (*Theobroma cocoa*) is an agricultural commodity which is a source of foreign exchange for the country when the product is suitable for export. The feasibility of agricultural products to enter international and national markets is determined by the quality and quantity of production. The quality of cocoa pods begins with their physical appearance, rotten pods are certainly not suitable for consumption or for export.

Decay of cocoa pods is caused by a fungal infection which causes the pods to rot, dry out and turn black. There are various types of fungi that cause agricultural fruit, including: *Fusarium* sp, *Rhizoctonia* sp, *Phytium* sp, *Colletotricum* sp, *Amerosporium* sp and *Phomopsis* sp and *Phytopthora* sp. Fungi that cause rotting of cocoa pods in Indonesia have been studied in North Sumatra, Lampung, West Java, East Java, South Sulawesi and Southeast Sulawesi .

In the province of Papua there is a focus area for smallholder cocoa plantations, namely in the Yapsi area, Jayapura district, which is located at an astronomical position of 129°00'16''BB-141°01'47"E and 2°23'10"N-9°15'00"S. The harvest of cocoa pods has decreased due to pests that disturb the cocoa pods in the form of fungi.

Handling of the fungi that damage the cocoa pods has been carried out but is not on target, this is because the type of fungus is not known with certainty. Various types of fungi have the ability to damage cocoa pods, with external morphology that has the necessary resemblance. determined by a more convincing identification method at the molecular level. Morphological identification and characterization is the first step before doing it molecularly. A molecular approach can be carried out by analyzing the barcoding results of the DNA sequencing of the fungal cells that have the greatest decay power.

In the DNA strands there are genes that are used as markers to classify certain types of fungi in their reference fungi groups. In the ribosomal DNA gene, there are 18SrDNA, 5,8SrDNA and 28SrDNA regions, between the three areas there is an ITS (Internal Transcribed Spacer) fragment. Analysis of the results of ITSrDNA gene sequencing has become a common method for determining the diversity of variations within and among fungi that causes damages to both plants and animals. Once identified molecularly, the fungi that cause rotting of cocoa pods can become material for studying ways to inhibit their growth properly and immediately. Quick and precise identification both morphologically and molecularly can help overcome the spread and infection of the fungi that cause cocoa pod rot in Jayapura in particular and Papua in general.

THEORETICAL REVIEW

National Education Policy

The implementation of national education policies in many countries has been a major focus for improving education standards. A review of the various policies and initiatives that have been implemented to improve the quality of education can provide an in-depth understanding of the global and national contexts Barber, M., & Mourshed, M. (2007).

SWOT Analysis in the Education Context

Studies applying SWOT analysis in education contexts can provide a relevant framework for evaluating education policies. This analysis helps identify strengths, weaknesses, opportunities and threats that may affect the implementation of education policies Henry, A., & Kashif, M. (2019)

METHODOLOGY

Fungal isolates that cause damage to cocoa pods were obtained from cocoa pods and cocoa leaves infected with the fungus, originating from smallholder cocoa plantations in the Transmigrant Placement Unit, Yapsi District, Jayapura Regency, Papua Province by taking a small portion of the cocoa and leaves which had been washed with alcohol 70 % and clean water. Samples were inoculated into Potato Dextrose Agar (Oxoid) medium and incubated for seven days.

Then the appearance and morphology of the colony were observed and isolated into pure culture until spores were formed.Spores from pure cultures of the fungus were inoculated into the liquid medium of potato dextrose broth and incubated for seven days at 37^{o}C with a shaker at 100 rpm, until spores formed on the surface of the medium. The culture in Spores was tested for its damaging ability to cocoa pods, by injecting 1 ml of cultured spore suspension into uninfected cocoa pods that had been washed with 70% alcohol and rinsed with clean water and then drilled holes with a 5 mm drill. The drilled cacao pods were covered with sterile cotton and inoculated with 1 ml of suspension of the fungal isolate contain 10^5 spores, then covered with plastic and incubated at room temperature for seven days.

During the incubation period, changes were observed in the cocoa pods, after seven days it appeared that the cocoa pods were damaged. The selected isolates for molecular identification were isolates with the criteria of causing faster damage to healthy cocoa pods inoculated with the isolated fungi. The isolated samples were analyzed using the codes SP4 DYAPSI-1, SP4 DYAPSI-2, SP3 DYAPSI-1 and SP6 Daun-3 with PCR primers: ITS 1/4 PCR products, namely barcoding of fungal species with a nucleotide length of ˜600bp. Genome DNA extraction was performed using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, D6005). PCR amplification was carried out 2 times with My Taq HS Red Mix reagent (Bioline, BIO-25048). PCR REACTION 1 x 25µl PCR Master Mix consisting of 9.5 µl ddH2O, MyTaq HS Red Mix, 2x 12.5 µL, 10 µM ITS1 Primer* 1µl, 10 µM ITS4 Primer** 1µl and 1µl DNA template. *Sequence ITS1 Primary : 5' –TCCGTAGGTGAACCTGCGG– 3'**Sequence ITS4Primary: 5'TCCTCCGCTTATTGAT ATGC– 3' . PCR Condition: Step Temperature (^{o}C) Duration Cycles Initial Denaturation 95^{o}C 3 min 1 cycle; Denaturation 95^{o}C 15 sec, Annealing 52^{o}C 30 sec, Extension 72^{o}C 45 sec with 35 cycles and Final Extension 72^{o}C 3 min 1 cycle; maintained at 4^{o}C for 1 cycle. After PCR was stopped, 1 µL of PCR product was analyzed by electrophoresis containing 1% TBE Agarose M, 100 bp DNA ladder (2.5 µL); and control without a template (Non Template Control / NTC). The electrophoretic gel was viewed with a UV Transilluminator to see DNA bands

and to determine its purity was determined with a UV-VIS Spectrophotometer at a wavelength of 260 nm and 280 nm. The absorbance ratio of A 260/280 is used to determine the level of DNA purity.

Good DNA purity is 1.8 – 2.0. Sequencing was carried out using the Bi-directional Sequencing method. The sequenced data were then aligned with the DNA sequences of various reference strains accessed from the genbank database at the National Center for Biotechnology Information (NCBI) in the USA. Then processed with the ClustalX program, a phylogenetic tree construction was obtained which described the kinship relationship between the tested isolates and their reference strains.

RESULTS

Hyphae morphology and shape of conidia from fungal isolates onbtained from infected cocoa pods showed that hyphae were not insulated and white in color, while macroconidia were cano in structure and microconidia were oval in structure.

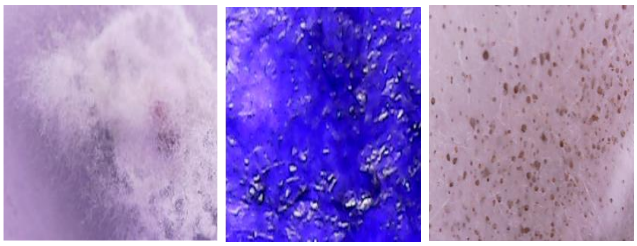

Figure 1. Morphology of hyphae, macroconidia and microconidia of fungal isolates from cocoa pods

Fungal isolates from infected cocoa pods after being inoculated back into healthy cocoa pod showed a similar effect to the infected cocoa pods, namely black pod color, drying and silvery white conidia formation, like in figure 1. In figure 2 it can also be seen that the fungal in still in the cocoa pod flesh.The fungus isolated from infected cacao pods was inoculated bach into fresh cocoa pods by making a holenin the skin of the fruit with a drill and 1 ml of culture was injected into the cacao pod and covered with cotton, observed day after day until the seventh day looked like in figure 2 below.

Figure 2. Reinfected with Isolate Fungi from Rotten Cocoa

There are four isolates of fungi that have the ability to decompose cocoa pods, namely SP4DYAPSI-1 code (a), SP4DYAPSI-2 code (b), SP3DYAPSI-1 code (c) and SP6 Daun-3 code (d). Furthermore, the four isolates were identified molecularly. The first stage of identification were extraction to isolate genomics DNA on ITS 18 SrDNA genes from each isolates by ITS gene marker (M) with a nucleotide length of ˜600bp and non template control (NTC). The DNA of each isolate is pure with a purity level of absorbance ratio of wavelength 260/280 between 1.0 – 2.0 for isolate (a) 1.81, (b) 1.06, (c) 1.76 and (d). 1.92. The results of the electrophoresis of the PCR product were recorded in the photo gel of the PCR product from DNA sample each isolates shown in figure 3.

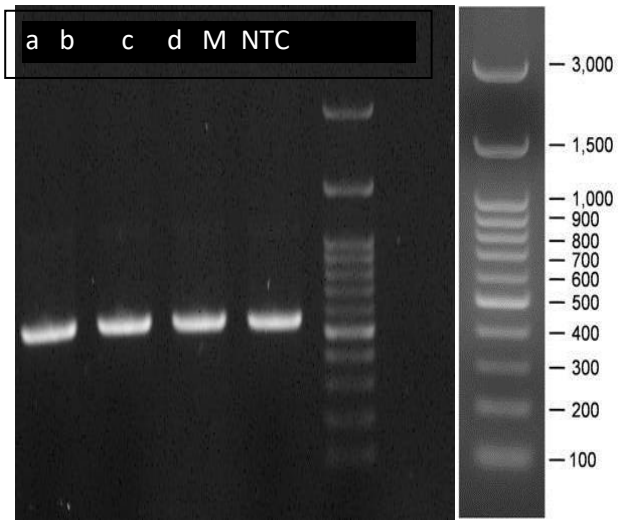

Figure 3. Gel photo-PCR Products of DNA from Four Isolates of Cocoa Pod Rot Fungi with ITS Gene Marker

The PCR product from the DNA of each isolate was then sequenced to obtain a string of nucleotide bases which were then analyzed using the Clustal X program by aligning the sequencing results compared with the nucleotide sequence accessed from the NCBI database. The results of the analysis barcoding DNA are the construction of a phylogenetic tree that describes the relationship between the test isolate and the reference showed that at Figure 4.

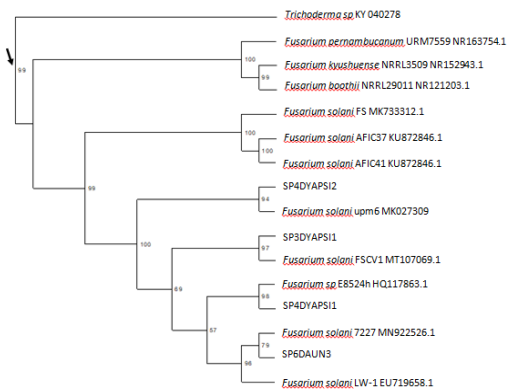

Figure 4. Reconstructed phylogeny tree based on the 18SrRNA gene sequence, partial sequence

Internal transc ribed spacer 1, 5.8SrRNA gene, and internal transcribed spacer 2, complete sequence; and 28 SrRNA gene, partial sequence, using the Neighbor-joining algorithm (Saitou&Nei, 1997) which showed a kinship relationship between 11 reference strains belonging to the genus Fusarium and 4 isolates of fungi that cause damage to cocoa pods. The number on the branch indicates the bootstrap value (%) based on Neighbor-Joining analysis with 1000 replications. The arrows indicate the root position of the phylogenetic tree using *Trichoderma sp* (KY040278) as the outgroup. The scale indicates a substitution rate of 1 per 100 nucleotides in the 18S rRNA gene sequence.

DISCUSSION

The quality of cocoa beans is determined by the condition of the cocoa pods, whether they are healthy or infected by fungus. The fungal isolate that causes rot in cacao fruit isolated from people's core plantations in the Yapsi area has the character of white hyphae, showing a pink color at the base of the colony, cano-shaped macroconidia and oval-shaped microconidia , these characters are similar to the genus Fusarium[11]. The presence of Fusarium on cacao plants can compete with Phytophtora palmivora, but Fusarium is also a fungal pathogenic for cacao and several other plants.

Endophytic fungi in cocoa pods are from the genera Aspergillus, Trichoderma, Verticillium, Colletrotrichum, Botryodiplodia. Apart from that, there are also several fungi that are associated with the fungi that cause cocoa pod rot, including *Aspergillus aculeatus, Fusarium sp, Mucor sp and Rhizopus sp.*.

In general, the fungus that causes rot in cocoa pods is caused by *P. palmivora*, Likewise in Papua New Guinea and in Southeast Sulawesi, Indonesia [4]. Therefore, to determine the type of fungus that causes cocoa rot disease in an area, this has been carried out on a massive scale, not only based on phenotypic data of cells and colonies, but also carried out at the molecular level at the genotypic level, using DNA Barcoding analysis.

DNA Barcoding Analysis is a fast and reliable fungal identification method for determining fungal species, using the inter transcribed spacer (ITS) ITS1 and ITS2 as metabarcoding markers and its position between the 18S rRNA and 28S rDNA gene markers.

Molecular analysis has been carried out on four fungal isolates that cause rot in cacao fruit, showing that each isolate is related to Fusarium. In Figure 4, shows that the fungal isolates that cause rot in cocoa pods, namely isolate SP4DYAPSI1, isolate SP4DYAPSI2, isolate SP3DYAPSI1 and isolate SP6DAUN3, are members of the species and are related to *Fusarium solani*. Then in table 1 it is explained that the results of the nucleotide similarity analysis for each isolation and reference strain used with the Phydit program from the 750 nucleotides that have been analyzed. The results of the analysis stated that isolate SP4DYAPSI1 was related to *Fusarium sp*. E8524h HQ117863.1 at 99.64%, this reference strain has the ability to produce the enzyme manganese peroxidase which plays a role in the lignin hydrolysis process closely related to the ability to damage cocoa pod skin which contains lignin. Isolate SP4DYAPSI2 was related to isolate *Fusarium solani* upm6 MK027309.1 Isolate SP3DYAPSI1

99.64% was related to *Fusarium solani* FSCV1 MT107069.1 at relationship rate of 100%. Isolate SP6DAUN3 is related to *Fusarium solani* isolate 7227 MN92256.1 with 99.82%, this type of fungus is a Fusarium from the marine environment and produces

Fusarin as an anti-inflammatory substance. The DNA similarity level of the test above 97%, indicating that the test isolates are a member of the same species with reference strain. Fusarium has quite variety of types, the presence of fungus in rotten will produce mycotoxins (deoxynivelanol, nivalenol dan T-2 toxin) which cause tremendous loss in human who consume it. Apart from that, it is also known as a fungus that has the ability to infect almost all plant in various climatic zones in the words.

CONCLUSIONS AND RECOMMENDATIONS

Based on DNA Barcoding analysis using the ITS gene marker, the fungus thacauses rot in cocoa pod from the people core cocoa plantation area in Yapsi district, Jayapura residence, Papua Province is from the genus Fusarium.

FURTHER STUDY

It is hoped that the research will provide an in-depth understanding of the factors that influence the implementation of national standards policies and provide a basis for better policy recommendations.

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