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Progress in a LAMP-PCR (Loop-Mediated Isothermal Amplification) assay for detecting the Sigatoka fungus in *Musa* sp. plants

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Abstract*–The cultivation of bananas and plantains ranks fourth in global food importance after rice, wheat, and corn. The fungus *Mycosphaerella sp.*, known as Sigatoka, is a pathogen that mainly affects these crops. In Colombia, black and yellow Sigatoka are among the nine most significant diseases impacting *Musa sp.* production. This study aimed to characterize the fungal microbiota associated with *Musa sp.* using microbiological and molecular methodologies to identify the phytopathogenic fungus. The molecular technique LAMP-PCR (Loop-mediated Isothermal Amplification Polymerase Chain Reaction) was proposed for nucleic acid amplification at a constant temperature. DNA extraction was performed using the "Wizard® Genomic DNA Purification Kit" (PROMEGA), following the manufacturer's instructions. The LAMP-PCR assay was conducted with the "WarmStart® LAMP Kit (DNA & RNA)" (New England Biolabs). Microbiological processing allowed for the isolation of pure and viable cultures, essential for studying target microorganisms. The LAMP-PCR technique proved to be effective for detecting and amplifying specific genetic material, providing rapid and accurate results. The study continues the standardization process to optimize this test and implement it routinely for Sigatoka pathogen identification.

Keywords*– Loop-mediated isothermal amplification, Phytopathogenic fungi, *Mycosphaerella sp.*, Black Sigatoka, Yellow Sigatoka.

I. INTRODUCTION

Banana and plantain crops rank fourth in global food importance, following rice, wheat, and maize [1][2]. In Colombia, plantain farming is primarily subsistence-based and small-scale, in contrast to banana cultivation, which is more highly mechanized. Both crops have a wide geographical distribution and are crucial for food security and employment generation [3][4]. In Colombia, Black Sigatoka and Yellow Sigatoka are among the nine major diseases affecting banana and plantain production [4],[5],[6].

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Black Sigatoka is caused by the phytopathogenic fungus *Mycosphaerella fijiensis* Morelet (Mf), anamorph *Paracercospora fijiensis* (Morelet) Deighton. Yellow Sigatoka is caused by the fungus *Mycosphaerella musicola* Leach ex Mulder (Mm), anamorph *Pseudocercospora musae* (Zimm.) Deighton [6],[7]. Regarding the suitable conditions for disease development, the incidence and severity of Black and Yellow Sigatoka outbreaks in plantain crops in Caldas, Colombia, were determined through symptom records, disease evolution, scales, and development time, in relation to the area's meteorological conditions [8]. These observations showed stable disease behavior, initially with incidences greater than 70%, reaching 100%, and defined five phenological stages: sprouting and emergence, seedling, pre-flowering, flowering, and bunch filling and physiological maturity.

Sigatoka occurred from the seedling stage to physiological maturity, with Black Sigatoka being more aggressive in the study area. A higher incidence of the disease was recorded during periods of heavy rainfall, followed by dry periods with slight temperature increases, conditions favorable for fungal spore germination [9]. In other studies, the relationship between climate, edaphic properties, and the incidence of Black Sigatoka in the banana-growing region of Magdalena department, Colombia, was evaluated using Geographic Information Systems (GIS), digitized data from meteorological stations, and soil maps from the Instituto Geográfico Agustín Codazzi (IGAC). This study once again demonstrated that precipitation incidence is favorable for the onset and spread of the disease, as well as the increase of other variables such as magnesium content, exchangeable clay Mg^{+2}, microporosity and soil clay, and evaporation [10]. In order of importance,

Black Sigatoka, caused by the ascomycete *M. fijiensis* [11], is one of the most devastating pathogens, leading to significant economic losses in export bananas and plantains [12],[13].

Finally, the development and adoption of DNA-based methods for the detection and typing of phytopathogens are of great practical interest for real-time disease quantification and epidemic forecasting [14]. For example, a rapid diagnostic model for Sigatoka was developed and optimized through molecular detection and quantification by real-time PCR, using DNA extracted from banana leaves [15],[16]. A set of primers based on the β-tubulin gene was designed, species-specific for *M. fijiensis*, *M. musicola*, and *M. eumusae*, addressing the need for a reliable and robust system to detect and differentiate the three fungal species present in Sigatoka-affected plants [17]. During the validation stage, this study also demonstrated the presence of *M. fijiensis* in samples from higher altitudes in Costa Rica, suggesting a potential ability of this fungus to adapt to colder environments, posing a threat to banana crops in non-tropical regions. On the other hand, an initial study conducted to surveil the polycyclic dispersal of asexual conidia used the fungus *Mycosphaerella graminicola* from wheat as a model [18],[19]. The aim was to investigate the role of ascospores in the air involved in the spread and dispersion of the pathogen. What was interesting about this work was the integration of spore trapping combined with the development of real-time PCR assays [15].

The loop-mediated isothermal amplification (LAMP) technique has been applied in various fields, including medical diagnostics, food safety assessments, and the detection of bacteria, fungi, viruses, and nematodes in plants and insects. This method provides a rapid, accurate, and cost-effective approach for diagnosing infectious diseases. LAMP can amplify a target gene from a single copy to 10^9 copies within one hour at a constant temperature, typically between 60 and 70°C [20]. Due to its ability to function under stable temperature conditions, it only requires basic equipment such as a water bath or a heating block [20]. The method relies on Bst polymerase, which possesses strand displacement activity [21], and uses internal primers (FIP, BIP) and external primers (F3, B3) that recognize six distinct regions of the target DNA sequence [22]. A key advantage of LAMP is that amplified products can be easily detected by visual inspection, eliminating the need for gel electrophoresis. LAMP has been successfully used for the detection of fungal pathogens in bananas. A real-time fluorescence LAMP technique was developed for the efficient quantification of Fusarium oxysporum f. sp. cubense (Tropical Race 4) in soil samples [23]. Additionally, a rapid detection method for *Pseudocercospora eumusae* in both pure fungal cultures and infected leaf tissues was implemented in [24]. Furthermore, LAMP has been applied for detecting *Mycosphaerella fijiensis*, the causative agent of Black Sigatoka in banana plants [25], and a similar approach has been used to detect *Pseudocercospora musae* in bananas [26]. Moreover, a novel LAMP assay for detecting *Fusarium solani*, another fungal pathogen affecting bananas, was also developed [27].

In this context, the present study aims to characterize the fungal microbiota associated with *Musa* sp. using microbiological and molecular methodologies to identify phytopathogenic fungi presents in the analyzed samples.

II. METHODOLOGY

The main methodological stages developed during this study are shown below:

A. Sampling and collection site

This research took place in Turbaco, a municipality in the Bolívar department of Colombia, which has a tropical savanna climate with moderate to high humidity and an average temperature of 30°C. The region experiences a rainy season from May to November, characterized by intense precipitation, while the dry season extends from December to April with little to no rainfall (IDEAM). Samples were collected at Agrícola Camelias, located at coordinates 10° 20' 0.08" N - 75° 22' 51.33" W. In this preliminary study, two samplings were conducted using the purposive sampling methodology. Seven leaflets of *Musa* sp. The specific sampling locations are illustrated in Fig. 1.

Figure 1. Location of the sampling site (https://online.mapcreator.io).

B. *Processing and isolation of microbiological material*

To disinfect the foliar tissue, an affected leaf segment was treated with a 70% ethanol solution for 30 seconds. Subsequently, it was rinsed with distilled water and dried in a laminar flow chamber. Finally, sterilization was performed by flame exposure using a Bunsen burner. Samples of culturable fungi were collected on Petri dishes containing PDA agar and incubated at 26 °C for 8 days. The visible characteristics of the colonies (color, texture, shape, and size) were recorded daily during the 5 days of growth. Additionally, a colony count was performed, and the data were recorded in an Excel spreadsheet corresponding to the three collected replicates. Subsequently, fragments of the colonies were stained with methylene blue and observed under an optical microscope to evaluate fungal structures such as hyphae, spores, and conidia. The methodology used was the one proposed by [14]. [15].

C. *Implementation of the LAMP-PCR molecular assay*

DNA extraction was performed using the "Wizard® Genomic DNA Purification Kit" provided by PROMEGA, following the manufacturer's instructions. For the LAMP-PCR assay, the "WarmStart® LAMP Kit (DNA & RNA)" supplied by New England Biolabs was used, following with the manufacturer's guidelines. The LAMP-PCR amplification products were visualized through agarose gel electrophoresis (1%), using the "Invitrogen E-Gel SYBR Safe" fluorophore for visualization. Meanwhile, the methodology for the LAMP assay targeting *P. fijiensis* involved the use of specific primers for amplification. Below are the primers used for PCR-LAMP amplification:

Table 1. Primer sequences for the *Paracercospora fijiensis* LAMP assay.

Primer name	Sequence 5'-3' *
PFF3	ATAGGATTAGATACCCTAGT
PFB3	GTCTAATGATTTCAGTTCCT
PFFIP	GCTGCGTTTCTAATATGATATTAATTGTCCAGGCAGAAAATTATGAATG
PFBIP	ATATGCTCTTGTTAATTAATGTATATGCCACATTACTCTTGAGG

*The following primers were developed in the master's thesis of Luania Koi Bob, 2020.

These primers include the outer primers (PFF3 and PFB3) and inner primers (PFFIP and PFBIP), which facilitate the amplification of a specific region of the *P. fijiensis* genome. The LAMP reaction was carried out under isothermal conditions, and amplification products were monitored through gel electrophoresis for subsequent visualization and confirmation of the presence of the pathogen. This method ensures rapid and specific detection of *P. fijiensis* in plant tissue samples.

III. RESULTS AND ANALYSIS

The results obtained in this study present our preliminary findings on the LAMP-PCR technique for detecting the Sigatoka fungus in *Musa* sp. plants in a region of the Colombian Caribbean (Fig. 2-A). The results obtained at each stage of the procedure are described below:

A. *Macroscopic and Microscopic observation of fungi*

From *Musa* sp. leaves, after a surface disinfection process and incubation on PDA (Potato Dextrose Agar) medium, fungal cultures exhibiting heterogeneous morphologies and colors were observed. The cultures showed variations in size, shape, and color of the colonies, which were initially classified based on their macroscopic characteristics, such as texture (cottony, woolly, or radicular) and color (white, yellow, gray, or brown), (Fig. 2-B).

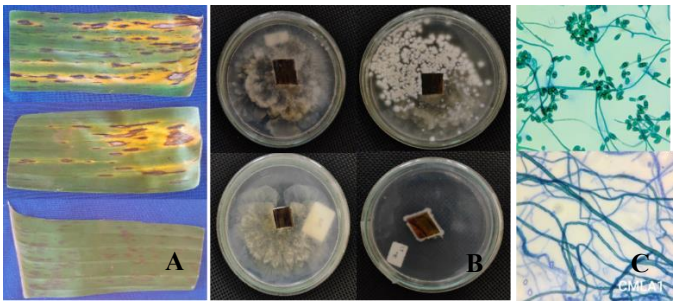

Figure 2. (A) The symptomatic observation of the infected plants revealed characteristic signs of fungal presence, such as necrotic spots on the leaves and discoloration ranging from yellowish to brown. (B) At the macroscopic level, the cultured fungi showed colonies ranging from white to gray, with diffuse borders and a cotton-like or filamentous growth on the surface of the medium, typical features of fungi. (C) At the microscopic level, fungal infection was observed through the presence of typical fungal structures, such as hyphae, spores, and conidia.

Microscopically, observations were made under a light microscope to examine the reproductive structures of the fungi, such as spores, conidia, and sporangia. Septate and non-septate hyphae were observed (Fig. 2-C). The fungal cultures and plant leaves are being subjected to molecular identification through methods like LAMP-PCR and ribosomal gene sequencing to

verify the species involved. Additionally, the morphology of the spore-forming structures is being evaluated, which will allow for precise identification and comparison with reference databases. This initial macroscopic and microscopic analysis is crucial for understanding the fungal diversity associated with *Musa* sp. plants and for establishing a more accurate diagnostic approach for the fungal diseases affecting this species.

B. Optimization of LAMP reaction

Our initial results indicate that the application of the LAMP-PCR molecular assay has proven to be an effective tool for the detection and amplification of specific genetic material. In this study, we are continuing with the standardization process to optimize this assay, aiming to improve its sensitivity, specificity, and overall reliability. Once fully optimized, we plan to implement it routinely for the accurate and rapid identification of the Sigatoka fungus. This will enhance diagnostic capabilities, allowing for early detection and better management of the disease, ultimately contributing to more effective control measures in banana plantations.

These findings demonstrated that the DNA extraction procedure used in this study successfully recovered genetic material from plant samples, particularly from Musa leaves. However, it is necessary to carefully assess the presence of contaminants that may arise during the extraction process, as they could interfere with amplification during the LAMP-PCR molecular assay, inhibiting the reaction and affecting the accuracy of the results. Regarding the obtained results, colorimetric reactions were observed, suggesting a possible amplification of the fungal DNA, although these results require confirmation through conventional PCR to ensure their reliability. These initial experiments have been essential in establishing a solid foundation for implementing the LAMP-PCR assay as a diagnostic tool in the field (Fig. 3).

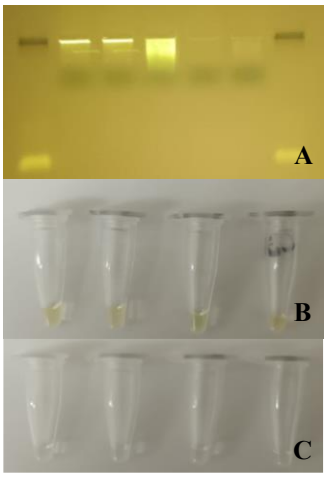

Figure 3. (A) DNA extraction from *Musa* sp. leaf samples, demonstrating the successful recovery of high-quality genetic material for further analysis. (B) LAMP-PCR assay results, where four samples were analysed. A color change was observed in the reaction tubes corresponding to the positive samples. (C) The negative controls remained unchanged, confirming the absence of contamination.

The next steps in the research will focus on developing additional experiments to further assess the sensitivity and specificity of the LAMP-PCR assay. This includes evaluating its ability to detect the pathogen at different stages of infection and its performance in samples with low fungal DNA content. Additionally, direct comparisons with other diagnostic techniques are planned to confirm its efficiency and establish its routine application for the early identification of the Sigatoka fungus in *Musa* sp. plantations, which would contribute to more timely and effective disease management.

V. CONCLUSIONS

The LAMP-PCR molecular assay has proven to be an effective tool for the detection and amplification of specific genetic material. This technique provides rapid and accurate results, enabling efficient pathogen identification and a detailed assessment of its presence in the samples analyzed. In this study, we are advancing the standardization process of the assay to optimize it and facilitate its routine implementation for the specific identification of the Sigatoka fungus.

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