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Molecular detection of *Nigrospora sphaerica* (Sacc.), an associated microfungi in diseased mulberry (*Morus alba* L.) leaf using qPCR based approach

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ABSTRACT

Background: Mulberry (*Morus alba* L.) is susceptible to fungal pathogen attack and early detection of the pathogen is important for proper disease management. Moreover, precise identification of pathogens was considered essentials in terms of health care, agriculture and environmental monitoring and plant disease management. This study reported the isolation and molecular identification of *Nigrospora sphaerica* an associated microfungi in diseased mulberry leaf using the rDNA-ITS region. Furthermore, a specific primer (NigroF and NigroR) was designed from rDNA-ITS region of the microfungi facilitating its rapid detection using the qPCR platform. By absolute quantification analysis, the amplification was detected as early on the 15th cycle while there was no amplification registered from the control set up. The C_T value (treshhold value) corresponding to the real-time PCR data, at which the detectable signal using the dye was achieved. This strategy was rapid compared to the conventional PCR, since no sequencing was required for the analysis. Moreover, the fungal pathogen has not been reported found in mulberry as a causal pathogen, an association studies must be established on its significance in the occurrence and its presence in the diseased leaf of mulberry.

KEYWORDS: *PCR, rDNA-ITS, mulberry, plant disease, qPCR*

INTRODUCTION

Mulberry belongs to the family *Moraceae* [1], it was generally propagated through stem-cuttings that are planted either directly to the fields, or in the nurseries to raise saplings for transplantation [2]. Previous surveys and observations undertaken in India in the year 1993 and 1994 revealed that several diseases attack mulberry cuttings and saplings in the nurseries as well as in the fields which cause 30-35% mortality of cuttings and death of saplings [3]. Mulberry fruit tree has tremendous potential due to its many uses. For a very long period of time, mulberry has been used for sericulture in most part of the world. Mulberry leaves is the only crop consumed by silkworm, making it the most important plant for sericulture industry.

Mulberries are susceptible to pathogen attack and early and precise detection of the pathogen is important for proper diseases management and to protect the plant for further attack. Detection of the pathogen using PCR based approach will address this issue. Significantly, early detection and precise identification of pathogens are considered essentials in health care, agriculture, environmental monitoring and plant disease management.

Study shows the identity of two *Nigrospora* species have been revealed to be as *N. oryzae* and *N. sphaerica* and showed that both pathogens were found to be true pathogens on different date palm cultivars thus, fungal pathogens are considered as one of the most serious pathogens causing a significant reduction in date palm growth, development and production [4, 5].

In terms of morphology, *N. sphaerica* is known as leaf pathogen for several hosts [6, 7, 8] including *Glycyrrhiza glabra*, blueberry and date palm but no record to be found in mulberry. *N. sphaerica* is a well-known saprophyte on many plant species but has been mentioned as pathogen on many hosts [9]. In the field, the fungus would have gained access to the plant through wounds caused by insects or frost after long-term wetness duration [10].

Researches revealed that symptoms of the disease in its host appear in the form of small (2-5 mm), circular to irregular, red coloured spots on leaflets, covering major area of the leaf. Occasionally, the spots are seen delineated by the midrib. In advanced stages of the disease, some spots cracked at the centre. Eventually, leaves dry and the plant defoliates. The fully developed colonies were flat and grey coloured, having dark brown, septate, branched mycelium. Conidia were black, single celled, globose (12.4-18.5µm), borne on a hyaline vesicle at the tip of the conidiophores [11].

The present study is therefore design to investigate new fungal pathogen of the diseased mulberry leaf through PCR based molecular approach.

MATERIALS AND METHODS

Fungal Isolation and Purification:

Diseased mulberry leaves were randomly collected from the mulberry plantation at Sapilang, Bacnotan, La Union, Philippines. These mulberries are planted and maintained under field condition which are intended for silkworm research and sericulture industry. The diseased leaf samples was subjected for pathogen isolation in Molecular Biology and Biotechnology Laboratory at the Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines. The diseased samples were surfaced sterilized in 1% NaOCl for 30s and washed under running tap water and washed twice with sterilized distilled water. Approximately 1x1 cm tissues of the diseased leaf were directly inoculated to freshly prepared potato-dextrose agar (PDA) dishes (HIMEDIA®) for 5 days at room temperature with a 12-h photoperiod. A loopful of fungal growth from the revived culture was aseptically transferred into the sterilized slant test tube with PDA for the stock culture. Mycelial sample from the stock culture was inoculated using three-point inoculation method (three mycelia inoculums plated equidistantly from each other) in a sterilized Potato Dextrose Agar (PDA) and incubated until profuse growth was observed for cultural characterization [11]. Previous studies revealed that effective maintenance of stock cultures is essential for quality control and research purposes and it also serve as a reference strains for quality control of culture media and methods. These cultures was incubated and stored as pure cultures readily available prior to molecular identification.

DNA extraction, PCR amplification, sequencing and identification of species

For DNA extraction, about 50 mg of mycelia was used using CTAB method [12], with minor modification. The genomic DNA was evaluated and confirmed using gel electrophoresis (Enduro Gel XL, Labnet International Inc.) by loading 1µl mixed with 1µl of gel red (GelRed™ Nucleic Acid, Biotium) in 1% gel and observed using gel documentation system (Enduro™ GDS) for imaging and quantified using Quantus ™ Fluorometer (Promega, Madison, USA).

To identify the fungal organism that was isolated from the mulberry leaf, a molecular technique was performed using conventional PCR. The genomic DNA was be diluted 1:100 using sterilized distilled water. The rDNA ITS region was amplified using the ITS3R (5'-ATCGATGAAGAACACAG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') using PCR machine (2720 Thermal Cycler). A volume of 2µl of diluted DNA was mixed with PCR components (volume depends on brand's instruction). The PCR amplification conditions was set as follows: 94 °C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 30 sec, with final extension step of 72°C for 7 min. The 1µl amplified product was mixed with 1µl of gel red (GelRed™ Nucleic Acid, Biotium), and observed using gel doc system (Enduro™ GDS) in 1% gel and was sent to 1st BASE Laboratory for PCR purification and sequencing. The generated sequence was used for sequence similarity using the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) particularly the BLAST (Basic Local Alignment Search Tool) program.

For the detection of microfungi, a primer pair NigroF (5'- ACGGCTTCGTAGTTCCTCAA-3') and NigroR (5'-GATCCGAGGTCAACCAGAAA-3') targeting the specific 135 bp of the rDNA-ITS region of the micro-fungi was designed using Primer3 [13] and was used for qPCR analysis. In a 0.2 ml sterile PCR tube, the 10 µl PCR components per reactions was prepared. The components were the following; 5.0 µl of Itaq™ Universal SYBR® Green Supermix (2x), Forward primer [(10 µM) (75-150 bp)] 0.3 µl, Reverse primer [(10 µM) (75-150 bp)] 0.3 µl, 3.4 µl of Nuclease free water and 1.0 µl of genomic DNA as template. After mixing all the components using vortex, next was to centrifuge the sample tube in order to collect the contents at the bottom of

the tube. Nine microliter (9 µl) of the master mix were properly dispensed into a PCR microtube plates with one microliter (1 µl) of DNA template to complete a 10 µl volume reaction per well selector of the microtube plates, this was loaded on the CFX™Real-Time Detection System with the following reaction protocol. The qPCR initial denaturation was at 95°C for 3 minutes; denaturation with 95°C for 10 sec; annealing/extension with 56-58°C for 20 sec, and then returned to step 2 (denaturation) for 39 more times, 50 °C for 30 sec and finally, a melt curve with 65-95°C, increment of 0.5 °C for 5 sec (based on qPCR protocol by Lifeline) with slight modifications. Absolute quantitative data after qPCR were evaluated on the basis of the amplicon detection of the specific rDNA-ITS region of the specific fungal organism during the analysis.

RESULTS AND DISCUSSIONS

The disease may appear in the form of small (2-5 mm), circular to irregular, red colored spots on leaflets, covering major area of the leaf but in advanced stages of the disease, some spots cracked at the center and eventually the leaves dry and the plant defoliates.

Species identification:

Based on cultural and morpholological characteristics *N. sphaerica* pathogen has flat colonies, grey coloured, having dark brown and septate branched mycelium. Conidia were black, single celled, globosely (12.4-18.5µm), borne on a hyaline vesicle at the tip of the conidiophores [14] (Figure. 1).

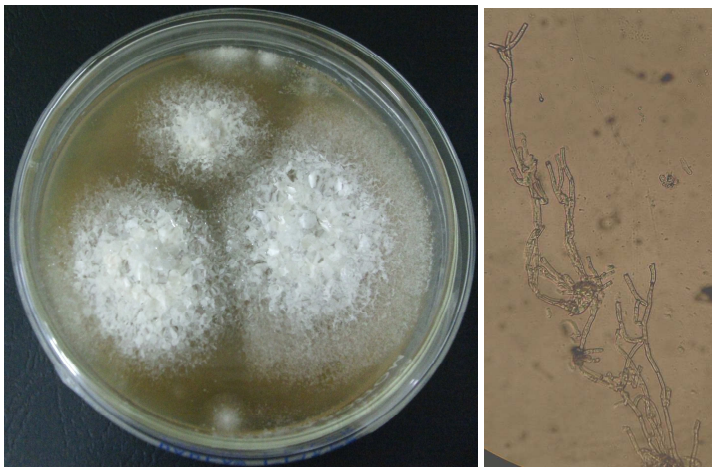

Fig. 1: Triple plated inoculation of identified *Microspore spherical* (Sac.) pathogen and the multi-septate hyphae (1000X)

Using the partial rDNA-ITS sequence, the fungi has 100% identity to *Nigrospora spaherica* with and NCBI accession number of KM999230.1. The *Nigrospora sphaerica* (Sacc.) fungal pathogen is one of the most serious pathogens causing a significant reduction in date palm growth, development and production [4, 5]. It is known as leaf pathogen for several hosts [6, 7, 16], including *Glycyrrhiza glabra*, blueberry and date palm. *N. sphaerica* was a well-known saprophyte on many plant species and has been mentioned as pathogen on many hosts [9,10]. In the field, the fungus would have gained access to the plant through wounds caused by insects or frost after long-term wetness duration (Farr *et al*., 1989). Isolation of pathogen from mulberry leaf indicated the presence of *N. sphaerica* in the infected leaf of mulberry.

Absolute quantification by linear regression analysis using qPCR:

The accumulation of the amplicon was detected and measured as the reaction progresses in real time. The Y axis on the presented Figure 2 represent the proportion of the amplified product of the target region as detected by the dye using the specific primers and with the different DNA concentrations. On the other hand, the cycles were shown on X-axis. To construct the standard curve, five different concentrations of DNA from the sample (1.0, 0.8, 0.6, 0.4, 0.2 ng/µl) were analyzed using RT-PCR including the test sample and the control. The resulting C_T values are plotted in the Y-axis against the log quantity of sample. The QPCR data for *N. sphaerica* was shown in Figure 2. The amplification for the sample was detected started as early at the 15 cycles while there was no amplicon registered from the control sample (distilled water). The C_T value (threshold value) was corresponds to the real-time PCR data (Table 1), at which the detectable signal using the dye was achieved.

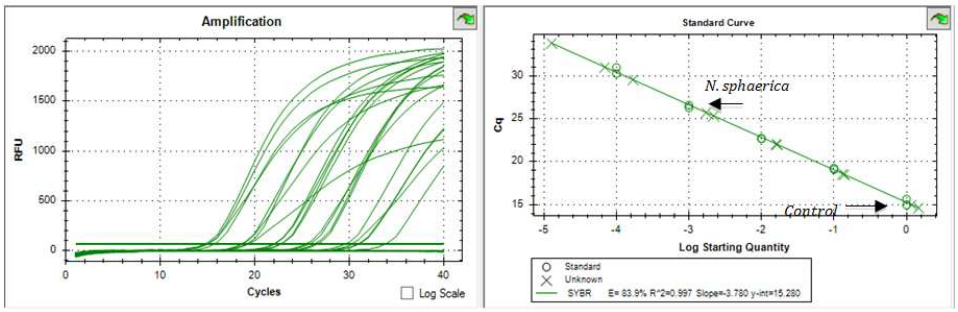

Fig. 2: Fluorescence dye detection of A1 using qPCR analysis (A) Detectable signal by fluorescence indicating amplicon progression (B) Standard curve for linear regression analysis

It shows that the C_T values of the sample during the 30, 35 and 40th cycle with 56°C of annealing temperature was significant as compared with the control as shown in Figure 2 and Table 1 having mean C_T values of 24.75 (with a regression value of 0.997), 25.24 (r^2 = .998) and 25.52 (r^2 = .998) respectively. The results showed that *N. sphaerica* can be detected using the 0.4 ng/µl and can provide an accurate result using the designed primer in this study.

Table 1: The real-time PCR data analysis for *N. sphaerica* (~.4 ng/µl).

Sample	Replicates	Cycles and C_T Values		
		30	35	40
Control	1	0.00	0.00	0.00
	2	0.00	0.00	0.00
Mean		0.00	0.00	0.00
A1	1	24.53	25.07	25.33
	2	24.94	25.41	25.72
Mean		24.74**	25.24**	25.53**
R^2		0.997	0.998	0.997

Comparisons of mean (30th, 35th and 40th) using student t-test at 0.05% level of significance

Moreover, the r^2 showed almost perfect, specifically at 35th cycles with an r^2 value of 0.998. By looking at the C_T values of the sample and the control, it falls within the range of the standard curve, predicted that the analysis performed was indeed a valid analysis. This strategy was rapid compared to the conventional, since no sequencing was required for the analysis

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