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Characterization and pathogenicity of *Aspergillus carbonarius* on *Coffea excelsa* causing berry rot and premature fall in Southern Philippines

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Abstract

Mycotoxigenic fungi in the family Aspergillaceae are among the most popular pathogens associated with post-harvest conditions. However, a recent discovery of a disease in 30–year–old trees of Excelsa coffee that caused black rot and resulted in the premature falling of the coffee berries in the Philippines. This study aims to characterize the pathogen of Coffea excelsa berries under pre-harvest conditions. *Aspergillus carbonarius* was ascertained through the combined results of cultural, morphological, molecular, and phylogenetic analyses of the rDNA ITS region of the isolate obtained from thirty diseased berries of twenty coffee trees examined. The results of the pathogenicity test also verified the virulence of the fungus. Hence, this study confirms that the disease infection of *A. carbonarius* is not only limited to post-production but also initiates pre–harvest disease infection by causing black rot and premature fall of coffee berries in the Philippines.

Keywords – Aspergillaceae – coffee trees – fungal disease – rDNA ITS – Rubiaceae

Introduction

Excelsa coffee (*Coffea excelsa*) is grown largely in Southeast Asian countries, including the Philippines. This coffee variety is popular in the country due to its nutty, fruity flavor and as a less bitter alternative to traditional coffee that suits the country's sweet palate. Excelsa coffee ranks third in overall coffee production in the country. However, in 2021, a decline in production was reported with several factors associated, including the incidence of plant diseases (Philippine Statistics Authority 2023). Despite findings of insignificant or zero susceptibility of excelsa variety against *Colletotrichum kahawae* which causes coffee berry disease (Gaitán et al. 2015), in July 2022, black rot of coffee berries and premature fall of young green berries were discovered at Pangao–an, Magpet (7.1168°N; 125.1682°E), Cotabato, Philippines.

Aspergillus carbonarius is prevalently known as a post–harvest pathogen associated with a variety of major crop commodities and by–products in the Philippines, including grains, pulses, and groundnuts. It is also considered a mycotoxigenic fungus containing carcinogenic metabolites like aflatoxin and ochratoxin that are harmful to animals and humans (Alvindia DG & De Guzman 2016, Balendres et al. 2019).

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However, there is little information about pre-harvest infection of the pathogen in the early stages of *Coffea excelsa*, given that the pathogen is not commonly associated with pre–production. The recorded incidence of the disease is critical information that must be addressed in order to develop disease management strategies, knowing that its significance remains unknown in the future. Hence, the present study aims to characterize the identity and pathogenicity of the pathogen associated with black rot and premature fall of *C. excelsa* berries.

Materials & Methods

Sampling and Fungal Isolation

A total of twenty trees of excelsa coffee were examined in Pangao–an, Magpet, Cotabato, Philippines (7.1168°N; 125.1682°E). Thirty diseased samples were collected and brought to the laboratory for disease diagnosis and fungal isolation. Small pieces of tissue were taken from the infected area's edge and cut into 2–3mm sections. These sections were then dipped in a solution of 10% sodium hypochlorite (NaOCl) for one minute, rinsed in sterile distilled water three times, and dried using sterilized tissue paper. After that, the pieces were planted equidistantly onto the potato dextrose agar (PDA) medium and kept in an incubator at a temperature of 28 ± 1°C. The pure culture was obtained through subsequent transfers of active mycelial discs, which were then cultured for seven days.

Morphological and molecular characterization

Following a seven–day incubation period, the fungal isolates were mostly identified using their combined cultural and morphological characteristics. Afterwards, using the Zymo Quick–DNATM Fungal/Bacterial Miniprep Kit, the genomic DNA of the representative isolate was extracted from the seven–day–old culture cultured in PDA broth (Zymo Research, California, USA). The primer sets ITS4/ITS5 were used to amplify the rDNA's ITS region (White et al. 1990). The PCR products were delivered to Apical Scientific Sdn. Bhd. in Malaysia for DNA sequencing (formerly known as First BASE Laboratories Sdn Bhd). Additionally, the sequence of the rDNA ITS region that was obtained using BLASTn analysis was compared to those that had been deposited in GenBank (http://www.ncbi. nih.gov/). Furthermore, a phylogenetic tree was inferred using the data of rDNA ITS sequence from *Aspergillus carbonarius* (OR141607) isolate and other reference *Aspergillus* species deposited in GenBank. The bootstrap values of 1000 replicates were provided to indicate support levels for tree nodes and the maximum–likelihood tree analysis was inferred using MEGA 11 software to confirm the identity of the fungus.

Phylogenetic Analysis

The Staden Package was used to edit DNA sequence alignment following the bioinformatics methods and protocols (Staden et al. 1999). The Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) was used to analyze the edited DNA sequences to determine and ascertain the evolutionary relationships and members of gene families. The ITS sequence of the fungal isolate was deposited in the NCBI and given an accession number (https://www.ncbi.nlm.nih.gov/). The ITS sequence obtained in this study was supplemented with further sequences of *Aspergillus* species retrieved from GenBank based on BLAST searches. The phylogenetic tree was constructed using MEGA 11 Software (https://www.megasoftware.net/).

Pathogenicity Test

Pathogenicity tests were performed thrice on immature and mature detached berries of excelsa coffee. Healthy berries were dipped in a 10% solution of NaOCl for five minutes and then blot-dried with aseptic tissue paper. Thirty wounded (pin–pricked) and unwounded berries were sprayed with 10 µl conidial suspension ($3x10^7$ spores/ml) taken from a seven–day–old pure culture (Nikolic et al. 2016). For the control group, sterile distilled water was sprayed. The samples were

then incubated at 27 ± 0.5°C for seven days. Berries showing diseased symptoms were re–isolated to attest to Koch's postulate.

Results

Disease symptoms

The results of the examination revealed that a significant proportion of *Coffea excelsa* trees, specifically 95%, had been afflicted by black and sunken lesions that were irregular in shape. The infection was seen to initiate on green young berries and subsequently progressed to red berries, where the entirety of the berries were covered with black necrotic patches and eventually mummified. Mummification occurred in both immature and mature berries, and the mummification process was accompanied by visibly black conidial masses (Fig. 1a–b).

Fig. 1 – Berries of excelsa coffee infected by black rot and premature fall in Pangao–an, Magpet, Cotabato, Philippines. a Infected trees. b Infected coffee berry.

Morphological characterization

After seven days of incubation, colonies measure 20–30 mm in diameter. Colony morphology was inconspicuous and white; conidial areas were black; reverse hyaline. Colony texture was dominantly granular to floccose, medium dense without zonation (Fig. 2a–b). Conidia (n = 50) were brown, globose to sub–globose (4.44–8.88 × 4.44–8.88 µm), typically, conidial heads radiate stipes and biseriate (77.70 × 71.04 µm), and conidiophores were long, smooth, thick–walled, hyaline to yellowish or slightly brown (444–488 µm) (Fig. 3a–c). The result of the combined morpho–cultural characterizations was following that described by Al–Musallam (1980).

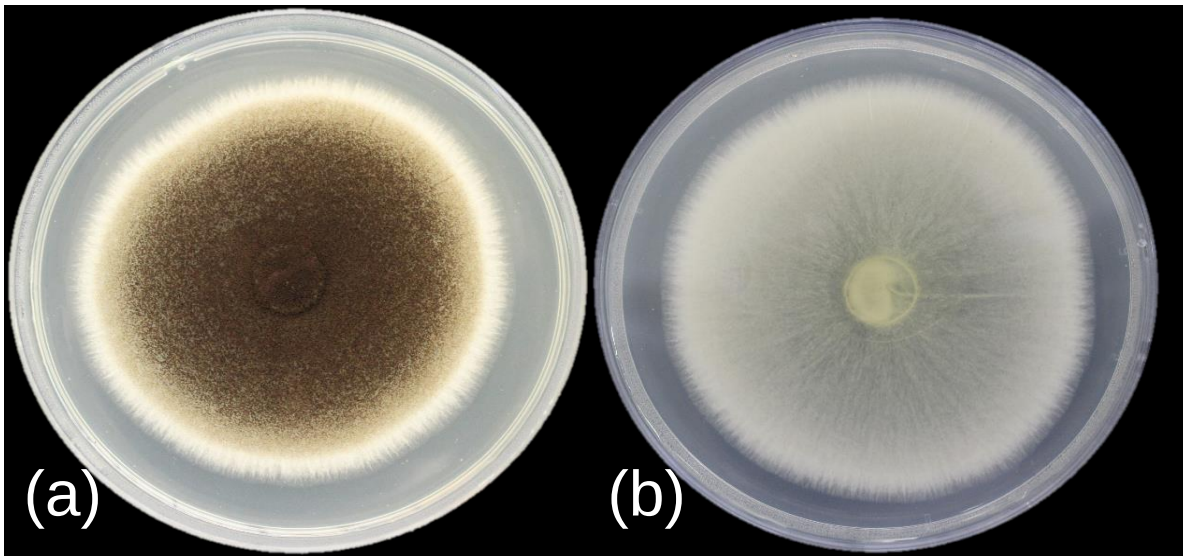

Fig. 2 – Colonies of *Aspergillus carbonarius* (OR141607) on PDA after seven days. a Obverse. b Reverse.

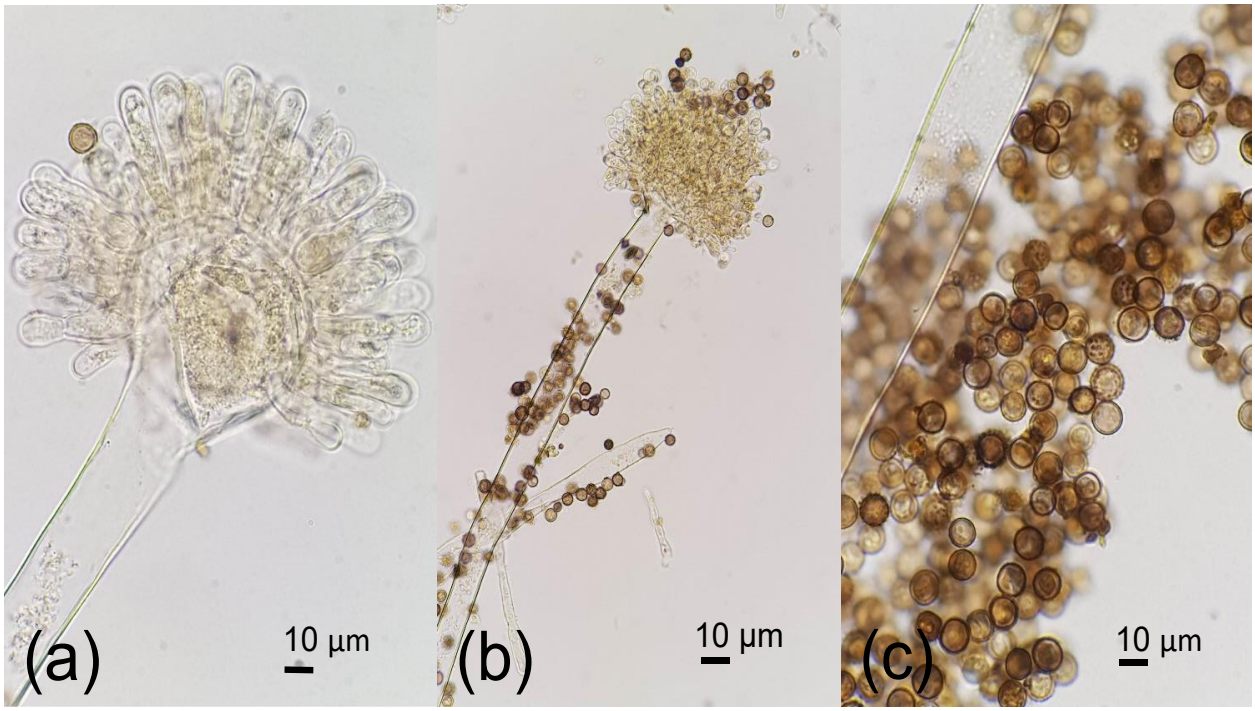

Fig. 3 – Morphological characteristics of *Aspergillus carbonarius* (OR141607). a Conidiophores, along with vesicle and phialides. b Conidiophores along with conidial head. c Conidia seven days after incubation under 4000x magnification. Scale bars = 10 µm.

Molecular and Phylogenetic Analyses

The rDNA ITS region of the fungal isolate was amplified in PCR reactions. BLASTn search analysis of the resulting sequence showed 100% identical to *Aspergillus carbonarius* isolate strain CBS: 128800 (MH865150.1). The sequence was deposited in GenBank under the accession no. OR141607. A phylogenetic analysis for the sequence along with other *Aspergillus* species was inferred by using the maximum-likelihood method (Fig. 4). This showed that the sequence of *Aspergillus carbonarius* obtained in this study align alongside other *Aspergillus carbonarius* strains and is closely related to *Aspergillus niger*.

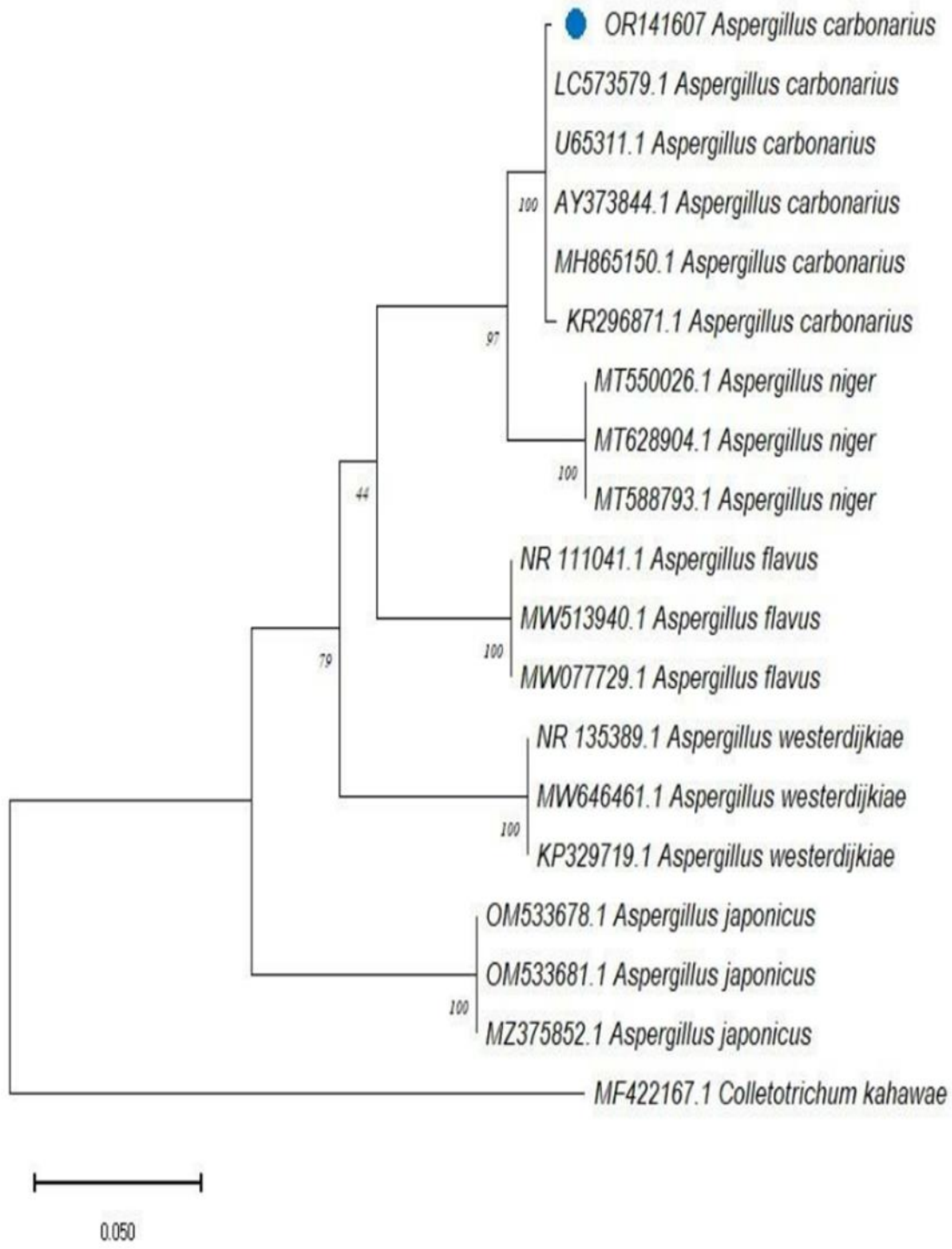

Fig. 4 – Phylogenetic tree generated from maximum likelihood analysis based on ITS. Bootstrap support values over 100% are given at the nodes. *Colletotrichum kahawae* (PL12–1A) was used as the outgroup taxon. The isolate in this study is indicated with blue dot.

Pathogenicity assay

Pathogenicity tests on both young and red detached berries of excelsa coffee ascertained that *A. carbonarius* was pathogenic in both wounded and unwounded berries, where symptoms manifested at three- and five-days post-inoculation, respectively. Meanwhile, uninoculated coffee berries did not manifest any disease symptoms (Fig. 5).

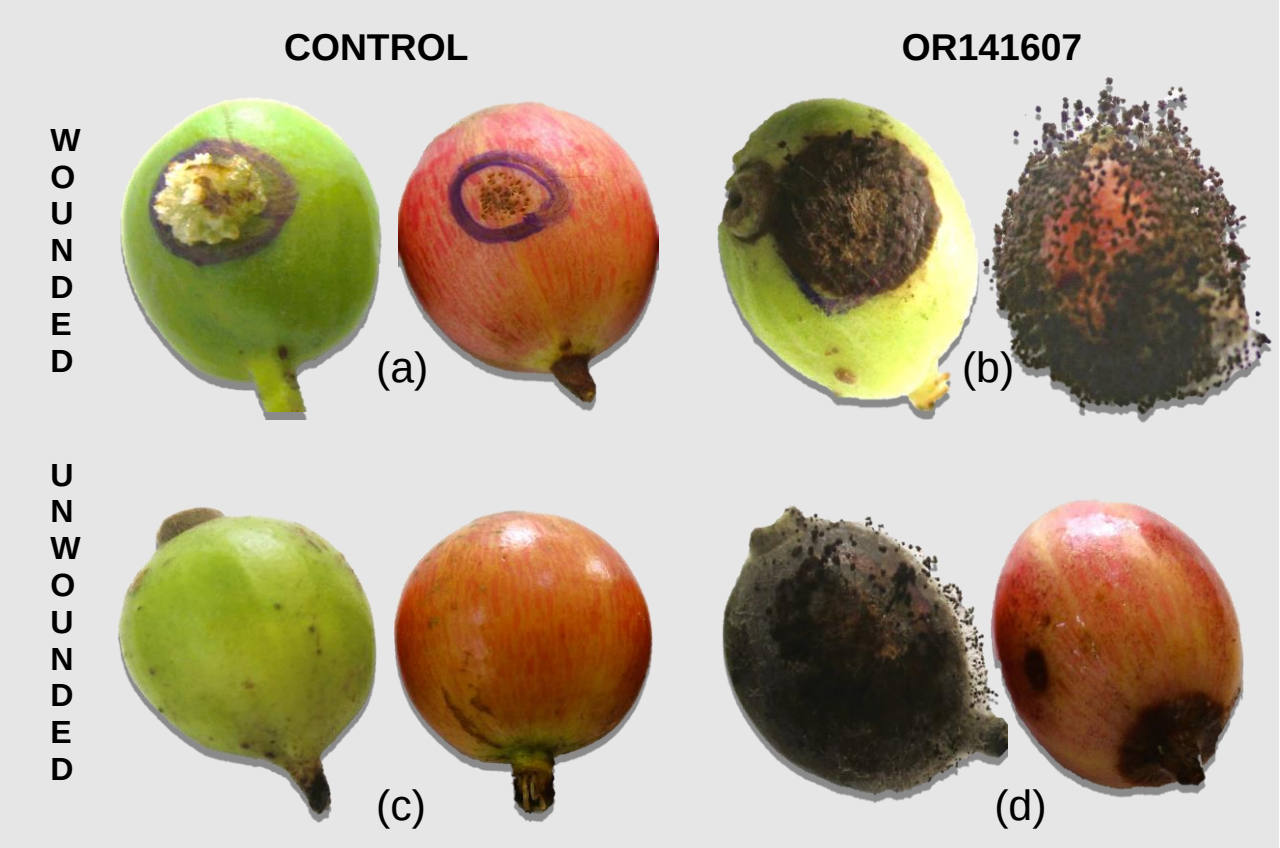

Fig. 5 – Pathogenicity test of *Aspergillus carbonarius* (OR141607): (b) three-day infection on wounded berries and (d) five–day infection on unwounded berries. Control berries of (a) wounded and (c) unwounded showed no disease symptom.

Discussion

Aspergillus carbonarius was reported to infect the dried green beans of excelsa, liberica and arabica coffee varieties in the provinces of Abra, Cavite and Davao, Philippines (Alvindia DG & De Guzman 2016). Based on the findings of Culliao & Barcelo (2015), *Aspergillus* species are contaminants of post-harvest coffee samples of both arabica and robusta coffee but not the green and ripe coffee cherries. However, this study ascertained that *Aspergillus carbonarius* is not only limited to being a post-harvest pathogen but could also initiate pre-harvest disease infection by causing black rot and premature fall of coffee berries up to a significant extent. Further studies about the potential impact of this mycotoxigenic fungal agent should also be emphasized and potential control of this pathogen must be explored.

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