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# MORPHOLOGICAL AND ITS MOLECULAR CHARACTERISATION OF PATHOGENIC MOULDS ISOLATED FROM CASHEWS (*Anacardium occidentale* L.), TOGO

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***Abstract:*** *Fungal diseases are among the factors limiting cashew nut production in Togo. This study aimed to isolate and characterise the diversity of mould species associated with 8 fungal diseases of cashews. Therefore, samples (leaves, buds, apples, nuts and bark) showing symptoms of one of these fungal diseases were collected from cashews. Explants taken from symptomatic samples were disinfected and then transferred to PDA medium. The resulting moulds were purified and identified using morphological (growth rate, texture, topography, colour, pigmentation, spore and asca) and ITS molecular methods. A total of 248 moulds were isolated after fungal analysis. Based on morphological characteristics, 33 fungal clusters were formed, of which 8 were identified. Molecular analysis confirmed the results of morphological identification and enabled the identification of moulds that had not been identified due to their non-sporulation. As a result, a total of 27 mould species belonging to 13 genera were identified for alignment with various sequences available from the National Centre for Biotechnology Information (NCBI). Colletotrichum, Pestalotiopsis, Lasiodiplodia, Aspergillus, Fusarium, Curvularia and Diaporthe were the most represented genera. This study revealed a specific diversity of pathogenic moulds infecting cashews in Togo. The use of agroecological control methods could contribute to the effective management of the eight fungal diseases studied.*

***Key words:*** *Anacardium occidentale L., fungal diseases, mould identification, PCR-ITS, Togo.*

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## 1. Introduction

The stability of agricultural production is linked to a number of factors, including the control of bioaggressors in host crops. The diagnosis of Togo's cashew chain revealed the harmful impact of bioaggressors on cashews productivity [44]. The appearance and emergence of disease outbreaks in agricultural ecosystems are linked to favourable agropedoclimatic conditions and can lead to production losses in the field or postharvest losses. Research on cashew pests and diseases has shown that cashews are susceptible to various biological invasions [17, 35, 49]. From 1971 to 2022, moulds were by far the most frequently mentioned, as they were cited in a significantly higher number of studies (90) than bacteria (9) [34]. Thus, *Colletotrichum gloeosporioides*, *Pestalotia heterocornis*, *Diaporthe anacardii* (Syn: *Phomopsis anacardii*), *Erysiphe quercicola* (Syn: *Oidium anacardii*) and *Cryptosporosis* spp. have been reported to cause anthracnose, pestalotiopsis, bud's dieback, powdery mildew and black rust on cashews, respectively [8, 31, 32, 35, 49]. Fusarium wilt is caused by *Fusarium oxysporum*, which can wilt cashews within 3 to 4 weeks of the first symptoms appearing [31]. Cashew leaves infected by *Fusarium oxysporum* lose their natural green colour and turn yellow, and yield losses can reach 100% if nothing is done [45]. In their study, Domedjui et al. [17] reported that, in addition to pathogenic moulds, superinfecting moulds are also associated with cashew fungal diseases. The presence of *Penicillium* spp. inside immature or mature cashew nuts represents a threat during the postharvest period [35]. According to Freire et al. [23], moulds belonging to the genera *Aspergillus* and *Penicillium* are frequently isolated from immature cashew nuts kernels. These infections can lead to losses of up to 20 million dollars due to the strict limits on mycotoxins in kernels. Moulds are capable of producing a wide variety of secondary metabolites and are rich in genes that encode proteins involved in their biosynthesis [22]. These secondary metabolites include mycotoxins, which are small organic molecules with diverse chemical structures and biological activities. Mycotoxins are toxic compounds that are occasionally very hazardous to animals and humans [42].

The detection and identification of plant pathogens provide a deeper understanding of their diversity and the appropriate control measures to be adopted. According to Blaize et al. [5], techniques for identifying moulds have developed considerably since the 2000s. Morphological identification methods, including the classic method, are based on macroscopic and microscopic characteristics. However, morphological identification relies heavily on subjective judgment due to limitations such as non-sporulation and morphological similarity between isolates. This approach has been supplemented by molecular biology approaches based on comparisons of Deoxyribonucleic Acid (DNA) sequences. Molecular biology techniques are widely used for species identification because they are rapid, specific, sensitive and relatively accurate [50]. Molecular methods are based on the characteristics of DNA sequences. In addition, a promising technique that has emerged in recent years is Matrix-Assisted Laser Desorption/Ionization Time-of-Flight

(MALDI-TOF) mass spectrometry, which allows mass spectra to be established within a defined range from a culture or spore suspension and compared with a database of spectra from reference strains of different species [9].

In previous phytosanitary surveys in cashew orchards in the prefectures of Anie, Est-Mono, Tchamba and Tchaoudjo in Togo, areas of high cashew nut production, 8 fungal diseases were reported [15]. Their epidemiological parameters revealed their dynamic progression from one locality to another. Therefore, this study's aim was to isolate and characterise the pathogenic moulds diversity associated with each cashew fungal diseases to develop appropriate biocontrol measures.

## 2. Materials and Methods

### 2.1. Technical Material

Camera (Infinix Hot mobile), Silica gel (VWR Chemicals), Self-stick adhesive envelope (Sotimex), plastic envelope (Domedia), Horizontal laminar flow hood (Air Science Multiplex™), Refrigerator-freezer (REX®), Petri dishes (DiaTrust Medical Co.,Ltd), Scalpel Handle (Swann Morton), Sodium hypochlorite 12° chlorine (Roi detergents), Ethanol 96° (ITW Reagents), Scalpel Chirurgical Lame (Swann Morton), Chloramphenicol (Zymo Research corporation), Potato Dextrose Agar (PDA, Thermo Fisher Scientific™ Oxoid™), Malt Extract Agar (MEA, Thermo Fisher Scientific™ Oxoid™), Microblue microscope (EUROMEX®), Microscope slides (Menzel-Glaser), Coverslip (Deckglaser), LactoPhenol Cotton Blue (LPCB, Thermo Fisher Scientific™), Potato Dextrose Broth (PDB, Thermo Fisher Scientific™ Oxoid™), Quick-DNA™ Fungal/bacterial Miniprep Kit (Zymo Research corporation), SpectraMax® QuickDrop™ (Molecular Devices®), Mini Personal Thermal Cycler (MultiGene™), Standard PCR reagents and Kits (HhaI, Hin1II(NIaIII), RsaI) (Thermo Scientific™), Mini Gel Electrophoresis System, Model B2 | eBay (ThermoScientific™), EThidium Bromide (BET, Thermo Fisher Scientific™), Agarose gel (Thermo Fisher Scientific™) were used.

### 2.2. Sample Collection

As part of this study, samples of cashew's leaves (Figure 1), buds and bark (Figure 2) and fruits (Figure 3) were obtained from the Mycology Unit of the Laboratory of Botany and Plant Ecology (LBEV) at the University of Lomé for fungal analysis [15]. Samples were taken from individuals showing fungal symptoms, and the varieties present were red, light red, yellow and golden yellow apples. They were placed in self-stick adhesive envelopes containing silica gel, wrapped in plastic envelope and stored at 4°C.

### 2.3. Isolation and Purification

Using a sterile scalpel, 3 mm explants were taken from the edge of the necrotic spots on the samples. These explants were then rinsed thoroughly with tap water and soaked for five minutes in a 1% sodium hypochlorite solution followed by one minute in 96° ethanol, before being rinsed three times with sterile distilled water. They are then dried on sterile absorbent paper and five transferred onto sterile PDA medium supplemented with 5% chloramphenicol (w/v). Finally, the cultures were incubated at 28°C for alternating cycles of twelve hours light and twelve hours dark for seven days [49]. The mould isolation was repeated three times

(03). The cultures were monitored regularly every 24 hours until day 7. Any mould that emerged was isolated and subcultured onto a fresh sterile PDA or MEA medium and then incubated at 25°C for seven days.


Fig. 1. *Fungal diseases symptoms on cashew leaves: a. Anthracnose; b. Yellow leaf spot; c. Pestalotiopsis; d. ramularia*


Fig. 2. *Fungal diseases symptoms on cashew buds and bark: a. bud's dieback; b. gummosis*

**2.4. Morphological Characterisation**

It was performed on the basis of macroscopic characteristics (growth rate, texture, topography, colour and pigmentation) and microscopic characteristics (Hyphae, asci and spores) of purified mould isolates. The identification keys contained in reference works [6, 11, 19, 46] were used. Mould isolates with the same phenotypic characteristics were selected and identified.


Fig. 3. *Fungal diseases symptoms on cashew fruits: a. Black rust; b. Anthracnose; c. Pestalotiopsis; d. Powdery mildew*

### 2.5. Molecular Characterisation

#### 2.5.1. Maintenance of Mould Isolates

Sample preparation for molecular analysis was carried out under aseptic conditions, and approximately 50 mg of fungal material was needed. Woolly textured mould isolates were obtained from seven-day cultures and then conditioned in sterile 1.5 ml Eppendorf tubes containing glass beads. Those with a powdery texture were collected in sterile 1.5 ml Eppendorf tubes using sterile distilled water. In contrast, mould isolates with a fluffy or hairless texture were maintained in sterile PDB medium for 72 hours and then collected in sterile 1.5 ml Eppendorf tubes containing glass beads. Finally, the samples were stored in a freezer at -20°C for the following steps.

#### 2.5.2 Total DNA Extraction

DNA was extracted from mould isolates using the Quick-DNA™ Fungal/bacterial Miniprep Kit. After extraction, the quality of the DNA extracts was verified by electrophoresis on a 2% (w/v) agarose gel. In addition, the concentration and quality of the DNA extracts were assessed before each use by spectrophotometry using a SpectraMax® QuickDrop™.

#### 2.5.3. PCR Amplification of ITS DNA

The primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), described as universal primers by Gargas and Taylor [24], were used in this study. The composition of the reaction mixture (final volume 50 µL) was as follows (Table 1).

Amplification was performed in a

thermal cycler according to the program described by Phalip et al. [38]. Its first step consists of an initial denaturation of the target DNA at 94°C for 3 minutes, followed by a second step comprising 34 cycles, including denaturation at 94°C for 1 minute and 30 seconds; hybridisation at 55°C for 1 minute and 30 seconds; and elongation at 72°C for 2 minutes per cycle. The final elongation marks the last step and is completed at 72°C for 15 minutes. Amplicon size was analysed on a 2% agarose gel containing ethidium bromide (BET). The amplicons were stored at -20°C until further use.

*Composition of the PCR–ITS mixture* Table 1

| Reagents | Initial concentration (I.c.) | Final volume (F.v.) | Final concentration (F.c.) |
|---|---|---|---|
| Eau Milli-Q 2 | – | 28,75 | – |
| 10X DreamTaq Green Buffer with $MgCl_2$ 20 mM | – | 5 | – |
| dNTP | 10 | 1 | 0.2 mM |
| Primer forward | 10 µM | 2.5 | 0.5 µM |
| Primer reverse | 10 µM | 2.5 | 0.5 µM |
| DreamTaq polymerase | 5 U. $\mu L^{-1}$ | 0.25 | 0.025 U. $\mu L^{-1}$ |
| ADN | 100 to 150 ng. $\mu L^{-1}$ | 10 | 10 ng. $\mu L^{-1}$ |

### 2.5.4. DNA Sequence Analysis

Amplicon concentration and purity control were carried out spectrophotometrically using SpectraMax® QuickDrop™. The amplicons were sequenced by Cellular and Molecular Immunological Application (CEMIA, Greece) and submitted to BLASTN tools for alignment with different sequences available in the NCBI database (http://www.ncbi.nlm.nih.gov, accessed on 3 December 2022) based on percent identity (PID).

### 2.6. Isolation Frequency

This was evaluated according to the number of times a mould sample was isolated per symptom, according to Equation (1).

$$IF = \frac{N_f}{N_t} \cdot 100 \quad (1)$$

where:

IF is the isolation frequency [%];

$N_f$ – the number of mould isolates exhibiting the same phenotypic characteristics;

$N_t$ – the total number of mould isolates from samples showing same symptoms.

### 2.7. Data Analysis

The Paint 3D graphic design software (Microsoft) was used to process the pictures of the samples and mould isolates. The R software version 4.5.1. (R foundation) was used to enter and process isolation frequency data. The FinchTV software (Geospiza, Inc) was used to process DNA sequence data.

## 3. Results

### 3.1. Isolated Mould Characterised Morphologically

Following fungal analysis, a total of 248 mould isolates were purified and the cultural characteristics enabled to form a total of 33 mould clusters (Figures 4 to 6).

Based on microscopic appearance, 8 mould species were identified (Figure 7) among the 33 mould clusters. These were *Colletotrichum gloeosporioides*, *Pestalotia heterocornis*, *Phomopsis anacardii*, *Curvularia lunata*, *Fusarium oxysporum*, *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus ochraceus* (Table 2).

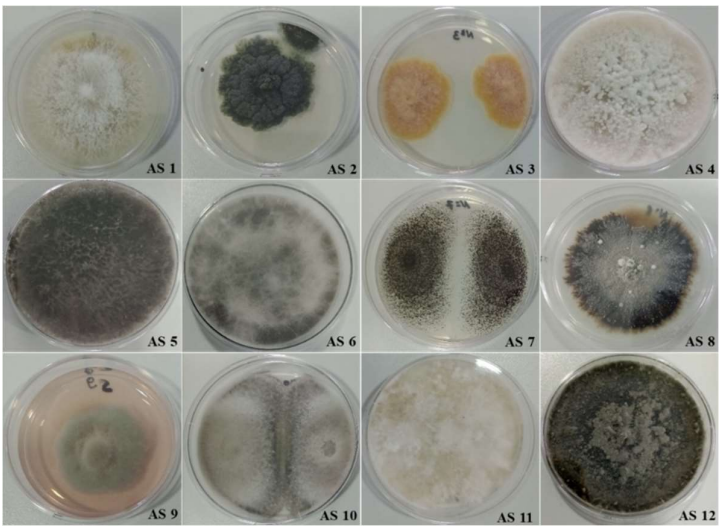


Fig. 4. *Illustration of mould clusters identified using cultural characteristics (AS 1 to 12)*

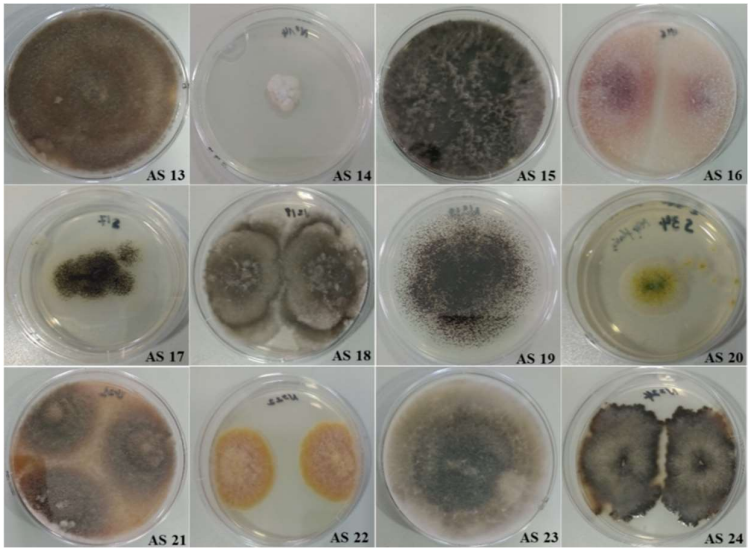


Fig. 5. *Illustration of mould clusters identified using cultural characteristics (AS13 to 24)*

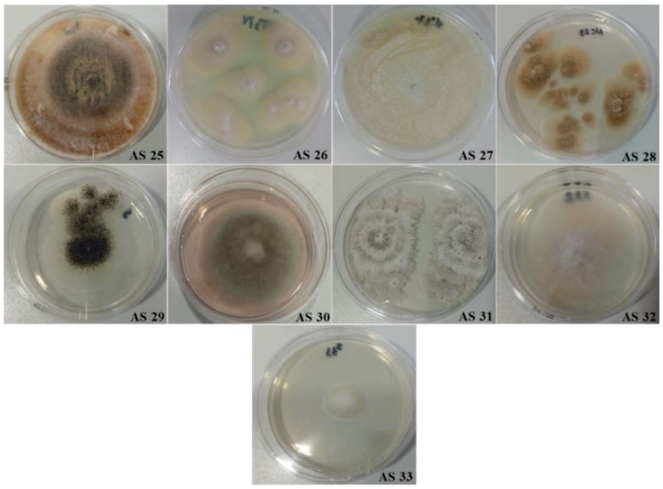


Fig. 6. *Illustration of mould clusters identified using cultural characteristics (AS25 to 33)*

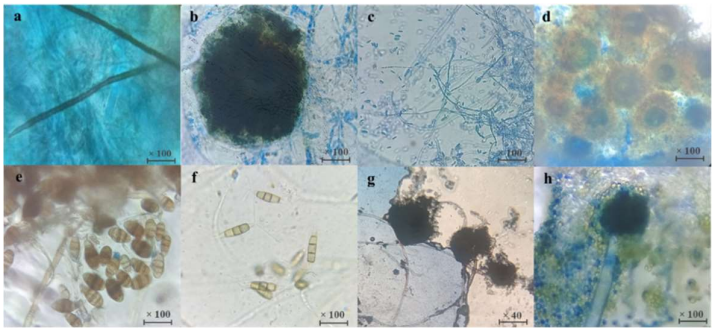


Fig. 7. *Microscopic appearance of mould species: a. Brown, septate hyphae with the presence of acervuli bearing bristles. The spores are unicellular, hyaline, cylindrical with rounded edges; b. Hyaline, septate and branched hyphae and asci present. The asci are subglobose to globose, smooth, dark brown to black, without ostioles, with a very thin membrane containing 4 to 8 ascospores; c. Septate, branched and thin hyphae. Presence of short conidiophores bearing unicellular microconidia and elongated, slightly curved macroconidia (2 to 5 lodges); d. Presence of brown conidiophores at the ends of which are aspergillate heads bearing hyaline unicellular spores erected in two rows of sterigmata; e. Brown, septate and branched hyphae. The spores are brown, multicellular (5-celled), with two large cells in the centre; f. Septate, branched, non-pigmented and thin hyphae. The spores are brown, multicellular (3 chambers) and ellipsoidal in shape with heterogeneous edges (1 and 2 tentacles spread out at the edges); g. Brown conidiophores bearing round, hyaline, unicellular spores arranged in very long chains erected on two rows of sterigmata on the aspergillum head; h. Brown conidiophores with globular ends bearing sterigmata on which round, hyaline, unicellular spores are arranged in chains*

### 3.2. DNA Samples Quantification and Purity

As shown in Table 3, the concentrations of all DNA samples analysed ranged from 14 to 130 µg/ml. The purity analysis of the 33 DNA samples reveals that the ratio of absorbance at 260 and 280 nm (A260/280 ratio) of 13 of the DNA samples is below the normal range of 1.8-1.9. In contrast, the A260/230 nm ratio of the 33 DNA samples is below the normal range of 2.0-2.2. Despite the suboptimal purity ratios, PCR amplification was successful, suggesting sufficient DNA quality for ITS analysis. The 33 amplicon samples appear as clear bands and have a molecular weight of approximately 600 bp each (Figure 8).

Table 2

*Eight cashew's pathogenic moulds identified by morphological characterization*

| Isolates | Species | Growth rate [mm] | Texture | Topography | Colour | Pigment | Spore | Asca |
|---|---|---|---|---|---|---|---|---|
| 1 | *Colletotrichum gloeosporioides* | rapid (47) | woolly | raised | gray – brown | absent | unicellular, cylindrical with rounded edges | absent |
| 2 | *Pestalotia heterocornis* | rapid (55) | downy | cerebriform with radial streaks | white | absent | pluricellular, ellipsoidal with heterogeneous margins (presence of tentacles) | absent |
| 3 | *Phomopsis anacardii* | rapid (68) | downy | cerebriform with radial striae | brown – gray | absent | Not reported | subglobose to globose (4 to 8 ascospores) |
| 4 | *Fusarium oxysporum* | rapid (34) | downy | flat | purplish – white | absent | elongated microspore and macrospore (2 to 5 lodges) | absent |
| 5 | *Aspergillus flavus* | rapid (55) | powdery | flat | yellowish – white | absent | round unicellular | absent |
| 6 | *Aspergillus niger* | rapid (45) | powdery | flat | black | absent | unicellular round | absent |
| 7 | *Curvularia lunata* | rapid (85) | woolly | raised | olive – brown | absent | curved spore with 4 cells | absent |
| 8 | *Aspergillus ochraceus* | slow (16) | powdery | flat | ochre | absent | unicellular round | absent |

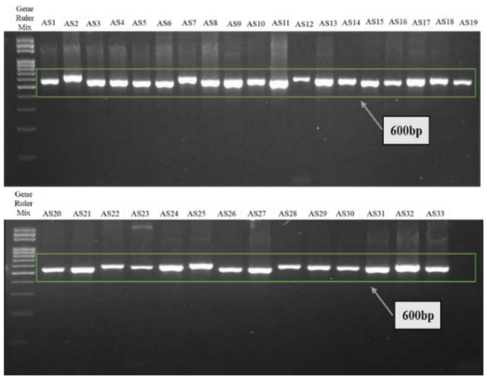


Fig. 8. *Representative illustrative of a 2% agarose gel highlighting the amplicons from mould samples nested PCR*

*DNA concentration and purity of thirty-three mould clusters* Table 3

| Mould DNA clusters | Concentrations [µg/ml] | A260 | A260/280 | A260/230 | Fungal DNA clusters | Concentrations [µg/ml] | A260 | A260/280 | A260/230 |
|---|---|---|---|---|---|---|---|---|---|
| AS 1 | 22 | 0.019 | 1.833 | 0.361 | AS 18 | 39 | 0.041 | 1.696 | 0.886 |
| AS 2 | 101 | 0.119 | 1.712 | 0.093 | AS 19 | 110 | 0.118 | 1.746 | 1.019 |
| AS 3 | 84 | 0.112 | 1.527 | 0.535 | AS 20 | 83 | 0.082 | 1.844 | 1.361 |
| AS 4 | 47 | 0.061 | 1.382 | 0.362 | AS 21 | 85 | 0.086 | 1.848 | 0.109 |
| AS 5 | 50 | 0.064 | 1.562 | 0.625 | AS 22 | 14 | 0.013 | 1.556 | 0.359 |
| AS 6 | 95 | 0.152 | 1.638 | 0.646 | AS 23 | 38 | 0.039 | 1.727 | 0.442 |
| AS 7 | 46 | 0.054 | 1.704 | 0.939 | AS 24 | 31 | 0.055 | 1.550 | 0.316 |
| AS 8 | 85 | 0.95 | 1.848 | 1.000 | AS 25 | 15 | 0.016 | 1.667 | 0.500 |
| AS 9 | 56 | 0.054 | 1.806 | 1.191 | AS 26 | 73 | 0.086 | 1.825 | 1.304 |
| AS 10 | 35 | 0.037 | 1.842 | 0.795 | AS 27 | 19 | 0.034 | 1.462 | 0.260 |
| AS 11 | 130 | 0.144 | 1.781 | 0.396 | AS 28 | 99 | 0.121 | 1.833 | 1.338 |
| AS 12 | 31 | 0.03 | 1.824 | 0.193 | AS 29 | 59 | 0.079 | 1.735 | 0.527 |
| AS 13 | 64 | 0.064 | 1.829 | 1.032 | AS 30 | 83 | 0.505 | 1.886 | 0.033 |
| AS 14 | 15 | 0.022 | 1.667 | 0.517 | AS 31 | 126 | 0.149 | 1.800 | 0.380 |
| AS 15 | 35 | 0.041 | 1.750 | 0.921 | AS 32 | 66 | 0.069 | 1.737 | 0.286 |
| AS 16 | 58 | 0.057 | 1.812 | 1.487 | AS 33 | 74 | 0.089 | 1.609 | 0.474 |
| AS 17 | 73 | 0.087 | 1.659 | 0.529 | | | | | |

Note: A (absorbance), AS (mould clusters)

### 3.3. BLASTn Sequence Analysis

The percent identity exhibited high similarity between the 33 target mould sequences and the sequences already present in the NCBI database, with a percent identity exceeding 98%. This analysis enabled the identification of 27 mould species belonging to 13 genera (Table 4).

*Mould species identified using BLASTn* Table 4

| ITS | Code no. | Mould identity after BLASTn | nbp | PID [%] |
|---|---|---|---|---|
| 1 | AS1 | *D. discoidispora* (Ref. MH371253.1) | 517 | 98.81 |
| 1 | AS2 | *Phyllosticta capitalensis* (Ref. OR742009.1) | 562 | 100 |
| 1 | AS3 | *F. decemcellulare* (Ref. MH857667.1) | 389 | 98.46 |
| 1 | AS4 | *Pestalotiopsis aeruginea* (Ref. KM111479.1) | 384 | 99.74 |
| 1 | AS5 | *Lasiodiplodia theobromae* Ref. OR018404.1) | 379 | 100 |
| 1 | AS6 | *L. pseudotheobromae* (Ref. OP999707.1) | 382 | 100 |
| 1 | AS7 | *A. niger* (Ref. OP737648.1) | 438 | 100 |
| 1 | AS8 | *Chaetomium* sp. (Ref. MT261910.1) | 397 | 100 |
| 1 | AS9 | *Corynespora cassiicola* (Ref. MT470607.1) | 387 | 100 |
| 1 | AS10 | *C. gloeosporioides* (Ref. MK311219.1) | 475 | 99.79 |
| 1 | AS11 | *P. microspora* (Ref. MT568595.1) | 479 | 100 |
| 1 | AS12 | *Pseudofusicocum violaceum* (Ref. OL414950.1) | 503 | 99.6 |
| 1 | AS13 | *C. cassiicola* (Ref. OR459957.1) | 483 | 100 |
| 1 | AS14 | *Chaetomium nigricolor* (Ref. MN180855.1) | 495 | 100 |
| 1 | AS15 | *L. crassispora* (Ref. KJ450853.1) | 481 | 100 |
| 1 | AS16 | *F. verticillioides* (Ref. CP079906.1) | 473 | 100 |
| 1 | AS17 | *A. aculeatus* (Ref. MT541884.1) | 511 | 100 |
| 1 | AS18 | *Curvularia eragrostidis* (Ref. MK886805.1) | 523 | 100 |
| 1 | AS19 | *A. sydowii* (Ref. MT582755.1) | 504 | 100 |
| 1 | AS20 | *A. flavus* (Ref. OR509893.1) | 530 | 100 |
| 1 | AS21 | *C. cassiicola* (Ref. OR517239.1) | 493 | 100 |
| 1 | AS22 | *F. decemcellulare* (Ref. MH857667.1) | 499 | 100 |
| 1 | AS23 | *D. schini* (Ref. MT470617.1) | 515 | 99.61 |
| 1 | AS24 | *Daldinia eschscholtzii* (Ref. MT507818.1) | 508 | 99.8 |
| 1 | AS25 | *Hypoxylon bellicolor* (Ref. MN056425.1) | 521 | 99.81 |
| 1 | AS26 | *F. fujikuroi* (Ref. OP392556.1) | 480 | 100 |
| 1 | AS27 | *F. oxysporum* (Ref. MN871804.1) | 479 | 100 |
| 1 | AS28 | *A. ochraceopetaliformis* (Ref. MH857406.1) | 542 | 100 |
| 1 | AS29 | *A. japonicus* (Ref. MT602615.1) | 512 | 100 |
| 1 | AS30 | *C. cassiicola* (Ref. MT470606.1) | 486 | 99.58 |
| 1 | AS31 | *D. sennae* (Ref. OM536197.1) | 517 | 98.46 |
| 1 | AS32 | *F. verticillioides* (Ref. CP079906.1) | 481 | 100 |
| 1 | AS33 | *F. oxysporum* (Ref. MN871804.1) | 481 | 100 |

Note: nbp (Molecular weight), PID (Percent identity); ITS (Internal Transcribe Sequences), BLASTn (Basic Local Alignment Search Tool for nucleotides)

### 3.4. Isolation Frequency

The results in Table 5 indicate the frequency of mould species isolated for each fungal disease. Anthracnose leaf spot was associated with 9 fungal species, followed by bud's dieback (8 fungal species), fruit anthracnose and pestalotiopsis (6 fungal species), yellow leaf spot, gummosis and ramularia (5 fungal species), powdery mildew (3 fungal species) and black rust (1 fungal species). In addition, *Diaporthe sennae* had the highest isolation frequency for leaf anthracnose (29.31%) and bud's dieback (31.58%). *Colletotrichum gloeosporioides, Pestalotiopsis microspora, Phyllostica capitalensis, Lasiodiplodia pseudotheobromae* and *Phyllostica capitalensis* had the highest isolation frequencies for fruit anthracnose (23.23%), pestalotiopsis (46.15%), yellow leaf spot (50%), gummosis (30.23%) and ramularia (30%), respectively. Similarly, *Daldinia eschscholtzii* and *Diaporthe schini* had the highest isolation frequency for powdery mildew (38.46%). Only *Aspergillus niger* was isolated from black rust samples (100%).

*Isolation frequency of mould species* Table 5

| Fungal diseases | Mould species | Isolation frequency [%] |
|---|---|---|
| Anthracnose leaf spot | 1. *D. sennae* | 29.31 |
| | 2. *P. capitalensis* | 25.86 |
| | 3. *A. niger* | 3.44 |
| | 4. *Chaetomium* sp | 3.44 |
| | 5. *C. cassiicola* | 6.89 |
| | 6. *C. gloesporioides* | 13.79 |
| | 7. *F. verticillioides* | 6.89 |
| | 8. *F. fujikuroi* | 6.89 |
| | 9. *F. decemcellulare* | 3.44 |
| Anthracnose fruit spot | 1. *C. gloesporioides* | 23.3 |
| | 2. *P. aeruginea* | 6.7 |
| | 3. *L. crassispora* | 40 |
| | 4. *A. flavus* | 10 |
| | 5. *A. niger* | 10 |
| | 6. *H. bellicolor* | 10 |
| Black rust | A. niger | 100 |
| Bud's dieback | 1. *C. cassiicola* | 5.26 |
| | 2. *C. eragrostidis* | 21.05 |
| | 3. *D. sennae* | 31.58 |
| | 4. *D. discoidispora* | 21.05 |
| | 5. *A. ochraceopetaliformis* | 5.26 |
| | 6. *F. verticillioides* | 5.26 |
| | 7. *C. corynespora* | 5.26 |

| | | |
|---|---|---|
| | 8. *A. niger* | 5.26 |
| Yellow leaf spot | 1. *P. capitalensis* | 50 |
| | 2. *P. violaceum* | 20 |
| | 3. *C. corynespora* | 10 |
| | 4. *C. nigricolor* | 10 |
| | 5. *D. eschscholtzii* | 10 |
| Pestalotiopsis | 1. *P. microspora* | 46.15 |
| | 2. *P. aeruginea* | 19.23 |
| | 3. *C. nigricolor* | 7.69 |
| | 4. *C. corynespora* | 11.54 |
| | 5. *F. decemcellulare* | 7.69 |
| | 6. *F. oxysporum* | 7.69 |
| Powdery mildew | 1. *D. eschscholtzii* | 38.46 |
| | 2. *D. schini* | 38.46 |
| | 3. *A. japonicus* | 23.08 |
| Gummosis | 1. *L. pseudotheobromae* | 30.23 |
| | 2. *L. theobromae* | 25.58 |
| | 3. *L. crassispora* | 11.63 |
| | 4. *P. violaceum* | 27.91 |
| | 5. *A. ochraceopetaliformis* | 4.65 |
| Ramularia | 1. *A. sydowii* | 20 |
| | 2. *A. aculeatus* | 10 |
| | 3. *A. ochraceopetaliformis* | 20 |
| | 4. *D. discoidispora* | 20 |
| | 5. *P. capitalensis* | 30 |

## 4. Discussion

The isolation of moulds from samples of infected cashew organs confirmed the diagnosis of fungal diseases. Morphological characteristics were used to identify 24.24% of the 248 moulds isolated in this study. This low identification rate was linked to the lack of sporulation in most mould isolates. This observation corroborates that of Santos et al. [40], who reported that classifying fungi based on morphological characteristics has major limitations, such as the non-sporulation of fungi and/or morphological similarities between different species. The non-sporulation of these mould isolates is often linked either to their physiological state and/or to the photoperiod. In their study, Latge and Sanglier [28] reported that physical and nutritional factors influenced the growth and sporulation of *Conidiobolus obscurus*. These authors also reported that a temperature of 20°C, total darkness and a culture medium with a neutral pH of approximately 6.5 could be more conducive to mold sporulation. The effectiveness of morphological characteristics in identifying fungal isolates was relative. This analysis is in agreement with that of Wei et al. [47], who reported that the morphological identification method relies heavily on subjective judgments. Although the morphological identification method is simple, morphological characteristics can be

influenced by environmental and growth factors and are sometimes variable, vague and confusing [51]. In contrast, molecular biology is widely used for species identification because it is rapid, specific, sensitive and relatively accurate [50]. The variation in DNA concentration per mould isolate may be related to the amount of fungal biomass used or the DNA extraction method employed. The constraints associated with collecting fungal biomass from moulds belonging to the *Fusarium* and *Aspergillus* genera could explain the low concentration of their DNA samples.

The results of the A260/280 nm and A260/230 nm ratios provided information on the purity of the DNA samples. An A260/280 ratio of less than 1.80 indicated contamination of the DNA extracted by proteins, whereas an A260/230 ratio of less than 2 indicated contamination of the DNA extracted by phenolic compounds, polysaccharides and Ethylenediaminetetraacetic Acid (EDTA). These results are similar to those of González-Mendoza et al. [25], who suggested that the DNA extract was pure when the A260/280 absorbance ratios of the isolated DNA were approximately 1.9 and negligible polysaccharide contamination when the A260/230 values were above 1.8. For good nucleic acid preparation, the A260/280 ratio, which represents protein contamination, should be between 1.8 and 2.0, whereas the A260/230 ratio, which represents carbohydrate contamination, should be greater than 2.0 [27].

The high specific diversity of pathogenic moulds infecting cashews was revealed through molecular identification using PCR-ITS. Three species of mould belonging to *Lasiodiplodia* genus (*L. theobromae*, *L. crassispora* and *L. pseudotheobromae*) were isolated from gummosis-affected stems and twigs. These results are consistent with those obtained by Monteiro et al. [33] who isolated *L. theobromae, L. pseudotheobromae* and *L. caatinguensis* from samples of cashew organs infected by gommosis. A previous study on cashew gommosis identified *L. theobromae* as the causal agent [35]. Furthermore, Cardoso et al. [7] and Coutinho et al. [13] established, through pathogenicity tests, that *L. theobromae* is the causal agent of gommosis. *D. discoidispora* and *Diaporthe sennae* were isolated from the bud's dieback of cashews. These results are similar to those of Dhanushka et al. [14], who reported *D. anacardii* (syn. *Phomopsis anacardii*) as the mould causing cashew bud's dieback. This means that different species of the genus *Diaporthe* (syn. *Phomopsis*) could be responsible for this disease. The attack of cashew by *D. anacardii* is thought to be facilitated by damage caused by mirids (*Helopeltis* spp.) or coconut bugs (*Pseudotheraptus wayii*) [31]. The pathogen attacks buds and flowers and then spreads within infected organs. The symptoms of bud's dieback are similar to those of damage caused by fire on trees [16]. Shoot necrosis may cause total defoliation in the most severe cases of infection.

*P. aeruginea* and *P. microspora* species were isolated from cashew samples infected by pestalotiopsis. Similar studies on cashew organs infected by pestalotiopsis have isolated several species belonging to the genus *Pestalotiopsis* (Syn. *Pestalotia*) [2, 41]. These findings suggest that different species of the genus *Pestalotiopsis* are associated with cashew pestalotiosis. A pathogenicity test using a suspension of *P. heterocormis* spores

revealed reddish-brown spots on the leaves of inoculated cashew trees [18]. The study's results revealed that the specific diversity of the *Fusarium* and *Aspergillus* genera was greater than that of the other genera identified. The involvement of species of *Fusarium* and *Aspergillus* in the infection of cashew nuts, leaves and flowers has been revealed by several authors [3, 17, 21, 49]. *F. oxysporum* has been implicated in cashew nut rot [1, 26] and is responsible for fusarium wilt in cashews [45]. In their study, Monteiro et al. [34] reported that, of the moulds infecting the aerial parts of cashew trees, only those belonging to the *Fusarium* genus were found in infected roots. According to Castillo et al. [10], the main mycotoxin-producing species belong to *Fusarium*, *Aspergillus* and *Penicillium*. Owing to their mycotoxinogenic capacity [11], *A. flavus, A. ochraceopetaliformis* and *F. verticillioides* isolated in this study could, under favourable conditions, produce mycotoxins in cashew fruits. This would reduce the organoleptic quality of the fruits and lead to post-harvest losses.

*Chaetomium* sp. and *C. nigricolor* are associated with anthracnose and pestalotiosis, respectively. Madhukeshwara et al. [30] and Parlindo et al. [37] reported different strains of *Chaetomium* among the endophytic fungi of cashew. *C. brassiliense* is associated with cashew nut blight Khatoon et al. [26] and *C. nigricolor* is known to produce beneficial secondary metabolites, such as those with antitumour, cytotoxic, antimalarial and enzyme inhibitory activities [20]. *C. gloeosporioides* strains were isolated from anthracnose-infected leaf and fruit samples. These results are in agreement with those of Dooh et al. [18], who showed through pathogenicity tests that anthracnose is caused by *C. gloeosporioides*. Different strains of this pathogenic fungus have been reported in numerous studies [23, 35]. *Glomerella cingulata* is the teleomorphic form of *C. gloeosporioides* [48]. It is common in other tropical fruit trees, such as avocado, banana, citrus, mango and papaya [23].

In this study, *P. capitalensis* is associated with anthracnose, yellow leaf spot and ramularia. Several species belonging to the *Phyllostica* genus have been isolated from samples of organs infected with anthracnose [26]. *P. anacardiacearum* (syn. *Phyllosticta capitalensis*) is the anamorph of *Guignardia mangiferae,* which is responsible for Guignardia leaf spot on mango [39]. The association of *P. capitalensis* with anthracnose suggests that this mould could also cause cashew rot. *P. violaceum* was associated with the species *L. theobromae* and *L. pseudotheobromae*, which were isolated from samples of cashew organs infected by gummosis. A similar observation was made by Netto et al. [36], who reported that *L. theobromae*, although the causal agent of cashew gummosis, was associated with other Botryosphaeriaceae species, such as *Neofusicoccum batangarum* and *Pseudofusicoccum stromaticum*. Moulds belonging to the *Curvularia* and *Corynespora* genera have been associated with various fungal diseases of cashew [17, 23, 49]. *D. eschscholtzii* is known to be a lignicolous endophyte or saprophyte that is widespread in warm tropical climates [43]. Chigozie et al. [12] and Liu et al. [29] isolated *D. eschscholtzii* from *Pogostemon cablin* and *Musa paradisiaca* leaves, respectively.

The results of the isolation frequency analysis revealed that, for each fungal disease, other fungal species were isolated

alongside the disease agent. The presence of the latter would have favoured infection of the disease agent, or they would have become established following the onset of the disease agent. Similar observations were made by Domedjui et al. [17], who reported the presence of superinfection strains in many cashew fungal infections. Furthermore, the absence of *O. anacardii*, *R. areola* and *Cryptosporoiodes* spp., which are causal agents of powdery mildew, ramularia and black rust, respectively, among the mould species isolated may be related to the advanced infectious state of the cashew nut samples used for fungal isolation. Although using BLASTn to characterise the nucleotide sequences of ITS1 primer amplicons improved the results of morphological identification, the results would be even better if the ITS4 primer were also taken into account. In their study, Baturo-Ciesniewska et al. [4] reported that identification based on rDNA ITS region is usually supplemented by morphological observations and analysis of additional genes, as this region is not equally variable in all fungal groups.

## 5. Conclusion

This study's aim is to isolate and characterise the diversity of pathogenic moulds associated with eight (08) cashew fungal diseases. A total of 248 pathogenic moulds were isolated from infected organ samples. Morphological identification of these isolates was limited because of 75.76% were non-sporulated. Molecular identification using PCR-ITS revealed a large diversity of cashew's pathogenic mould species. *Phyllostica*, *Xylariaceae*, *Curvularia*, *Corynespora*, *Colletotrichum*, *Pseudofusicoceum*, *Daldinia*, *Diaporthe* (syn: *Phomopsis*), *Pestalotiopsis* (Syn: *Pestalotia*), *Lasiodiplodia*, *Fusarium*, *Aspergillus*, and *Chaetomium* genera have been reported. Under favorable conditions, these mould species could lead to major losses in cashew nut production, as well as postharvest losses through the production of mycotoxins in the fruit. The use of plant extracts or microorganisms antagonistic to the phytopathogenic fungi isolated could be a promising approach to control cashew fungal diseases.

## Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Lucien Komi Domedjui, Camelia Filofteia Diguta and Koffi Apeti Gbogbo. The first draft of the manuscript was written by Lucien Komi Domedjui and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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