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Morphological, pathological and phylogenetic analyses identify a diverse group of *Colletotrichum* spp. causing leaf, pod and flower diseases on the orphan legume African yam bean

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Abstract

African yam bean (AYB; *Sphenostylis stenocarpa*) is an underutilized legume indigenous to Africa with great potential to enhance food security and offer nutritional and medicinal opportunities. However, low grain yield caused by fungal diseases, including pod blight and leaf tip dieback, deters farmers from large-scale cultivation. To determine the prevalence of fungal diseases affecting leaves, pods and flowers of AYB, a survey was conducted in 2018 and 2019 in major AYB-growing areas in Nigeria. Leaf tip dieback, flower bud rot and pod blight were the most common symptoms. Morphological and molecular assays were conducted to identify the causal agents of the observed diseases. In all the samples examined, fungi from eight genera were isolated from diseased leaves, buds and pods. Koch's postulates were fulfilled only for fungi belonging to the *Colletotrichum* genus. Fungi from the other seven genera did not produce disease symptoms in healthy AYB tissues. Several *Colletotrichum* isolates were characterized by sequencing the rDNA internal transcribed spacer (ITS), *glyceraldehyde-3-phosphate dehydrogenase*, *calmodulin* and *ApMAT* loci. A combined phylogenetic analysis revealed four *Colletotrichum* species: *C. siamense*, *C. theobromicola* and *C. fructicola*, which were recovered from diseased leaves, and *C. truncatum*, recovered from diseased pods and buds. Our results are useful to gear efforts to develop integrated management strategies to control diseases affecting AYB in Nigeria and elsewhere. Availability of such strategies may stimulate greater AYB cultivation, which can contribute to diet diversification, something repeatedly advocated by a range of stakeholders to increase food security and prosperity of smallholder farmers.

KEYWORDS
African yam bean, anthracnose, integrated management, orphan crop, polyphasic approach

1 | INTRODUCTION

African yam bean (AYB; *Sphenostylis stenocarpa*) is a tuberous legume that belongs to the *Fabaceae* family. The crop has the capacity to withstand climatic stresses such as heat and drought, thrives well in marginal soils and improves soil quality, thereby possessing great potential to enhance food security in various African countries (Nnamani et al., 2017, 2021; Ojuederie & Balogun, 2019). However, AYB is underutilized. It produces two organs of economic importance, grains and tubers, although not all AYB accessions produce tubers (Adewale & Nnamani, 2022), which are consumed based on regional preferences and beliefs. In West Africa, grains are consumed, but many farmers are unaware that AYB can tuberize, and many of those who are aware believe that the tubers are poisonous. In contrast, in East Africa, the tubers are consumed, but many farmers believe the grains are poisonous (Adewale & Nnamani, 2022; Potter & Doyle, 1992). However, both grains and tubers have enormous benefits. They are safe for human and livestock consumption and have nutritional and medicinal benefits (Christopher et al., 2013; Nwankwo et al., 2018). The tubers are rich in crude protein, total ash and fat (Konyeme et al., 2020). The grains are rich in minerals, vitamins (Ajibola & Olapade, 2016) and fibre (Anya & Ozung, 2019). In traditional medicine administered in Enugu state, Nigeria, AYB grains are used to treat insomnia, measles and diabetes (Nnamani et al., 2021).

Fungal diseases are one of the factors deterring farmers from large-scale cultivation and germplasm regeneration of AYB (Afolabi et al., 2019; Ameh & Okezie, 2005). Several diseases of AYB including powdery mildew, leaf spot, stem rust, wilt (Ameh & Okezie, 2005), pod blight, flower bud rot and tip dieback have been reported (Afolabi et al., 2019). In AYB fields managed by researchers of the International Institute of Tropical Agriculture (IITA), located in south-west Nigeria, tip dieback, wilt, flower bud rot and pod blight are diseases associated with AYB (authors' unpublished observations). These observations raised questions on whether these diseases are common to AYB in all active AYB-growing regions of Nigeria and what pathogens are responsible for these diseases.

Most reports on fungal diseases associated with AYB have been informal or have not focused on the identification of the causal agents of the diseases. For example, Afolabi et al. (2019) found that fungi from 13 genera were associated with AYB flower bud rot and pod rot diseases, but Koch's postulates were not fulfilled. Accurate identification of causal agents of important diseases of AYB is crucial for disease management and breeding purposes. To accurately identify microorganisms associated with a disease, a polyphasic approach is required. Relying on a single identification method may not provide sufficient information to correctly identify the causal agent of a disease (Cai et al., 2009; Simões et al., 2013). Thus, the objectives of the current study were to identify diseases associated with AYB in major AYB-growing areas in Nigeria and to characterize the pathogenic fungi employing a polyphasic approach composed of morphological, phylogenetic and pathogenicity assays.

2 | MATERIALS AND METHODS

2.1 | Sample collection

A survey was conducted between 2018 and 2019 in major AYB-growing areas in Nigeria to investigate fungal diseases associated with AYB. Samples of diseased AYB tissues were collected from 36 farmers' fields in Enugu, Ebonyi and Abia (located in south-east Nigeria) and Cross River states (located in south Nigeria; Figure 1), as well as from two AYB research fields in IITA-Ibadan in Oyo state (south-west Nigeria; Figure 1). At each survey site, a zigzag transect (area 3 m^2) was used to randomly select 10 plants of about 5-months old for visual examination and sample collection. Pods, leaves and flower buds were visually examined for the signs of fungal infection: necrotic lesions or discolouration on leaves, browning on flower buds and rot or lesions in pods. A total of 360 leaf samples (10 per field) were collected from farmers' fields, placed in appropriately labelled bags, and transferred to a plant press for preservation. Due to the distance from the laboratory and lack of resources to keep plant materials fresh, only leaf samples were collected from farmers' fields in the south-east/south. Information on the specific AYB accessions sampled was not available. IITA research fields in Ibadan were closer to the laboratory and had access to ice to preserve the samples, hence various sample types (20 leaves, 20 diseased pods and 10 flower buds) were collected from 20 AYB accessions in these fields.

2.2 | Fungal isolation

Fungal isolates were recovered from infected AYB tissues (buds, pods and leaves). Appropriate pieces of material (about 6 mm^2, comprising 1/3 diseased and 2/3 healthy tissue) were excised with a sterile scalpel and surface sterilized in 50% (vol/vol) NaOCl for 30 s. Samples were then triple rinsed with sterile distilled water (SDW), and then blotted dry using sterile paper towels in a Class II biosafety cabinet. With the aid of a mounting needle, four segments of the sterilized plant tissues were placed in Petri dishes containing acidified potato dextrose agar (PDA + 0.1% lactic acid). Petri dishes were incubated for 3 days at room temperature (25–28°C). A maximum of four discrete colonies of fungi per sample were subcultured onto PDA and incubated at 25–28°C for 5 days. Then, cultures of each fungal isolate recovered were single-spored using the method of Goh (1999), with minor modifications. The procedure entailed transferring spore masses with the aid of a sterile toothpick and suspending them into SDW. The suspensions were then diluted 1000-fold. Subsequently, 80 μL of each diluted suspension was spread evenly onto the surface of water agar plates that were incubated overnight at 25°C.

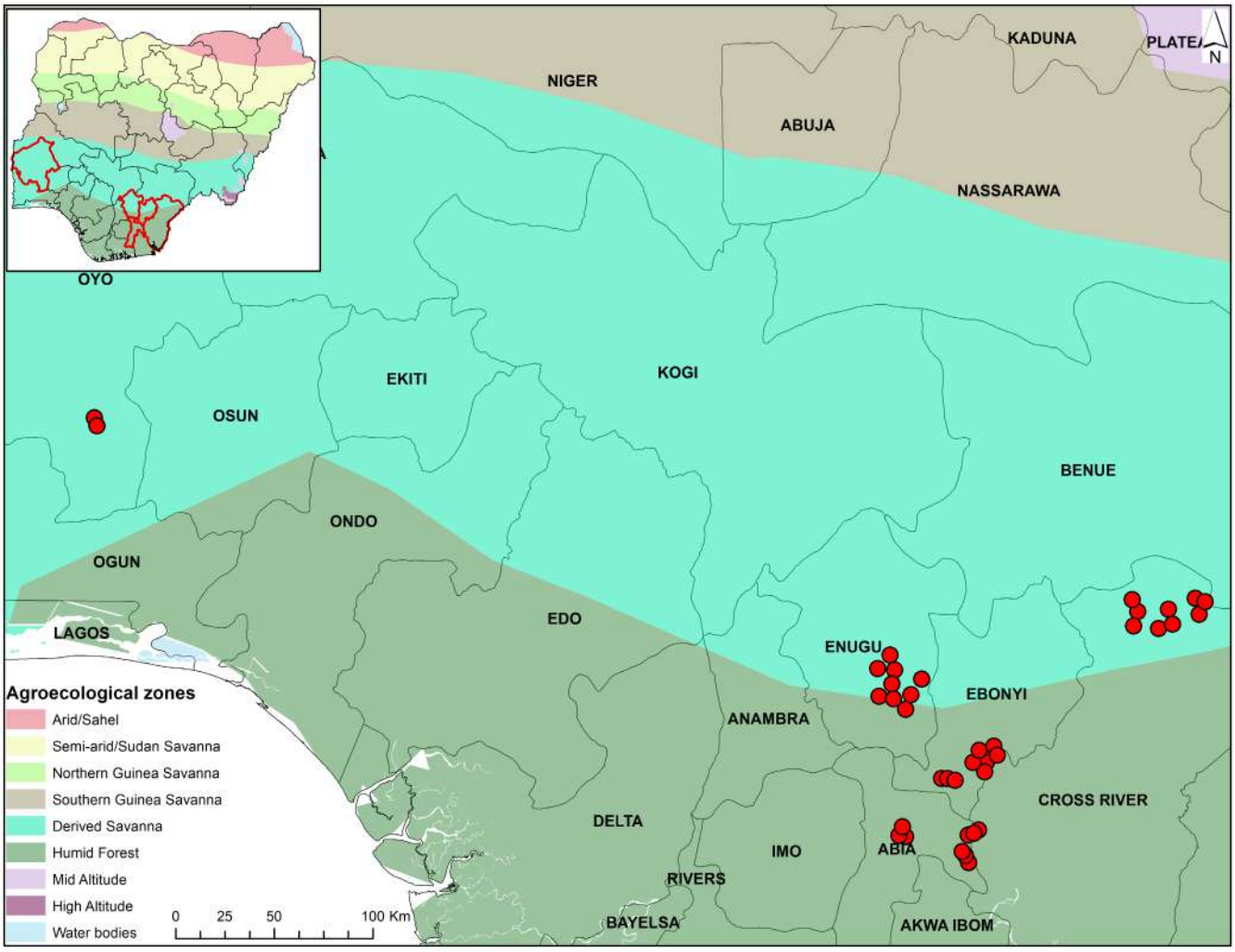

FIGURE 1 Area of study in Nigeria, with red circles representing the locations of African yam bean fields sampled between 2018 and 2019 with the aim of identifying diseases prevalent in these regions and elucidating their respective causal agents.

Under the dissecting microscope (Wild Heerbrugg), single germinated spores were identified. Each individual spore, along with its surrounding agar block, was carefully excised using a sterile scalpel, and carefully transferred onto new PDA plates. Plates were incubated at 25°C to allow for fungal growth and sporulation.

Axenic cultures were identified through their morphology on PDA after 8 days of incubation and then conidia were viewed using a compound microscope (BX51; Olympus) at 40× magnification. The pure cultures of each isolate were maintained in the refrigerator at 4°C on Petri dishes and slopes containing PDA for about 14 days. Separate cultures were made for saving in 20% glycerol and stored at −80°C for long-term preservation.

2.3 | Planting material for pathogenicity assays

One accession of AYB, TSs 1, was used for the experiments. TSs 1 was selected because it is frequently used in diverse experiments in IITA and because of its known susceptibility to fungal diseases in on-station experiments. Seeds of TSs 1 were obtained from the Genetic Resources Centre (GRC) of IITA. Two seeds were sown in 8-kg pots filled with sterilized field soil; no fertilizer was added. The plants were grown in a screenhouse at IITA. Two months after planting, healthy leaves were carefully excised, placed in transparent bags, and SDW was slightly sprinkled on the leaves in the bags for immediate transportation to the laboratory.

2.4 | Box preparation for detached leaf assay

Clear plastic boxes (23×31×10 cm; Pioneer Plastics) with lids were used for the detached leaf assay (DLA). Boxes were sterilized in 50% NaOCl for 5 min, rinsed twice with SDW and immediately transferred into a Class II biosafety cabinet. The boxes were left to properly drain, after which the UV light in the cabinet was turned on for 10 min for further decontamination. Sterilized paper towels and cotton wool were laid in the base of the sterilized boxes and soaked with 100 mL SDW amended with 200 µL Hexacal (0.02% fungicide; Farmpays) to inhibit growth of saprophytic fungi. Detached leaves remained viable for up to 16 days.

2.5 | Inoculum preparation

Fungal isolates recovered from diseased AYB tissues (leaves and pods) were grouped based on their morphological features. From these groups, 25 representative isolates recovered from leaves and six representative isolates from pods were selected to be included in the DLA (Table 1). Conidia of each of the 31 isolates were washed off from 7-day-old PDA cultures by adding 2 mL SDW amended with Tween 20 (0.01% vol/vol). These suspensions were transferred to sterile 10 mL vials. The concentrations were adjusted to 10^6 conidia/mL using a haemocytometer (Improved Neubauer Bright Line; Hausser Scientific) and a compound microscope (Leitz Laborlux S; magnification 40×) with a graduated eyepiece.

2.6 | African yam bean leaf surface sterilization, inoculation and disease assessment

For the DLA, leaves were submerged for 30 s in SDW amended with an acaricide (100 ppm Vertimec; Syngenta) to prevent mite

TABLE 1 Morphological features and pathogenicity response of *Colletotrichum* spp. isolates from diseased African yam bean tissues collected in several locations in Nigeria.

Isolate code	State, local government area	Morphological characteristics on potato dextrose agar medium after 8 days of incubation	Isolate identification based on morphological characteristics[a]	Plant part origin[b]
S4L2F3	Enugu, Enugu South	Olivaceous aerial mycelia with rings of abundant orange conidia masses	*C. gloeosporioides* sp. complex	Leaf
S4L1F3	Enugu, Nkanu West	Olivaceous aerial mycelia	*C. gloeosporioides* sp. complex	Leaf
S4L2F1	Enugu, Enugu South	Grey aerial mycelia with rings of orange and black conidia masses	*C. gloeosporioides* sp. complex	Leaf
S4L1F1	Enugu, Nkanu West	Olivaceous aerial mycelia	*C. gloeosporioides* sp. complex	Leaf
S3L1F2	Ebonyi, Onicha	Grey aerial mycelia with no conidia mass	*C. gloeosporioides* sp. complex	Leaf
S3L1F1	Ebonyi, Onicha	Grey aerial mycelia with black conidia masses	*C. gloeosporioides* sp. complex	Leaf
S3L1F3	Ebonyi, Onicha	Olivaceous aerial mycelia with rings of black conidia masses	*C. gloeosporioides* sp. complex	Leaf
S3L2F2	Ebonyi, Afikpo North	Dark grey aerial mycelia with orange conidia masses	*C. gloeosporioides* sp. complex	Leaf
S3L3F3	Ebonyi, Afikpo South	Dark grey aerial mycelia with black conidia masses	*C. gloeosporioides* sp. complex	Leaf
S3L2F1[c]	Ebonyi, Afikpo South	Dark grey aerial mycelia with black conidia masses	*C. gloeosporioides* sp. complex	Leaf
S2L1F2	Cross River, Bekwarra	Olivaceous aerial mycelia with rings of orange and black conidia masses	*C. gloeosporioides* sp. complex	Leaf
S2L2F2	Cross River, Yala	Olivaceous aerial mycelia with rings of orange and black conidia masses	*C. gloeosporioides* sp. complex	Leaf
S2L3F2	Cross River, Yala	Olivaceous-grey aerial mycelia with few orange conidia masses	*C. gloeosporioides* sp. complex	Leaf
S2L1F3	Cross River, Yala	Olivaceous aerial mycelia with abundant black and few orange conidia masses	*C. gloeosporioides* sp. complex	Leaf
TSs 25	Oyo, Akinyele	Aerial mycelia are dark grey and white with black conidia masses	*C. gloeosporioides* sp. complex	Pod
TSs 29B, TSs 61, TSs 98, TSs 421, TSs 432, Pod 6	Oyo, Akinyele	Whitish-grey aerial mycelia	*C. truncatum*	Pod

Note: The table contains information of isolates that were later found to be pathogenic on African yam bean tissues. Isolates from the seven genera that did not produce disease symptoms (*Fusarium*, *Curvularia*, *Pestalotia*, *Botryodiplodia*, *Seridium*, *Exserohilum* and *Drechslera*) are not listed in the table.

[a]All *C. gloeosporioides* species complex isolates had conidia with rod shape and no presence of setae. The *C. truncatum* isolates had conidia with falcate shape and had presence of setae.

[b]All isolates recovered from leaves were tested in pathogenicity detached leaf assays while the isolates recovered from pods were tested in both detached leaf and pod assays.

[c]S3L2F1 was the sole isolate that did not produce disease symptoms in the pathogenicity tests.

infestation in the laboratory. Leaves were then surface sterilized with 1% NaOCl and dried, as described above in the fungal isolation section. Thereafter, four leaves were carefully placed in a labelled sterile clear plastic box with the adaxial side placed on wet paper towel while the abaxial side was inoculated using a pipette tip without wounding the leaves (Bankole et al., 2022). For each of the 25 evaluated isolates, 10 μL of the 10^6 conidia/mL suspension was inoculated onto the top and bottom regions of the leaf abaxial surface as described by Bankole et al. (2022). The experiment included mock-inoculated controls using Tween 20 (0.01% vol/vol). The boxes were sealed with plastic wrap cling film to help keep the chamber humid and incubated at room temperature (about 25°C) on the benchtop with a cycle of 12 h light/12 h dark for 8 days. Inoculated leaves were examined every 2 days for disease progression. Disease scoring was done in the biosafety cabinet, with plastic containers remaining sealed to avoid contamination. Necrotic lesions on leaves were visually assessed by the percentage area of leaf infected and scored on a scale of 1–5, where 1 = no disease symptom, 2 = <10%, 3 = 10%–25%, 4 = 26%–50%, 5 = >50% of leaf surface area showing symptoms. This scale was adapted from the one reported by Nwadili et al. (2017). At the end of the 8 days of incubation, sections of diseased AYB leaves were transferred to PDA to isolate and identify the causal agent of the observed disease. The experiments were set up in a complete randomized design (CRD) with four replications per isolate. The DLA was conducted twice on the same set of isolates.

2.7 | African yam bean pod collection, inoculation and disease assessment

In this experiment, 50 AYB seeds of TSs 1 were sown in a research field at IITA. Plots were formed by single 4-m ridges spaced 0.75 m apart. The seeds, treated with Mancozeb 80% wettable powder (WP), were planted 0.5 m apart on the ridges. Three weeks after seedling emergence, triple superphosphate fertilizer was applied at a rate of 50 kg/ha. Every 2 weeks, a mixture of Cypermethrin 30 g/L + Dimethoate 250 g/L EC and Mancozeb 80% WP was applied at a rate of 10 mL/L and 10 g/L, respectively. Standard agronomic practices such as weeding (when necessary) and staking (3 weeks after planting) were conducted at the appropriate time.

Healthy AYB pods (5 months old) were carefully excised and placed in a transparent bag that was lightly sprinkled with SDW before transporting to the laboratory. In the laboratory, the detached pods were sterilized for 1 min in distilled water amended with a drop of Vertimec, rinsed with SDW and blotted dry as described above. Thereafter, three pods were carefully placed in labelled sterile clear plastic boxes. Conidial suspensions for each of the six isolates recovered from pods, belonging to representative groups, were prepared as above, and for each isolate, 10 μL of 10^6 conidia/mL suspension was inoculated, without wounding, using a pipette tip onto three different points of a healthy pod (top, middle and bottom regions). The experiment included mock-inoculated controls using sterile Tween 20 (0.01% vol/vol). The boxes were sealed with plastic clingwrap film to help keep the chamber humid and incubated at room temperature (about 25°C) on the benchtop with a photoperiod cycle as above for 9 days.

Inoculated pods were examined every third day for disease progression and scoring under a sterile biosafety cabinet, with containers remaining sealed until the end of the experiment to avoid contamination. Necrotic lesions on pods were assessed by the percentage area of pod infected and scored on a scale of 1–5, where 1 = no disease symptom, 2 = 1%–10%, 3 = 11%–20%, 4 = 21%–50%, 5 = >50% of pod area showing symptoms (adapted from Nwadili et al., 2017). At the end of the 9 days of incubation, sections of diseased AYB pods were transferred to PDA to isolate and identify the causal agent of the observed disease. The experiments were set up in a CRD with four replications per isolate. The detached pod assay was conducted twice.

2.8 | Tissue specialization: Pathogenicity test of the recovered *Colletotrichum truncatum* on African yam bean leaves

The six isolates of *C. truncatum* were inoculated on AYB leaves following the DLA protocol described above, to test their ability to cause disease on leaves. Four isolates (S2L1F2, S2L1F3, S2L3F2 and S2L2F2) belonging to the *C. gloeosporioides* species complex were included in the test as positive controls, in addition to negative, mock-inoculated controls using Tween 20 (0.01% vol/vol). This assay was conducted twice.

2.9 | Extended characterization of pathogenic fungi

Isolates that were pathogenic in the detached leaf and pod assays, all showing characteristics of the *Colletotrichum* genus, were subjected to additional morphological characterization. Mycelial plugs were aseptically collected from actively growing edges of 7-day-old single-conidium subcultures using sterile 5-mm diameter plastic cork borers and placed individually at the centre of PDA plates. The cultures were incubated at 25–28°C, with three replicates per isolate. The colony growth rate was recorded every 2 days for 8 days. The conidia and appressorium shape were examined using a compound microscope (BX51; Olympus). Appressoria were produced using the slide culture technique (Cai et al., 2009). Conidial size (length and breadth) was measured using a compound microscope (Laborlux S; Leitz) and conidial shape was visually inspected.

2.10 | Statistical analysis

The disease score values of *Colletotrichum* spp. isolates evaluated in pathogenicity assays were subjected to Kruskal–Wallis test and uncorrected Dunn–Bonferroni tests (post hoc test) were used for

the pairwise multiple comparisons using GraphPad Prism v. 7.0. Student's *t* tests were used to compare the growth rate and spore size of *Colletotrichum* isolates. Graphs depicting the mean values with corresponding *SEM* were produced using GraphPad Prism v. 7.0.

2.11 | Genomic DNA extraction

Mycelia plugs (about 3 mm; one per isolate) of 5-day-old monosporic cultures of isolates found to be pathogenic were independently inoculated in potato dextrose broth (Difco) and shaken at 300 rpm for 5 days using a dual-action KL 2 orbital shaker (Edmund Buhler 7400). Mycelia were harvested from broth using vacuum filtration. The setup was as follows: a sterile porcelain Buchner funnel was placed on top of a sterile conical filtering flask to collect the filtrate. A vacuum pump was connected to the filter flask with appropriate tubing. Sterile filter paper of appropriate size was cut to fit the funnel. The mycelia were gently poured into the filter funnel and filtered until most of the liquid passed through. Thereafter, the mycelia were rinsed with SDW to remove residual medium. Then, a sterile spatula was used to collect the mycelia into a sterile Falcon tube and stored at 20°C. Two different methods were used for DNA extraction: Zymo Research-Quick DNA Fungal/Bacterial Miniprep Kit following the manufacturer's recommendation and Shorty buffer method (Edwards et al., 1991; Harrison & Thompson, 2020).

The Shorty buffer method, with minor adaptations as described by Harrison and Thompson (2020), is as follows: about 40 mg of mycelia were collected into Eppendorf tubes and subsequently pulverized in liquid nitrogen using sterile micropestles. Then, 500 μL sterile Shorty buffer (200 mM Tris–HCl pH 8, 400 mM LiCl, 25 mM EDTA pH 8, 1% wt/vol SDS) were added to the powdered mycelia. The mixture was vortexed for 10 s and then centrifuged at 16,000 *g* for 5 min. In separate Eppendorf tubes, 350 μL isopropanol was carefully added. Then, 350 μL of the supernatant obtained from the centrifugation step was pipetted into the tubes containing isopropanol. The mixture was gently inverted for 30 s to allow DNA precipitation and then centrifuged at 16,000 *g* for 15 min to pelletize the DNA. The supernatant was removed without disturbing the DNA pellet, and 500 μL 70% ethanol was added for DNA washing. A subsequent centrifugation step at 10,000 *g* for 2 min facilitated the separation of the ethanol, after which the supernatant was carefully pipetted off. To ensure the removal of residual isopropanol and ethanol, tubes were inverted and incubated at 37°C for 15 min. Finally, the DNA pellets were resuspended in 30 μL sterile TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8) to complete the extraction process.

2.12 | PCR, gel electrophoresis, DNA purification and sequencing

The primers used in the current study targeted the rDNA internal transcribed spacer (ITS), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *calmodulin* (*CAL*) and *Apn2-MAT 1–2* intergenic spacer (*ApMAT*) regions of the examined isolates. These primers have proven effective in identifying *Colletotrichum* species (Hassan et al., 2018). The primer pairs are listed in Table S1. For all primer pairs, the PCR mix (25 μL) consisted of 12.5 μL OneTaq Quick-Load (2× Master mix; New England Biolabs), 1.5 μL each of forward and reverse primers (10 μM), 1 μL DNA template and 8.5 μL nuclease-free water.

Initially, 42 isolates were subjected to PCR followed by sequencing of the ITS locus to select isolates for the multilocus sequence analysis. The PCR conditions for ITS involved 94°C for 3 min; followed by 30 cycles at 94°C for 45 s, 54°C for 30 s, 72°C for 45 s; a final 72°C for 7 min, and hold at 4°C. Amplicons were visualized on 1% agarose electrophoresis gels run at 100 V for 1 h in Tris-acetate-EDTA buffer. Amplicons were purified using a Monarch Genomic DNA purification kit following the manufacturer's recommendation, and sequenced bidirectionally by either IITA Bioscience Centre, Source Bioscience (UK) or Eurofins Genomics (Germany).

Based upon the ITS phylogenetic analysis, 13 isolates were further selected for analysis through sequencing portions of *GAPDH* and *CAL* loci. Selected isolates were chosen randomly from various locations to ensure representation from each state. The *ApMAT* locus was examined for the 10 isolates in the *C. gloeosporioides* species complex but not for the *C. truncatum* isolates due to its specific design for the *C. gloeosporioides* species complex (Silva et al., 2012). The PCR conditions were the same as those mentioned above, except for the annealing temperatures: *GAPDH* at 55°C, *CAL* at 59°C and *ApMAT* at 62°C.

2.13 | Phylogenetic analysis

The raw nucleotide dataset generated from sequencing the ITS locus for each isolate was reviewed, edited and assembled into consensus sequences using BioEdit Sequence Alignment Editor v. 7.2.5e (Hall, 1999). The ITS sequences of type *Colletotrichum* species were retrieved from NCBI for use as reference. MEGA X v. 10.2.6 (Kumar et al., 2018) was used to perform multiple sequence alignments and the statistical selection of best-fit models. The nucleotide substitution model with the lowest Bayesian information criterion (BIC) score was considered to describe the best substitution pattern and used for subsequent phylogenetic analysis. A maximum-likelihood phylogenetic tree was constructed using MEGA X v. 10.2.6 and the analysis was performed with 1000 bootstrap replicates to assess support for the resulting phylogenetic clades. The phylogenetic tree was exported and visualized using Figtree v. 1.4.4 (Rambaut, 2018).

The raw nucleotide datasets generated from sequencing the *CAL* and *GAPDH* regions of the 13 selected isolates were subjected to sequence editing and assembly to generate consensus as above. From NCBI, the *CAL* and *GAPDH* sequences of the type *Colletotrichum* isolates used above were also retrieved. The nucleotide datasets generated from sequencing the *ApMAT* region of the 10 *C. gloeosporioides* species complex isolates were subjected to post-sequencing analysis as above and the *ApMAT* region

TABLE 2 Origin, host crop and GenBank accession numbers of sequences of four loci of *Colletotrichum* isolates used in the current study.

Fungal species	Isolate code	Origin	Host	GenBank accession number[a]			
				ITS	*GAPDH*	*CAL*	*ApMAT*
C. aenigma	CG56	Japan	Strawberry	NW-023500921	NW-023500908	NW-023500911	NW-023500914
	ICMP 18608	Israel	Avocado	JX010244	JX010044	JX009683	KM360143
C. aeschynomenes	ICMP 17673	USA	Virginia jointvetch	JX010176	JX009930	JX009721	KM360145
C. fructicola	CGMCC3	China	Strawberry	NW-0224744931	NW-022474561	NW-022474237	NW-022474248
	ICMP 18581	Thailand	Arabian coffee	JX010165	JX010033	FJ917508	JQ807838
	S2L3F2	Nigeria	Africa yam bean	OQ586194	OQ581979	OQ581966	OQ581961
	S3L3F3	Nigeria	Africa yam bean	OQ586191	OQ581987	OQ581972	OQ581958
C. gloeosporioides	MTCC 10323	India	Orange	KC790935	–	–	JQ807843
	LC1	China	Chinese tulip tree	NW-025544752	NW-025544729	NW-025544669	NW-025544710
C. higginsianum	C5	China	Sorrel	MF033888	MF033889	MF033893	–
C. hymenocallidis	ICMP 18642	China	Beach spider lily	JX010278	JX010019	JX009709	JQ899283
C. jasmini-sambac	LC921	Vietnam	Arabian jasmine	HM131511	HM131497	HM131492	JQ807841
	S3L2F1	Nigeria	Africa yam bean	OQ586196	OQ581982	OQ581977	OQ581963
	S2L1F2	Nigeria	Africa yam bean	OQ586192	OQ581988	OQ581971	OQ581959
	S2L1F3	Nigeria	Africa yam bean	OQ586193	OQ581992	OQ581967	OQ581960
	TSs 57	Nigeria	Africa yam bean	OQ586189	OQ581994	OQ581974	OQ581956
	TSs 25	Nigeria	Africa yam bean	OQ586190	OQ581995	OQ581973	OQ581957
	S3L1F1	Nigeria	Africa yam bean	OQ586197	OQ581989	OQ581975	–
	S2L2F2	Nigeria	Africa yam bean	OQ586188	OQ581986	OQ581968	OQ581955
	S3L2F2	Nigeria	Africa yam bean	OQ586195	OQ581980	OQ581965	OQ581962
C. musae	ICMP 18701	Philippines	Banana	JX010145	JX010047	JX009687	–
C. nupharicola	CBS 470	USA	Yellow pond lily	JX145173	JX009972	JX009663	JX145319
	CBS 469.96; ICMP 17938	USA	Yellow pond lily	JX010189	JX009936	JX009661	–
C. siamense	ICK-3	Korea	Persimmon	LC260488	LC260529	LC260532	LC307171
	ICK-22	Korea	Persimmon	LC260489	LC260530	LC260533	LC307172
	ICK-23	Korea	Persimmon	LC260490	LC260531	LC260534	LC307173
	ICK-47	Korea	Persimmon	LC208833	LC208834	LC208835	LC307174
	Cg363	Japan	Strawberry	NW-023501017	NW-023501015	NW-023501018	NW-023501021
C. theobromicola	ICMP 17958	Australia	Stylo	JX010291	JX009948	JX009598	–
	S4L1F1	Nigeria	Africa yam bean	OQ586187	OQ581990	OQ581976	OQ581954
C. tropicale	CMM3767	Brazil	Mango	KC702985	KC702960	KC992378	KJ155464
C. truncatum	CBS 151.35	USA	Lima been	MH855611	GU228254	KY856132	–
	TSs 432	Nigeria	Africa yam bean	OQ586200	OQ581985	OQ581978	–
	Pod 6	Nigeria	Africa yam bean	OQ586198	OQ581991	OQ581969	–
	TSs 29	Nigeria	Africa yam bean	OQ586199	OQ581984	OQ581970	–

Note: Isolates in bold are those identified in the current study.

[a]*ApMAT*, Apn2-MAT 1–2 intergenic spacer; *CAL*, calmodulin gene; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase gene; ITS, rDNA internal transcribed spacer.

sequences of the same type *Colletotrichum* isolates were retrieved from NCBI (Table 2).

The consensus sequences of the 13 isolates were aligned and concatenated using Geneious Prime v. 2022.2.2 (Biomatters Ltd), to generate a composite consensus sequence for each isolate that contained the ITS region as well as the *CAL* and *GAPDH* loci, in addition to *ApMAT* for the 10 *C. gloeosporioides* species complex isolates. In the multigene phylogeny analysis, RAxML (Stamatakis, 2014), integrated

within Geneious Prime, was employed to partition the data by genetic region. Specifically, the K2 + G model was applied to ITS, *ApMAT* and *CAL*, while the K2 model was applied for *GAPDH*. These partitioning choices were based on the distinct evolutionary models best fitting each region, as determined by model selection criteria such as Akaike information criterion (AIC) and BIC. A maximum-likelihood phylogenetic tree was constructed and exported as above.

3 | RESULTS

3.1 | Field observations

A total of 380 plants were examined across locations. Healthy AYB tissues are shown in Figure 2a. The most common disease symptoms were pod blight, flower bud rot, blight and leaf tip dieback (Figure 2b–e). The frequency of these symptoms varied among locations. However, pods were most likely to show blight (mean = 53%, $N = 380$) whereas leaf tip dieback (mean = 38%, $N = 380$) and bud rot (mean = 37%, $N = 380$) were noted in fewer of the examined samples. The characteristic symptom of leaf tip dieback was a necrotic lesion at the leaf margin that progressed inwardly. Pod blight disease was characterized by irregular-shaped necrotic lesions with distinguishing signs of acervuli (ringed black mass of fruiting bodies) on pods. In addition, some infected pods were twisted and some contained few or no seeds. All diseased flower buds were completely brown and nonviable.

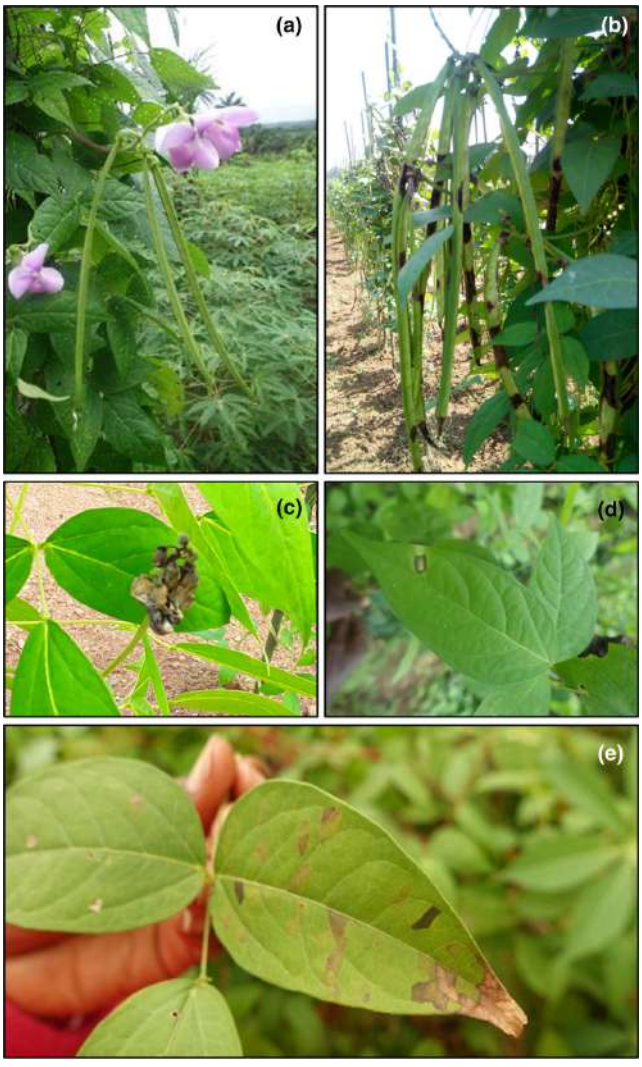

FIGURE 2 African yam bean plants in the visited fields. (a) Healthy pods, leaves and flowers; (b) blighted pods; (c) flower showing bud rot; (d) leaf blight; (e) leaf tip dieback.

3.2 | Fungal isolation from AYB tissues

A total of 231 fungal isolates were recovered from infected AYB tissues: 15 from diseased flower buds, 62 from diseased pods and 154 from diseased leaves. Based on morphological characteristics, eight fungal genera were identified among the recovered isolates. The most frequently isolated fungi from symptomatic leaves were isolates of the *C. gloeosporioides* species complex (52%; Figure 3). *Fusarium* spp., *Curvularia* spp. and *Pestalotia* spp. were also recovered from infected leaves at frequencies ranging from 12% to 18%. *C. truncatum* composed about 98% and 90% of the fungi recovered from the pods and flower buds, respectively, but was never recovered from leaves (Figure 3). *C. gloeosporioides* species complex isolates composed 2% of the fungi recovered from the pods (Figure 3). Other fungal genera occurring at frequencies equal to or less than 6% include *Botryodiplodia* (6%), *Seridium* (5%), *Exserohilum* (2%) and *Drechslera* (1%).

3.3 | Pathogenicity assays on detached leaves and pods

Only isolates with morphological characteristics of the *Colletotrichum* genus (21 in total) were able to cause disease on AYB leaves. Fourteen of the 15 isolates belonging to *C. gloeosporioides* species complex and all six *C. truncatum* isolates (all from the research field in Ibadan) caused disease symptoms on the inoculated leaves and pods, respectively, in the laboratory assays (Table 1). None of the other 10 isolates, belonging to other genera, produced disease symptoms by 8 days post-inoculation (dpi) and therefore did not satisfy Koch's postulates. The disease scores for all isolates that produced symptoms were significantly ($p < 0.0001$) different from the symptomless noninoculated control leaves (Figure 4).

In the DLA, all *C. gloeosporioides* species complex isolates produced a characteristic irregular black lesion on leaves (Figure 5). However, there were significant (Kruskal–Wallis test, $H = 47.18$, $p < 0.0001$; Figure 4) differences in pathogenicity of 10 of the 15 *Colletotrichum* isolates (S3L3F1, S4L2F3, S3L2F2, S4L1F1, S2L3F2, S3L3F3, S3L1F2, S2L1F3, S2L1F2 and TSs 25; Figure 4). The post hoc test revealed that while the majority of the isolates differed in pathogenicity, five isolates (S3L1F1, S4L2F1, S3L2F1, S4L1F3 and S2L2F2) were not significantly different (p from ≥0.3054 to 0.9999) from the symptomless, noninoculated control leaves. Isolate S3L2F1

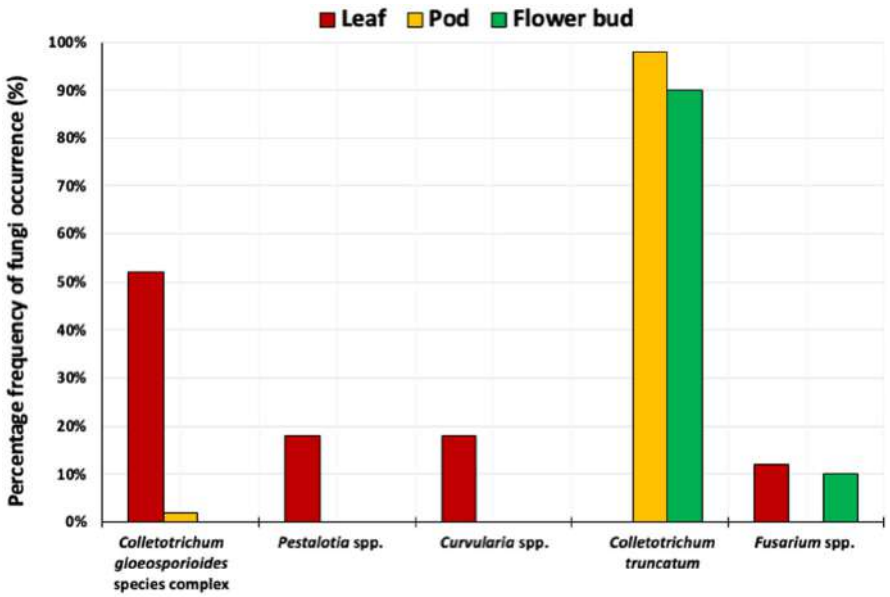

FIGURE 3 Fungi associated with African yam bean leaves, pods and flower buds sampled in Nigeria in 2018/2019.

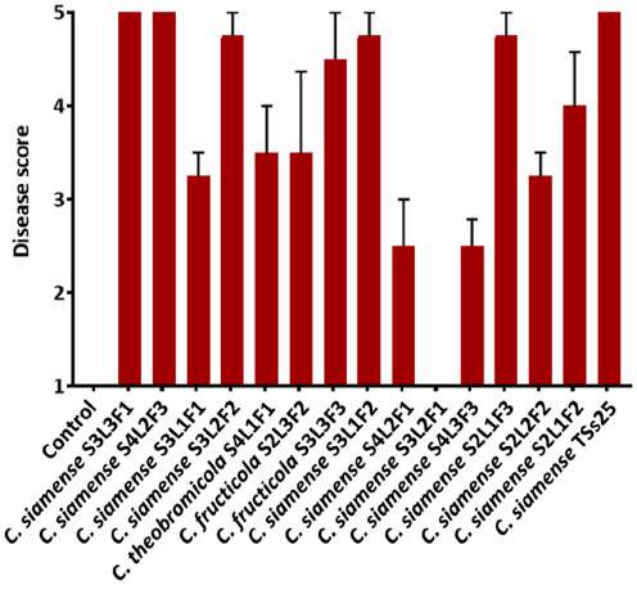

FIGURE 4 Disease severity scores of 15 isolates belonging to the *Colletotrichum gloeosporioides* species complex when inoculated on African yam bean leaves. The origin of the isolates is provided in Table 1. The data were analysed using the Kruskal–Wallis test ($p < 0.0001$). The bars represent the mean disease severity scores, and the error bars indicate the *SEM*.

did not produce disease symptoms on unwounded leaves (Figure 4) but it was able to cause disease when inoculated on wounded leaves (data not shown).

In the detached pod assay, all six *C. truncatum* isolates produced a characteristic black lesion on pods (Figure 5e). *C. truncatum* was the sole fungal species included in this assay because it was the predominant fungus found in pods (Figure 3) and there were limited pods for the assay. The lesions produced by five isolates (TSs 98, TSs 29B, TSs 421, TSs 61 and Pod 6) were significantly different (Kruskal–Wallis test, $H = 14.40$, $p = 0.0255$; Figure 6) from the control. Isolate TSs 432 had the least disease score and was not significantly different ($p = 0.5271$) from the symptomless control pod.

When testing for tissue specialization, the six *C. truncatum* isolates recovered from pods induced disease symptoms on AYB leaves but were in general less virulent on AYB leaves (post hoc test, $p = 0.0034$) than *C. gloeosporioides* species complex isolates (Figure 7). Indeed, the symptoms caused by *C. truncatum* (except TSs 421) were generally minor and not significantly different from the noninoculated control leaves (Table S2). In both detached leaf and pod assays, pathogens isolated from diseased leaves and pods exhibited the same morphological features as those used for the inoculations, fulfilling Koch's postulates.

3.4 | Extended characterization of *Colletotrichum* species

The encountered *Colletotrichum* species were grouped based on conidia shape, rod-shaped in Group A and falcate-shaped in Group B (Figure S1). Group A were all *C. gloeosporioides* species complex, predominantly isolated from the leaves, while Group B were all *C. truncatum* isolated from pods and flower buds.

There were varying conidia sizes, with isolates in Group B having significantly longer (unpaired *t* test, $p < 0.0001$; $t = 14.80$) conidia than those in Group A. The conidia size of Group B isolates ranged from 7.5 to 22.5 μm × 2.5–7.5 μm, whereas in Group A isolates it ranged from 5.0 to 17.5 μm × 2.5–5.0 μm (Figure S2). In addition, Group A grew faster than Group B isolates, with mycelial growth rate for Group A isolates ranging from 31 to 72 mm at 8 dpi compared to 25–57 mm for Group B isolates, but these differences were not statistically significant ($p = 0.187$; $t = 1.334$). The colony morphology of Group A (Figure S3) and Group B (Figure S4) isolates on PDA was inconsistent, with some isolates within the same group exhibiting two distinct cultural types on PDA.

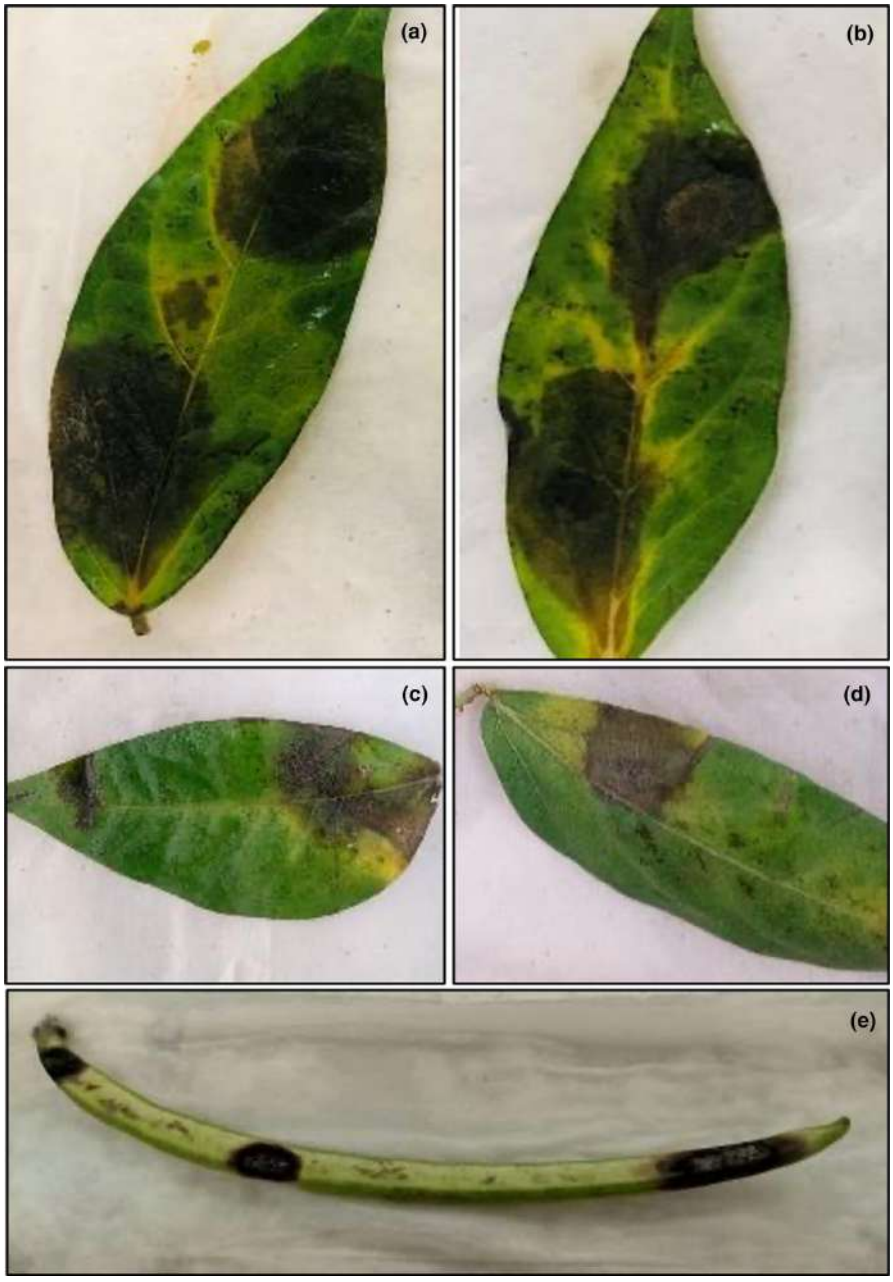

FIGURE 5 Disease reaction of *Colletotrichum* species inoculated on detached African yam bean leaves and pods. (a–d) Leaf lesions caused by isolates from the *C. gloeosporioides* species complex (*C. siamense* S2L1F2, *C. siamense* S2L1F3, *C. fructicola* S2L3F2 and *C. theobromicola* S4L1F1, respectively); (e) pod lesions caused by *C. truncatum* TSs 29.

Minor differences were observed among the groups in terms of appressoria shape and colour. In both groups, the appressoria were brown to dark brown in colour and lobed to irregular in shape. In addition, only Group B isolates possessed setae. The setae were commonly septate and cylindrical with sharp tips (Figure S5).

3.5 | Phylogenetic analysis

The phylogenetic analysis revealed three species among the *Colletotrichum* spp. isolates causing leaf diseases, while members of a single *Colletotrichum* species produced the pod disease. Initially, the ITS locus was amplified and sequenced for 42 isolates, with the phylogenetic analysis revealing two clades. However, the ITS phylogeny, while effective in distinguishing the 17 *C. truncatum* isolates from the 25 isolates within the *C. gloeosporioides* species complex clade, provided inadequate resolution to differentiate between species within the *C. gloeosporioides* species complex.

A multilocus analysis (ITS-*GAPDH*-*CAL*) resolved 13 selected *C. gloeosporioides* species complex isolates into four clades (Figure 8). Isolates S3L2F1, S2L2F2, S3L2F2, S2L1F2, S2L1F3, TSs 57, S2L3F2 and TSs 25 clustered in the *C. siamense* group. Isolates S3L3F3 and S4L1F1 clustered in the *C. fructicola* and *C. theobromicola* group, respectively. The *C. truncatum* isolates TSs 29, TSs 432 and Pod6 were grouped in the same clade, as in the ITS phylogeny tree (data not shown). However, in the four loci analysed (ITS-*GAPDH*-*CAL*-*ApMAT*), S2L3F2 grouped in the *C. fructicola* clade (Figure 9). Furthermore, within the *C. siamense* clade, S2L1F2, S2L2F2 and S2L1F3 (from different local government areas in Cross River) and TSs 57 (from Oyo) formed subclade 1

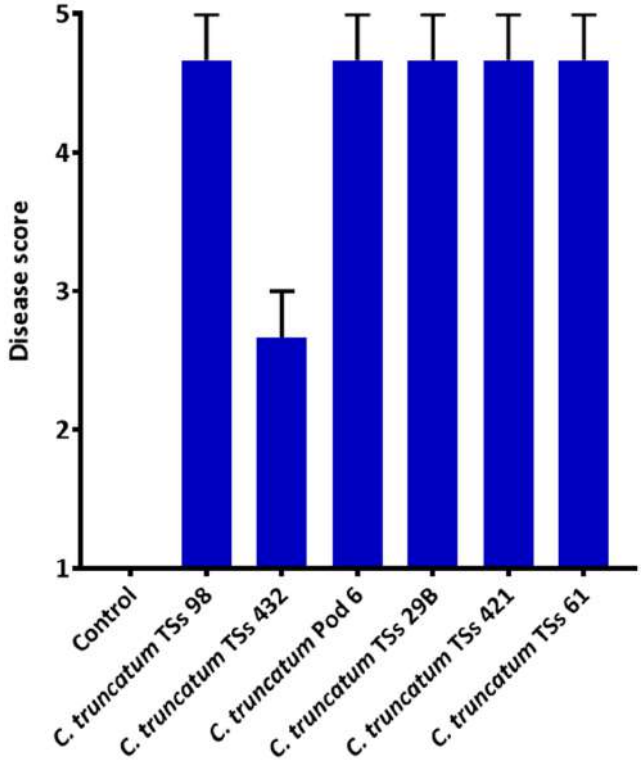

FIGURE 6 Disease severity scores of six isolates of *Colletotrichum truncatum* when inoculated on African yam bean pods. The origin of the isolates is provided in Table 1. The data were analysed using the Kruskal–Wallis test ($p=0.0255$). The bars represent the mean disease severity scores, and the error bars indicate the *SEM*.

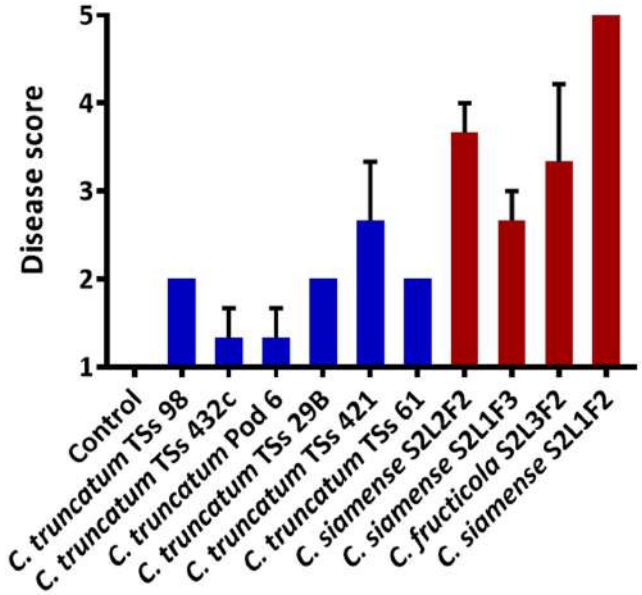

FIGURE 7 Variation in pathogenicity among *Colletotrichum truncatum* (blue bars, all recovered from pods) and *C. gloeosporioides* species complex isolates (red bars, all recovered from leaves) inoculated on African yam bean leaves. Isolates of *C. truncatum* were less pathogenic on leaves. The data were analysed using the Kruskal–Wallis test ($p=0.0034$). The bars represent the mean disease severity scores, and the error bars indicate the *SEM*.

while S3L2F1 and S3L2F2 (from the same local government area in Ebonyi) formed subclade 2.

4 | DISCUSSION

A disease survey was conducted to identify fungal diseases associated with African yam bean (AYB) in five states of Nigeria (Oyo, Enugu, Ebonyi, Abia and Cross River). A polyphasic approach was employed to identify the causative pathogens of the encountered diseases through morphological, molecular and pathogenicity assays. During the field survey, leaf tip dieback, flower bud rot and pod blight diseases were consistently observed. Farmers who were interviewed during the survey testified to the adverse impact of these diseases on AYB production. Overall, this laboratory investigation determined that the observed diseases are caused by *C. siamense*, *C. fructicola*, *C. theobromicola* and *C. truncatum*.

Fungal isolation revealed the presence of eight fungal genera associated with AYB diseases: *Botrydiplodia*, *Colletotrichum*, *Curvularia*, *Drechslera*, *Exserohilum*, *Fusarium*, *Pestalotia* and *Seridium*. However, *Colletotrichum* spp., *Curvularia* spp., *Pestalotia* spp. and *Fusarium* spp. were more frequently isolated from the symptomatic tissues. Oyedele et al. (2024) conducted a study to identify fungi associated with AYB leaf and pod diseases in south-west Nigeria. Their study revealed *Phoma*, *Botryodiplodia*, *Fusarium* and *Colletotrichum* as the main fungal pathogens. Afolabi et al. (2019) identified *Colletotrichum*, *Curvularia*, *Fusarium* and *Pestalotia* as fungi associated with AYB flower bud rot and pod blight. In the study of Afolabi et al. (2019), AYB accessions were grown in a research field in Abeokuta, Nigeria, during 2016/2017 and evaluated for their susceptibilities to flower bud and pod rot under natural conditions.

While *Curvularia* spp. and *Pestalotia* spp. are recognized as pathogens in Barbados lily and tea plants (Liang et al., 2018; Liu et al., 2017) and *Fusarium* spp. are known as soilborne pathogens in cultivated plants (Arie, 2019), our findings indicate that the isolates of these fungi did not exhibit pathogenicity towards the examined AYB accession. However, Oyedele et al. (2024) reported *Pestalotia* and *Fusarium* to be pathogenic on AYB leaves. In contrast, the main causal agents of leaf and pod blights in our studies were *Colletotrichum* spp. Nearly all isolates from the *C. gloeosporioides* species complex and all *C. truncatum* isolates tested in our laboratory assays induced disease symptoms. These findings implicate *Colletotrichum* spp. as significant causal agents of pod and leaf blight in AYB within Nigeria.

The presence of *Curvularia* spp., *Pestalotia* spp. and *Fusarium* spp. in our samples raises questions about their roles in the AYB environment. They may exist as endophytes, potential sources of secondary infections or saprophytes. Also, it is unclear if fungi not found in the current study but detected by Afolabi et al. (2019) (i.e., *Aspergillus*, *Cercospora*, *Macrophomina*, *Mucor*, *Pestalotia*, *Penicillium*, *Phomopsis*, *Pythium* and *Rhizopus*) are also pathogenic to AYB. To fully ascertain the pathogenicity of these fungi towards AYB, future studies should aim to inoculate AYB grown under field conditions with *Curvularia*, *Pestalotia* and *Fusarium* isolates using diverse inoculation

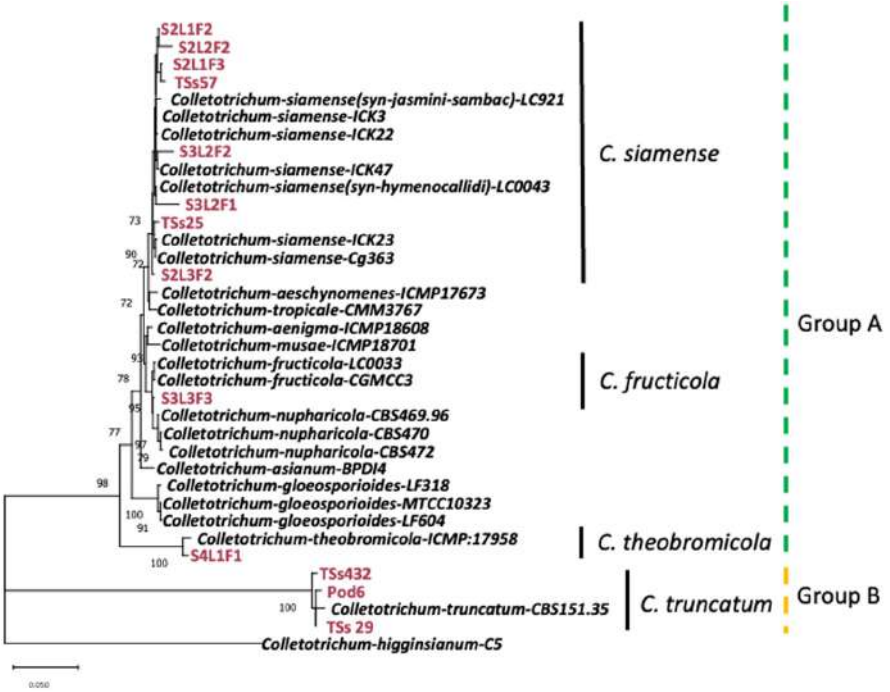

FIGURE 8 *Colletotrichum* species phylogeny inferred by maximum parsimony from concatenated sequences of the rDNA internal transcribed spacer (ITS), *GAPDH* and *CAL* loci. The 13 isolates from the current study are highlighted in red. Labels in black are for isolates for which sequences were retrieved from GenBank. The image represents the most parsimonious tree. The bootstrap support values are shown at the nodes. Bootstrap values lower than 70% are not shown. The *C. gloeosporioides* species complex isolates (Group A) and *C. truncatum* isolates (Group B) are indicated by the green and yellow dashed lines, respectively. The tree is rooted with *C. higginsianum* as the outgroup.

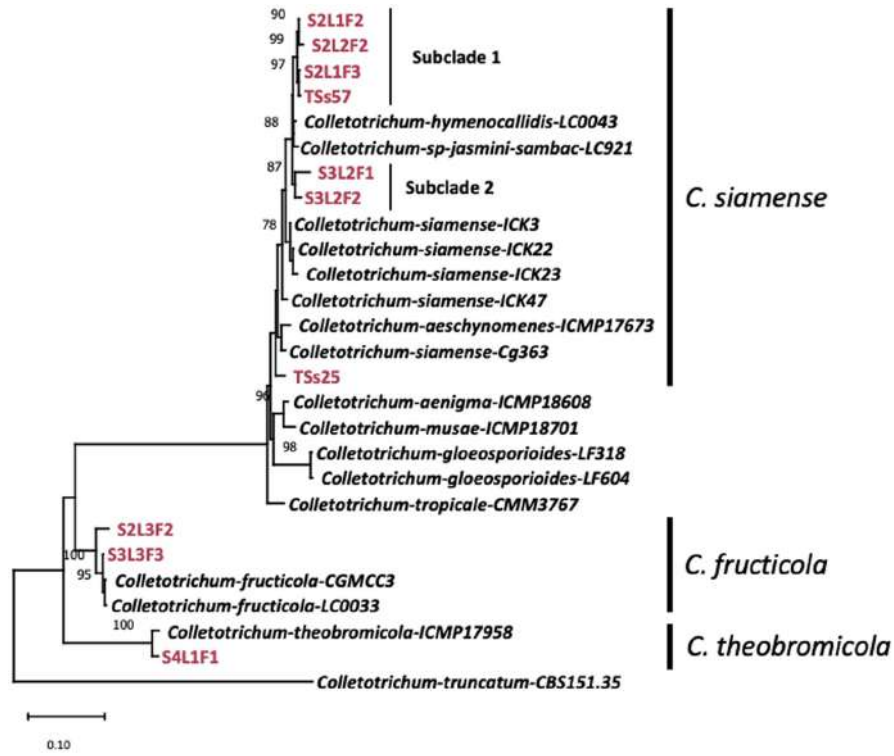

FIGURE 9 *Colletotrichum gloeosporioides* species complex phylogeny inferred by maximum parsimony from concatenated sequences of the rDNA internal transcribed spacer (ITS), *ApMAT*, *GAPDH* and *CAL* loci. The 10 isolates from the current study are highlighted in red. Labels in black are for isolates for which sequences were retrieved from GenBank. The image represents the most parsimonious tree. The bootstrap support values are shown at the nodes. Bootstrap values lower than 70% are not shown. The tree is rooted with *C. truncatum* as the outgroup.

methods. These methods could include soil drenching, foliar spraying or seed inoculation. It is crucial to note that detached leaf and pod assays, while informative, may not fully capture the complexity of interactions between these pathogens and AYB. Therefore, further investigation under field conditions or controlled environment (e.g., greenhouse) using diverse inoculation methods is necessary to thoroughly understand the risk posed by these fungi to AYB crops. Additional research efforts are also needed to improve the understanding of AYB pathogen diversity in Nigeria and elsewhere.

Colletotrichum is a genus composed of destructive pathogens responsible for immense losses in crop production. They cause diseases in leaves, stems, tubers and fruits (Cannon et al., 2012; Dean et al., 2012). Different complexes within the *Colletotrichum* genus exist, such as the *C. gloeosporioides* species complex (Cai et al., 2011;

Weir et al., 2012). Employing morphological characters alone to delineate *Colletotrichum* spp. is inadequate as it does not provide enough information to effectively differentiate them. For instance, in the current study, variations in colony morphology on PDA were observed within a species (*C. siamense* S2L1F3 and S2L1F2). Also, conidial size and radial growth measurement were similar among the isolates from the *C. gloeosporioides* species complex, although conidial shape, size and the presence of setae were useful in discriminating between *C. truncatum* and the *C. gloeosporioides* species complex. The appressorium of examined isolates was not informative in differentiating the species, as reported by Cai et al. (2009) and Sanders and Korsten (2003).

C. truncatum was observed to be a relatively weak pathogen on leaves compared to the *C. gloeosporioides* species complex (Table S3). Given high disease symptoms in pod pathogenicity assays and prevalence during pod isolations, the results suggest that *C. truncatum* from the current study are specialized at infecting AYB pods. Previous studies have shown that tissue-specific infection patterns are common in fungal pathogens and may be caused by their adaptation to plant organs, available nutrients (Abrahamian et al., 2016), the developmental stages of the host (Barrett & Heil, 2012) and pathogen, or the plant defence response (Lacaze & Joly, 2020). Reasons for *C. truncatum* to preferentially infect AYB pods requires further investigation.

The ITS phylogenetic analysis allowed resolution of isolates into *C. gloeosporioides* species complex and *C. truncatum* but was inadequate to discriminate isolates within the *C. gloeosporioides* species complex. Similar observations were reported by Cai et al. (2009). The ITS, *CAL* and *GAPDH* phylogenetic analysis, on the other hand, revealed that the isolates responsible for the observed AYB diseases belong to four *Colletotrichum* species, namely *C. siamense* (comprising the largest number of isolates), *C. theobromicola*, *C. fructicola* and *C. truncatum*. The three-loci analysis was useful in separating *C. truncatum* and *C. theobromicola*. However, the four-loci analysis provided a finer resolution, as it was effective in differentiating *C. fructicola* from the *C. siamense* sensu lato group. It also provided branches with overall good bootstrap support values. The phylogenetic analysis revealed that in the *C. siamense* clade, isolates from Cross River (S2L1F2, S2L1F3 and S2L2F2) formed a distinct subclade (subclade 1) while isolates from Ebonyi (S3L2F1 and S3L2F2) clustered in subclade 2. Interestingly, isolate TSs57 from Oyo clustered with isolates in subclade 1 despite originating from relatively far away. Long distance dispersal of a plant pathogen can occur through various mechanisms, including human-mediated transportation, air, large water bodies and plant transmission (Golan & Pringle, 2017; Nathan, 2001).

There was no consistent trend in disease scores among the evaluated isolates of *C. theobromicola*, *C. fructicola* and *C. siamense*. Furthermore, the two *C. siamense* isolates forming subclade 2, S3L2F1 and S3L2F2, had contrasting abilities to cause disease in the unwounded leaves used in the DLA. S3L2F1 did not cause disease while S3L2F2 had the highest score. On the other hand, S3L2F1 had a high disease score when inoculated in wounded leaves (data not shown). Isolates of some *Colletotrichum* species are found to be pathogenic only when a host is wounded (Pring et al., 1995; Than et al., 2008). Testing a larger collection of *C. gloeosporioides* species complex isolates in their abilities to cause leaf blight in a diverse set of AYB accessions, using both wounded and unwounded leaves, warrants investigation to understand pathogen variability and mechanisms of resistance among AYB accessions.

The current study revealed the presence of four *Colletotrichum* species as causative pathogens of AYB foliar and pod diseases. These species have been characterized and reported on several hosts, highlighting their adaptability and potential impact across different cropping systems. For instance, *C. siamense*, *C. theobromicola* and *C. fructicola* can cause anthracnose on coffee and mango, among other crops (Sharma et al., 2013; Weir et al., 2012) while *C. truncatum* causes anthracnose on soybean, pepper and papaya, among other crops (Boufleur et al., 2021; Torres-Calzada et al., 2018). To the best of our knowledge, this study is the most thorough examination of *Colletotrichum* species that impact AYB. Notably, it is the first documentation of *C. siamense*, *C. theobromicola* and *C. fructicola* as the causative agents of AYB leaf diseases, as well as the first identification of *C. truncatum* as the pathogen that causes pod blight disease in AYB. To accurately delineate *C. gloeosporioides* species complex isolates, multilocus sequencing has been the standard approach used. However, this method is time-consuming and requires refinement. Results from previous and the current study suggest that the *ApMAT* locus is a reliable marker for delimiting *C. gloeosporioides* species complex compared to other markers (Sharma et al., 2013). Therefore, we recommend screening the *ApMAT* gene for classifying isolates within the *C. gloeosporioides* species complex.

Accurate identification of diseases is the first crucial step in designing disease management strategies. The presence of several *Colletotrichum* species associated with AYB diseases investigated in the current study may hinder decisions relating to disease management, with species possibly differing in their response to different management strategies or crop-resistance. Thus, further understanding of the plant–pathogen interactions involved in infection is critical to understand host resistance. Furthermore, understanding the response of these taxa to fungicides and alternative treatments will support the development of integrated management strategies to control the diseases that they cause. Ultimately this will allow greater cultivation of this underutilized security legume crop which has substantial nutritional and nutraceutical promise.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

Sequences generated in the current study are available from GenBank at https://www.ncbi.nlm.nih.gov/genbank/. Accession numbers are provided in Table 2. Other data that support the findings reported in this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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