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RESEARCH ARTICLE

Molecular identification and characterization of pathogenicity of fungal pathogens associated with crown rot disease in banana from Odisha, India

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Abstract

Crown rot disease on banana varieties (Champa, Patakpura and G9) was identified in Odisha's 10 coastal districts during the post-harvest storage stage in 2022-23. Five crown fragments were taken for isolation from each variety of bananas based on symptom developments. Agar plate and standard blotter technique isolation procedures have been used to isolate 1210 fungal colonies, of which 584 representative colonies were purified from banana crown fragments. Different fungal colonies were purified, characterized and identified using morphological and molecular methods from G9 (244), Patakpura (205) and Champa (135) fungal colonies. Morphological identification of *Fusarium* and *Colletotrichum* isolates was madeusing colony morphology, pigmentation, the growth rate on the PDA plate and microscopic observation. Molecular identification of *Fusarium* and *Colletotrichum* isolates was done by amplifying the internal transcribed spacer (ITS) region of the conserved ribosomal DNA, which includes 5.8S rDNA. *Fusarium verticillioides*, *Fusarium equiseti*, *Fusarium oxysporum* and *Colletotrichum gloeosporioides* were the 4 fungal isolates identified. The sequences of these fungi were deposited in the GenBank database (NCBI) under the accession numbers OQ363325 (*Fusarium verticillioides*), OP735534 (*Fusarium equiseti*), OQ438654 (*Fusarium oxysporum*) and PP448030 (*Colletotrichum gloeosporioides*). The neighbor-joining method was used to conduct phylogenetic analysis on the gene sequences. Pathogenicity test results revealed that all 4 isolated fungal strains produced similar crown rot symptoms on 3 varieties of banana fruits. Disease severity index and AUDPC indicated that the *Fusarium* strain is the most aggressive crown rot pathogen and the G9 is the more susceptible banana variety to crown rot pathogens in Odisha.

Keywords

banana; crown rot; *Fusarium equiseti*; molecular identification; pathogenicity test

Introduction

In India, postharvest infections cause the loss of 20-50 % of perishables, with bananas suffering the most significant losses of 20-80 % (1). Bananas can be affected by various environmental factors during the ripening process and are susceptible to the attack of pathogenic microorganisms, causing several diseases that lead to the deterioration of fruit quality.

Crown rot is one of the most common postharvest fungal complex diseases caused by several pathogenic fungi. The frequency and presence of these fungal species vary depending on the farming area. As a result, the fruit quality is negatively impacted, adversely affecting banana exports from all producing nations (2, 3). The crown rot disease is caused by *Fusarium* spp., *Colletotrichum musae* (Berk. & Curt.) Arx., *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. and more than 5 other genera that vary depending on the geographic region (4). Fungi like *Fusarium equiseti, F. incarnatum, F. oxysporum, F. solani, F. verticillioides, Colletotrichum musae, C. gloeosporioides, Curvularia lunata, Glomerella cingulata, L. theobromae, Phomopsis sp., Phyllosticta musarum* and *Thielaviopsis paradoxa* were also known to be associated with banana fruits diseases (5). Seasonal fluctuations influenced by several pre-harvest factors influence banana fruit susceptibility to crown rot (6). Crown rot is mainly caused by *Fusarium* spp., which causes significant postharvest losses in worldwide banana production (7). This genus, particularly *Fusarium*, significantly reduces yields in several perennial crops, including bananas (8). *Fusarium* crown rot and fruit rot are 2 major postharvest banana diseases that pose challenging issues (9, 10). *Fusarium* species can be distinguished primarily by their different morphological traits, especially the sizes and forms of their macro and microconidia and the way they form chlamydospores. Identification also considers pigmentations produced and agar media growth rates (11). Due to the similarities between *Fusarium* species, morphological identification may be complicated. The most reliable analysis is performed on nucleotide sequences from conserved gene regions, such as the internal transcribed spacer (ITS). The molecular technique avoids the advantages of morphological similarities between *Fusarium* species, which offers a more precise means of species identification than micro and macroscopic studies (12). The sequence data utilizing ITS regions has dramatically benefited the phylogeny and taxonomy of *Fusarium* species since these regions help identify species (12, 13). PCR-based methods are frequently used to identify, characterize and treat microorganisms and diseases early. Fungal identification has been done using random amplified polymorphic DNA (RAPD) analysis (14). RAPD was observed to have a high level of variability among many isolates (15).

In comparison to other molecular approaches such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and inters small sequence repeats (ISSRs) provides simplicity, speed and cost-effectiveness while demonstrating high variability among isolates (16). RAPD is the preferred technique for molecular fungal characterization because of its low cost and simplicity of analysis (17). As a result, this technique has been widely applied in the molecular characterization of fungi (18). Therefore, the present study was designed to identify the fungi responsible for crown rot infections in different banana cultivars in Odisha, i.e., isolation of fungal pathogens, morphological and molecular identification of *Fusarium* and *Colletotrichum* strain. In addition, pathogens and banana cultivars were screened based on pathogenicity test findings, as described by Koch's postulate.

Materials and Methods

Sample collection

Ten districts of Odisha: Mayurbhanj, Balasore, Bhadrak, Jajpur, Kendrapara, Jagatsinghpur, Cuttack, Puri, Ganjam and Khordha (latitude 19.38 N to 22.00 N, longitude 85.05 E to 86.91 E) were the target areas of our study on the banana crown rot disease from March 2022 to February 2023 taking into consideration 3 varieties of banana (Champa: AAB, Patakpur: AAB and Grand Nain: AAA). Each variety had 50 symptomatic fruits that were selected randomly from each district. A total of 4500 samples of 3 different banana varieties were collected from markets, storage spaces and farmers' storage facilities and wrapped up quickly in sterile plastic bags, then delivered within 48 h to the laboratory of Plant Pathology. The diseased fruits were examined under a compound microscope (Nikon T169864, Tokyo, Japan) before being placed in plastic bags with wet filter paper to encourage sporulation.

Fungal Isolation, Purification and Preservation

Depending on the fungal sporulation, 2 methods of isolation were used. Following a modified procedure (19), agar plate and standard blotter technique procedures were used to isolate related pathogens of banana fruits. Based on the development of symptoms, 5 crown fragments from each variety of banana were isolated. Mercuric chloride ($HgCl_2$) solution (0.1 %) was used to surface-disinfect pieces (5 × 5 mm^2) of diseased fruit tissue that were exhibiting symptoms. The tissues were cleaned with sterile distilled water and inoculated on Potato Dextrose Agar (PDA) media containing streptomycin sulfate (25 mg/L) to inhibit the growth of bacteria. Fungal colonies were observed under a binocular microscope after being incubated for 10 days at 28 ± 2 °C. In the standard blotter technique, 3 blotting papers, each cut to suit a 90 mm petri dish, were sterilized in 70 % ethanol and dried before being placed on presterilized petri plates. The filter paper was wet with 10-15 mL of sterilized water. Fruit fragments were surface sterilized, dried using filter paper and then plated on moistened blotter paper. The petri plates were then incubated at 28 ± 2 °C for 10 days. After incubation, fungal growth was detected using a binocular microscope. The associated fungi were identified and purified using the hyphal tip and/or single spore techniques described in the previous work (9). Colonies with identical characteristics were chosen as representatives and purified on PDA. Pure cultures were acquired through frequent sub-culturing every 45 days for later research and the sterile cultures (PDA slants) were refrigerated at 4 °C.

Fungal Identification

Morphological characterization

Morphological and cultural characterizations were conducted using the procedures (11) for isolating MRR1 and for isolating MRR2 (20). The morphological characteristics of the fungal isolates were evaluated for ten days after inoculation on PDA. These characteristics included colony morphology, pigmentation, growth rate and diameter of isolated fungus. Semi-permanent slides prepared under a digital light microscope (Nikon Eclipse Ni-U,

Tokyo, Japan) were used to identify micromorphological characteristics such as mycelium, conidia, conidiophore and chlamydospore at 40 x magnification. One sample from each group of isolates with similar features was randomly selected for morphological identification at the genus level and forwarded for molecular identification.

Molecular characterization of the isolated fungus

Extraction of genomic DNA and Molecular characterization using ITS

DNA was extracted from each fungal isolate pure culture using a 500 µL extraction buffer that is identical to that suggested by the previous study (21). Fungal mycelia mats were harvested from isolates cultured in potato dextrose broth and incubated in an orbital shaker at 200 rpm at 28 °C for 3-5 days. The fungal biomass was filtered using Whatman No. 1 filter paper and 50 mg of freeze-dried mycelium was crushed in liquid nitrogen with a pestle and mortar to obtain a fine powder of mycelium. The mycelial powder was transferred into 1.5 mL Eppendorf tubes. After adding 500 µL of extraction buffer, the resulting slurry was incubated at 60 °C for 20 min in a water bath. An equivalent volume of phenol: the incubated slurry was gently mixed with chloroform: isoamyl alcohol (25:24:1) before being centrifuged at 10000 rpm at 4 °C for 60 min. Transfer the supernatant to new sterile Eppendorf tubes. Add 1 µL of RNase solution (10 mg/mL) and incubate for 10 min at 37 °C. An equal volume of chloroform-isoamyl alcohol (24:1) was added and gently mixed. The tubes were centrifuged for 10 min at 10000 rpm at 4 °C. The supernatant was transferred to a new, sterile Eppendorf tube and an equal amount of isopropyl alcohol was added. The centrifugation stage was performed twice. The supernatant was collected, and then ice-cold 70 % ethyl alcohol and 3 M sodium acetate (pH 5) were added and kept overnight at 0 °C, followed by centrifugation at 13000 rpm for 5 min, draining the supernatant and air-dried the pellet. The pellet was resuspended in 20-100 µL of TE buffer (pH 8). The DNA was kept at -20 °C. Finally, the quality and amount of DNA were evaluated on a 1.5 % agarose gel. The conserved ribosomal internal transcribed spacer (ITS) region served as the molecular identification basis. We used the universal primer pairs ITS1 (5'-TCCTAGGTGAACCTGCGG -3') and ITS4 (5'-TCCTCC GCTTATTGATAT GC -3') that have been reported (22) to amplify the ITS regions of the ribosomal nucleic region, which includes the 5.8S rDNA of the fungus. PCR amplification was performed in a 25 µL reaction mixture containing 10 pmol/mL of forward and reverse primers, 2.5 mM $MgCl_2$, 200 µM each of the 4 deoxyribonucleotide triphosphates (dNTPs), 0.5 U of Taq DNA polymerase, 1 x concentration of PCR buffer (Invitrogen, Life Technologies, Brazil) and 50 to 100 ng genomic DNA. The mixture was pre -denaturated at 95 °C for 5 min. Then, 39 cycles were run: denaturation at 95 °C for 30 sec, annealing for 45 sec at 36 °C and elongation at 72 °C for 1 min. The reaction was terminated with a final extension step of 8 min at 72 °C. Amplified products were separated on a 1.5 % agarose gel in 1 x TAE buffer at 65 V for 150 min and visualized with a gel documentation system. The size of the amplified fragments was determined by comparing the 600-bp DNA ladder.

Sequencing, Nucleotide alignment and Phylogenetic analysis

Commercial service provider Heredity Biosciences Labs carried out the PCR sequencing results. Sequencing was done with the same primers that were used for PCR amplification. PCR products (sequences) were assembled using the DNA Baser V.4 software to create whole contigs. BioEdit software's CLUSTAL W technique was used to align these contigs. Using the Basic local alignment Search Tool for nucleotide sequences (BLASTN software), each isolate's ITS nucleotide sequences were compared to those in the public domain databases NCBI (www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed using the MEGA v.11 (Molecular Evolutionary Genomics Analysis Version 11) software program (23, 24). MEGA 11 generated a dendrogram using the 30 isolates in the current research and reference strain genomes from GenBank. Phylogenetic analysis was performed using 3 types of combination datasets. The first dataset compared isolated *Fusarium* spp. to other species of *Fusarium*, the second dataset compared isolated *Colletotrichum* spp. to different species of *Colletotrichum* and the third phylogenetic dataset compared isolated *Fusarium* spp. and *Colletotrichum* spp. to other related genera of *Fusarium* and *Colletotrichum*. Phylogenetic trees were created using the neighbour-joining (NJ) method with bootstrap analysis with 1000 replications to determine the confidence in branching (25).

Pathogenicity test and screening of the banana varieties

Three varieties of bananas obtained from OUAT Farm, Barmunda, Bhubaneswar were inoculated with the fungal isolated toconfirm the pathogenicity. Asymptomatic fruits were completely washed and disinfected by immersing them in a 1.5 % (v/v) sterile sodium hypochlorite solution for 5 min, then rinsed with sterile distilled water 3 times. The sterilized fruits were air-dried at room temperature (25 ± 2 °C) for 10 min (26). Using a sterile cork borer, uniform wounds (1 pore, 3 mm deep and 5 mm wide) were made at the equator crown part of each air-dried fruit (9). Conidial suspensions were prepared from fungal cultures grown on PDA at 28 °C for 2 weeks. The suspensions were then placed in sterile distilled water and filtered through 2 layers of sterile cheesecloth. The spore suspension was (1×10^6 conidia/mL) prepared using a hemacytometer after being diluted in distilled water containing 0.05 % Tween 20 (27). The wounded banana fruits were drop-inoculated with 500 µL of conidial suspension. Sterilized distilled water was used to treat wounded control fruits as well. To prevent contamination, inoculated samples were placed in sterile containers and sealed with transparent plastic bags. The inoculated fruits were incubated at 28 °C, 85 % relative humidity, with 12 h light cycles and observation was taken in 2 days intervals up to 10 days. Each treatment was replicated 5 times and the studies were independently repeated twice. The crown rot index scale (1-7), established (28), was used to measure disease severity, with 1 indicating no symptoms and 7 indicating the rot on the entire crown part and peels. To establish the presence

of the infection, inoculation fungi were re-isolated from all infected crowns. The formula suggested was used to express the disease severity (DS) (29):

$$\text{Disease severity (\%)} = \frac{\sum (\text{Severity rating} \times \text{number of fruit clusters in that rating})}{(\text{Total number of fruit clusters examined} \times \text{highest scale})} \times 100$$

Using the formula given (30), the area under the disease progress curve (AUDPC) was calculated:

$$AUDPC = \sum [(y_i + y_{i+1})/2 \times (t_{i+1} - t_i)]$$

where, y_i = Disease severity at time t_i; y_{i+1} = Disease severity at time t_{1+1}; t = Time in days

Statistical analysis

The laboratory experiment used the Completely Randomized Design (CRD) design. The recorded data were analyzed using the Analysis of Variance (ANOVA) techniques. The Critical Difference (CD) test or Least Significant Difference (LSD) test was used as a post hoc test to compare the treatments and determine the significant difference between the treatments. R Software Version V 4.2.2 was used to analyze the data. The LSD values were calculated at a 5 % probability level of significance (P=0.05).

Results and Discussion

Sample collection and Disease symptoms

The 4500 banana samples of 3 distinct varieties collected from 10 Odisha districts had greenish-yellow peels and mild crown rot symptoms. Every district was surveyed using 50 samples and the percentage of disease incidence varied from 12 % to 100 %. The district of Jajpur had the highest disease incidence in the summer (March to June 2022) and Mayurbhanj had the lowest in the winter (November to February 2022-23). According to a research, crown rot disease in bananas in Honduras is usually higher in the summer and lower in the winter (31). Crown rot symptoms included tissue blackening and softening and irregular-shaped dark brown patches appearing on the crown surface, which spread to the peduncle and fruit and caused the fruit to drop. The diseased tissue was covered in white mycelium or orange moulds (Fig. 1). This observation is consistent with earlier reports documented in the existing literature (32).

Fungal isolation

Different fungal colonies (1210) were found after examining 900 crown fragments for a study on banana crown rot disease in Odisha. At a temperature of 28 °C, 584 pure cultures of four fungal species from 1210 fungal colonies were isolated using agar plates and blotting paper method. Fungal colonies were purified from G9 variety 244, Patakpura 205 and Champa 135. *Fusarium* strain (MRR1) presented *Fusarium verticillioides* (MRRS1), *F. equiseti* (MRRS2) and *F. oxysporum* (MRRS3). *Colletotrichum gloeosporioides* (MRRS4) was also identified within the *Colletotrichum* strain (MRR2). NCBI allocated accession numbers to the fungal isolates MRRS1, MRRS2, MRRS3 and MRRS4 (Table 2). *Fusarium* was the most common genus isolated, which is consistent with Kamel's findings (9), in which *Fusarium* formed 55 % of the fungal community and was found in more than 80 % of banana crown rot samples. This emphasizes the presence of *Fusarium* in the examined fungal community linked with banana crown rot.

Morphological characterization and Identification of the isolated fungi

The morphological characteristics of the causative agents were examined on the artificial culture medium and the host to determine the associated pathogen studied on the PDA medium. A total of 584 purified fungal colonies were

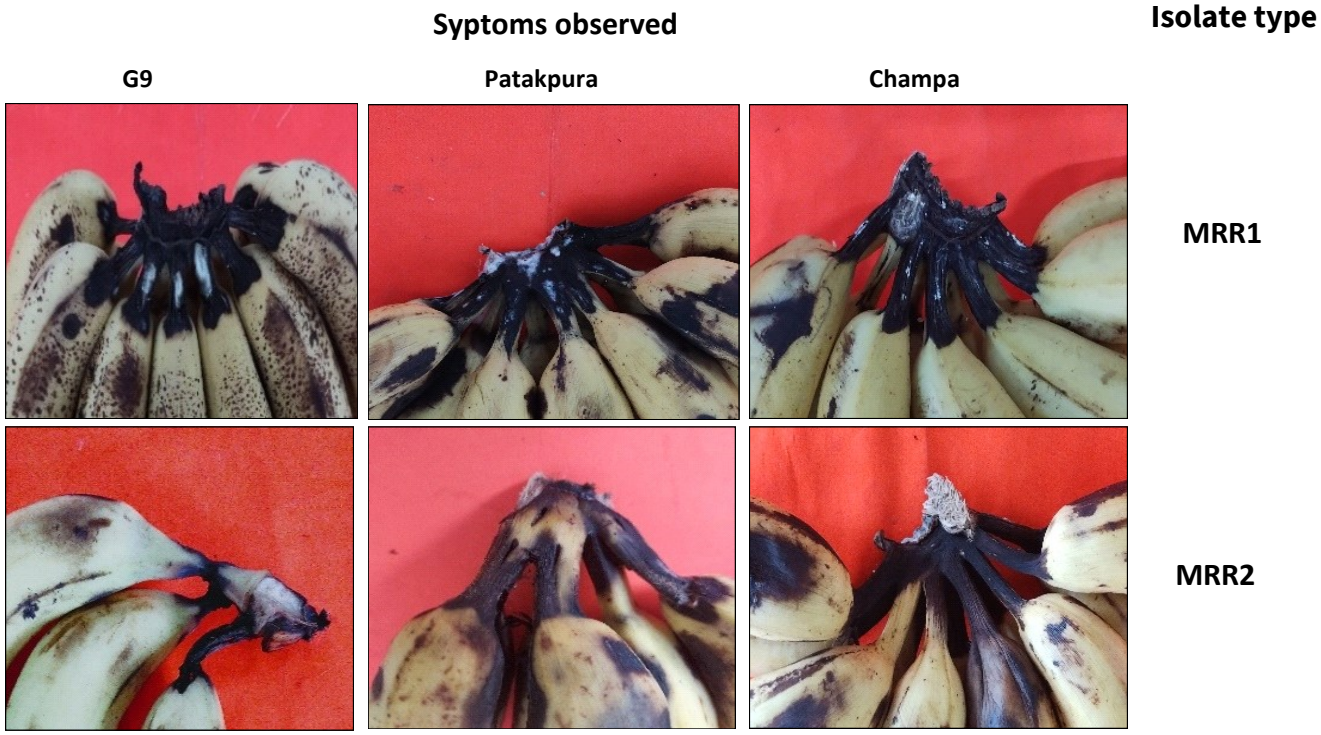

Fig. 1. Crown Rot Symptoms of three banana varieties.

divided into 2 groups. Based on the 584 fungal pure cultures analysis, 491 pure cultures exhibited conidiophores, macroconidia, microconidia, chlamydospores and mycelium colonies that initially appeared white before changing colour on the agar media. These features led to their preliminary identification as members of the *Fusarium* genus (11). Three isolates from the *Fusarium* (MRR1) genus were initially assigned the names MRRS1, MRRS2 and MRRS3 based on their different morphological and cultural characteristics. MRRS1 has been found in 167 out of 491 pure cultures of the *Fusarium* genus. After ten days of incubation, the medium plates were covered entirely with white mycelium, which was the first indication of cottony flat mycelium development in these cultures and gradually orange colouration pigmentation appeared. Microscopic inspection indicated the existence of macroconidia, which have three septa and are thin-walled, slender and slightly straight. Apical cells were flattened with slight notching, while basal cells measured 21.18- 46.35 µm × 4.25–6.12 µm. Chlamydospores were conspicuously absent and the microconidia ranged in shape from oval to obovoid, measuring 6.65–22.48 µm × 2.45–4.32 µm (Fig. 2). Out of 491 *Fusarium* genera, 182 pure cultures shared identical phenotypic characteristics, beginning with white colonies before transitioning to a peach-orange tint near the agar base. A microscopic examination revealed smooth, branching, cylindrical and septate mycelium. The macroconidia were curved hyaline and 2-5 septate, measuring

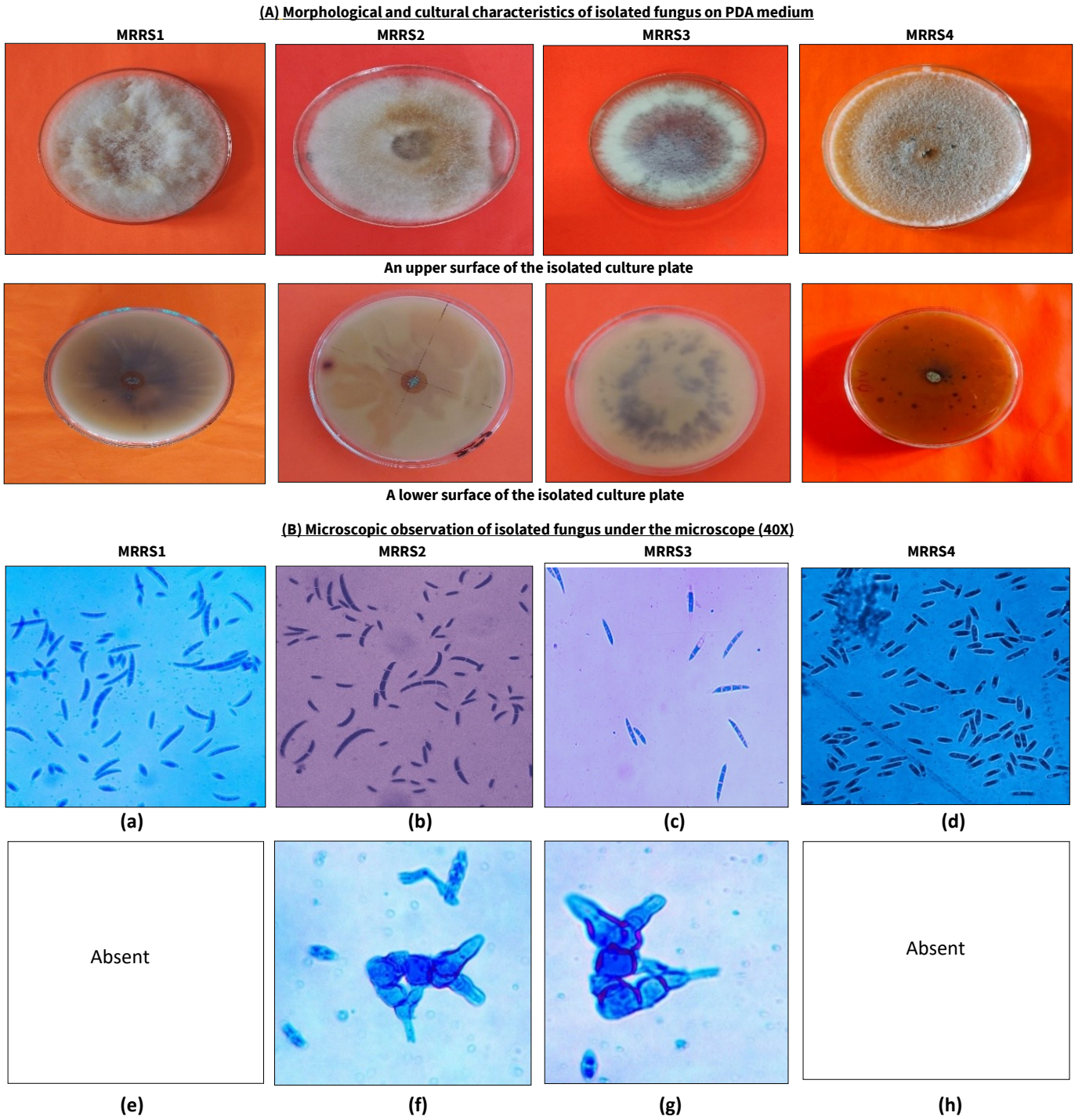

Fig. 2. (**A**) Morphological and cultural characteristics of isolated fungus on PDA medium. (**B**) Microscopic observation of isolated fungus under the microscope (40 X); (**a-c**) Macroconidia and microconidia of MRRS1, MRRS2 and MRRS3 isolates; (**d**) Conidia of MRRS4 isolate containing oil globule; (**f, g**) Chlamydospores of MRRS2 and MRRS3.

20.15–37.25 × 3.86– 6.55 µm. The microconidia were round, hyaline and 0-1 septate, measuring 12.20–16.72 × 3.80–5.83 µm. Chains of spherical, hyaline chlamydospores measuring 7.51 – 12.10 µm in diameter were observed in MRRS2 (Fig. 2). The agar base of the colony that MRRS3 imparted, changed in colour with time, from pale orange to dark magenta, which was initially white. A common feature 142 of the 491 pure cultures of the *Fusarium* genus shared was its smooth, cylindrical and septate mycelium. Most isolates produced oval-shaped, kidney-shaped microconidia that were mainly single-celled. A small number of isolates had single septations from short mono phialides. These microconidia were visible as false heads with aerial mycelium appearance and measured 5.17–14.52 × 3.58–5.11 µm. In addition, macroconidia were found to measure 29.46–38.66 × 4.34–6.41 µm. They had pointy ends, a straight to sickle-shaped morphology, pedicellate basal cells and 2-3 septa. Cylindrical, hyaline chlamydospores with a diameter varying from 4.52–9.64 µm were generated in chains (Fig. 2). Only 93 of the original 584 pure cultures were found to have characteristics similar to PDAs, such as white to pale grey mycelium around the fungal colony with black, acervulus-like masses with orange conidial masses near the inoculation site. The isolates known as MRR2 were found to belong to the *Colletotrichum* genus (33) because of their unique morphological characteristics, which included hyaline, mostly ellipsoid, aseptate conidia with oil globules and measuring between 13.11 – 3.34 µm. (Fig. 2). The growth rates of colony cultures were constant between genera, with no significant variations between MRRS1 (9.08 mm day^{-1}), MRRS2 (9.12 mm day^{-1}) and MRRS3 (8.98 mm day^{-1}). However, there was a slight statistical difference in the growth rate of the MRRS4 genus (8.48 mm day^{-1}). MRRS2 isolates grew significantly quicker than the others, followed by MRRS1 (Table 1). After 10 days of incubation, all MRRS1, MRRS2, MRRS3 and MRRS4 isolates developed on PDA at room temperature (28 ± 2 °C) with an average diameter of 92-94 mm, 93-95 mm, 89-91 mm and 83-88 mm respectively. The morphological and cultural identification of MRRS1, MRRS2 and MRRS3 was supported by similar morpho-cultural isolated fungal traits described by previous studies (34-36).

Table 1. Growth rate of different isolated fungus from crown rot disease

Treatment No.	Isolated Fungus	Growth Rate (mm day^{-1})
T1	MRRS1	9.08
T2	MRRS2	9.12
T3	MRRS3	8.98
T4	MRRS4	8.48
SE(m)		3.37
CD 5 %		10.38

*****Mean(± SD) of 5 replications. Numerical values followed by the same letter are not significantly different at the 5 % level ($p < 0.05$).

Identification based on molecular methods based on ITS and Phylogenetic results

Internal transcribed spacer (ITS) sections of four fungal isolates, including the 5.8S rDNA gene, were amplified using universal ITS1 and ITS4 primers, producing fragments that ranged in size from 517 bp to 596 bp (Table 2, Fig. 3). All sequences showed 100 % sequence homology with GenBank sequences following PCR product sequencing and BLASTn analysis. BLAST 2.15 was used to compare *Fusarium* and *Colletotrichum* sequences that were obtained from the amplification of the conserved ribosomal ITS region with sequences from the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were deposited in NCBI GenBank and identified MRRS1 as *F. verticillioides* (Accession No. OQ363325), MRRS2 as *F. equi-*

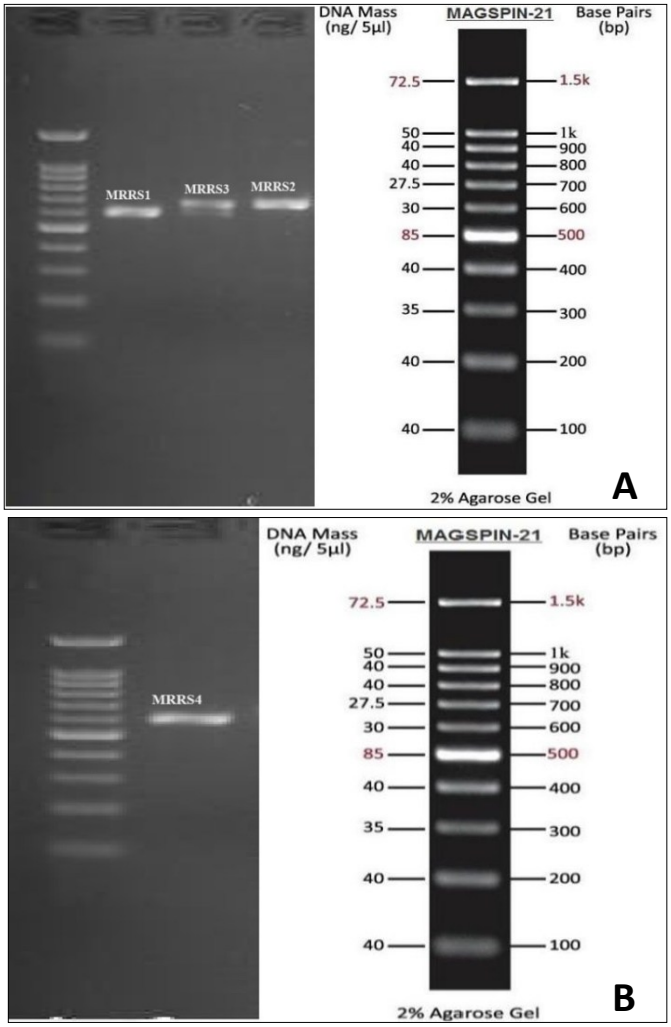

Fig. 3.PCR product amplified from ITS Region in *Fusarium* sp.(**A**) and *C. gloeosporioides* (**B**) L-100 bp ladder, the expected band size has been significantly amplified in all samples.

Table 2. List of *Fusarium* and *Colletotrichum* isolates with their GenBank accession number, primer name, band length and sequence similarity

Fungus (Isolate name)	GenBank Accession Number	Primer Name	Band Length	Sequence similarity (%)
F. verticillioides (MRRS1)	OQ363325	ITS1-ITS4	517	100
F. equiseti (MRRS2)	OP735534	ITS1-ITS4	549	100
F. oxysporum (MRRS3)	OQ438654	ITS1-ITS4	531	100
C. gloeosporioides (MRRS4)	PP448030	ITS1-ITS4	596	100

seti (Accession No. OP735534), MRRS3 as *F. oxysporum* (Accession No. OQ438654) and MRRS4 as *C. gloeosporioides* (Accession No. PP448030) (Table 2). According to earlier reports (9, 37, 47), molecular identification was done by PCR using the ITS1 and ITS4 primers for *Fusarium* and *Colletotrichum* strains in banana crops and other tropical fruits. Strong support was seen at internal nodes in the phylogenetic study, which used the neighbour-joining approach with 1000 bootstrap replicates. The dendrogram in MEGA-11 that established the relationships between the various species of *Fusarium* represented that the species was divided into 3 groups: I, II and III (Fig. 4). Similarly, the species of *Colletotrichum* were divided into 10 general groups: I-X of related species (Fig. 5). *Fusarium* phylogenic

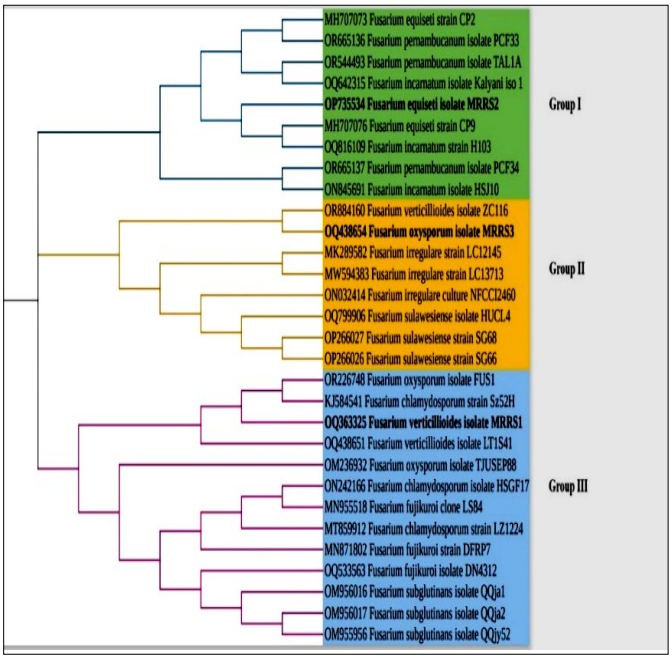

Fig. 4. Phylogenetic tree of *Fusarium* genus based on ITS nucleotide sequences constructed using the neighbor-joining method in MEGA 11.0. The *Fusarium* isolate of the present study is marked in bold black color in the phylogenetic tree.

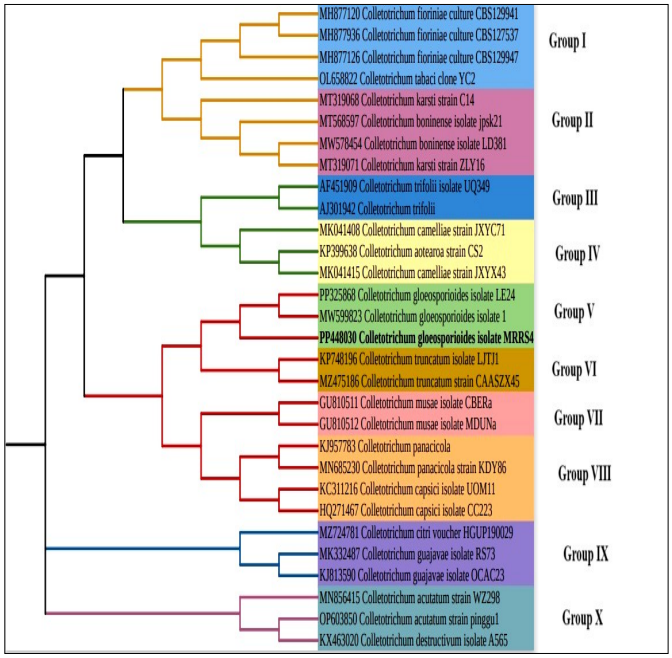

Fig. 5. Phylogenetic tree of *Colletotrichum* genus based on ITS nucleotide sequences constructed using the neighbor-joining method in MEGA 11.0. The *Colletotrichum* isolate of the present study is marked in bold black color in the phylogenetic tree.

analysis indicating that the new isolate MRRS1 (*F. verticillioides*, Acc. No. OQ363325) directly falls into group III along with *F. chlamydosporum* strain Sz52H (Acc. No. KJ584541), *F. oxysporum* isolate FUS1 (Acc. No. OR226748) and *F. verticillioides* isolate LT1S41 (Acc. No.OQ438651). Isolate MRRS2 (*F. equiseti*, Acc. No. OP735534) directly share with *F. equiseti* strain CP9 (Acc. No. MH707076) and *F. incarnatum* strain H103 (Acc. No. OQ816109) under group I and isolate MRRS3 (*F. oxysporum*, Acc. No. OQ438654) makes up under *F. verticillioides* isolate ZC116 (Acc. No. OR884160) under group III. This observation is consistent with other research (38, 39) documented in this research. In the case of *Colletotrichum* phylogenic analysis, the new isolate MRRS4 (*C. gloeosporioides*, Acc. No. PP448030) pertains under group VI, *C. truncatum* (Acc. No. MZ475050) and *C. gloeosporioides* (Acc. No. PP325868, MW599823) under group V (40). The phylogenetic study illustrates the evolutionary relationships between *Fusarium*, *Colletotrichum* and closely related genera. It implies that these genera have common ancestors with *Phomosis*, *Verticillium*, *Cercospora*, *Pyricularia*, *Lasiodiplodia*, *Rhizoctonia*, *Alternaria* and *Botrytis*, which are classified into clusters I -VIII based on relatedness (Fig. 6). *Fusarium* and *Colletotrichum*, both in Group VI, share a more recent progenitor and are closely linked to *Lasiodiplodia theobromae* in Group V. These findings support previous studies (41), which share information on the evolutionary history of these plant pathogenic fungus and their relationships with

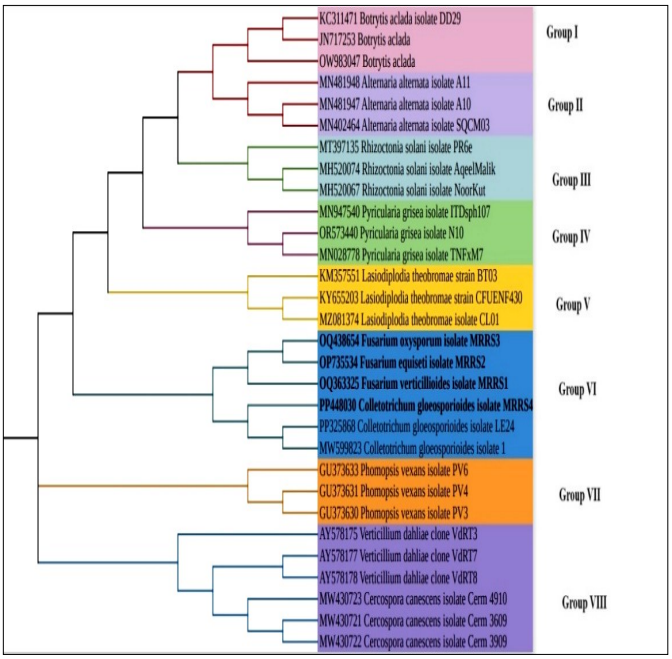

Fig. 6. Neighbor-joining tree representing the phylogenetic relationship of *Fusarium* and *Colletotrichum* genus and its closely related genera derived from the ITS region in MEGA 11.0. *Fusarium* and *Colletotrichum* isolate of the present study are marked in bold black color in the phylogenetic tree.

other genera.

Pathogenicity test and screening of the banana varieties

Four fungal isolates, *F. verticillioides* (MRRS1), *F. equiseti* (MRRS2), *F. oxysporum* (MRRS3) and *C. gloeosporioides* (MRRS4) were selected for pathogenicity testing following wounding methods. Banana fruit was inoculated with conidial suspensions from each isolate. All inoculated fruits

developed the first symptoms after 2 days. Fruits that were treated with *Fusarium* spp. (*F. verticillioides, F. equiseti* and *F. oxysporum*) exhibited rapid lesion enlargement, producing brown to dark brown patches on the fruit crowns that were ultimately covered in white mycelia (Fig. 7). However, in the case of *C. gloeosporioides*, symptoms such as crown tissue necrosis with orange colour mould growth were seen (Fig. 7). Disease lesion measurements (mm) were taken every 2 days interval up to 10 days. There was no fungus growth on the crown lesion of banana fruits on day 2. On day 4, the development of white fungal mycelia was observed in G9, Patakpura and Champa varieties with 6.21– 24.44 %, 3.99 – 19.10 % and 1.77 –15.11 % disease severity (4 isolates) respectively (Table 3). Banana varieties were found moderately infected with 9.32-75.99 % DS on day 6 and day 8, while severe infection was observed on day 10 with 64.88-96.88 %, 58.21-86.21 % and 43.99-72.88 % DS (4 isolates) in G9, Patakpura and Champa varieties of banana fruits respectively (Table 3). The disease progressed over time based on the area under disease progress curve (AUDPC) was highest in the G9 (399.07 – 206.19) followed by Patakpura (337.74 – 163.08) and Champa (300.41–

Fig. 7. Pathogenicity test using all isolated pathogens on G9 (**A**), Patakpura (**B**) and Champa (**C**) varieties of banana fruits after ten days of inoculation.

Table 3. Disease Severity Index (DSI) for each 2 days interval up to 10 days of *Fusarium* and *Colletotrichum* isolates, including (A) G9, (B) Patakpura and (C) Champa varieties

A. Disease severity of pathogens on G9 variety				
Isolates	**Day 4**	**Day 6**	**Day 8**	**Day 10**
F. equiseti	24.44	50.66	75.99	96.88
F. verticilloids	19.55	43.99	67.10	89.77
F. oxysporum	12.88	37.77	61.77	83.95
C. gloeosporioides	6.21	21.77	42.66	64.88
SE(m)	0.94	0.87	1.19	1.16
CD 5 %	2.82	2.62	3.58	3.49
B. Disease severity of pathogens on Patakpura variety				
Isolates	**Day 4**	**Day 6**	**Day 8**	**Day 10**
F. equiseti	19.10	42.66	63.99	86.21
F. verticilloids	14.21	40.88	55.99	77.32
F. oxysporum	11.55	36.88	53.77	75.99

C. gloeosporioides	3.99	14.21	34.21	58.21
SE(m)	0.86	1.17	1.04	0.83
CD 5 %	2.58	3.52	3.12	2.49
C. Disease severity of pathogens on Champa variety				
Isolates	**Day 4**	**Day 6**	**Day 8**	**Day 10**
F. equiseti	15.10	39.10	59.55	72.88
F. verticilloids	11.55	35.55	55.99	69.77
F. oxysporum	8.43	33.33	52.00	64.88
C. gloeosporioides	1.77	9.32	20.44	43.99
SE(m)	0.83	0.99	1.15	1.15
CD 5 %	2.49	2.97	3.46	3.46

*****Mean(± SD) of 5 replications. Numerical values followed by the same letter significantly differ at the 5 % level ((p < 0.05).

107.08) (Table 4, Fig. 8). The lesions subsequently spread to the entire fruit and merged on the banana sample within 12 to 14 days, which were inoculated by *Fusarium* but *C. gloeosporioides* taken 15 to 17 days spread to the entire fruits after the occurrence of necrosis. Pathogenicity tests indicated that all species exhibited symptoms associated with crown rot, but the disease severity with AUDPC varied and made it prominent which species was the most virulent. The most virulent species were *F. equiseti, F. verticillioides* and *F. oxysporum* followed by *C. gloeosporioides*. Following the screening of banana cultivars, G9 was most susceptible to crown rot pathogens, followed by Patakpura and Champa. However, no symptoms of crown rot disease were observed in the inoculation treatments involving sterile distilled water among all wounded control fruits. The fungi were re-isolated from symptomatic fruit tissue and then cultured on PDA to fulfil Koch's postulates. The re-isolated fungi were identified as *F. verticillioides*, *F. equiseti*, *F. oxysporum* and *C. gloeosporioides*. The results of other earlier investigations corroborate our findings that *Fusarium* is the most common pathogen in banana crown rot disease and an economically important plant pathogen in the postharvest stage (9, 27). According to previous reports (42), *F. equiseti* is a common species isolated from many fruits and vegetables during the postharvest stages. Fruit rot disease is caused by *F. equiseti* on watermelon specimens collected frequently in China (43) and Malaysia (44). *F. verticillioides* is also frequently associated with banana crown rot disease (45, 46).

Table 4. Area under disease progress (AUDPC) of *Fusarium* and *Colletotrichum* isolates on each variety of banana after ten days of inoculation

Isolates	**AUDPC**		
	G9	**Patakpura**	**Champa**
F. equiseti	399.07	337.74	300.41
F. verticilloids	351.08	299.52	275.96
F. oxysporum	308.82	280.41	252.41
C. gloeosporioides	206.19	163.08	107.08
SE(m)	4.11	3.65	3.42
CD 5 %	12.33	10.96	10.25

*****Mean(± SD) of 5 replications. Numerical values followed by the same letter significantly differ at the 5 % level ((p < 0.05).

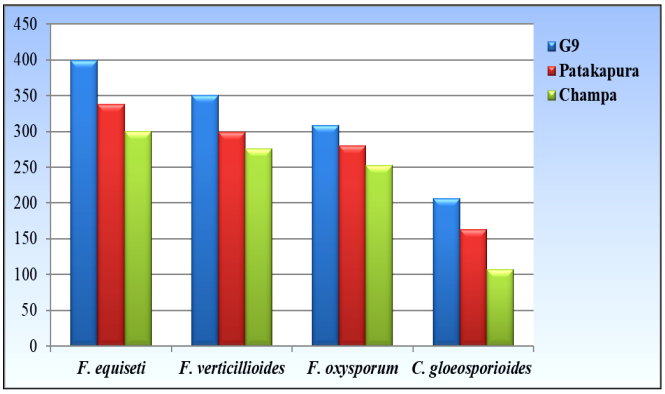

Fig. 8. The mean AUDPC values for all *Fusarium* and *Colletotrichum* isolates over 3 banana varieties (G9, Patakpura and Champa).

Conclusion

Fusarium species cause crown rot disease, one of the most prevalent postharvest diseases affecting bananas in Odisha's coastal areas. In this investigation, the diseased crown portion of the bananas was used to isolate 3 pathogenic *Fusarium* species: *F. verticillioides*, *F. equiseti* and *F. oxysporum* as well as one *Colletotrichum* species, *C. gloeosporioides*. Fungi associated with crown rot disease were found in all analyzed samples, dominated by *F. equiseti* (37.06 %), *F. verticillioides* (34.01 %), *F. oxysporum* (28.92 %) and *C. gloeosporioides* (15.92 %). Both morphological characteristics and molecular analyses were used to identify isolated fungi—the identification of *Fusarium* spp. causing crown rot disease in bananas reflects the importance of further research on *Fusarium* taxonomy to mitigate the risk to the banana fruit industry. More research will need to be conducted on the distribution of these diseases in other regions of India and the control of crown rot diseases will need to be undertaken. To address the significant economic losses caused by this disease, developing effective monitoring and preventative strategies in the future will be essential.

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Authors' contributions

MR had done all the experiments in the laboratory of the Department of Plant Pathology, Institute of Agricultural Sciences, Sikhsha 'O' Anusandhan (Deemed to be) University. SSM guided all the experiments overall. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no competing interests.

Ethical issues: None

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