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RESEARCH

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Fungal endophytes as potential biocontrol agent of Panama disease of banana

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Abstract

Background *Fusarium odoratissimum* (*Foc* TR4) is a devastating fungal pathogen that causes Panama wilt in Cavendish banana, a high value crop that generates significant revenue. The excessive use of synthetic fungicides for disease control poses risks to both human health and the environment. Consequently, there is a pressing need for eco-friendly alternatives to manage this disease. One potential approach is the utilization of biocontrol agents, which have shown promise in disease control. This study aimed to evaluate the biocontrol potential of fungal endophytes isolated from the naturally-resistant cultivar Cardaba banana against *Foc* TR4. These fungal isolates were subjected to a dual culture assay, characterized based on morpho-cultural characteristics, and confirmed molecularly using the internal transcribed spacer (ITS) region of rDNA.

Results Of the 15 fungal endophytes isolated from Cardaba; *Blakeslea trispora, Pseudopestalotiopsis theae*, *Xylaria badia, Nigrospora hainanensis, Colletotrichum gloeosporioides, Xylaria feejeensis, Gymnoascus reessii, Fusarium subglutinans, Rigidoporus vinctus,* and *Macrophomina phaseolina* showed potential antagonistic activity against *F. odoratissimum*. Isolates *X. feejeensis* (USMJMTBL10), *G. reesii* (USMJMTBL11), and *M. phaseolina* (USMJMTBR1) had the highest antagonistic activity of 87.8–96.6% against *F. odoratissimum* in vitro.

Conclusion This study presents evidence of the potential of Cardaba-associated endophytes isolated and identified in this research, as effective biocontrol agents against *F. odoratissimum*, the causal agent of Panama disease in Cavendish banana. Our findings suggest that these endophytes hold promise as a biocontrol agent for managing *Foc* TR4-infected banana plants. Future research will focus on in planta testing, validation in greenhouse and field trials, as well as development of formulations and application protocols to optimize the utilization of these biocontrol agents in an integrated management approach for *Foc* TR4-infected bananas.

Keywords Antagonistic activity, Biocontrol, *Foc* TR4, Internal transcribed spacer (ITS), Panama disease of banana

Background

Banana (*Musa* spp.) continues to dominate the global fruit market, with over 135 countries involve in its production (FAO 2021). Over the years, its production and cultivated areas have increased (FAO 2021). Aside from being a major cash crop, bananas are grown in tropical and subtropical regions for local consumption (Olaoye and Ade-Omowaye 2011). In 2020, banana exports reached an estimated 22.2 million tons, with 1.7% increase compared to 2019. This growth was primarily attributed to the successful implementation of disease mitigation strategies in banana plantations of the top

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exporting countries, namely Ecuador, Costa Rica, and Colombia (FAO 2021). In Asia, 90% of exported bananas originate in the Philippines (Department of Agriculture 2019). However, the country experienced a significant decline of 46% in banana exports due to a decrease in production caused by Panama wilt, attributed to *F. odoratissimum* (Thangavelu et al. 2019).

Banana cultivars are highly susceptible to diseases (Molina et al. 2008). While the Cavendish banana, a triploid (AAA) cultivar of *Musa acuminata*, exhibits resistance to *Fusarium oxysporum* f. sp. *cubense* (*Foc*) race 1 (R1), which destroyed 'Gros Michel' cultivar, it remains susceptible to *Foc* tropical race 4 (TR4), now known as *F. odoratissimum* (Thangavelu et al. 2019). *Foc* TR4 is a virulent strain of *Foc* that poses a severe threat to the global Cavendish banana industry. In the Philippines, the presence of *Foc* TR4 was first confirmed in 2005, affecting numerous Cavendish farms in the Mindanao provinces of the Southern Philippines (Molina et al. 2008).

Chemical soil fumigation and the use of resistant cultivars are the two methods commonly employed for managing Panama wilt (Fravel et al. 2003). Broad-spectrum biocides such as methyl bromide have been used for soil fumigation before planting. However, these are harmful to human health and detrimental to the environment. Alternatively, genetic resistance provides a sustainable long-term approach for managing and controlling Panama wilt. The use of resistant cultivars is cost-effective and environment-friendly technique for pathogen control (Fravel et al. 2003). Yet, there are currently no commercially viable cultivars available that exhibit resistance against *Foc* TR4 (Chen et al. 2019).

Considerable efforts have been done to search for resistant cultivars against *Foc* TR4 (Zuo et al. 2018). However, transferring the favourable alleles from the resistant cultivar into a commercially viable cultivar with good agronomic traits is challenging (Dita et al. 2018). Resistant traits to *Foc* have been identified in wild banana species (Li et al. 2014) such as in *Musa acuminata* ssp. *Malaccensis*, which exhibits resistance to *Foc* race 1 (Ahmad et al. 2020). Notably, the Cardaba cultivar (Saba subgroup) in the Philippines showed an absence of *Foc* TR4 infection (Molina et al. 2016), indicating its resistance to Panama disease. Additionally, Solpot et al. (2016) found that Cardaba cultivar of banana, a triple hybrid that originated from the Philippines, remains free from *Foc* TR4 infection in South-Central Mindanao.

Another influential factor contributing to the resistance of certain cultivars is the diverse microbial community associated with the banana plant (Catambacan and Cumagun 2021). Several studies have demonstrated that the synergistic and cumulative effects of microbial communities enhance a plant's resistance to biotic and abiotic challenges while promoting growth. Specifically, the application of microbial antagonists and/or organic fertilizers has shown promising results in altering the microbiota of the banana rhizosphere, leading to effective control against *Foc* TR4 (Xue et al. 2015).

The challenge of effectively controlling Panama wilt has prompted research in biological control to manage soilborne pathogens (Khan et al. 2018). Fungi possess several characteristics as candidates for biological control agents, as they help control pathogenic organisms that attack plants. For instance, fungi can inhibit or reduce the infection of the pathogen in host plants or impede the initial establishment of the pathogen (Thambugala et al. 2020). Additionally, fungi can also colonize tissues of plants as endophytes (Arnold 2007). Endophytic fungi not only contribute to reducing disease severity but also promote plant growth, induce plant defense mechanisms, and produce compounds with anti-herbivore properties (Rudgers et al. 2007). Therefore, the aim of this study was to isolate fungal endophytes from the naturally-resistant Cardaba variety and evaluate their potential as a biocontrol agent against *F. odoratissimum* (*Foc* TR4) causing Panama wilt in Cavendish banana.

Methods

Culturing and molecular analysis of *F. odoratissimum*

The *Foc* isolate was obtained from the Plant Pathology Research Laboratory, University of Southern Mindanao Agricultural Research and Development Center, USM, Kabacan, Cotabato, Philippines. The isolate was subcultured in PDA (Potato Dextrose Agar) medium supplemented with ampicillin and incubated for 14 days. Figure 1A and B show the *F. odoratissimum* isolate at 14 days of incubation in PDA medium. The colony growth of *F. odoratissimum* isolate exhibited a white cottony and/or fluffy aerial mycelium, which corresponds to the description of Leslie and Summerell (2006) on *Foc* mycelia in PDA. Figure 1C shows the chlamydospores in pairs at 3 weeks of incubation, and in single and/or in chains at 2 months of incubation (Fig. 1D). The macroconidia are falcate to upright to practically straight and the microconidia appeared oval to kidney-shaped (Fig. 1E) typical for the description of *F. odoratissimum* isolate (Perez-Vicente et al. 2014).

To validate the isolate as *Foc* TR4, genomic DNA of *Foc* isolate was extracted following the DNEasy Powersoil kit protocol. The extracted DNA was quantified using a spectrophotometer (MultiSkan Sky, ThermoFisher). The PCR amplification was carried out through Promega Go Taq 2×MasterMix using universal primers F-ITS1 (5′-TCCGTAGGTGAACCTGCG G-3′) and F-ITS4 (5′-TCCTCCGCTTATTGATAT GC-3′) by White et al. (1990). The following conditions

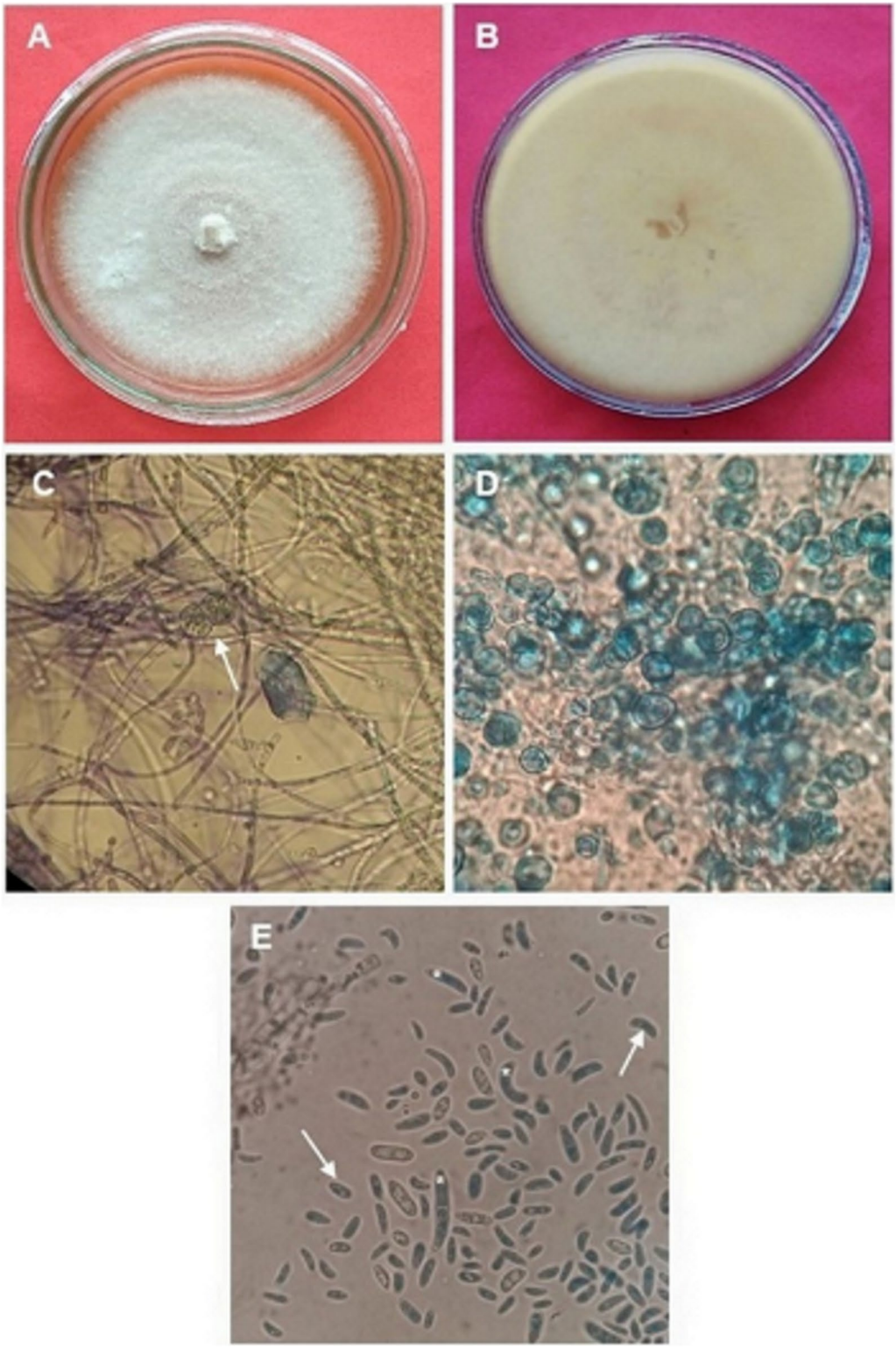

Fig. 1 Cultural and morphological characteristics of *Fusarium odoratissimum* grown in PDA plate. **A** Obverse view. **B** Reverse view. **C** Chlamydospores under the microscope appear in pair (see arrow). **D** Chlamydospores (single and/or in chains). **E** Oval to kidney-shaped microconidia (in arrow) and falcate to upright to practically straight macroconidia (in asterisk)

were followed: initial denaturation at 94 °C for 2 min, followed by 35 cycles of each denaturation at 94 °C for 30 s, primer annealing at 57 °C for 30 s, extension at 72 °C for 2 min, and at last 1 cycle final extension at 72 °C for 10 min. The PCR product was purified following the protocol of Vivantis Gel/PCR Purification kit. Sequencing was done using Genetic Analyzer SeqStudio (Thermo Fisher) kit: Big Dye Terminator kit V3 (Philippine Genome Center). The forward and reverse sequences were trimmed, aligned, and cleaned up using FinchTV, BioEdit, and Molecular Evolutionary Genetics Analysis (MEGA). The generated DNA sequence of the *Foc* TR4 isolate used in this study was analysed using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI), where functional and evolutionary relationships between the DNA sequences can identify members of gene families (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The DNA sequence homology from the BLASTn search revealed that the *Foc* isolate used in this study had a significant BLAST hit with the fungal organism belonging to *F. oxysporum* f. sp. *cubense*, which corresponds to the deposited *Foc* TR4 sequence in NCBI GenBank (Nakkeeran et al. 2021).

Pathogenicity test of *F. odoratissimum* isolate

The pathogenicity test was conducted following the technique of Catambacan and Cumagun (2021) with some modifications. Six tissue-cultured Cavendish cv. Grand Naine banana plants, aged four weeks, were transplanted into 350 cm^3 pots filled with sterile clay loam. Prior to inoculation, two root hairs of each plantlet were exposed and wounded using a sterile scalpel. A two-week-old pure culture of *Foc* TR4 was then inoculated to the base of the three plantlets by drenching 50 ml spore suspension (10^6 spores per ml). The inoculated roots were immediately covered with soil. Another three plantlets were designated as the uninoculated control treatment and were applied only with sterile PDA media. At two and four weeks post-inoculation, the banana plantlets were assessed for the presence of typical symptoms associated with Panama wilt. Yellowing of old leaves (Fig. 2A) and vascular discoloration (Fig. 2B) were observed two weeks after inoculation. The symptoms of *Foc* TR4 infection in this study correspond to the study of Catambacan and Cumagun (2021). The uninoculated plantlets were all healthy with no visible yellowing of leaves nor pseudostem discoloration (Fig. 2C, D). After the pathogenicity testing, the materials and the soil were heat-sterilized, and the infected banana plantlets were properly disposed by burning.

Isolation and culturing of the fungal endophytes

Healthy leaves, pseudostem, and roots were randomly collected from five young (≤6-months) and five old (>6-month-old) Cardaba banana which were not sprayed with chemicals from a backyard banana farm at Hagonoy, Davao del Sur, Philippines for the isolation of fungal endophytes. The leaves were obtained by directly cutting a section of the lamina near the petiole, extending to the edge of the midrib. The pseudostems were collected by cutting a triangular shape with a depth of 3 inches from the outer to the inner portion of the pseudostem, while the roots near the corm were collected. Subsequently, the samples were placed inside the sterile plastic bags and processed in the laboratory to isolate the fungal endophytes. Three replications of each plant part were considered for a total of 90 plant samples.

Endophytes were isolated immediately after sampling following the standard procedure described by Pujade-Renaud et al. (2019). Banana leaves and pseudostems were divided into three segments, each measuring about 3×3 mm. These segments were briefly immersed in solutions consisting of 2% (vol/vol) sodium hypochlorite solution, 70% (vol/vol) ethanol, and sterilized water. Afterwards, they were planted equidistantly on flat bottles containing Malt Extract Agar (MEA). For root samples, 5 g of fresh root sample was soaked in a 100 ml solution containing 5% sodium hypochlorite and agitated for 5 min. Subsequently, the roots were immersed in 70% ethanol for 10 min to ensure complete surface sterilization. The root surface was thoroughly rinsed three times with sterilized water to remove any residual sterilizing agents. The surface-sterilized roots were cut into 1 cm sections and was placed equidistantly on flat bottles with MEA supplemented with ampicillin (Catambacan and Cumagun 2021). These were incubated for 3–7 days at 28 °C. Subsequent transfer to PDA medium was performed to purify the fungal endophytes. In this study, a sterile PDA medium served as the negative control.

Dual culture test

Pure cultures of fungal endophytes were subjected to a dual culture method (Rahman et al. 2009) to assess the interaction and antagonism between the isolated fungal endophytes and the pathogen *Foc* TR4 within the same Petri plate. Five mm diameter of mycelial plugs from a 7-day-old *Foc* TR4 culture and fungal antagonist were obtained using a sterile cork borer. The mycelial plug was placed two cm from the edge of the plate, while the pathogen was positioned at the opposite side of the antagonist. The setup for the dual culture assay was replicated three times. A PDA plate inoculated solely

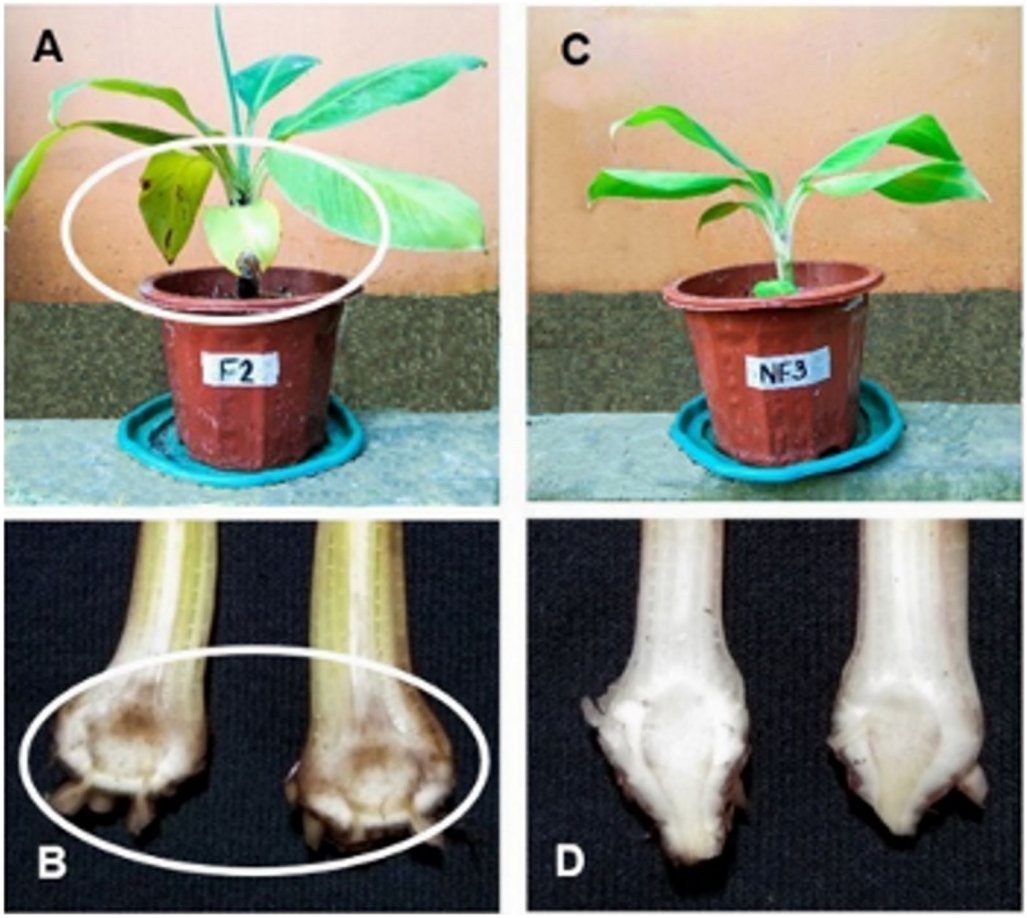

Fig. 2 Banana plantlets subjected to the pathogenicity of *Foc* TR4 isolate. **A** Yellowing of old banana leaves at 2 weeks after inoculation. **B** Vascular discoloration of banana pseudostem at 4 weeks after inoculation. **C** and **D** Control treatment without inoculation of *Foc* TR4

with the pathogen served as the control. The plates were then incubated at 28 °C for a period of 7 days.

The fungi were evaluated using the rating scale developed by Bell et al. (1982) with stages 1–5 representing the overgrowing capabilities of the fungal endophytes, where Stage 1 = the antagonist completely outgrew the pathogen and took up the entire surface of the medium, Stage 2 = the antagonist outgrew the pathogen by at least two-thirds, Stage 3 = the antagonist and the pathogen each colonized half of the medium surface, Stage 4 = the pathogen outgrew the antagonist by at least two-thirds, and Stage 5 = the pathogen completely outgrew the antagonist and took up the entire surface of the medium. If the mean score, rounded to the closest whole stage, for a specific comparison was less than or equal to two, a fungal endophyte (FE) was considered antagonistic to the pathogen.

Percentage inhibition of pathogen was calculated by the following formula (Fokkema 1973):

$$\text{Percent inhibition} = \frac{R1 - R2}{R1} \times 100$$

where $R1$ = the radial growth of *Foc* TR4 in the control plate (mm), and $R2$ = the radial growth of *Foc* TR4 towards the antagonistic fungi (mm).

The fungal endophytes' antagonistic activity was described using the technique of Soytong (1988) where > 75% means very high antagonistic activity, 61–75% as high antagonistic activity, 51–60% being moderate antagonistic activity, and > 50% as having low antagonistic activity.

Morpho-cultural characterization of the fungal endophytes

The stock cultures of the purified fungal endophyte isolates were grown in PDA plates and incubated at room temperature in an inverted position. Fungal growth was observed daily for a duration of 7 days, then growth diameter was measured and various colony morphology

parameters such as shape, texture, surface, and reverse color were recorded. For microscopic examination, the scotch tape method was used wherein a piece of scotch tape was placed on the top of each fungal endophyte isolate. One drop of lactophenol cotton blue was added to the slide and the tape with fungal endophytes was positioned on top. The slide was examined under a 100–1000 × light microscope. Identification of fungal endophytes was based on their morphological characteristics, primarily at the genus level (Barnett and Hunter 1998). Endophytic fungal isolates which did not produce any spores in culture media were categorized as non-sporulating fungal endophytes.

Molecular identification of the fungal endophytes

DNA extraction

Fungal endophytes belonging to stages 1–3 of Bell's rating and exhibiting moderate to very high inhibitory activity against *Foc* TR4, as determined by the dual culture assay were further identified using molecular analysis. The 7 days old fungal isolates grown in PDA at 28 °C were carefully scraped using a scalpel to isolate fungal DNA. Genomic DNA was extracted using the Norgen Biotek Corp plant/fungi DNA isolation kit, following the manufacturer's protocol. The concentration and purity of DNA samples were assessed and quantified using the SPECTROstar Nano instrument.

PCR amplification

Amplification of the internal transcribed spacer (ITS) region of the fungal isolates was performed using the universal forward primer ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) as described by White et al. (1990) with a target size of 550–700 bp of the amplicon. PCR amplification was carried out on a programmable Veriti 96 Well Thermal Cycler in 0.2 mL PCR tubes containing 5 µl of DNA template, 25.5 µl ddH2O, 14 µl 5 × MyTaq reaction buffer, 1.5 µl of Taq, and 2 µl of each primer, a total volume of 50 µl PCR reaction. Conditions that were used for PCR amplification included initial denaturation at 95 °C for 1 min followed by 35 cycles of each denaturation at 95 °C for 15 s, primer annealing at 52 °C, 54 °C, 56 °C, and 58 °C (via VeriFlex) for 15 s, and an extension at 72 °C for 10 s. A non-template negative control was included in each PCR run to monitor for potential contamination.

Gel electrophoresis and visualization

The integrity and quality of PCR products were assessed using Labnet's ENDURO™ Gel XL Electrophoresis System. A volume of 4 µl of each PCR product was loaded onto a 0.8% agarose gel supplemented with 2 × loading dye. After which, the gel was stained with ethidium bromide for 5 min, and DNA bonds were visualized under the Azure 200 Gel Imaging Workstation. After checking for single bond amplifications of fungal endophyte isolates, PCR products (50 µl) were prepared for sequencing through Noveaulab Asia Corporation.

Bioinformatics and statistical analyses

Chromatograms were processed using BioEdit software. The universal primers and paired-end reads were first removed. Forward and reverse sequences were aligned and a consensus sequence was generated. The resulting DNA sequences of the fungal endophyte isolates were analyzed using BLAST. Nucleotide sequences of the fungal endophytes were identified by comparison to those in the GenBank database. Maximum score and percent identity are the basis of determining the top three blast hits for each fungal endophyte isolate. Evolutionary analyses were conducted using MEGA11. The relationship among the isolates was inferred using the neighbor-joining method. A consensus tree was generated from 1000 replicates, with bootstrapping performed from the taxa analyzed.

All data were statistically analysed using analysis of variance (ANOVA) and the computed antagonistic activity of Cardaba fungal endophytes were compared using the Tukey Honestly Significant Difference (HSD) test at 0.05 level of significance using IBM SPSS statistical software.

Results

Fungal endophytes from healthy Cardaba banana

A total of 15 fungal endophytes isolates were obtained from the leaves, pseudostem, and roots of Cardaba banana. There were 11 fungal endophytes isolated from Cardaba banana leaves, three isolates from the pseudostem and one from the roots (Fig. 3). In the dual culture test, 10 out of the 15 fungal endophyte isolates grew faster than *F. odoratissimum,* while five isolates were slow-growing (Fig. 4). Four of the 10 fungal endophyte isolates occupied half of the plate and two isolates overgrew the pathogen by at least two-thirds. Four isolates USMJMTBL1, USMJMTBL11, USMJMTBP3, and USMJMTBR1 completely overgrown the pathogen at 7 days after incubation (DAI).

In terms of growth inhibition, all fungal endophyte isolates displayed antagonistic activity against *F. odoratissimum*, albeit at varying degrees of antagonism (Table 1). Within 14 DAI, the growth of *F. odoratissimum* was suppressed by all fungal endophyte isolates, except for USMJMTBL1. Moderate inhibition, exceeding 50%, was observed in three isolates, while 7 fungal endophyte isolates exhibited high inhibition percentages ranging from 61 to 75% against *F. odoratissimum*. Notably, the growth

Fig. 3 Photographs of the 15 fungal endophytes isolated from healthy Cardaba banana leaves (**A**–**K**), pseudostem (**L**–**N**), and roots (**O**) at 7 days after incubation

of *F. odoratissimum* was greatly reduced by the isolates USMJMTBR1 (96.56%), USMJMTBL10 (93.33%), and USMJMTBL11 (87.77%). However, isolates USMJMTBL1 and USMJMTBL2 exhibited low inhibition against the pathogen with only 46.67%. At 7 DAI, USMJMTBL1 exhibited faster growth compared to *F. odoratissimum*, but by 14 DAI, regrowth of *F. odoratissimum* was observed, indicating that USMJMTBL1 was unable to effectively inhibit the growth of the pathogen.

Morpho-cultural identity of the fungal endophyte isolates

The ten fungal endophyte isolates were characterized through combined morphological and cultural traits. Most of the fungal colonies had a circular shape while others had semi-circular or irregular shape (Table 2). More than 50% of the fungal endophyte isolates had cottony and/or fluffy to powdery or granular texture, while others had a web-like appearance. Majority of the isolates had white to pale white mycelium. Some isolates

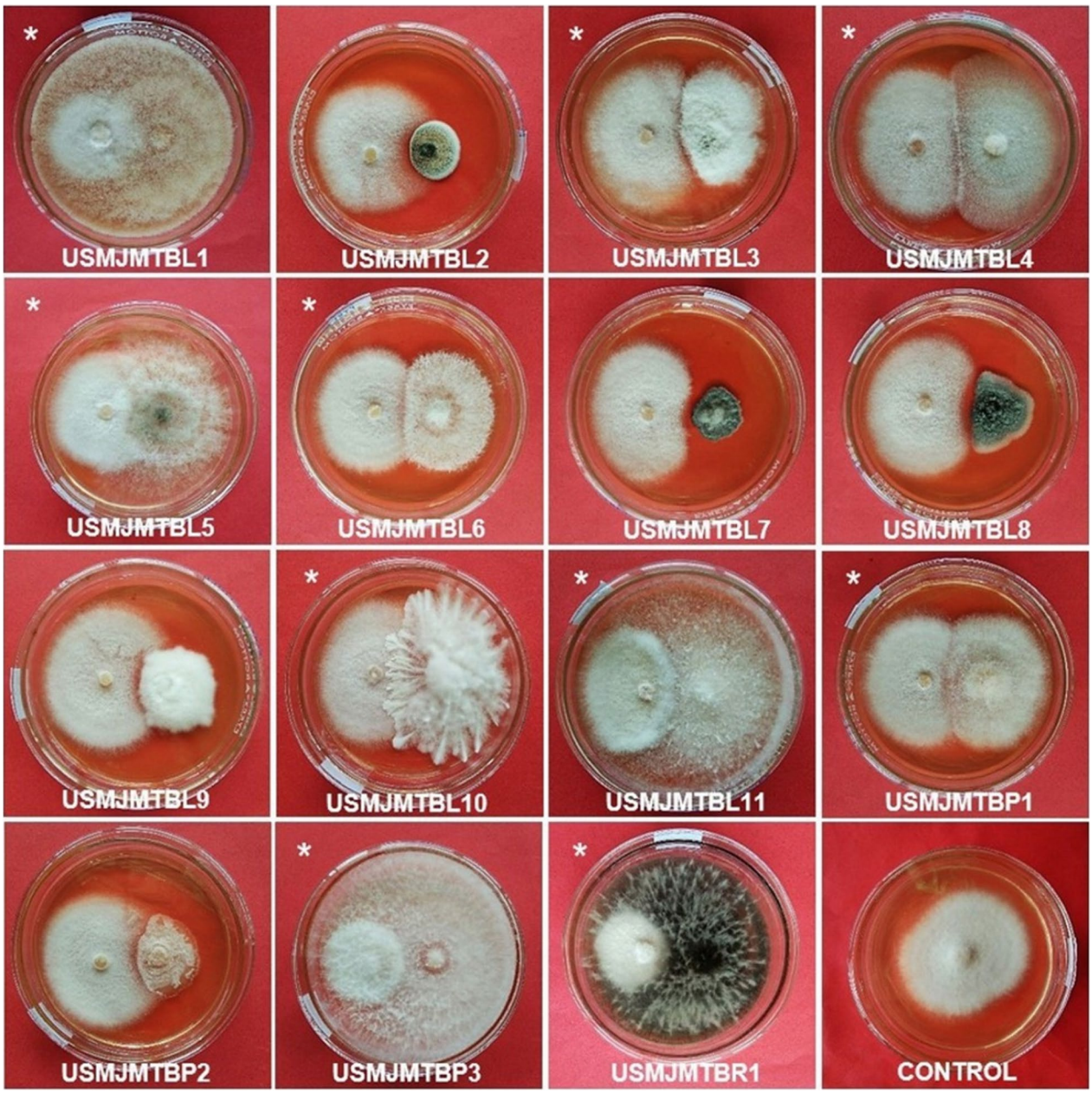

Fig. 4 Antagonistic activity of fungal endophytes (right) against *Fusarium odoratissimum* (left) at 7 days after incubation. Fast-growing fungal endophyte isolates are indicated with asterisks (*)

exhibited scattered black spots and pale white patches at the edge when viewed in reverse, while one isolate had dark grey mycelium. The average growth diameter of the fungal colonies ranged from 70 to 90 mm at 7 DAI on the PDA plate.

On morphological characteristics shown in (Table 2 and Fig. 5), the shape of conidia varied from spherical to cylindrical to elliptical, and from micro to macroconidia. Most fungal hyphae were hyaline or clear/non-pigmented to pale and dark brown. The majority had septa and varied from branched, highly branched to highly coiled hyphae. Based on the cultural and morphological characteristics, isolates were identified as *Blakeslea*, *Colletotrichum*, *Fusarium, Gymnoascus*, *Macrophomina*, *Nigrospora*, *Pseudopestalotiopsis*, *Rigidoporus*, and *Xylaria* but were subjected to molecular confirmation.

Table 1 Degree of antagonism of fungal endophytes in co-cultivation with *Fusarium odoratissimum* in vitro conditions

Fungal endophyte isolates	Bell's rating (1–5)	Growth inhibition (%)	Degree of antagonism
USMJMTBL1	1	46.67	Low
USMJMTBL2	4	46.67	Low
USMJMTBL3	3	61.67	High
USMJMTBL4	3	53.33	Moderate
USMJMTBL5	2	70.00	High
USMJMTBL6	3	63.89	High
USMJMTBL7	4	56.67	Moderate
USMJMTBL8	4	54.43	Moderate
USMJMTBL9	4	61.11	High
USMJMTBL10	2	93.33	Very high
USMJMTBL11	1	87.77	Very high
USMJMTBP1	3	61.67	High
USMJMTBP2	4	63.33	High
USMJMTBP3	1	63.89	High
USMJMTBR1	1	96.56	Very high

Molecular identity of the fungal endophyte isolates

Optimization of the PCR amplification of the fungal endophytes revealed successful amplification of all PCR products of the pathogen using annealing temperatures (VeriFlex) of 52 °C for USMJMTBL5, 54 °C for USMJMTBP1, USMJMTBP3, and USMJMTBR1, 56 °C for USMJMTBL3, USMJMTBL4, and USMJMTBL6 and 58 °C for USMJMTBL1, USMJMTBL10, and USMJMTBL11. Table 3 shows the closest similar organisms with significant BLAST hits to the fungal endophyte isolates, at 86–99% similarity, and their corresponding biological roles.

Comparison of the antagonistic activity of the fungal endophytes against *F. odoratissimum*

Among the 10 fungal endophyte isolates (Table 4), *B. trispora* (USMJMTBL1) had the least antagonistic activity but comparable to *X. badia* (USMJMTBL4). The isolates *X. feejeensis* (USMJMTBL10), *G. reesii* (USMJMTBL11), and *M. phaseolina* (USMJMTBR1) had the highest antagonistic activity and were significantly different from *B. trispora* (USMJMTBL1), *P. theae* (USMJMTBL3), *X. badia* (USMJMTBL4), *N. hainanensis* (USMJMTBL5), *C. gloeosporioides* (USMJMTBL6), *F. subglutinans* (USMJMTBP1), and *R. vinctus* (USMJMTBP3).

Discussion

Majority of the fungal endophytes were isolated from the leaves of Cardaba while a small number was obtained from the pseudostem and roots. The variation in the abundance of fungal endophytes among the different plant tissues could be influenced by environmental conditions which may affect their functional activity (Horner-Devine et al. 2007). The PCR amplification using the ITS1 and ITS4 primer pair resulted in ~650 base pair (bp) product from all fungal endophytes isolated, which falls within the typical size range of rDNA ITS amplicon size for fungal isolates (Catambacan and Cumagun 2021). Some of the fungal endophytes isolated in this study demonstrated fast growth compared to the growth of the *F. odoratissimum* pathogen, suggesting that they may have competitive advantage when coexisting with other fungi in a plant host. This often leads to a high isolation frequency for fast-growing endophytes in various habitats (Huang 2020). One such isolate, USMJMTBL1 (*B. trispora*) exhibited rapid growth but failed to inhibit *F. odoratissimum* at 14 DAI. Its relationship with the pathogen remains unclear and warrants further investigation.

Out of the 15 fungal endophytes examined, 10 isolates exhibited notable antagonistic activity against *F. odoratissimum*. These isolates demonstrated the ability to overgrow the pathogen and displayed varying degrees of antagonism, ranging from moderate to very high. The variability in the degree of antagonism of the 10 fungal endophyte isolates against *F. odoratissimum* could be attributed to the different mechanisms of antagonistic activity they employ, such as competition, antibiosis, and parasitism (Nuraini et al. 2017). Competition with the pathogen was observed in endophytic fungal isolates including USMJMTBL4 (*X. badia*), USMJMTBL6 (*C. gloeosporioides*), USMJMTBP1 (*F. subglutinans*), and USMJMTBR1 (*M. phaseolina*). These isolates demonstrated dominance over the pathogen by occupying a larger surface of the culture medium. USMJMTBR1 (*M. phaseolina*) displayed the highest level of dominance over the pathogen, as evidenced by the growth inhibition and Bell's ratings. This suggests that the nutritional needs of *F. odoratissimum* may have been disrupted, leading to a decline in the germination percentage of the fungal spores (Berlian et al. 2013). Endophytes employing the competition mechanism act by colonizing and foraging nutrients, as well as occupying the space that pathogens would typically utilize for their activities (Rodriguez et al. 2009).

The mechanism of antibiosis was observed in the case of USMJMTBL3 (*P. theae*). Notably, a narrow demarcation line was observed between this isolate and *F. odoratissimum*. This inhibition could be attributed to the production of volatile organic compounds or antibiotic substances by *P. theae*, which hindered the further growth of the pathogen (Mahendran et al. 2021). Fungal endophytes are known to alleviate the consequences and severity of pathogenic infections by producing various effective secondary metabolites (Gao et al. 2010). In addition, Kurnia et al. (2014) explained that active

Table 2 Morphological and cultural characteristics of the fungal endophyte isolates from Cardaba banana

Isolate code	Cultural characteristics			Morphological characteristics			Genus
	Shape	Texture	Surface color	Reverse color	Conidia	Hyphae	
USMJMTBL1	Circular	Powdery, granular	Milky white to yellowish	Creamy yellowish	–	Aseptate, and highly branched	*Blakeslea*
USMJMTBL3	Irregular	Cottony	White	Pale white	Elliptical, slightly curved, septate	Hyaline, highly branched	*Pseudopestalotiopsis*
USMJMTBL4	Circular	Cottony, fluffy	White	Pale white	Hyaline, smooth to finely roughened	Hyaline, branched	*Xylaria*
USMJMTBL5	Irregular	Granular, cottony	Ashy white with black mycelium at the middle	Black mycelium in the middle and dark grey patches at the edge	Spherical, black, shiny, smooth	Smooth, hyaline to pale brown, branched, septate	*Nigrospora*
USMJMTBL6	Circular	Cottony, web-liked	Inner black mycelium with pale white patches at the edge	Dark grey mycelium	Straight cylindrical conidia	Highly branched	*Colletotrichum*
USMJMTBL10	Irregular	Feathery, web-liked	Creamy white	White mycelium	Hyaline, smooth	Branched, septate, highly coiled	*Xylaria*
USMJMTBL11	Circular	Cottony, fluffy	White	Pale white	Unbranched, cylindrical shaped with flattened ends	Branched, aseptate peridial	*Gymnoascus*
USMJMTBP1	Semi circular	Cottony, granular	White	White mycelium	0–1 septate microconidia three-septate macroconidia	Hyaline, septate	*Fusarium*
USMJMTBP3	Circular	Granular	Pale white	Pale white mycelium	–	Thin- walled, frequently branched, hyaline, simple-septate	*Rigidoporus*
USMJMTBR1	Irregular	Web-liked	Dark gray	Dark mycelium	Dark brown to black, rough, globose, beaked and ostiolated	Thin walls hyaline to dark brown, septate	*Macrophomina*

– = not detected at 7 days after incubation

compounds produced by endophytic microbes cause the hyphae of fungal pathogens to undergo malformations. Endophytic *M. phaseolina* was reported to produce secondary metabolites and volatile compounds (Singh et al. 2022) and in this study, this isolate exhibited a very high degree of antagonism against *F. odoratissimum*.

Two species of *Xylaria* in this study have varying degrees of antagonism. *Xylaria* species are known to produce a diverse array of secondary metabolites including volatile and non-volatile compounds, and aromatic compounds. These metabolites have demonstrated potential activity as herbicides, fungicides, and insecticides, while others possess antibacterial, antimalarial, and antifungal properties (Macías-Rubalcava and Sánchez-Fernández 2016.) The *X. feejeensis* isolate in this study displayed a growth inhibition of 93.33%, indicating a very high degree of antagonism against *F. odoratissimum.* The crude extract of *X. feejeensis* isolated from a mangrove tree was reported to inhibit *F. oxysporum*, the causative agent of Fusarium wilt and *Alternaria solani* which causes early blight in tomato (Brooks et al. 2022). The *X. badia* isolate in this study showed a growth inhibition of 53.33%, indicating a moderate degree of antagonism against the pathogen. Oppong et al. (2010) reported the isolation of secondary metabolites benzoquinone and naphthol glucoside from *X. badia*.

The *G. reessii* isolate in this study completely overgrew the pathogen and displayed a very high degree of antagonism. Liu et al. (2017) reported that the *G. reessii* isolated from the rhizosphere of tomato plants produced metabolite known as gymnoascole acetate, which significantly reduced root-knot nematode population. Furthermore, an endophytic *N. hainanensis* isolate from Carbada banana demonstrated a high degree of

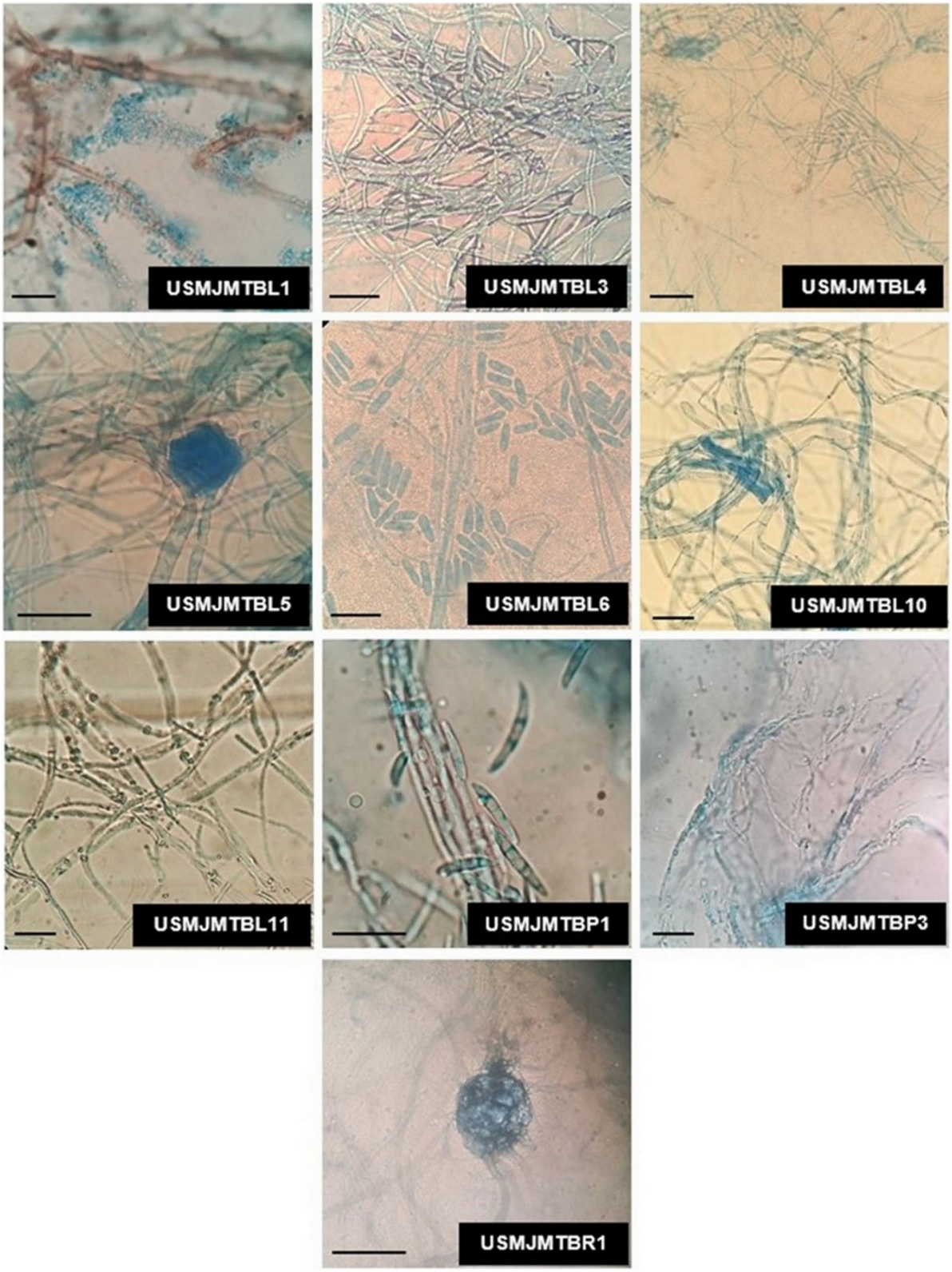

Fig. 5 Photomicrographs of fungal endophytes isolates from Cardaba banana, at 7 days after incubation. Scale bar: 50 µm

Table 3 Identity of the fungal endophyte isolates from Cardaba banana and their reported biological roles

Isolate code	Top BLAST hits (https://blast.ncbi.nlm.nih.gov/Blast.cgi)			Biological roles
	Organisms with significant BLAST hits	NCBI accession number	Percentage similarity (%)	
USMJMTBL1	*Blakeslea trispora* Thaxt. 1914	LN609581.1	86.01	An industrial fungal species used for large-scale production of carotenoids (Luo et al. 2020)
USMJMTBL3	*Pseudopestalotiopsis theae* (Sawada) Maharachch Hyde & Crous 2014	MH472583.1	99.64	Produce polyketides, pestalotheols and cytosporins with clinical importance (Yu et al. 2020)
USMJMTBL4	*Xylaria badia* Pat. 1891	GU322446.1	98.64	Produce secondary metabolites namely benzoquinone and naphthol glucoside (Oppong et al. 2010)
USMJMTBL5	*Nigrospora hainanensis* Mei Wang & Cai 2017	OK083685.1	99.47	Its vital bioactive secondary metabolites such as griseofulvin, dechlorogriseofulvin, and mellein have antifungal activity (Zhao et al. 2012)
USMJMTBL6	*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. 1884	KX022505.1	99.66	Showed strong antagonistic activity against tea pathogens *Pestalotiopsis theae* and moderate activity against *C. camelliae* (Rabha et al. 2014)
USMJMTBL10	*Xylaria feejeensis* Berk.1881	GU322453.1	99.32	Produce mellein derivatives which inhibited *F. oxysporum* causing Fusarium wilt and *Alternaria solani* causing early blight in tomato (Brooks et al. 2022)
USMJMTBL11	*Gymnoascus reessii* Baran. 1972	JQ387570.1	92.31	Produce nematotoxic indoloditerpenoid metabolite against *Meloidogyne incognita* (Liu et al. 2017)
USMJMTBP1	*Fusarium subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas 1983	OM955951.1	94.91	Produce subglutinol A and B considered as immunosuppressive agents (Mishra et al. 2021)
USMJMTBP3	*Rigidoporus vinctus* Berk. 1852	KF494814.1	98.30	Induce agarwood formation (Chen et al. 2019)
USMJMTBR1	*Macrophomina phaseolina* (Tassi) Goid. 1947	MK757624.1	100.00	Important source of bioactive secondary metabolites (Singh et al. 2022)

Table 4 Percentage antagonistic activity of the 10 fungal endophyte isolates from Cardaba banana against *Fusarium odoratissimum*

Fungal endophyte isolate code	Molecular identity	Antagonistic activity (%)
USMJMTBL1	*Blakeslea trispora*	46.67[a]
USMJMTBL3	*Pseudopestalotiopsis theae*	61.67[bc]
USMJMTBL4	*Xylaria badia*	53.33[ab]
USMJMTBL5	*Nigrospora hainanensis*	70.00[c]
USMJMTBL6	*Colletotrichum gloeosporioides*	63.89[c]
USMJMTBL10	*Xylaria feejeensis*	93.33[d]
USMJMTBL11	*Gymnoascus reesii*	87.77[d]
USMJMTBP1	*Fusarium subglutinans*	61.67[bc]
USMJMTBP3	*Rigidoporus vinctus*	63.89[c]
USMJMTBR1	*Macrophomina phaseolina*	96.56[d]

Means with the same letter superscript are not significantly different at 0.05 level in Tukey's HSD

antagonism against the banana pathogen. It was reported that the endophytic fungus *Nigrospora* sp. isolated from the roots of *Moringa oleifera* Lam produces antifungal secondary metabolites such as griseofulvin which exhibited clear growth inhibition against 8 plants' pathogenic fungi (Zhao et al. 2012).

The *C. gloeosporioides* and *R. vinctus* isolates from Cardaba banana in this study demonstrated a growth inhibition of 63.89% and displayed a high degree of antagonism against *F. odoratissimum*. Previous research has indicated that *C. gloeosporioides* has antagonistic effect against tea pathogen *C. camelliae* in vitro (Rabha et al. 2014). Additionally, *C. gloeosporioides* has been found to produce compounds against various bacterial, fungal, and parasitic protozoan pathogens (Denise et al. 2008). In the case of endophytic *R. vinctus*, it has shown activity against *Cladosporium oxysporum* (Renuka and Ramanujam 2016).

This study also isolated *P. theae* and *F. subglutinans* from Cardaba banana, both of which exhibited a growth inhibition of 61.67% and displayed a high degree of antagonism against *F. odoratissimum*. In the study of Yu et al. (2020), endophytic fungus *P. theae* produced secondary metabolites which showed moderate antibacterial activity against drug-resistant *Acinetobacter baumannii*. The endophytic *F. subglutinans* with a high degree of antagonism against *F. odoratissimum* in this study showed no records of biological control agents, but can be subjected to further investigation for prospecting of bioactive compounds. Despite the lowest degree of antagonism observed in *B. trispora* in this study, it is widely used for large-scale carotenoid production (Luo et al. 2020).

The mechanism of parasitism was observed in several fungal endophyte isolates, namely USMJMTBL1 (*B. trispora*), USMJMTBL5 (*N. hainanensis*), USMJMTBL10 (*X. feejeensis*), USMJMTBL11 (*G. reessii*), and USMJMTBP3 (*R. vinctus*), as indicated by the visual overlapping of these endophytes with the surface of the pathogen. Fungal endophytes can develop hooks around the hyphae of fungal pathogens prior to penetration, and/or some cases, they can directly enter the fungal pathogens (Dolatabadi et al. 2012).

Conclusions

Only the in vitro assay was conducted in this study with the aim is to screen and evaluate potential endophytes against the *F. odoratissimum*. Our findings suggest that Cardaba fungal endophytes had the potential to be utilized as a biocontrol agent against *F. odoratissimum* (*Foc* TR4). The fungal endophytes isolated from Cardaba banana have potential mechanisms of competition, antibiosis, and parasitism. Further investigation into the specific control mechanisms of these isolates would allow for a deep understanding of their potential as a biocontrol agent. Future research will focus on the in planta testing, validation in greenhouse and field trials, as well as the development of formulations and application protocols to optimize the utilization of the biocontrol agent in an integrated management approach for *Foc* TR4-infected bananas. The development of sustainable and environmentally-friendly biocontrol agents based on these findings could help address the economic challenges faced in banana production caused by this pathogen.

Abbreviations

BLAST	Basic Local Alignment Search Tool
DAI	Days after incubation
HSD	Honestly Significant Difference
MEA	Malt Extract Agar
MEGA	Molecular Evolutionary Genetics Analysis
NCBI	National Center for Biotechnology Information
PDA	Potato Dextrose Agar

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Author contributions
JMFT performed the experiment, analyzed the data and wrote the manuscript with MTNC. BTB and MTNC designed and supervised the experiment. BLPB and MENT collaborated in the editing and review of the manuscript and analysis of the data. All authors read and approved the final manuscript.

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