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Research Article

Identification and Prevention of Microbial Contaminants in *Musa paradisiaca* Tissue Culture

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ABSTRACT

Banana (*Musa* spp.) is an economically vital fruit crop in Malaysia and therefore, micropropagation was applied to meet the market demand for disease-free seedlings. However, microbial contamination is a significant obstacle in micropropagation techniques. In this research, the microorganisms that were present in the banana culture were characterized and the efficacy of antimicrobial and antifungal agents to inhibit contaminants was evaluated. Three bacterial and two fungal isolates were isolated from the contaminated culture. *Klebsiella pneumoniae*, *Klebsiella quasipneumoniae*, and *Klebsiella variicola* were identified by molecular identification based on the 16S rDNA sequence. The gram-staining method confirmed all three bacteria were gram-negative. Oxidase and catalase tests showed the presence of cytochrome oxidase system and catalase enzyme in all bacteria. The bacteria can also hydrolyze starch, ferment sugars, and reduce sulfur from the amylase test. Phenotypic identification of fungi revealed the presence of conidia and hyphae, indicating the presence of *Colletotrichum* spp. and *Aspergillus* spp. In fungi characterization, *Colletotrichum gloeosporioides* and *Aspergillus flavus* were detected. Chloramphenicol was identified as an effective antibacterial agent from the disc diffusion method. Fluconazole was a potent antifungal agent by screening the sterilizing agents. The findings may potentially lead the way for the implementation of reducing the contamination rate in banana micropropagation.

Key words: Antibiotics, antifungal, contamination, *Musa paradisiaca*

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INTRODUCTION

Plantain and banana belong to the family of Musaceae, order Zingiberales, and genus *Musa*, but in separate genome groups (De Langhe *et al*., 2009). Plantain is a fruit that is starchy even when mature (Adeniyi *et al*., 2019), meanwhile banana contains a relatively low amount of starch and can be eaten raw (Bhuiyan *et al*., 2020). In Malaysia, there are several banana cultivars such as Mas, Berangan, and Rastali, which are eaten raw, while others, like Raja, Awak, Abu, Nangka, and Tanduk, which are consumed cooked or processed into food products such as cake or chips (Abd Shukor *et al*., 2000). The current study concentrates on the Tanduk cultivar (*Musa* spp. cv Tanduk) that shows widespread consumption in Malaysia and across Southeast Asia. In Malaysia, Tanduk bananas demand the highest wholesale (RM 3.09/kg) and retail prices (RM 4.48/kg) in 2020 (Jabatan Pertanian, 2020). Tanduk bananas provided a gross income of RM 225,000.00 with an output of around 75,000 kg (Jabatan Pertanian, 2021). This cultivar has a high economic value due to its excellent flavor and crispy texture for making chips.

Micropropagation of bananas was applied to meet market demand for a higher yield of fruit and disease free-seedlings. However, microbial contamination is a significant obstacle to tissue culture applications and is frequently attributed to ineffective explant sterilization or insufficient sterile equipment (Kim *et al*., 2017). Banana micropropagation also faces a similar issue, although the majority of the worldwide banana industry relies on tissue culture techniques to

generate uniform plants that enable efficient farm management, increased banana fruit yield, and better systems to control pests and diseases (Hamill & Rames, 2018). The majority of the bacteria contamination in banana tissue culture is caused by *Brenneria* sp., *Erwinia* sp., *Klebsiella* sp., *Lactococcus* sp., *Proteus* sp., *Salmonella* sp., *Staphylococcus* sp. and *Stenotrophomonas* sp. (Sharma *et al*., 2017; Liu *et al*., 2019). The most prevalent fungal contaminants in banana cultures are *Aspergillus* sp., *Candida* sp., *Cladosporium* sp., *Colletotrichum* sp., *Fusarium* sp., and *Penicillium* sp. (Msogoya *et al*., 2012; Eunice *et al*., 2019). These microorganisms can be exogenous or endogenous. The endogenous bacteria and fungi are specific microorganisms that inhabit healthy plant tissue in nature, most commonly the roots (Rodriguez *et al*., 2009; Orlikowska *et al*., 2017) without exhibiting disease symptoms during all or part of their life cycle (Tekielska *et al*., 2019). Almost every plant appears to contain these endophytic fungi (Sharma & Singh, 2021). Furthermore, certain endophytic bacteria or fungi can generate phytotoxins, which contain high quantities that will cause plant deterioration (Seliem *et al*., 2021).

Since exogenous bacteria reside on plant surfaces, sterilizing agents such as ethanol and sodium hypochlorite are typically successful in eliminating them (Hodkinson & Murphy, 2019). Ethanol is a potent sterilizing agent, but it is also highly phytotoxic at high concentrations (Getnet, 2017). Therefore, it must be applied below 80% (v/v) of concentration to avoid the excessive coagulation of the bacterial cell wall that prevent the disinfectant from entering the cell (Yoo, 2018). Sodium hypochlorite is a halogen that acts by denaturing proteins in microorganisms; for a disinfectant to be effective, it must pass through the bacteria's outer membrane and reach the target site (Acsa *et al*., 2021). However, surface sterilization does not sufficiently eliminate endophytic microorganisms that exist in the explant tissues. Therefore, the elimination of endophytic contaminants can be achieved through the necessitates of antibiotics. After surface sterilizing of the explant, antibiotics can be applied in a growth medium to control latent contamination (Liang *et al*., 2019). Antibiotics including carbenicillin, cefotaxime, cephalothin, gentamicin, polymyxin, rifampicin, and streptomycin are bacteriostatic or bactericidal, which make them effective to reduce adverse effects of endophytic bacteria (Bohra *et al*., 2014). However, it is critical to identify the endogenous microorganism that allows the researchers to choose the appropriate types, combination, and concentration of antibiotics to eliminate specific bacterial or fungal growth without affecting plant regeneration (Shehata *et al*., 2010). To characterize the microorganism, it is essential to isolate the single bacterial or fungal from the mixed populations and then identifies it molecularly. Then the researchers may decide the most effective antibiotics depending on the species of bacteria or fungi.

Despite the implementation of reliable aseptic techniques, it is estimated that the total losses due to microbial contamination in bananas and plantains were cultured between 40% and 60%, resulting in substantial impacts on the agricultural industry (Msogoya *et al*., 2012). Microorganisms from the contamination will compete with the explant for nutrients and it will be challenging to identify the contaminants if they remain dormant for extended periods (Bohra *et al*., 2014). The presence of latent infections, consequently will increase the rate of culture mortality, tissue necrosis, and variable growth because of decreased shoot proliferation and rooting. This circumstance prevented the subculturing of bananas culture into plantlets (Dangariya, *et al*., 2020; Agbadje *et al*., 2021). Given this condition, the purpose of this study is to isolate and characterize endogenous bacteria and fungi that cause contamination of banana suckers culture, as well as to determine the most efficient antibiotics and sterilizing agents for contamination-free tissue culture products.

MATERIALS AND METHODS

Plant materials and explant inoculation

This study employed 3 to 4 months old, disease-free suckers of *M. paradisiaca* grown from a banana farm in Kampung Dal, located at Kuala Kangsar, Perak (4°45'56.9"N 100°53'16.3"E). The suckers were excised from the mother plant and thoroughly washed under running tap water for 2 h. The explants were trimmed until the length was 6 to 10 cm and the diameter at the base was 10 to 15 cm. Under laminar airflow, trimmed suckers containing the apical meristem were surface sterilized by dipping in 70% (v/v) ethanol for 3 min and then rinsed with sterile distilled water. The suckers were sterilized with 20% (v/v) of sodium hypochlorite with the addition of Tween 20 for 20 min and then rinsed with sterile distilled water thrice. The outer layer of the sterilized explants was removed until the three innermost midribs remained and trimmed to a 5 cm length. Following that, explants were inoculated into Murashige and Skoog (MS) media (Murashige & Skoog, 1962) supplemented with benzyl aminopurine (BAP) (1.0, 2.0, 4.0, & 8.0 mg/L) and naphthaleneacetic acid (NAA) (1.0, 2.0, 3.0, & 4.0 mg/L) at varying concentrations. Before the explant inoculation, the Phytagel (Sigma, USA) (0.25% w/v) and sucrose 30% (w/v) were added into the MS media before it was autoclaved (Tommy, Japan) at 121 °C for 15 min after adjusting the pH to 5.8 with 1.0 M of sodium hydroxide (NaOH). The explants were

incubated at 18 ± 2 °C under a light intensity of 1000 lux for 16 h. Each BAP and NAA treatment had three replicates.

Isolation of bacteria and fungi from contaminated banana culture

The contamination of banana culture from bacteria and fungi occurred after 2 weeks of incubation (Figure 1). The bacteria and fungi colony growing on the explant surface was selected for isolation. To determine the presence of endogenous bacteria, the isolated colony was plated on a Nutrient Agar (NA) medium and incubated overnight at 28 °C. The obtained bacterial colonies were further purified by repeated streaking on NA medium and the cultures were maintained on NA slants. The pure bacteria cultures were stored in a chiller at 4 °C until further used. For fungi isolation, two samples were collected randomly from contaminated banana sucker culture and plated on Potato Dextrose Agar (PDA) medium for growth. The fungus colony was obtained from repeated culture on PDA media that was incubated at 25 °C for five days. The pure fungus single colony was stored in a chiller at 4 °C until further used.

Morphological characterization of bacterial

Gram staining was done following the method of Tripathi and Sapra (2022). The bacteria slide was heat-fixed and then stained with crystal violet. The crystal violet stain was removed with distilled water and iodine. The crystal violet-iodine complex (VIC) was then washed with distilled water to remove the purple color. Finally, a drop of safranin was added to act as a counterstain. Under a light microscope with oil immersion (100×), the morphology and color of the bacteria stained were observed.

Biochemical characterization of bacterial

Oxidase test

The presence of cytochrome C in isolated bacteria was determined using an oxidase assay. On a filter paper saturated with 1% (v/v) oxidase reagent, the tested single colony bacteria were smeared by using a wire loop and examined for purple formation. The positive test result was recorded as a purple color formation within ten sec (Omemu *et al*., 2018).

Catalase test

Catalase tests were carried out by adding one to two drops of 3% (v/v) of hydrogen peroxide solution onto a glass slide containing purified bacteria culture. A positive catalase test result was detected from the formation of gas bubbles within 10 s of the mixture (Islam *et al*., 2020a).

Amylase test

The amylase test was applied to determine the bacteria's capacity to hydrolyze starch by α-amylase and oligo-1,6-glucosidase. The bacteria were streaked directly onto the starch agar plate

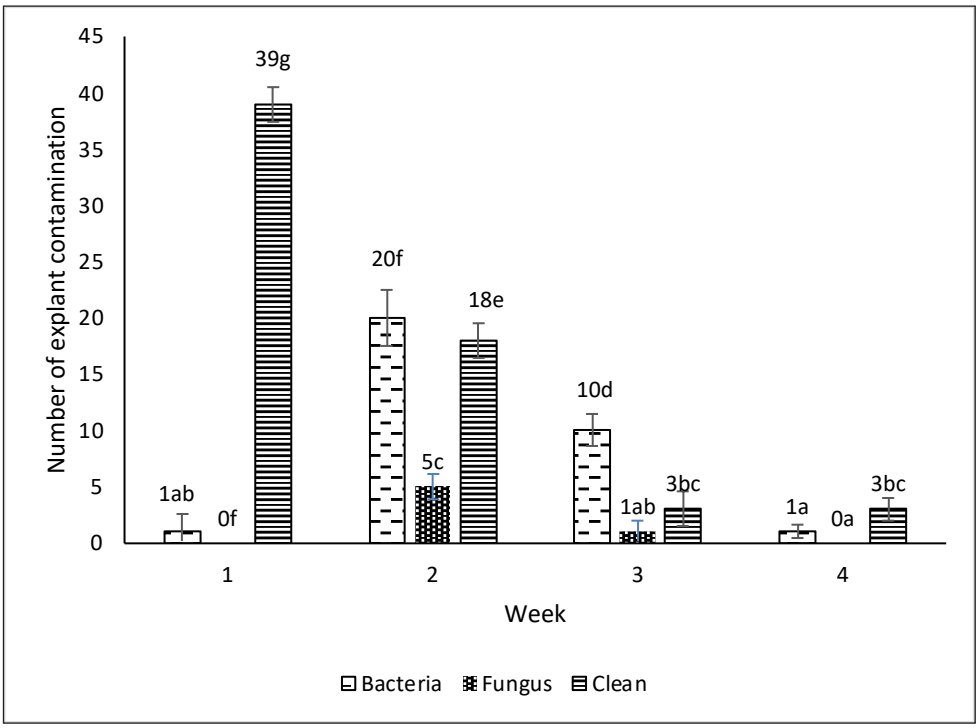

Fig. 1. The number and types of explants contamination per incubation period. The vertical bar represents the standard deviation of the mean and different letters indicate significant differences (P<0.05), n=3.

(3.27 mg/mL Bushnell Haas Broth, 20 mg/mL starch, and 15 mg/mL nutrient agar) and incubated at 37 °C for 48 h. Following incubation, the starch agar plates were flooded with iodine solution for 30 s. The clear zone surrounding the bacterial growth line indicated hydrolyzed starch, whereas the colored zone indicated a negative result.

Triple sugar iron (TSI) test

The Triple Sugar Iron (TSI) test is designed to distinguish between bacteria based on their carbohydrate fermentation and hydrogen sulfide (H_2S) emission patterns. The triple sugar medium containing 3 g/L of beef extract, 3 g/L of yeast extract, 15 g/L of peptone, 5 g/L of sodium chloride, 10 g/L of lactose, 10 g/L of sucrose, 1 g/L of dextrose, 0.2 g/L of ferrous sulfate, 0.3 g/L of sodium thiosulfate, 0.24 g/L of phenol red and 15 g/L of agar. The bacteria colony was stabbed through the center of the TSI agar with a sterile needle and then streaked on the surface of the agar slant. The culture was incubated at 37 °C for 24 h. The change in color of the agar slant after incubation revealed whether there was the production of hydrogen sulfide (H_2S) and carbohydrate fermentation occurred (Aung & Oo, 2020).

Sulfide indole motility (SIM) test

The needle covered with isolated bacteria was inserted approximately two-thirds of the way into the SIM agar and then pulled out in the same direction as the entrance. The media was incubated at 37 °C for 24 h. The presence of turbidity around the puncture site indicated the mobility of the bacteria. Three to five drops of Kovac's reagent were placed on the agar slant to determine indole availability. The pink color formation indicates that the reagent was indole positive, whereas the appearance of yellow indicated that the reagent was indole-negative (Alam *et al*., 2019).

Molecular identification of bacterial contaminants

The genomic DNA of the isolated bacteria was extracted following the manual instructions of the Wizard® Genomic DNA Purification kit (Promega, USA). The quality of DNA was determined by running 1% (w/v) agarose gel electrophoresis. Then, the targeted DNA strands were amplified using a polymerase chain reaction (PCR). The PCR was performed with the following 16S primer pairs: 16SF2: 5'- GAG TTT GAT CCT GGC TCA -3' for the forward primer, and 16SR2: 5'- ACG GCT AAC TTG TTA CGA CT-3' for the reverse primer. The template of rDNA was used as a template for the PCR by adding the following reagents: 1 µL of template DNA, 25 µL of MasterMix, 20 µL of deionized distilled water, and 2 µL of forward and reverse primer. PCR was performed with an initial denaturation at 95 °C for 1 min, and 95 °C for 30 s followed by annealing a short oligonucleotides primer to a single DNA strand at 50 °C for 1 min and synthesizing a new strand at 72 °C for 2 min for 40 cycles. A final extension ended the PCR cycles at 72 °C for 10 min. To detect the amplified amplicon of the 16S rDNA fragment, it was separated and stained with FloroSafe DNA Stain on a 1.5% (w/v) agarose gel. The gel image was analyzed using the Gel-Doc Imaging System (Bio-Rad). The PCR sequence was compared with the databases in the National Centre for Biotechnology Information (NCBI).

Lactophenol Cotton Blue (LPCB) staining of fungal identification

A drop of Lactophenol Cotton Blue Solution and a fragment of a fungal specimen were placed on a clean microscopic glass slide and covered with a coverslip. The slide was examined under a light microscope at 10× and 40× lenses after the excess stain was removed by blotting to observe for fungal spores and other fungal structures.

Molecular identification of fungal contaminants

The seven days old fungi culture was extracted using the FavorPrep™ Genomic DNA Extraction Mini Kit (Favorgen, Taiwan). The DNA extraction of the collected fungi culture was performed according to the manual provided. In the amplification of the 28S rRNA gene sequence, the targeted DNA strands were amplified using a PCR. First, 50 µL of the PCR reaction mixture was mixed in a PCR tube. The PCR reaction mixture included 1 µL of DNA Template, 25 µL of MasterMix, 20 µL of deionized distilled water, and 2 µL of forward and reverse primer. For the forward primer, LROR: 5'-ACC CGC TGA ACT TAA GC-3' was used, and for the reverse primer, LR7: 5'-TAC TAC CAC CAA CAT CT -3' was used. The following were the conditions for PCR amplification: initial denaturation at 95 °C for 1 min, denaturation at 95 °C for 30 s, annealing temperature at 50 °C for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. To achieve sufficient amplification, the PCR was run for 40 cycles. To visualize the PCR product, it was separated and stained with FloroSafe DNA Stain on a 1.5% (w/v) of agarose gel. The gel image was analyzed using the Gel-Doc Imaging System (BIO-RAD). The 1 kb DNA marker was used as a ladder. For DNA sequencing and blast analysis, the samples were delivered to the 1st Base DNA Sequencing Service. The obtained sequence was then blasted with nucleotide blast software from the Basic Local Alignment Search Tool (BLAST). The DNA sequences were then compared with the sequence from a database

available at the National Centre for Biotechnology Information (NCBI).

Determination of the antimicrobial agents for identified microbes

Disk Diffusion Method

The antibiotic susceptibility test was performed by the disk diffusion method. The bacteria and fungus inoculum were placed uniformly on the separate NA plate and leave it dried completely. Ten commercial antimicrobial susceptibility discs (kanamycin; 30 µg, streptomycin; 25 µg, ampicillin; 10 µg, cefotaxime; 30 µg, penicillin; 2 units, cephazolin; 30 µg, chloramphenicol; 50 µg, vancomycin; 5 µg, fluconazole; 25 µg, nystatin; 100 units) with fixed antibiotic amount were placed on the inoculated agar surface. Plates were incubated at 28 °C for 24 h for bacteria and seven days for fungus. The inhibition zone of each disc was measured to the nearest millimeter.

Screening of Sterilising Agents

Sterilizing agents were tested to reduce the growth of microbial contaminations. For bacteria, 1 mL of broth culture was first transferred into tubes with 20% (v/v) of bleach (Clorox®), 30% (v/v) of hydrogen peroxide, 70% (v/v) ethanol, and 3% (v/v) of bromine water, respectively. The culture tubes were vortexed and incubated at room temperature for 15 min. Then, a loop of bacteria was transferred into five nutrient broths and one broth served as a control. All the tubes were incubated at room temperature for 24 h. The turbidity of the bacteria culture was observed by absorbance reading at 600 nm using Eppendorf BioPhotometer® D30 to determine the cell number of growing microorganisms. Meanwhile, the above procedure was repeated for fungus with an incubation period of five days and an absorbance reading was obtained at 405 nm.

RESULTS

The rate of contamination from banana culture

The contaminants were detected from the formation of bacterial and fungal colonies on the MS media after two weeks of incubation. It was recorded that 50% of the contamination occurred from bacteria and 12.5% from fungus after 2 weeks incubation period (Figure 1). The bacteria contamination rate increased to 75%, while fungal contamination rose to 15% after 3 weeks of incubation. There was still 2.5% bacterial contamination, no fungal contamination, and only 7.5% of clean and surviving explants recorded at week 4 of the incubation period

Morphological characterization of bacterial and fungal

Bacterial isolation and identification

Three randomly selected bacteria isolated from the contaminated explants were streaked on NA media plates. After a series of subcultures, the pure culture of bacteria was obtained and labeled as S1, S2, and S3. The colony characterization of S1, S2, and S3 was opaque white, with smooth surfaces and abundant growth (Figure 2).

Gram Staining

Gram staining revealed that all three samples were gram-negative bacteria with pink appearances. Light microscopic at 100× magnification power observation revealed that all of the bacterial isolates were short rod-shaped (Figure 3).

Biochemical characterization of bacteria

The oxidase test is based on the presence of cytochrome oxidase, which is unique to saprophytic bacteria (Wikström *et al*., 2018). S1, S2, and S3 were identified as oxidase-positive bacteria from the formation of purple to dark blue bubbles within 10 sec (Table 1). The colored compound indophenol blue was formed when the tetramethyl-p-phenylenediamine dihydrochloride reagent was oxidized (Sanderson *et al*., 2018). The tetramethyl-p-phenylenediamine dihydrochloride reagent acted as an artificial electron acceptor for the enzyme cytochrome oxidase in this test while reducing oxygen to form water (Shields & Cathcart, 2010).

The catalase test was conducted to determine the presence of catalase that reacts with hydrogen peroxide (H_2O_2) produces oxygen and water. Rapid bubble production in all three samples indicated catalase oxidase positivity when mixed with H_2O_2 thus suggesting that these bacteria are also aerobic (Prastujati *et al*., 2019). While in the amylase test it was recorded that all bacteria samples can hydrolyze the starch from the formation of dark brown color after the addition of iodine to the medium containing bacterial culture (Table 1). This indicates that the bacteria managed to produce α-amylase to degrade the carbon source present in the MS media, thus competing with banana explant to grow. However, no bacteria were found to produce hydrogen sulfide (H_2S) gas.

Triple sugar iron (TSI) agar contains dextrose, lactose, and sucrose to detect the fermentation of carbohydrates by the bacteria tested. All the bacteria samples suggested that fermentation of dextrose, lactose, and/or sucrose occurs by changing color from red to yellow in all the slants

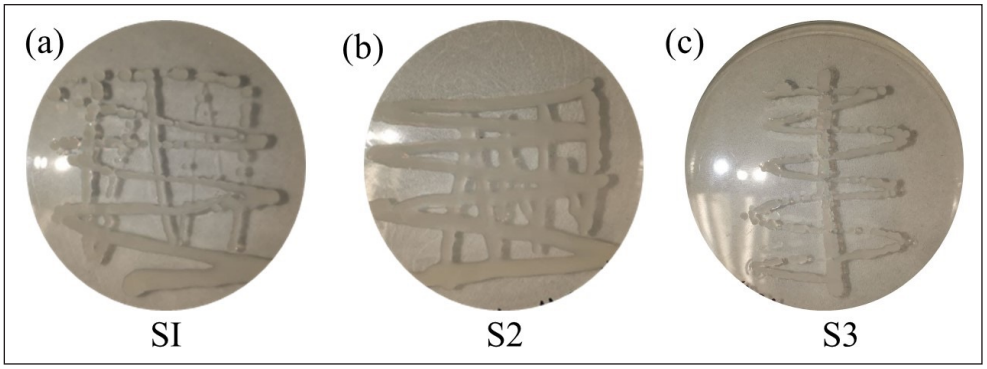

Fig. 2. The bacterial samples from banana culture after incubation in Nutrient Agar medium at 37 °C overnight. (a) S1; Sample 1 of bacteria, (b) S2; Sample 2 of bacteria, (c) S3; Sample 3 of bacteria.

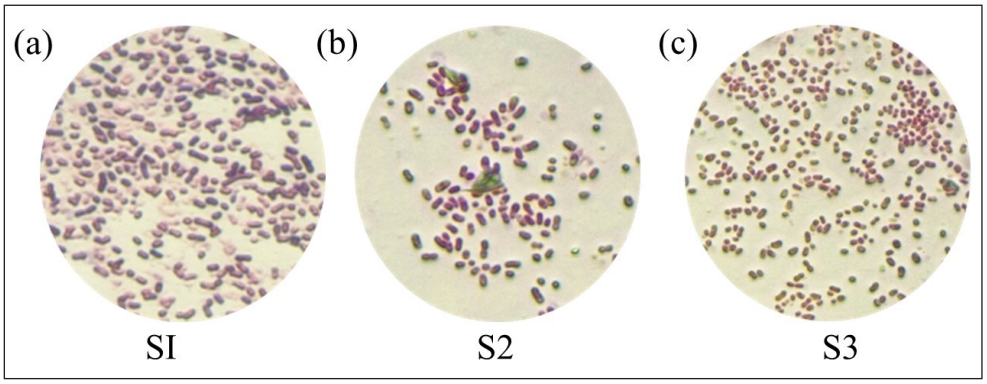

Fig. 3. Morphological characteristics of bacteria samples from Gram staining. All three samples showed gram-negative bacteria with rod shape observed under a light microscope with 100× magnification power. (a) S1; Sample 1 of bacteria, (b) S2; Sample 2 of bacteria, (c) S3; Sample 3 of bacteria.

Table 1. Biochemical test results from three isolated bacterial

Biochemical Test	Bacterial Strains		
	S1; ID Name *Klebsiella pneumoniae*	S2; ID Name *Klebsiella quasipneumoniae*	S3; ID Name *Klebsiella variicola*
Gram Staining	Gram-negative	Gram-negative	Gram-negative
Catalase test	+[#]	+	+
Oxidase test	+	+	+
Amylase Test	+	+	+
	Triple Sugar Iron (TSI) test		
H2S production	-	-	-
Gas production	+	+	+
Acid from TSI	Acid	Acid	Acid
	Sulfide Indole Motility (SIM) Test		
Sulfide	-	-	-
Indole	-	-	-
Motility	Non-Motile	Non-Motile	Non-Motile

[#]+ indicates positive, - indicates negative

and butt of TSI agar. This reaction is acidic and generates gases, as a result of the medium splitting and cracking. H_2S production gives negative results due to the absence of a black precipitate at the tube's bottom (Table 1).

As shown in Table 1, no sulfide or indole was formed by any of the bacteria tested and they did not exhibit motility characteristics. If the bacteria that produces H_2S is motile, the black precipitate would spread as it spread through the medium (Islam *et al*., 2020b). The non-motility properties obtained from the SIM test were because the growth was only along the line of inoculation. These results suggest that all the isolated bacteria did not have flagella, a filamentous structure that attaches to the cell surface and allow the movement of bacteria.

PCR amplification of bacteria

PCR amplification of 16S rDNA resulted in the formation of ~1,500 bp product for all the bacteria samples as identified by 1.5% agarose gel electrophoresis (Figure 4). The PCR product was further purified and sequenced. By using the 16S rDNA gene sequence comparison, these isolates were identified as *Klebsiella pneumoniae* (S1), *K. quasipneumoniae* (S2), and *K. variicol*a (S3). In S1, the similarity was 100%, and 99.93% for S2 and S3.

Phenotypic fungus identification

The fungal hyphae and spores were identified phenotypically using the dye lactophenol cotton blue (Figure 5). Direct microscopic examination revealed that F1 had a conidium shape that was cylindrical with rounded ends. F1 isolated colonies were pink to salmon in color with conidial masses. For F2, the fluffy mycelium on the conidial heads formed a cotton-like structure in each colony center. A microscopic examination (400×) of a lactophenol blue mount revealed that F2 was globose and ellipsoid in shape. F1 was identified as *Colletotrichum* spp. because it exhibited *Colletotrichum* spp. phenotypic characteristics.

PCR amplification of fungus

The PCR was performed using a universal primer set, and LROR and LR7 were used to amplify 28S large subunit ribosomal RNA genes. For all strains tested, a single PCR amplification product of the expected length (~1500bp) (Figure 6). Following that, DNA sequencing was performed to confirm the identity of the fungus. Sequence F1 was nearly similar (99.85% match) to *Colletotrichum gloeosporioides*, according to a comparison of the DNA sequences obtained in the GenBank database. The sequence F2 was determined to be 96.18 % identical to the A*spergillus flavus* from DNA sequencing.

Antibiotic susceptibility

The diameter of the inhibitory zone was measured to estimate antibacterial and antifungal susceptibilities. The bacterial and fungal isolates were categorized as resistant (9-14 mm), intermediately resistant (15-19 mm), or sensitive (>20 mm) based on inhibition zone widths (Msogoya *et al*., 2012). The most susceptible antibiotics for reducing bacterial growth were kanamycin, streptomycin, cephazolin, and chloramphenicol, according to the results (Table 2). Ampicillin was resistant to *K. pneumoniae* and *K. quasipneumoniae* but exhibited moderate resistance to *K. variicola* growth. Cefotaxime, penicillin, vancomycin, fluconazole, and nystatin resistance were found in all of the microorganisms. Fluconazole was

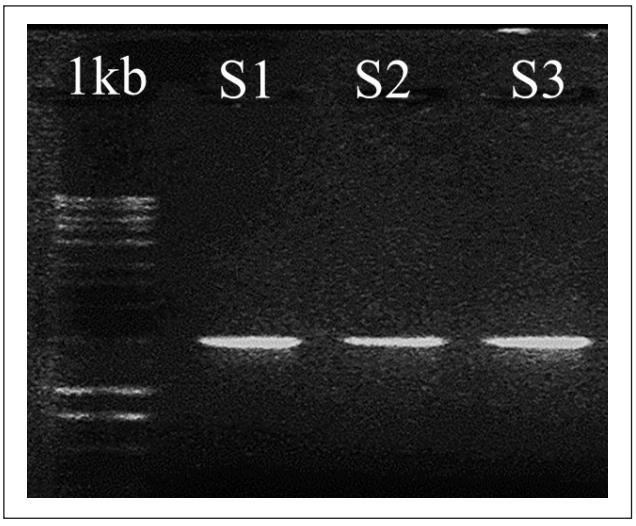

Fig. 4. Amplification of the targeted 16S rDNA from S1, S2, and S3 bacterial strains. The size of the amplified 16S rDNA gene was shown approximately 1500 bp on 1.5% agarose gel. S1: *Klebsiella pneumoniae,* S2: *Klebsiella quasipneumoniae,* S3: *Klebsiella variicola.*

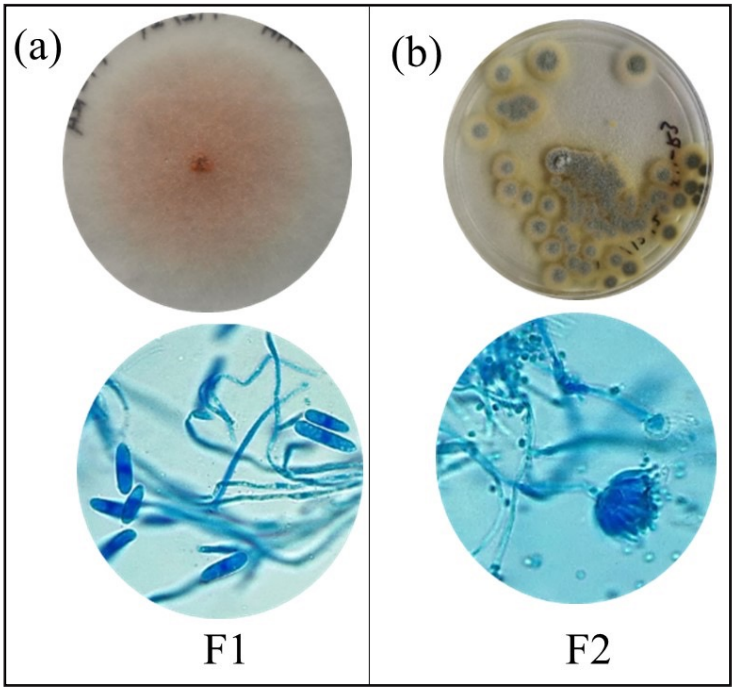

Fig. 5. Isolation of fungus from contaminated banana culture. (a top) The colony color of F1was pink to salmon conidial masses; (a, below) Microscopic (100 x) observation of a lactophenol blue mount revealed conidium shape is cylindrical with both ends rounded; (b, top) Colony color of F2 was yellow with grey to dark grey at the center; (a bellow) Microscopic (100 x) observation a lactophenol blue mount revealed the shape of F2 is globose vesicle with biseriate phialides and ellipsoid. F1: *Colletotrichum gloeosporioides,* F2: *Aspergillus flavus.*

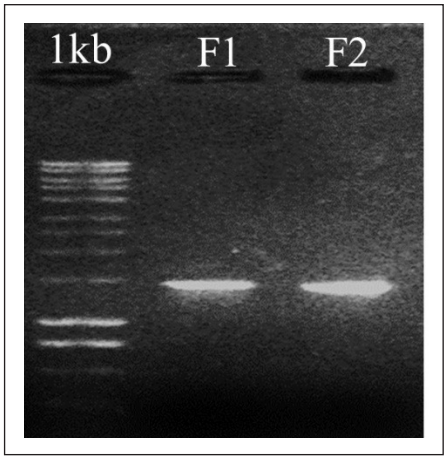

Fig. 6. PCR amplification of 28S rRNA gene for F1 and F2 strain using primer LROR and LR7 on 1.5% agarose gel electrophoresis. 1 kb DNA was used as a ladder. F1: *Colletotrichum gloeosporioides,* F2: *Aspergillus flavus*

Table 2. Antimicrobial Susceptibility Test (AST) of isolated bacteria and fungus from contaminated banana culture

Antibiotic	Discs content	Bacteria species			Fungi species	
		Klebsiella pneumoniae	*Klebsiella quasipneumoniae*	*Klebsiella variicola*	*Colletotrichum gloeosporioides*	*Aspergillus flavus*
Penicillin	2 units	R[#]	R	R	R	R
Cefotaxime	30 µg	R	R	R	R	R
Vancomycin	5 µg	R	R	R	R	R
Streptomycin	25 µg	S	S	S	R	R
Cephazolin	30 µg	S	S	S	R	I
Ampicillin	10 µg	R	R	I	R	R
Chloramphenicol	50 µg	S	S	S	R	S
Kanamycin	30 µg	S	S	S	R	R
Fluconazole	25 µg	R	R	R	S	S
Nystatin	100 units	R	R	R	S	R

[#]R = Resistant, S = Susceptible and I = Intermediate resistant

the most effective antibiotic against both fungi, whereas nystatin was only effective against *C. gloeosporioides*. Chloramphenicol susceptibility and intermediate resistance to cephazolin were found in *Aspergillus flavus*.

Sterilizing agents screening

Bacterial and fungal growth can be reduced by the application of sterilizing agents. From the turbidity measurement of microbial cultures, 30% (v/v) of hydrogen peroxide was the most effective sterilizing agent to inhibit the growth of bacteria and fungus isolated in this study. To inhibit bacterial growth, 20% (v/v) of bleach (Clorox®) and 70% (v/v) of ethanol showed moderate activity followed by the least effective, 3% (v/v) of bromine water. For fungal growth, 70% (v/v) of ethanol was less effective in suppressing fungus growth followed by 20% (v/v) of Clorox® and 3% (v/v) of bromine (Figure 7).

DISCUSSIONS

Bacterial and fungal contamination could slow the explant growth slower, delay rooting, and even cause death to a plant (Aduramigba-Modupe *et al*., 2017). The results in Figure 1 showed that microbial contamination was caused by bacteria and fungi with severe effects in banana tissue

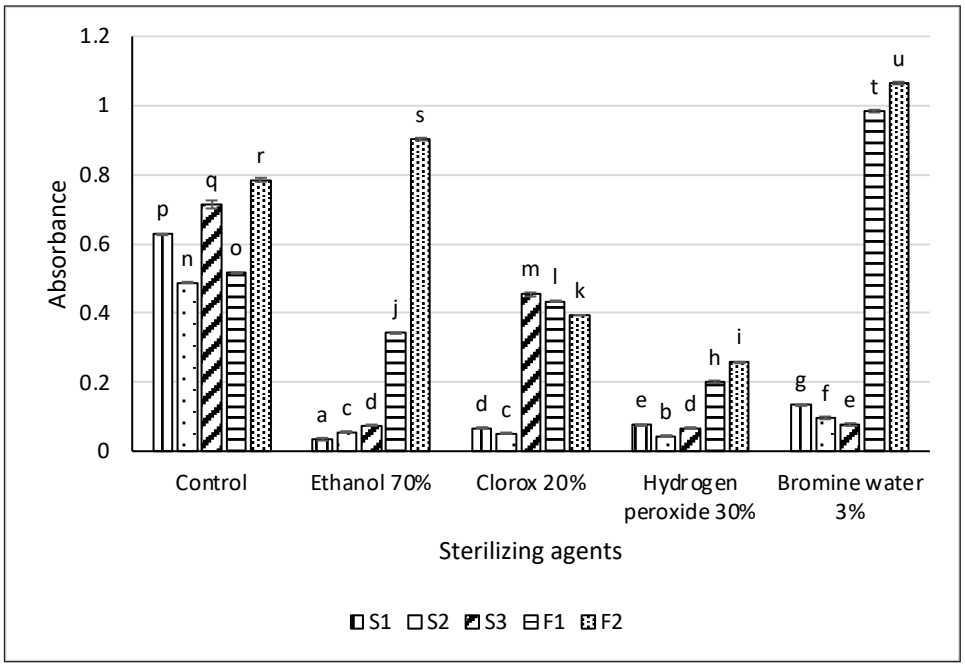

Fig. 7. Absorbance reading of bacteria and fungus on different types of sterilizing agents treatment. Low absorbance reading suggests a low rate of microbial growth in the culture. S1: *Klebsiella pneumoniae,* S2: *Klebsiella quasipneumoniae,* S3: *Klebsiella variicola*, F1: *Colletotrichum gloeosporioides,* F2: *Aspergillus flavus. The vertical bar represents the standard deviation of the mean. Different letters indicate significant differences* (P<0.05), n=3.

culture and could extinguish the entire process of micropropagation. Even though the banana suckers that were selected as an explant were symptomless, there was a possibility that there were endophytic microbes inside the meristematic tissue which might cause latent contamination. Additionally, contamination of the Tanduk cultivar was discovered at the base of plantlets after two weeks of incubation, indicating that it could be caused by endophytes. The endophytes remained latent and visible bacterial or fungi growth was developed in the medium weeks after incubation (Fang & Hsu, 2012). Besides, endophyte infection might be asymptomatic but could become symptomatic if the plant's immunity is impaired (Nsofor, 2021). The presence of latent infections could result in explant discoloration, browning, tissue necrosis, and an increased rate of culture mortality (Mizra *et al*., 2001; Liu *et al*., 2005), whereby these symptoms were equally observed in the in vitro cultures of bananas.

In this study, three isolated bacteria were identified, such as *Klebsiella pneumoniae*, *K. quasipneumoniae,* and *K. variicola*. Studies on population diversity have shown that *K. pneumoniae* is phylogenetically closely related to *K. quasipneumoniae* and *K. variicola* (Rodrigues *et al*., 2018). *K. pneumoniae* is an acute danger to global public health, producing substantial morbidity and death. *K. variicola* and *K. quasipneumoniae* are often regarded as opportunistic infections with lower pathogenicity in humans as compared to *K. pneumoniae* (Long, *et al*., 2017). Since these three bacteria are associated with human health, it is suggested that their presence in banana culture, which causes contamination, is the result of human error that occurs either in the sterilization method or inoculation of the banana during the experimentation. For fungi identification, *Colletotrichum gloeosporioides* and *Aspergillus flavus* were isolated. In general, *Colletotrichum* sp. develops hyaline, one-celled conidia with a range size of 10-15 µm long and 5-7 µm wide, from oval to rectangular, slightly curved, or dumbbell-shaped. *Colletotrichum* sp. reproduces asexually via the formation of conidia, which are pink or salmon in color (De Silva *et al*., 2017). The *Aspergillus* sp. hyphae were septate, and hyaline, and formed dense mycelia. Hyaline and coarsely roughened conidiophores conidia ranged in size from 3 mm to 6 mm and were sub-globose to globose in shape and yellowish-green in color. The conidial heads of *Aspergillus* sp. were uniseriate, with phialides born directly on the vesicle (Ali *et al*., 2021).

Colletotrichum sp. is a common endophytic and plant pathogenic fungus. *C. gloeosporioides* was identified in the symptomless banana leaves in Malaysia and Hong Kong (Brown *et al*., 1998; Zhou *et al*., 2002; Zakaria & Aziz, 2018). In addition, these fungi will induce anthracnose, which developed a brown to reddish spot on the surface of the banana fruit and gave a latent fruit infection (Riera *et al*., 2019; Alam *et al*., 2021). *Aspergillus* sp. is the most isolated endophytic fungus from banana roots, but less isolated from banana leaves. *Aspergillus niger* and *A. flavus* were able to produce toxic metabolites, such as malformins, naphthopyrones, oxalate, and aflatoxin, which could kill plant cultures. As well as bananas, *Aspergillus* sp. species may infect tomato, mango, and citrus fruit (Obuekwe & Osagie, 1989; Ali *et al*., 2021).

S1, S2, and S3 were identified as oxidase-positive bacteria (Table 1). All oxidase-positive bacteria are normally aerobic and utilize oxygen as a terminal electron acceptor in respiration (Wilber *et al*., 2018). This metabolism produces water or hydrogen peroxide (H_2O_2) as a by-product (Kano *et al*., 2021). The H_2O_2 is a reactive oxygen species (ROS) that can cause plant cell death because of the carcinogenic effects at the high cellular level of H_2O_2 (Khandaker *et al*., 2020). The catalase test suggested that these bacteria contain catalase that could neutralize hazardous forms of oxygen metabolites, such as H_2O_2 into oxygen and water (Khasabulli *et al*., 2017). However, excessive accumulation of water in the vessels will cause hyperhydricity of the plant tissue, causing oxidative stress and oxygen depletion in the cells (Ziv, 2005). All the bacteria samples managed to produce α-amylase (Table 1) from the amylase test. The α-amylase secreted by these bacteria can degrade the carbon source present in the MS media such as sucrose, fructose, maltose, or glucose, thus competing with banana explant that also needs carbon source as a source of energy (Saraswathi *et al*., 2016). The α-amylase would degrade starch molecules into smaller glucose subunits, which would enter the glycolytic pathway directly (Maskey, 2018). Based on the triple iron sugar test, all bacteria tested were able to ferment dextrose, lactose, and sucrose. After the depletion of the carbon source, the bacteria would release acid that rapidly oxidized, causing the medium to remain red (Kones *et al*., 2020). This reaction is undesirable since the acid will change the original pH value (5.8) of Murashige and Skoog's (1962) medium used for banana culture. It is crucial to maintain the pH of the media between pH 5.4- pH 5.8 because it affects the growth of plants and the bioactivity of plant growth regulators applied in the medium (Suman, 2017).

After identifying the contaminants, ten antibiotics including ampicillin (AMP100), cefotaxime (CTX30), cephazolin (KZ30), chloramphenicol (C50), fluconazole (FCA25), kanamycin (K30), nystatin (NS100), penicillin (P2), streptomycin (S25), and vancomycin (VA5) were

evaluated. These antibiotics have a broad spectrum of action against gram-positive and gram-negative bacteria (Romero-Soto *et al*., 2018). The antibiotic susceptibility of the identified bacteria and fungi was determined by the disc diffusion method according to the inhibition zone widths. The growth and control of gram-negative bacteria were found to be ineffectual with antibiotics according to De Nardo *et al*. (2013). However, isolated gram-negative bacteria isolated (*K. pneumoniae, K. quasipneumoniae, and K. variicola*) were found to be susceptible to cephazolin, chloramphenicol, kanamycin, and streptomycin tested in the current research, which can be an effective approach to control contamination during banana in vitro culture.

Amongst the antibiotics applied, chloramphenicol was the most effective antibiotic to inhibit all the isolated *Klebsiella* sp. and *Aspergillus flavus*, which can be added into the growth medium such as Murashige and Skoog (1962) to prevent latent contamination. This antibiotic is a synthetic antibiotic isolated from *Streptomyces venezuelae* strains (Tevyashova, 2021). Chloramphenicol inhibited bacterial protein synthesis by binding to the 50S ribosomal subunit and preventing the attachment of aminoacyl tRNA to the ribosome. It also inhibited mitochondrial and chloroplast protein synthesis and ribosomal formation of (p) ppGpp, which inhibited rRNA transcription (Tereshchenkov *et al*., 2018). Meanwhile, fluconazole is the most potent antifungal that can be applied to the growth media since it may suppress the development of both detected fungus, *A. flavus* and *C. gloeosporioides*. Fluconazole is a bis-triazole antifungal agent with a fluorine substitution. Its mechanism of action, like that of other azoles, involves interfering with the conversion of lanosterol to ergosterol by binding to fungal cytochrome P-450 and causing a membrane disruption (Anwar *et al*., 2018).

However, due to antibiotic phytotoxicity and the development of antibiotic resistance, the incorporation of antibiotics into the media must be regulated, particularly the concentration of each antibiotic. This is crucial to ensure that the administered antibiotics do not affect the growth and morphology of the in vitro plants (Fang & Hsu, 2012). Besides, antibiotics that are efficient against isolated microbes may be ineffective against contaminated plants owing to phytotoxicity or inadequate penetration into plant tissues (Reed *et al*., 1995; Shehata *et al*., 2010).

Contamination can also occur when a fungus enters a culture via an explant, is airborne, or enters directly into the vessel (Altan *et al*., 2010). Before incorporating antibiotics into the growth media of banana culture, the surface sterilization procedure of explants must be employed to eradicate any endophytes and epiphytes that exist on the explant's surfaces. Sodium hypochloride, ethanol, hydrogen peroxide, and bromine water are common sterilizing chemicals used to sterilize a variety of plant species. In general, banana sucker surface sterilization was carried out by immersing the explants in 70% (v/v) of ethanol for less than a min followed by rinsing with sterilized distilled water to remove traces of alcohol. Then, the explant will be immersed either in 20% (v/v) of sodium hyphochlorite, 10%-30% (v/v) of hydrogen peroxide, or 3% (v/v) of bromine water before it will be rinsed again with sterile distilled water (Ray & Ali, 2018; Kapadia & Patel, 2021). To determine the potential of which sterilizing agent could reduce the microorganism growth, the broth culture containing bacteria and fungi isolated would be added either with 20% (v/v) of sodium hyphochlorite, 30% (v/v) of hydrogen peroxide, 3% (v/v) of bromine water or 70% (v/v) of ethanol. Then after 24 hr of incubation, turbidity measurement would be taken to determine the approximate cell number of growing microorganisms in the culture. The absorbance value correlated to the approximate number of microbial cells in a solution; therefore, a low absorbance value implied a minimal growth of microorganisms and suggested that the applied sterilizing agent can prevent bacterial growth. The most effective disinfectant to inhibit bacterial and fungal development was 30% (v/v) of hydrogen peroxide (Figure 7). Additionally, 20% (v/v) of sodium hyphochlorite, 70% (v/v) of ethanol, and 3% (v/v) of bromine water were effective in inhibiting the development of *Klebsiella*.

The fact that hydrogen peroxide (H_2O_2) inhibited microbial growth the most might be attributed to its small, neutral molecule that could quickly penetrate microorganism membranes and cause the production of free hydroxyl radicals as well as the oxidation of DNA, proteins, and membrane lipids (Chen *et al*., 2017). Therefore, hydrogen peroxide was effective against a wide variety of organisms, including vegetative and bacterial endospores, fungi, and viruses (McEvoy & Rowan, 2019). While ethanol and sodium hypochlorite had only a mild inhibitory impact on fungal growth (Figure 7), they were the most efficient sterilizing agents to disinfect bacteria. The primary mode of action of ethanol was protein denaturation and coagulation, thereby it was also a potent phytotoxicant (Mahmoud & Al-Ani, 2016; Getnet, 2017). As a result, it should be given at a dosage of 60%–80% to avoid excessive coagulation of the bacterial cell wall, which would prevent the disinfectant from entering the bacterium cell. The sodium hypochlorite at 20% (v/v) of concentration was sufficient to significantly suppress the bacterial population (Arsogah *et al*., 2018).

The findings of this research indicated that in

vitro micropropagation of bananas would require sterilizing agents such as hydrogen peroxide, ethanol, and sodium hypochloride for surface sterilization of the explant from an exogenous microorganism. Ten antibiotics that had been tested in the disk diffusion test suggested that chloramphenicol and fluconazole could be incorporated into the banana culture medium to inhibit the growth of endogenous microorganisms. Hopefully, the combination of explant surface sterilization followed by the addition of antibiotics into the grow media would be effective at reducing microbial growth and increasing culture survival (Kapadia & Patel, 2021). This is because a single-step aseptic inoculation was ineffective in controlling the endophytic contaminants in the current banana culture.

CONCLUSION

Overall, *Klebsiella pneumoniae*, *K. quasipneumoniae*, *K. variicola*, *Colletotrichum gloeosporioides,* and *Aspergillus flavus* were identified by sequencing of 16S rRNA and 28S rRNA genes. The identification of these bacteria and fungi facilitated the identification of chloramphenicol and fluconazole as potential antibiotics to prevent their growth from the disk diffusion method. Hydrogen peroxide, ethanol, and sodium hypochlorite had demonstrated that these sterilizing agents have a high potential for controlling bacterial contamination in vitro banana culture. These findings are important foundations for optimized protocols of sterilizing banana explants and controlling bacterial contaminants for a large-scale in vitro micropropagation of the banana Tanduk banana cultivar.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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