Vol. 13(29), pp. 675-688, November, 2019
DOI: 10.5897/AJMR2019.9182
Article Number: DC785B462395
ISSN: 1996-0808
Copyright ©2019
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

ACADEMIC JOURNALS
expand your knowledge

African Journal of Microbiology Research

Full Length Research Paper

Screening of fungal contaminants in banana tissue cultures in Jkuat, Kenya

Eunice M. Kithaku[1]*, Anne W. T. Muigai[1], Johnstone O. Neondo[2] and Cecilia M. Mweu[2]

[1]Department of Botany, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.
[2]Institute of Biotechnology Research, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 62000-00200, Nairobi, Kenya.

Received 26 July, 2019; Accepted 2 October, 2019

Tissue culture is prone to high costs of production arising from losses incurred from fungal contamination. The aim of the study was to characterise fungal contaminants and elucidate the exhibited mode of resistance to most preferred sterilants. Twenty nine fungal samples were collected at the different stages of tissue culture growth, using purposive sampling technique. Morphology results were confirmed by molecular characterization using fungal 18S rRNA sequences. Biochemical and antibiosis tests, identification of genes for capsulation and ATP binding Cassete (ABC) transporters, were performed to show the relationship between the fungi and sterilants resistance. Amylases and proteases were highly expressed by all isolates while xylanases and lipases were moderately expressed and esterases were lowly expressed. Only fourteen isolates had antagonistic activity for *Candida albicans* while nine of them had antagonistic activity for *Pseudomonas aeruginosa.* Three isolates were both antagonistic for *Staphylococcus aureus* and for *Escherichia coli. Cunninghamella bainieri* (10R) recorded a unique antibiosis and extra cellular enzymatic activity ($p<0.05$). All the isolates were positive for mdr1 gene and three isolates had CAP64 capsule genes. *Aspergillus* sp., *Penincillium* sp., *Cladosporium* sp., *Cunninghamella* sp. and *Fusarium* sp. were identified to be the major fungal contaminants of tissue culture banana cultures in JKUAT laboratories.

Key words: Tissue culture, 18S rRNA, fungal contaminants, banana cultures.

INTRODUCTION

Bananas (*Musa* spp.) are ranked fourth as the world's most valuable crop after rice, wheat and are staple food in Uganda, Rwanda, Burundi and parts of Kenya and Tanzania (Rubaihayo, 2003; AHBFI, 2013). They are rich source of energy, with a banana fruit weighing 100 g containing approximately 350 Kilo Joules of energy (Hanumantharaya et al., 2009). Apart from containing carbohydrates, the banana fruit is rich in phosphorous, calcium, iron, potassium and magnesium and traces of zinc, copper, chlorine, cobalt and iodine (Rubaihayo, 2003). Most of the vitamins especially vitamin A, vitamin C and riboflavin are present in fair quantities (Rubaihayo,

*Corresponding author. E-mail: eunice.mutheu@gmail.com.

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

2003). Thus from the nutritive point of view, bananas can be considered as fruits that contains many nutrients and are highly nourishing (Mbaka et al., 2008).

Despite it being a valuable crop, its production worldwide has been decreasing. In Latin America, Caribbean and Asia the decline has been attributed to an increase in banana pests and diseases, climate change, rising cost of fertilizers among others (FAO, 2006; Dita et al., 2013). In Africa, its production has declined due to an increase in pests and diseases, reduced soil fertility and an increase of moisture stress (Nyombi, 2013).

The use of tissue culture (TC) to produce clean and disease free planting material has been adopted to address some of these problems. Tissue culture has been used to clean diseased planting materials obtained from infected soils and farms (Kahangi et al., 2004). Despite the technology being very profitable, it is prone to high costs arising from losses incurred due to bacterial and/or fungal contamination (Msogoya et al., 2012). Bacterial contamination is easily controlled by the available sterilants. However the available sterilants have proven to be ineffective in controlling fungal contamination. This might be because fungi have ABC transporters for active efflux of toxic substances from the fungal system (Del Sorbo et al., 2000).

Fungi have also been reported to form capsules around their spores that help them avoid stressful conditions including hostile host immunological reactions (Zaragoza et al., 2009). Fungi may also produce extra cellular enzymes or exhibit antibiosis for self-defence (Compant et al., 2012). *Fusarium* sp. for instance, produce lipases that enable the fungi to infect the host (Feng et al., 2005), while the *Aspergillus* species like *A. flavus* produce amylases, proteases, lipases that enable the fungi to attack and cause disease to the host (Amaike and Keller, 2011). Extracellular enzymes like lignases, esterases, glutathione 5-transferases, cytochrome are also involved in degradation of fungicides (Hernndez et al., 2013), providing another reason why fungi are resistance to most preferred sterilants. Fungi have enormous potential to produce metabolites (Zhao et al., 2014). Most microbes produce bioactive metabolites that act on the host by either depriving it of nutrients, space, lysing cells or blocking specific functions that are related to the hosts growth and metabolism, including shutting down hosts defence mechanisms and thus allowing pathogen to attack without any resistance (Habiba et al., 2002; Zhao et al., 2014).

In many TC laboratories, a large number of explants, estimated at between 40 to 60% are destroyed in the cultures due to both exogenous and endogenous microbial contaminants (Msogoya et al., 2012; Helaly et al., 2014). Aseptic techniques employed in tissue culture laboratories include surface sterilization using 0.3% Redomil® (fungicide) plus Tween® 20, 70% ethanol and 7% bleach, sterilizing the media and culture bottles, flaming the inoculating tools used until red hot among others (Odutayo et al., 2007). Surface sterilization eliminates the exogenous microbial contaminants only while endogenous ones emerge on nutrient-rich culture medium (Mng'omba et al., 2007). An application of systemic fungicides before collection of explants can eliminate the endophytic contaminants (Habiba et al., 2002). Despite following these aseptic procedures, microbial contamination still remains a major problem affecting banana *In vitro* propagation in tissue culture laboratories.

Limited research has been conducted on fungal contaminants in TC laboratories. The objective of this study was to identify the fungal contaminants in TC laboratories and whether produced extracellular enzymes, exhibit antibiosis, have ABC transporters or capsule genes for self-defence.

MATERIALS AND METHODS

Study site

Samples of contaminated banana cultures were obtained from banana tissue culture laboratories in the Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya.

Sample collection and isolation

Purposive sampling technique (Bernard, 2002) was used to collect twenty-nine samples of contaminated cultures at three developmental stages of tissue culture namely initiation, multiplication and rooting stages in 1st of October to 30th of November in 2017. Collected fungal cultures were assigned code names for easy traceability of their origin. To achieve this, samples from initiation, multiplication and rooting stage were labelled I, M and R respectively. The fungal contaminants were grown on sterile Potato Dextrose Agar (PDA) media. The medium was supplemented with 25 mg/l Streptomycin to prevent bacterial contamination (Neondo, 2017). Each of the fungal contaminant was cultured on this medium and incubated for seven days at 30°C. Pure fungal isolates were obtained after repeated subcultures in fresh PDA media (Msogoya et al., 2012) which were then used for subsequent studies.

Morphological characterization

Colony and cell morphology

The isolates were grown on fresh PDA media, descriptors such as colony colour, margin and elevation was observed and recorded. For cell morphology, wet mount slides of the isolates were prepared and covered carefully using a cover slip to avoid air bubbles. They were stained with lactophenol cotton blue and observed under Olympus BX43 light microscope at ×100 for identification of the appearance of conidiophores and conidia (Solano, 2011). The Saccardian System was then described and used to presumptively classify the fungal isolates morphologically (Barnett, 1962; Barnett and Hunter, 1998).

Biochemical characterization

Based on morphological characterization some isolates resembled

one another, thus in subsequent experiments these isolates were treated as one sample and the sample size was narrowed to 18.

Determination of amylolytic activity

Determination of amyolytic activity was done using the amylases test as described by Hankin and Anagnostakis (1975). The isolates were inoculated in nutrient agar containing 0.2% of soluble starch in grams per litre, pH 8.0 and incubated for seven days at 30°C. The plates were divided into four quadrants for replication purposes. The cultures were treated with 1 % Lugol's iodine. A positive results was recorded when brown iodine colour turned blue black while in negative isolates the brown colour of iodine remained (Castro et al*.,* 1993).

Determination of the esterase activity

Esterase activity was determined using media described by Sierra (1957), which was composed of peptone 10.0 g/l, NaCl 5.0 g/l, $CaCl_2$ $2H_2O$ 0.1 g/l, agar 18.0 g/l, pH 8.0. Sterilized Tween 80 in a final concentration of 1 % (v/v) was added to sterilized culture media. The plates were divided into four quadrants and incubated for seven days at 30°C. Positive results showed presence of a precipitation of calcium crystals around the colonies and no crystals for negative results (Solano, 2011).

Determination of the lipolytic activity

Lipolytic activity was determined using methodology described by Sierra (1957) with the exception that sterilized Tween 80 was replaced with Tween 20. The plates were marked into four quadrants and incubated for seven days at 30°C. Precipitation of calcium salts around the colonies was observed as an indication of the presence of lipase production while colonies without crystals for negative results (Solano, 2011).

Determination of the proteolytic activity

Proteolytic activity was determined using the protocol described by Zilda et al. (2012). The isolates were cultured on media comprising Nutrient Broth 8.0 g/l, glucose 1.0 g/l, agar 18.0 g/l, pH 8.0. Separately autoclaved 15 ml of skimmed milk was added to the autoclaved culture media. The plates were divided into four quadrants and incubated at 30°C for seven days. After the incubation period, 2.0 ml of HCl 0.1 Molar (M) was added to the plates. Positive isolates for protease production had clear halos around the colonies while the negative isolates did not have clear halos (Neondo, 2017).

Determination of the cellulolytic activity

Cellulolytic activity was determined using media that comprised of 7.0 g KH_2PO_4, 2.0 g K_2HPO_4, 0.1 g $MgSO_4.7H_20$, 1.0 g $(NH_4)_2SO_4$, 0.6 g yeast extract, 10 g microcrystalline cellulose and 15 g agar per litre (Stamford et al., 1998). The plates were marked into four quadrants and incubated at 30°C for seven days. The plates were then incubated at 50°C for an overnight after the incubation period. Clear zones around the colonies indicated presence of cellulase production while negative results did not have clear halos (Solano, 2011).

Determination of the xylanolytic activity

The fungal isolates were grown on Czapek's agar medium containing xylan as the sole carbon source to determine their ability to produce xylanase (Ruijassenaars and Hartmans, 2001). The medium contained Birch wood xylan 5.0 g/l, Peptone 5.0 g/l, Yeast extract 5.0 g/l, K_2HPO_4 1.0 g/l, $MgSO_4.7H_2O$ 0.2 g/l and agar 20 g/l. The plates were divided into four quadrants, inoculated with the fungal isolates and then incubated for seven days at 30°C. The plates were then flooded with 0.1% (w/v) Congo red dye. For positive results, there was colour change from red to orange but for the negative results the red colour remained (Solano, 2011).

Antibiosis test

The test organisms used were *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25922), *Escherichia coli* (ATCC 25923) and fungi *Candida albicans* (ATCC 90028). They were obtained from Kenya Medical Research Institute-Centre for Microbiology research. Fungal spore paste was prepared by mixing 10^6-spores per ml suspension with one drop of triton X-100 and used to saturate fungal inoculant discs (Sterile What man No.1 filter paper discs-1 cm diameter) following Kirby-Bauer disc diffusion method (Neondo, 2017). The discs were placed on the surface of the Mueller Hinton agar plates which had been freshly swabbed with 100 µl of each test organisms. The plates were sealed with parafilm and incubated at 30°C for a period of four days. Zones of inhibition around the colonies were measured and recorded (Neondo, 2017).

Molecular characterization

DNA extraction

The 18 fungal isolates were studied further using molecular tools. Fungal DNA was done using protocal reported by Gontia et al. (2014). Fresh fungus was put in sterile 1.5 ml eppendorf tubes that contained 0.5 g sterile sieved sand. The cultures were crushed using sterile micro pestles. A total of 600 µl of extraction buffer (0.1 M Tris HCL pH 8, 10 mM EDTA pH 8, 2.5M NaCl, and 3.5 % CTAB, and 150 µL of 20 mg/mL of proteinase k) was added. Incubation of the cells was at 65°C for one hour in a water bath with shaking after every 20 min. The samples were centrifuged for 10 minutes at 10,000 Xg. The supernatant was transferred to sterile 1.5 ml eppendorf tubes and equal volume of phenol chloroform-isoamylalcohol (25:24:1) was added to remove the proteins. The samples were centrifuged again at 10,000 Xg for 10 min. The supernatant was transferred to clean 1.5 ml eppendorf tubes.

To the supernatant, equal volume of chloroform isoamyl was added to wash the fatty tissues and phenol by gentle shaking. The sample was centrifuged at 8944 Xg[1] (times gravity) for 10 min. The supernatant was transferred in 1.5 ml eppendorf tubes and equal volume of isoamyl was added to completely remove fatty tissues and phenol from the DNA. The aqueous phase was transferred to a new 1.5 ml eppendorf tube and DNA precipitated with equal volume of absolute ethanol. Precipitation was done for overnight at -20°C. The precipitates were centrifuged for 15 min at 15115 Xg and the supernatant discarded. The pellet that contained the genomic DNA was rinsed with 70% ethanol twice to remove traces of salts. The DNA was dried in an incubator at 40°C for thirty minutes to completely remove ethanol and then dissolved in 100 µl TE buffer pH8. The genomic DNA was confirmed by gel electrophoresis using 0.8% agarose in 1x TAE buffer stained with 0.5 mg/L ethidium bromide. Three microliter of DNA and 1 µl loading dye was mixed and put on the agarose gel. The gel was run at 80 V for one hour

---

[1] Times gravity calculated using $g = (1.118 \times 10^{-5})$ R S^2 Where g is the relative centrifugal force, R is the radius of the rotor in centimeters, and S is the speed of the centrifuge in revolutions per minute

and DNA visualized under ultraviolet light. The DNA was then stored at -20°C for subsequent us.

Amplification of 18S rRNA gene

In order to identify 18S rRNA gene in isolates, polymer chain reaction was done using forward primer (566F) 5'-CAGCAGCCGCGGTAATTCC-3'and reverse primer (1200R) 5'CCGTGTTGAGTCAAATTAAGC-3' (Nayarisseri et al., 2013; Hadziavdic et al., 2014). Amplification was performed using Peqlab primus 96 PCR machine and the expected fragment size was 650 bp. Amplification was carried out in a 40 µl mixture containing 23.2 µl PCR water, 8 µl reaction buffer, 2 µl forward primer, 2 µl reverse primer, 0.4 µl BIOLINE Taq polymerase, 0.4 µl bovine serum and 2 µl template DNA. The DNA template was not added to the negative control (Solano, 2011). PCR conditions for 18s rRNA were; Initial denaturation (95°C for 5 min), 40 cycles of each denaturation (95°C for 30 s), annealing (58°C for 45 s), extension (72°C for 40 s) and final extension step (72°C for 5 min). After successful amplification, they were stored at 4°C (White et al., 1990). The PCR amplicons were confirmed using a 2% agarose gel. The unpurified PCR product was divided into two portions and 20 µl was put in 1.5 ml eppendorf tubes and sealed using parafilm. The tubes were placed in a box with interior holders and cotton wool was used to prevent the tubes from moving around inside the box ready for shipping to Macrogen in South Korea for purification and Sanger dideoxy sequencing. The other portion of PCR product was stored at -20°C for subsequent use.

Phylogenetic data analysis

After sequencing, the sequences were visually checked and edited using ChromasPro 2.6.6 software (Technelysium, 2018) to obtain consensus sequences. In this analysis, because of the inclusion of divergent sequences and the differences in length among the aligned DNA sequences, a large number of gaps (that is, insertions and deletions) were introduced in the aligned dataset. As a result, the gaps were treated as missing data. The consensus sequences were then compared with other DNA sequences in GenBank using basic local alignment search tool (BLAST) in National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Identification of the isolates was based on the highest similarity of the BLAST search (Shayne et al., 2003). The 18s rDNA gene sequences with the closest neighbour strains were aligned using MUSCLE software (Edgar, 2004). Phylogenetic analysis was performed using neighbor-joining method and maximum composite likelihood (MCL) methods (Tamura et al., 2004) of MEGA version 7.0 software, with 1,000 bootstrap replicates

Screening for ABC (ATP binding cassete) transporter and capsules genes

Amplification of ABC transporter was done using ABC1, mdr1 and CDR2 genes. Capsules genes were screened using CAP64 and CAP10 genes. They were designed using Primer3 Plus software version 0.4.0 (Untergasser et al., 2007). The primer sizes were set between 18 and 22b with optimum sizes of 20b. The melting temperatures of primers were set between 50 and 60°C with optimum temperatures of 55°C. The PCR was performed on a GeneAmp PCR system 9600 (Applied Biosystems). Amplification was carried out using 1 µl reverse primer, 0.2 µl BIOLINE Taq polymerase, 0.2 µl bovine serum and 1 µl template DNA. A total of 40 cycles of amplification was performed with template DNA and denaturation at 95°C for 5 min; primer annealing was between 44 and 54°C for 45 s and primer extension at 72°C for 40 s. The PCR amplicons were visualized through gel electrophoresis on a 2% agarose gel with 0.5 mg/L ethidium bromide added directly. The negative control did not have DNA template.

Statistical analysis

Data on antibiosis and extra cellular enzymatic experiments was noted and recorded. MINITAB software version 9.1 (Groebner et al., 2008) was used to perform analysis of variance (ANOVA) for all the measured data. Tukey's honest significant difference (HSD) test (Montgomery and Douglas, 2013) was used to compare and separate the means of diameter of zones of inhibition and clearance (presented in the form of alphabet letters in the tables). Correlation profiles of zones of inhibition, antibiosis with respect to selected isolates were visualized as heat maps generated by a hierarchical clustering R script using R version 3.3.1 software (Fox and Andersen, 2005). Genetic affiliation of the screened isolates was deduced from phylogenetic tree generated using Mega 7 (Kumar et al., 2016).

RESULTS

Colony and cell morphology

Most of the isolates top colour was grey with few having reddish white colour. The bottom colour of most of the isolates were cream with few been orange and white. Most of the isolates had entire margins with few having curled margin. The elevation differed with most of the isolates having risen and few having convex one (Table 1). On cell morphology, most of them had aseptate (Plate 1f) and septate hyphae (Plate 1c) and few of them had intertwined mycelia (Plate 1m). A dendrogram was drawn based on the colony and cell morphology characters giving 5 clusters. It was drawn using Ward D method and the distance between characters measured using Euclidean metric. This clustering shows that contaminants at given stage of culture are similar in that cluster 2 is comprised of contaminants from multiplication, cluster 3 rooting and one initiation (Figure 1).

Biochemical characterization

The ability of fungal isolates to produce extracellular enzymes was studied. The isolates were able to produce different enzymes, with amylases and proteases highly produced. The zones of clearance values were considered as indicators of enzyme activities in the isolates, which may play role in sterilants degradation. Amylases and protease were produced by all fungal isolates with 10R recording the highest (6.100±0.1915) and 11 lowest (3.650±0.0957) amylase activity (Figure 2 and Plate 2e). Isolate 10R recorded highest (7.550±0.0957) and 14 lowest (2.275±0.1797) protease activity (Table 2 and Plate 2a). Most of the isolates were able to produce lipases and xylanases. For lipolytic test, isolates 10R and I1 recorded high and low zones of

Table 1. Morphological characteristics of fungal isolates from JKUAT Banana Tissue Culture laboratory.

Isolate	Colour (top)	Colour (bottom)	Margin	Elevation
I1	Brown	Cream	Entire	Raised
I2	Grey, white	Cream	Entire	Flat
I3	Grey, white	Cream	Entire	Raised
I4	Grey	Cream	Entire	Convex
I5	Greenish/yellow	Cream	Undulated	Raised
I6	grey, white	Cream	Entire	Flat
I7	greenish/yellow	Cream	Undulated	Raised
I8	white(hairy)	Orange/white	Undulated	Raised
I9	grey/white	Cream	Undulated	Raised
M1	Greenish	Cream	Entire	Raised
M2	grey /white	Cream	Entire	Raised
M3	brown/white	Cream	Entire	Raised
M4	yellow/white	Cream	Curled	Raised
M5	green/yellow	Cream	Undulated	Raised
M6	grey/white	Cream	Undulated	Raised
M7	white/yellow	Cream	Undulated	Flat
M8	white/yellow	Cream	Undulated	Flat
M9	white/yellow	Cream	Entire	Raised
M10	white/yellow	Cream	Entire	Raised
R1	Grey	Yellowish	Entire	Convex
R2	white/yellow	Cream	Entire	Raised
R3	White	Cream	Entire	Raised
R4	reddish/white	Cream	Entire	Raised
R5	white/yellow	Cream	Entire	Raised
R6	white/yellow	Cream	Curled	Raised
R7	white/yellow	Cream	Curled	Raised
R8	grey/white	Yellow	Undulated	Raised
R9	white/yellow	Cream	Entire	Raised
R10	white(hairy)	Orange/white	Undulated	Raised

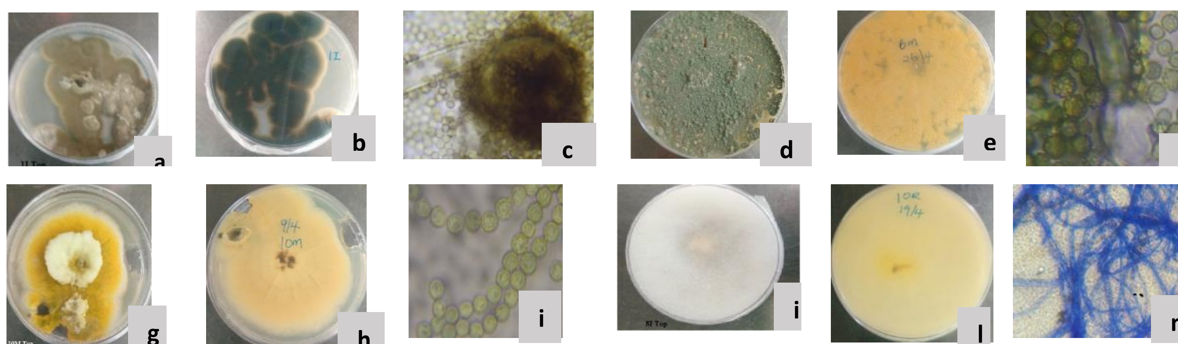

Plate 1. (a) Brown with cream margin; **(b)** Black with cream margin; **(c)** Brush like hyphae terminal with septate hyphae; **(d)** Grey granular; **(e)** Cream; **(f)** Aseptate hyphae; **(g)** Yellow white with white margin; **(h)** Cream bottom and margin; **(i)** Spores arranged in chain; **(k)** Hairy cotton; (**i)** Cream white; **(m)** Long intertwined branched mycelia.

clearance with average diameters of 5.050 ± 0.2223 and 4.850 ± 0.2217 mm, respectively (Table 2, Figure 2 and Plate 2d). Analysis of xylanase enzymatic test showed that isolates 10R and I4 recorded highest and least with a diameter of 5.95 ± 0.05 and 3.250 ± 1.2500 mm, respectively (Table 2, Figure 2 and Plate 1c). For

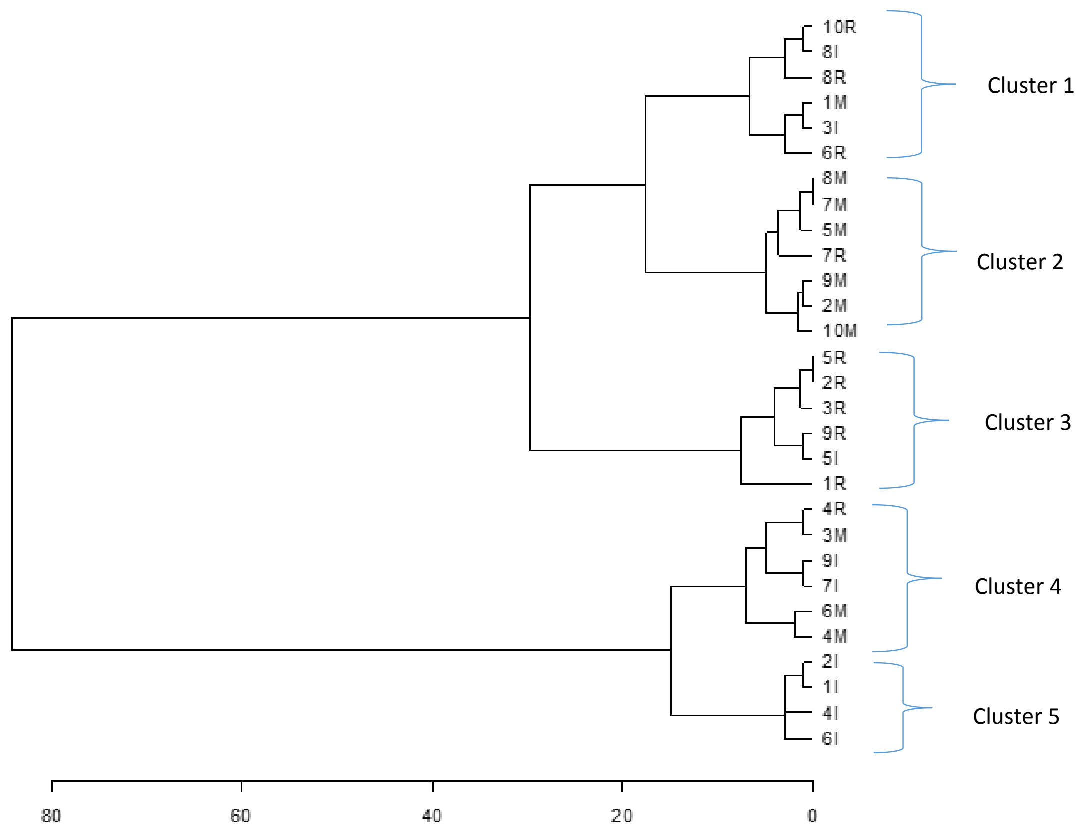

Figure 1. A dendrogram showing how the fungal isolates clustered morphologically.

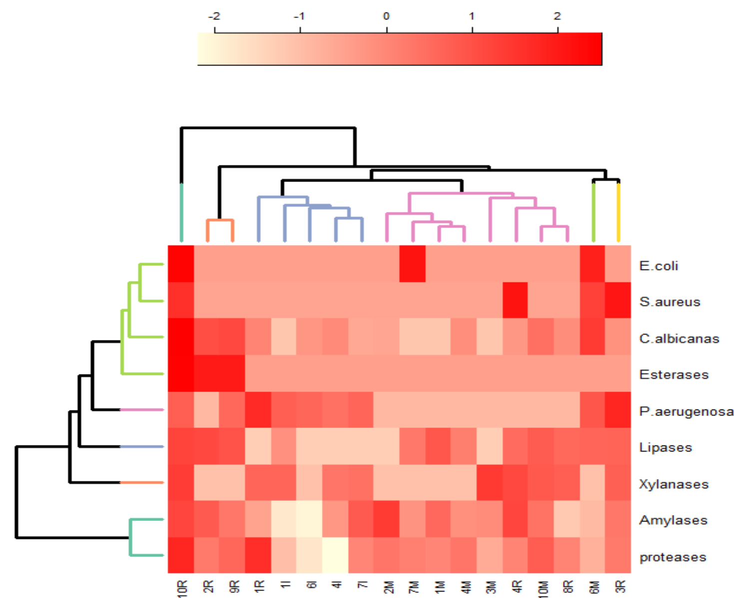

Figure 2. Hierarchical cluster gram of assayed fungal isolates. The heat map (based on Manhattan metric) shows relationship between fungal isolates and the measured morphometric descriptors. The coloured scale bar indicates the quantified strength of the assayed morphometric descriptor. Red and light red colours in the heat map indicate the highest and the least recorded significant mean values respectively at P≤0.05 for the assayed treatments.

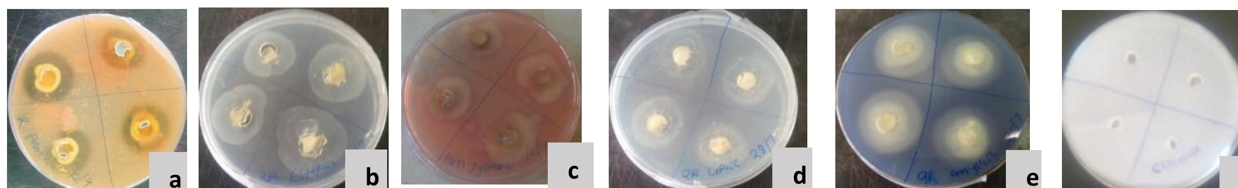

Plate 2. (a) R1 Positive results for protease **(b)** Positive results for esterase activity(precipitates of calcium salts around the colony) **(c)** Isolate M10 showing positive results for xylan utilization **(d)** Positive results for lipase tests (precipitates of calcium salts around the colony) **(e)** Positive results for amylase **(f)** Negative results of cellulose tests

Table 2. Mean diameters (mm) of inhibition zones of fungal growth by enzymes.

Isolate	Esterases (diameter in mm)	Proteases	Lipases	Amylases	Cellulases	Xylanases
11	-	3.850±0.0957[ef]	3.850±0.4500[bc]	5.650±0.1708[abc]	-	4.300±0.1[bcd]
I4	-	7.550±0.0957[a]	-	4.800±0.1414[cde]	-	5.700±0.1291[a]
I6	-	5.600±0.0816[bcd]	-	4.30±0.1915[defg]	-	-
I7	-	5.200±0.2[d]	-	3.650±0.0957[fg]	-	3.450±0.4193[de]
M1		5.200±0.2[gh]	4.350±0.1258[ab]	5.450±0.3202[abc]	-	-
M2	-	2.900±0.1915[h]	-	6.100±0.1915[a]	-	-
M3		7.350±0.0957[a]	-	4.950±0.1893[bcde]	-	5.850±0.0957[a]
M4	-	5.600±0.5354[bcd]	2.3±0.2041[de]	4.9±0.3[bcde]	-	-
M6	-	4.150±0.6702[e]	2.9±0.3873[de]	3.450±1.1701[g]	-	-
M7	-	5.350±0.15[cd]	3.150±1.05[cd]	4.850±0.5188[bcde]	-	-
M10	-	6.2±0.0816[b]	4.15±0.2250[ab]	5.300±0.1915[abcd]	-	4.5±0.3317[bc]
R1	-	4.3±0.2646[e]	-	4.650±0.3775[cdef]	-	4±0.1633[cde]
R2	5.35±0.2363[a]	5.5±0.1291[cd]	4.850±0.2217[a]	5.650±0.3304[abc]	-	-
R3	-	5.500±0.0577[cd]	3.900+0.1291[bc]	5.250+0.5560[abcd]	-	4.2±0.1896[cde]
R4	-	5.250±0.1258[d]	3.600±0.0645[e]	5.950±0.05[ab]	-	5.250±0.3862[ab]
R8	-	5.250±0.15[fg]	3.700±0.2287[de]	4.1±0.4796[efg]	-	3.950±0.0500[cde]
R9	-	5.950±0.1258[bc]	4.550+0.2217[ab]	5.250±0.35[bcd]	-	-
R10	4.5±0.1732[c]	2.275±0.1797[h]	5.050+0.2223[de]	5.95±0.05[ab]	-	3.250±1.2500[e]

esterase production, only isolates R2 and R10 were positive (Table 2, Figure 2 and Plate 2b). All isolates did not produce cellulose enzymes.

Analysis of extra cellular enzymatic activity for individual fungal isolate revealed that, isolate 10R recorded high extra cellular activity for esterases, protease, lipase amylase and xylanases tests of average clearance diameter of 5.35±0.2363, 7.550 ± 0.0957, 5.050 ± 0.2223, 5.95 ± 0.05 and 5.700 ± 0.1291 mm, respectively, p value = 0.000 (Table 2 and Figure 2).

Antibiosis

The effect of fungal isolates on different test organisms was studied. When the isolates were cultured in media containing *C. albicans,* antagonistic activity was observed in 14 isolates namely I4, I6, I7, M2, M4, M6, M10, R1, R2, R3, R4, R8, R9 and R10, with R1 recording the highest (3.0±0.2356 mm) and I1 lowest (0.5±0.8923 mm) diameters of zones of clearance (Table 3, Figure 2 and Plate 3d). The values of zones of clearance shows how efficient the fungal isolates are, in producing metabolites that may play role in sterilants resistance. About 11 isolates had antagonistic activity against *P. aeruginosa* that is I1, I4, I6, I7, M3, M6, M7, R1, R3, R9 and R10 with R3 recording the highest (5.800±0.4773) and I1 lowest (2.250±0.0500) antagonistic activity (Table 3, Figure 2 and Plate 3a). Few isolates had antagonistic activity against *S. aureus* and *E. coli.* Isolate R4 and M6 recorded highest and lowest antibiosis property of *S. aureus* of 3.700±0.1915 and 2.500±0.1315, respectively (Table 3, Figure 2 and Plate 3b). Isolate R10 and M6 recorded highest and lowest antagonistic activity against *E. coli* of 2.660±0.1314 and 2.175±0.0479, respectively (Table 3, Figure 2 and Plate 3c).

Correlation profile between morphometric descriptors (antibiosis and enzymatic activity estimates) and fungal isolates revealed that there are five functional clades (Figure 2). Isolate 10R formed solitary clade and the

Table 3. Antibiotic activity (mean of zone of inhibition) of the selected fungal isolates against four test organisms.

Isolate	*P. aeruginosa*	*S. aureus*	*E. coli*	*C. albicanas*
I1	3.600±0.2160^{ab}	-	-	-
I4	2.850±0.2363^{bc}	-	-	1.900±0.3456^{c}
I6	3.250±0.1258^{bc}	-	-	2.100±0.500^{b}
I7	3.300±0.3873^{abc}	-	-	1.300±0.4255^{e}
M1	-	-	-	-
M2	-	-	-	1.000±0.1256^{f}
M3	-			
M4	-	-	-	0.900±0.5678^{g}
M6	5.800±0.4773^{a}	2.500±0.1315^{c}	2.175±0.0479^{b}	3.000±0.2356^{a}
M7	-	-	2.400±0.1414^{a}	-
M10	-	-	-	0.460±0.7656^{i}
R1	5.600±0.1291^{ab}	-	-	0.500±0.8975^{i}
R2	-	-	-	1.800+0.9823^{d}
R3	3.870±0.1000^{ab}	3.600±0.0000^{a}	-	0.800±1.0500^{gh}
R4	-	3.700±0.1915^{a}	-	0.700±0.9367^{h}
R8	-	-	-	0.860±0.8923^{g}
R9	3.200±0.1414^{bc}	-	-	0.840±0.7345^{g}
R10	2.250±0.0500^{c}	2.960±0.2215^{ab}	2.660±0.1314^{a}	0.720±0.6500^{h}

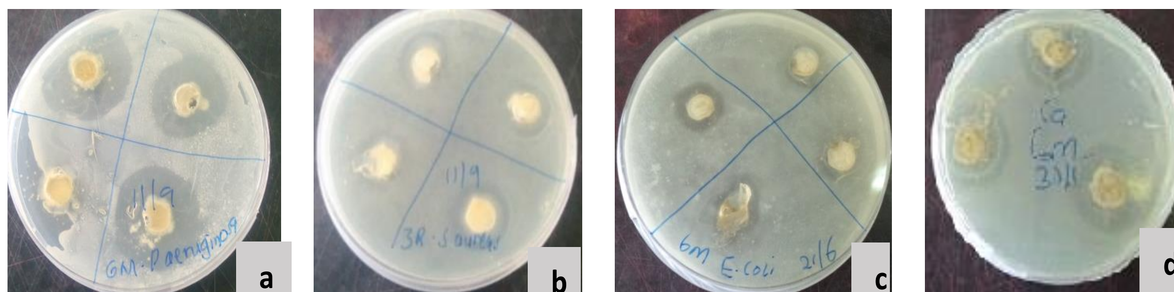

Plate 3. (a) Antagonistic activity of M6 on *P. aeruginosa*. **(b)** Antagonistic activity of R3 on *S. aureus* **(c)** Antagonistic activity of M6 on *E. coli* **(d)** Antagonistic activity of M6 on C. *albicans.*

remaining isolates formed four clades as shown in Figure 2. Isolate 10R recorded a unique antibiosis and extra cellular enzymatic correlation profile (the only member in this functional clade) as shown by colours dominating its column. On the contrary, isolates from initiation stage recorded relatively low expression values for most of the assayed tests among all isolates evaluated (Figure 2).

Phylogenetic analysis of the fungal isolates

After amplification, all the fungal isolates had 18S rRNA gene (Figure 3). After which phylogenetic analysis generated neighbour joining phylogenetic tree using 18s rRNA gene sequences with the closest neighbour strains which shows the phylogenetic relationships among the various fungal isolates (Figure 4).

Screening for ABC transporter genes

Only one set of the degenerate primer was able to amplify the ABC transporter gene producing a fragment of size 172 bps. All the isolates had this gene (Figure 5).

Screening for capsule genes

CAP64 was able to amplify only three isolates with an amplicon size of 260 bp, while CAP10 did not amplify any isolate. These isolates are *Penincillium citrinum* (R1), *Aspergillus fumigatus* (R2) and *Pencillium* sp. (R8) (Figure 6).

DISCUSSION

Based on morphological characterization, some isolates

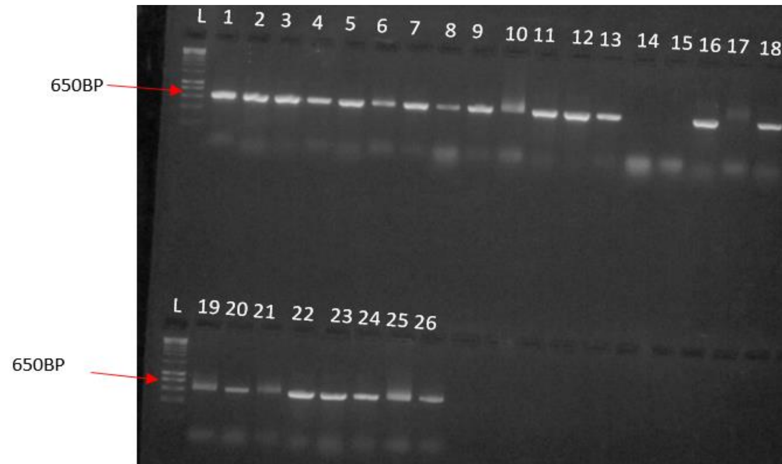

Figure 3. PCR products for the amplification of 18S rDNA for the fungal isolates using 1200R and 566F universal primers. The expected band size amplified is 650 bps. 2% agarose was used. L-Molecular marker (company name and catalogue number (1 kb plus ladder ThermoFisher scientific, Catalog number:10787018) 1-1I, 2-4I, 3-6I 4-7I, 5- MI, 6- M2, 7-M3, 8-M4, 9-M6, 10-M7, 11-M10, 12-R1, 13-R3, 14-Control, 15- Control, 16-R3, 17-R4, 18-R4, 19-R8, 20-R9, 21-R10, 22-I8, 23-M5, 24-I5, 25-I9, 26-R6, 27-R2.

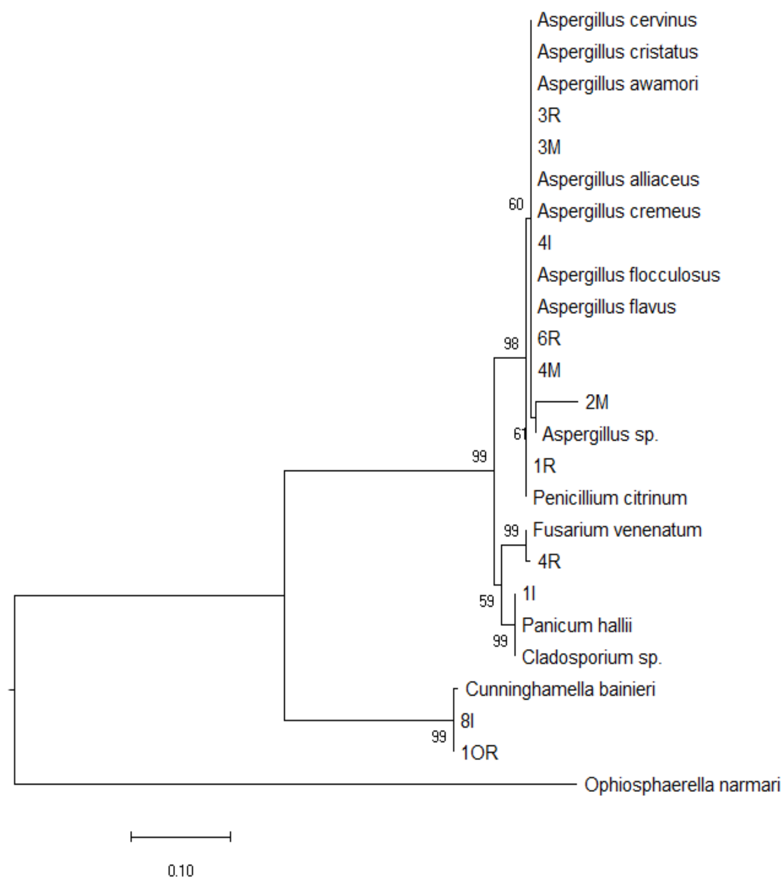

Figure 4. Neighbor joining phylogenetic tree displaying relationship between18s rRNA gene sequences and the closest neighbour strains. The scale bar refers to 0.1 substitutions per nucleotide position. Only boostrap values above 50 are shown.

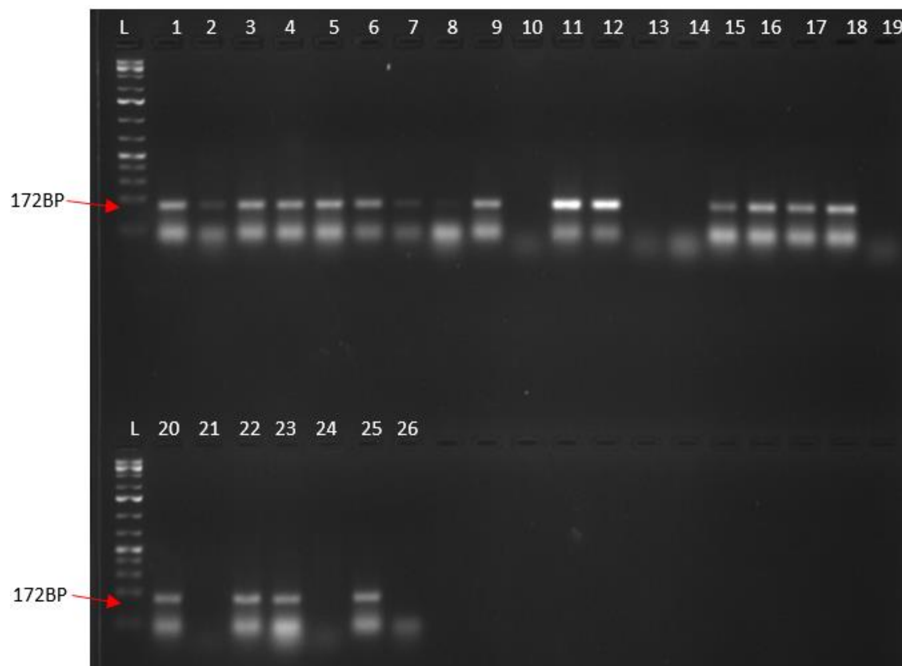

Figure 5. PCR products for the amplification of ABC transporter genes for the fungal isolates using degenerate primers set 1 primers. Lane 1 represents a 1 kb plus ladder. The expected band size amplified is 172 bps. L-Molecular marker (1 kb plus ladder, ThermoFisher scientific, Catalog number:10787018) 1-14, 2-M4, 3-R1, 4-R4, 5-M1, 6-I7, 7-M6, 8-R4, 9-R2, 10-Control, 11-M2, 12-I6, 13-Control, 14-Control, 15-R3, 16-R8, 17-M3, 18-R9, 19-Control, 20-M10, 21-Control, 22-10R, 23-M7, 24-M7, 25-I1, 26-Control.

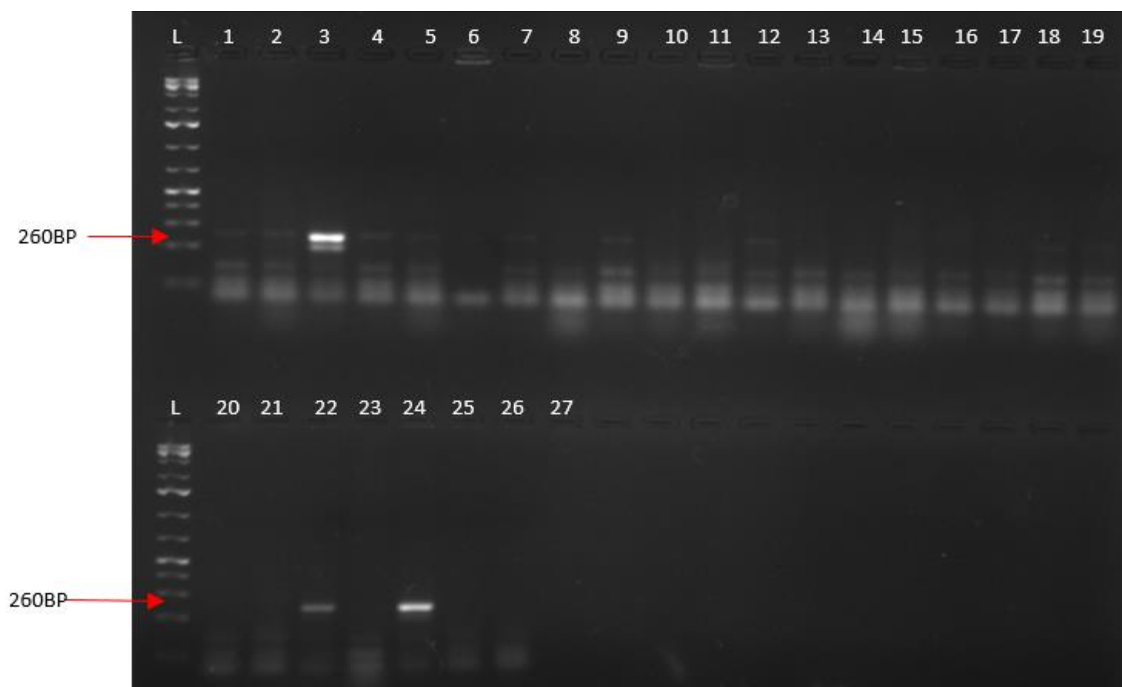

Figure 6. PCR products for the amplification of capsules genes for the fungal isolates using CAP64 primers. Lane 1 represents a 1 kb plus ladder. The expected band size amplified is 260 bps. L-Molecular marker(1 kb plus ladder); 1-14, 2-M10, 3-R1, 4-R4, 5-M1, 6-I9, 7-M6, 8-I7, 9-2, 10-M2, 11-I6, 12-I2, 13-R7, 14-R3, 15-10R, 16-M4, 17-I1, 18-M3, 19-R9, 20-R9, 21-M10, 22-R2, 23-M7, 24-R8, 25-I9, 26-I1, 27-Control.

resembled one another, thus in subsequent experiments these isolates were treated as one sample and the sample size was narrowed to 18. These fungal isolates belonged to genera; *Aspergillus*, *Penincillium*, *Cladosporium* and *Fusarium.* Other studies discovered the same isolates in TC laboratories (Odutayo et al., 2007; Msogoya et al*.,* 2012; Ankur et al., 2014). However*, Cunninghamella* sp. was the only fungus that was found in JKUAT Tissue laboratory occurred in both initiation and rooting stage which could be as a result of resistance mechanism. Morphological characterization was only possible up to genus level and therefore molecular characterization identified up to the species level and confirmed morphological results.

Production of extracellular enzymes plays an important role in degradation of sterilants through catalytic metabolic reactions like hydrolysis, oxidation, addition of an oxygen to a double bound, amino group oxidation to a nitro group, hydroxyl group added to a benzene ring, dehalogenation, reduction of a nitro group (NO_2) to an amino group, replacement of a sulphur with an oxygen, metabolism of side chains, ring cleavage (Hernndez et al., 2013). Other studies shows that metabolism of sterlilants may involve a three-phase process. In the first phase of metabolism, the initial properties of a parent compound are transformed through oxidation, reduction, or hydrolysis to generally produce a more water-soluble and usually a less toxic product than the parent (Hernndez et al., 2013). The second phase involves conjugation of a pesticide or pesticide metabolite to a sugar or amino acid, which increases the water solubility and reduces toxicity compared with the parent pesticide (Van et al., 2003). The third phase involves conversion of Phase II metabolites into secondary conjugates, which are also non-toxic (Ramakrishnan et al., 2011). In these processes fungal isolates are involved in producing extra cellular enzymes including hydrolytic enzymes, peroxidases, oxygenases, esterases, lipases, xylanases among others (Hernndez et al., 2013; Singh et al., 2016). In this study, production of extracellular enzymes, amylases, proteases, xylanases, cellulases and esterases were investigated. Isolate 10R recorded high extracellular activity and was both in initiation and rooting stage of *In vitro* culture. This could be attributed to its ability to produce higher amounts of extracellular enzymes which are involved in detoxification of sterilants.

Previous studies show that fungi mostly produce metabolites when under stress as a self-defence mechanism and some of them are produced to scavenge oxidizing agents like fungicides (Bertrand et al., 2014). Fungal isolates in this study were scrutinized for their ability to exhibit antibiosis and isolates in the rooting stage had the highest diameter of clearance due to antibiotic production. This could be attributed to their ability to produce higher amounts of antibiotics which are believed to play a role in shutting the immunity of the host for fungi to attack without any resistance (Compant et al., 2012). This resonates with earlier studies that showed that fungi produce metabolites that act on the host depriving it nutrients, space, lysing cells and block specific functions related to host growth (Bertrand et al*.,* 2014) hence their resistance to sterilants.

The tree displays three clades in which the isolates have been clustered. *Aspergillus* species clustered with *Pencillium citrinum* in one major clade with a score of 98% since they are from the same family Aspergillaceae (Hussain et al., 2017) while *Fusarium venenatum* and *Cladosporium* sp. were grouped in another clade with a score of 59% as they are not such closely related but are only from the same subphylum Pezizomycotina (Ashfaq et al., 2017). *Cunninghamella bainieri* formed separate cluster since it was from a different phylum from the rest of clades called Mucoromycota (Karim et al., 2007). Like in morphological characterization, the main clusters revealed by molecular characterization of fungi belonged to the genera *Aspergillus*, *Penincillium*, *Cladosporium* and *Fusarium* (Table 4). The genus *Aspergillus* separated into well supported eight sub groups belonging to eight species; *Aspergillus cervinus, Aspergillus cristatus, Aspergillus awamori, Aspergillus alliaceus, Aspergillus cremeus, Aspergillus flavus* and *Aspergillus* sp. (Table 4). In this study, morphological characterization was only possible up to generic level and it was difficult identifying *Aspergillus* species and *Penincillium* species since they are highly speciose lineages of fungi. Raja et al. (2017) had made similar proposition that morphological characterization may not perform well in lower level classification especially in highly speciose lineages of fungi since morphological characters may not always provide accurate groupings within an evolutionary framework, mainly at the species level. Due to the morphological characters presented, there was suspicion that 2M was *Penicillium* sp. but after molecular work, it was found to be *A. allieus.* Such morphological ambiguities have been experienced, as characters used in identification are often too variable and dependent on colony and cell morphology (Foltz et al., 2013). Therefore, using the two techniques (morphology and molecular) necessitates close to accurate identification of species. Most of the fungal contaminants were found to belong to the *Aspergillus* species and according to Cassells (1991) they are exogenous and their presence in this study may be due to inadequate surface sterilization. *Aspergillus* species are the main cause of aspergillosis in immunocompromised individuals and symptoms of infection by this species include is fever and coughing (Baysan et al., 2010). These species could have been introduced into the laboratory by insufficient asepsis among workers during tissue culture operations. *Fusarium* sp. have been reported as an endophytic fungus in banana (Suryanarayanan et al., 2000). The sterilization protocol used in the JKUAT tissue culture laboratory involves only surface sterilization using 7% bleach and 70% ethanol.

Table 4. Blast results of fungal isolates from IBR laboratory and their close relatives.

Sample ID	Length (BP)	Accession numbers	Affliated to	Closest match in BLAST	% identity	Reference
I1	650	MN549412	LT860211.1	*Cladosporium* sp. NS1 partial 18S rRNA gene, isolate NS1	99	Ashfaq et al. (2017)
I4	641	MN549413	MN326853	*Aspergillus* sp. isolate *7S6*	99	Hamed (2019)
I6	657	MN549414	JF895924.1	*Aspergillus* sp. *I16-3.*	99	Iniya et al. (2011)
I7	644	MN549415	MH539639.1	*Aspergillus orzae* isolate *RIB40*	99	Ramesh and Josephin (2019)
M1	628	MN549416	MN453363	*Aspergillus niger* strain *BA2*	99	Al-Talhi (2019)
M2	670	MN549417	KF018469.1	*Aspergillus alliaceus* strain *21.1*	96	Demirel et al. (2013)
M3	628	MN549418	KY307867.1	*Aspergillus cervinus* strain *JAPC9*	99	Abraham and Chakraborty (2016)
M4	641	MN549419	AB008399.1	*Aspergillus cremeus*	95	Nikkuni et al. (1998)
M6	657	MN549420	KU711809.1	*Aspergillus sojae* isolate *LF2_CPD_NRRI*	96	Pattanayak et al. (2019)
R1	639	MN549421	MK615877	*Penicillium citrinum*	99	Liu (2019)
R2	649	MN549422	MF563964	*Aspergillus fumigatus* strain *T3*	99	Liu (2017)
R3	632	MN549423	JN604547.1	*Aspergillus flocculosus* strain *OUCMDZ-730*	99	Wang and Zhu (2011)
R4	612	MN549424	XR003150050.1	*Fusarium vinenatum*	99	King et al. (2014)
R8	640	MN549425	KP872503.1	*Penicillium* sp. Y28	100	Li et al. (2015)
R9	631	MN549426	KY233188.1	*Aspergillus flavus Ya1*	99	Hussain et al. (2015)
R10	682	MN549427	EF562534.1	*Cunninghamella bainieri*	99	Karim et al. (2007)

This explains why *Fusarium* sp. was found in rooting stage in this study since it cannot be eliminated by surface sterilization. Earlier studies had made similar proposition (Cassells, 1991) that *Fusarium* sp. contaminates after many subculture or transfer since it cannot be destroyed by surface sterilization. Systemic sterilants can be used to get rid of endogenous contaminants (Omamor et al., 2007; Mng'omba et al., 2007).

ABC transporter genes are responsible for the production ABC transporter pumps that fungi use for efflux of toxic substances from the fungal system (Park and Williamson, 2015; Neelab and Singh, 2018) hence the resistance of fungicides. This explains why the current sterilization protocol in banana tissue culture laboratory is ineffective.

Capsule genes are responsible for formation of a capsule which provide resistance to stressful conditions (Zaragoza et al., 2009) and are very rare in eukaryotes but ubiquitous in prokaryotes (Park and Williamson, 2015). This isolates in this study with capsules genes were collected from rooting stage meaning they resisted sterilization at initiation and multiplication stages. This could be as a result capsule gene. This gene was found in *Aspergillus fumigatus* (2R). This supports earlier studies that *A. fumigatus* is capable of growing in temperatures over 50˚C, which is in contrast to other fungi that are mesophilic (Bhabhra and Askew, 2005). This might be attributed to protective coverage on the fungal system.

Conclusion

This study also indicates that JKUAT tissue culture laboratory harbour's diverse fungal species. *C. bainieri* (10R) was found both in initiation and rooting stage. This might be because of its ability to produce extracellular enzymes, ABC transporter genes and capsule gene which are responsible for sterilants resistance. It was ascertained that fungal isolates are capable of producing enzymes which have been confirmed to play a role in degradation of fungicides. They produce antibiotics which shut the immunity of the host for the fungi to attack without any resistance hence the sterilants resistance. They also have mdr1 genes that form ABC transporter pumps that are involved in efflux of any toxic substances from the fungal system. Some of the fungal isolates have capsules genes which are responsible for formation of capsules that protects fungi from stressful conditions. The main reason why fungi

contaminants in JKUAT laboratories are difficult to eliminate is because of their resistance mechanism. Therefore sterilants that inhibit production of extra cellular enzymes, antibiotics, ABC transporters and capsules should be introduced in tissue culture laboratories.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENTS

The authors appreciate National Research Fund for funding the work. The contribution of Amanuel Menghs Ghilamicael, Steve Ogada and Josiah Kuja is highly acknowledged.

REFERENCES

Abraham J, Chakraborty P (2016). Fungal isolate from sewage soil sample. https://www.ncbi.nlm.nih.gov/nucleotide/KY307867.1.

Africa Harvest Biotech Foundation International (AHBFI) (2013). Food, Nutritional Security and Sustainable Livelihoods of Smallholders Program in Kenya. Nairobi.

Al-Talhi HA (2019). Enhanced antioxidant enzymes production using alternative stimulatory waste medium. https://www.ncbi.nlm.nih.gov/nucleotide/MN453363.1

Amaike S, Keller N (2011). *Aspergillus flavus.* Annual Review of Phytopathology 49(1):107-133.

Ankur V, Meena B, Harsh N (2014). Identification and bioassay of fungal contaminants observed during in vitro propagation of Saraca asoca (Roxb.) De wilde Biotechnology Society 7:69-76.

Ashfaq M, Ali M, Haider MS, Shafiq M (2017). Fungal diversity from the soil of Punjab University. https://www.ncbi.nlm.nih.gov/nuccore/LT860211.1

Barnett H (1962). Illustrated Genera of Imperfect Fungi (2nd ed.). Minneapolis, Minnesota: Burgess Publishing Company.

Barnett H, Hunter B (1998). Illustrated Genera of Imperfect Fungi (4th ed.). New York, USA: Macmillan Publishing Company.

Baysan O, Izquierdo A, Saba R, Ogunc D, Ongut G, Timuragaoglu A, Tudela J (2010). *Aspergillus alliaceus* and *Aspergillus flavus* co-infection in an acute myeloid leukemia patient. Medical Mycology 48(7):995-999.

Bernard H (2002). Research methods in anthropology: Qualitative and quantitative approaches. (C. Creek, Ed.) (3rd ed). CA: Alta Mira Press.

Bertrand S, Bohni N, Schnee S, SchumppmO, Gindro K, Wolfender J (2014). Metabolite induction via microorganism co-culture: A potential way to enhance chemical diversity for drug discovery. Biotechnology Advances 32(6):1180-1204

Bhabhra R, Askew DS (2005). Thermotolerance and virulence of Aspergillus fumigatus: Role of the fungal nucleolus. Medical Mycology 43:87-93.

Cassells AC (1991). Problems in tissue culture:culture contamination. In: Micropropagation:Technology and Application, Derbergh, PC

Castro G, Ferrero M, Méndez B, Sineriz F (1993). Screening and selection of bacteria with high amylolytic activity. Acta Biotechnology 13:197-201.

Compant S, Brader G, Muzammil S, Sessitsch A, Lebrihi A, Mathieu F (2012). Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. BioControl 58(4):435-455.

Demirel R (2013). Fungal DNA barcoding of foodborne microfungi from Turkey. https://www.ncbi.nlm.nih.gov/nucleotide/KF018469.1.

Del Sorbo G, Schoonbeek H, De Waard M (2000). Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genetics and Biology 30(1):1-15.

Dita M, Garming H, Bergh V, Staver G, Lescot T (2013). Banana in Latin American and the Caribbean: Current State, Challenges and Perspectives. Proceeding of the international ISHS-Promusa symposium on Banana and plantains towards sustainable Global production. http://www.musalit.org/viewPostPrint.php?file=IN130316_pp.pdf&id=14818

Edgar RC (2004). MUSCLE: A multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5(13):1-19.

Feng J, Liu G, Selvaraj G, Hughes G, Hughes R, Wei Y (2005). A secreted lipase encoded by LIP1 is necessary for efficient use of saturated triglyceride lipids in Fusarium graminearum. Microbiology12(151):3911-3921

Food and Agricultural Organization (FAO) (2006). The state of food insecurity in the world. Rome. http://www.fao.org/3/a-a0750e.pdf

Foltz MJ, Perez KE, Volk TJ (2013). Molecular phylogeny and morphology reveal three new species of *Cantharellus* within 20 m of one another in western Wisconsin, USA. Mycologia 105:447-461.

Fox J, Andersen R (2005). Using the R statistical computing environment to teach social statistics. Department of Sociology M. Sc Master university.

Gontia MN, Tripathi, Tiwari S (2014). A simple and rapid DNA extraction protocol for filamentous fungi efficient for molecular studies. Indian Journal of Biotechnology 13:536-539.

Groebner R, David F, Mark L, Berenson S, David M, Levine D, Timothy C, Krehbiel, Hang L (2008). Applied management statistics custom ed. Boston; Pearson custom publishing.

Habiba U, Reja S, Saha ML, Khan MR (2002). Endogenous bacterial contamination during *in vitro* culture of table banana: identification and prevention. Plant Tissue Culture 12(2):117-124.

Hadziavdic K, Lekang K, Lanzen A, Jonassen I, Thompson M, Troedsson C (2014). Characterization of the 18s rRNA gene for designing universal eukaryote specific primers. PLoS One 9(2).

Hamed AA (2019). Endophytic fungi for biotechnological applications. Microbial Chemistry Department, National Research Center, Elbuhouth St., Giza, Dokki 12622, Egypt. https://www.ncbi.nlm.nih.gov/nucleotide/MN326853.1

Hankin S, Anagnostakis L (1975). The use of solid media for detection of enzyme production by fungi. Mycologia 67(3):597-607.

Hanumantharaya M, Kerutagi M, Patil B, Kanamadi C, Bankar B (2009). Comparative economic analysis of tissue culture banana and sucker propagated banana production in Karnataka. 22(4). http://14.139.155.167/test5/index.php/kjas/article/view/1507/1499

Helaly N, El-Metwally M, El-Hoseiny H, Omar S, El-Sheery N (2014). Effect of nanoparticles on biological contamination of in vitro cultures and organogenic regeneration of banana. Australian Journal of Crop Science 8(4):612-624.

Hernndez L, Salinas E, Gonzalez E, Godinez M (2013). Pesticide Biodegradation: Mechanisms, Genetics and Strategies to Enhance the Process-Life of science. https://www.intechopen.com/books/biodegradation-life-of-science/pesticide-biodegradation-mechanisms-genetics-and-strategies-to-enhance-the-process.

Hussain A, Al-Barakah FN, Shakeel M (2017). Isolation of polycyclic aromatic hydrocarbons (PAHs) degrading fungal strains from industrially polluted soils. International Journal of Biosciences 10(1):391-400.

Kahangi E, Muthee A, Chege B (2004). Constraints and sustainable solutions for adoption of TC banana technology and marketing. Acta Horticulturae 638:441-447.

Iniya KM, Xiao L, Zhan X (2011). Isolation and characterization of oleaginous fungi from Irish soil sample. Civil Engineering, National University of Ireland, University Road, NUIG, Galway, Glaway GLY, Ireland. https://www.ncbi.nlm.nih.gov/nucleotide/JF895924.1

Karim NA, Bakar FD, Zainal Z, Yusoff WM, Hamid AA, Mahadi NM,Murad AM (2007). Comparison Of Methods For Isolating High Quality DNA And RNA From An Oleaginous Fungus Cunninghamella Bainieri Strain 2a1. Malaysia Journal of Microbiology 3(1):7-13.

King R (2014). *Fusarium venenatum*. National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA. https://www.ncbi.nlm.nih.gov/nucleotide/XR_003150050.1

Kumar S, Stecher G, Tamura K (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution 33(7):1870-1874.

Li P (2015). *Penicillium* sp. Y28 18S ribosomal RNA gene, partial sequence. Key Laboratory of Marine Bioactive Substances. https://www.ncbi.nlm.nih.gov/nucleotide/KP872503.1.

Liu B (2019). Isolation and molecular identification of culturable filamentous fungi in fermented grains of Henan Nongxiang Liquor. https://www.ncbi.nlm.nih.gov/nucleotide/MK615877.1

Liu CY (2017). *Aspergillus fumigatus strain* T3 small subunit ribosomal RNA gene, partial sequence. https://www.ncbi.nlm.nih.gov/nucleotide/MF563964.1

Odutayo O, Amusa N, Okutade O, Ogunsanwo Y (2007). Sources of microbial contamination in tissue culture laboratories in southwestern Nigeria. African Journal of Agricultural Research 2(3):67-72.

Omamor IB, Asemota AO, Eke CR, Eziashi EI (2007). Fungal contaminants of the oil palm tissue culture in Nigerian Institute for Oil Palm Research (NIFOR). African Journal of Agricultural Research *2*(10):534-537.

Park D, Williamson P (2015). Masking the Pathogen: Evolutionary Strategies of Fungi and Their Bacterial Counterparts. Journal of Fungi 1(3):397-421.

Pattanayak A, Padhy SR, Dash PK, Routray S, Bhattacharyya P, Nayak AK (2019). Lignin decomposing fungus isolated from natural sources. https://www.ncbi.nlm.nih.gov/nucleotide/MK855472.1

Mbaka J, Mwangi M, Mwangi N (2008). The role of tissue cultured planting materials in Kenya. Applied Biosciences 9(1):354-368.

Nikkuni S, Nakajima H, Hoshina SI, Ohno M, Suzuki C, Kashiwagi Y, Mori K (1998). Evolutionary relationships among *Aspergillus oryzae* and related species based on the sequences of 18S rRNA genes and internal transcribed spacers. The Journal of General and Applied Microbiology 44(3):225-230

Mng'omba SA, Du Toit ES, Akinnifesi FK (2007). Effective preconditioning methods for *in vitro* propagation of Uapaca kirkiana Müell Arg. Tree species. African Journal of Biotechnology 6(14):1670-1676.

Montgomery R, Douglas C (2013). Design and analysis of experiments (eighth edition). Wiley. Section 3.5.7

Msogoya T, Kanyagha H, Mutigitu J, Kulebelwa M, Mamiro D (2012). Identification and management of microbial contaminants of banana in vitro cultures. Journal of Applied Biosciences 55:3987-3994.

Nayarisseri A,Yadav M, Bhatia M (2013). Impact of nextgeneration whole-exome sequencing in molecular diagnostics. Drug Invention Today 5(4):327-334.

Neelab, Singh K (2018). ABC and MFS Transporters : A reason for Antifungal drug resistance. Archives of Biotechnology and Biomedicine, pp. 1–7.

Neondo J (2017). Exploring biological control and transgenic weed management approaches against infestation by striga hermonthica in maize. PHD Thesis, JKUAT.

Nyombi K (2013).Towards sustainable highland banana production in uganda: opportunities and challenges. Africa Journal of Food, Agriculture, Nutrition and Development 2(13):7544-7546.

Ramakrishnan B, Megharaj M, Venkateswarlu K, Sethunathan N, Naidu R (2011). Mixtures of Environmental Pollutants: Effects on Microorganisms and Their Activities in Soils. Reviews of Environmental Contamination and Toxicology 211:63-120.

Ramesh K, Josephin D (2019). Biodegradation of polypropylene films by *Aspergillus* sp. from municipality solid waste contaminated soil. Microbial biotechnology, Bharathiar University, maruthamalai, Coimbatore, tamilnadu 641046, India. https://www.ncbi.nlm.nih.gov/nucleotide/MK564716.1

Raja A, Miller A, Pearce C, Oberlies N (2017). Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. Journal of Natural Products 80:756-770.

Rubaihayo P (2003). Agricultural technology interventions -bananas and plantains in sub-Saharan Africa. In: Report of Small Group meeting (SGM) on improved production of bananas and plantains in the Sub-Saharan Africa. In Abiotic stresses. Kampala, Uganda.

Ruijassenaars HJ, Hartmans S (2001). Plate screening methods for the detection of polysaccharide-producing microorganisms. Applied Microbiology 55(3):143-149.

Shayne J, Hugenholtz P, Sangwan P, Osborne C, Janssen H (2003). Laboratory cultivation of widespread and previously uncultured soil bacteria. Applied and Environmental Microbiology 69:7211-7214.

Solano O (2011). Isolation and Characterization of Fungi from Lake Magadi of the Kenyan Rift Valley. Msc Thesis, JKUAT.

Sierra G (1957) A simple method for the detection of lipolytic activity of micro-organisms and some observations on the influence of the contact between cells and fatty substrate. Journal of Microbiology 1(12).

Singh R, Kumar M, Mittal A, Mehta P (2016). Microbial enzymes: industrial progress in 21st Century. 3 Biotech 6(2):174.

Stamford T, Araújo J, Stamford N (1998). Atividade enzimática de microrganismos isolados de jacatupé (Pachyrhizus erosos L. Urban. Ciência E Tecnologia Dos Alimentos 18(4):382-385.

Suryanarayanan TS, Senthilarasu G, Muruganandam V (2000). Endophytic fungi from Cuscuta reflexa and its host plants. Fungal Diversity 4:117-123.

Tamura K, Nei M, Kumar S (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA)101:11030-11035.

Technelysium Pty Ltd. (2018). ChromasPro version 2.1.8. Retrieved from https://technelysium.com.au/wp/chromaspro/

Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JA (2007). Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Research 35(2):71-74. https://doi.org/10.1093/nar/gkm306

Van L, Hoagland E, Zablotowicz M, Hall J (2003).Pesticide metabolism in plants and microorganisms. Weed Science 51(4):472-495.

White T, Bruns T, Lee S,Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocols, a guide to methods and applications 18(1):315-322.

Wang J, Zhu W (2011). *Aspergillus flocculosus* strain OUCMDZ-730 18S ribosomal RNA gene, partial sequence. https://www.ncbi.nlm.nih.gov/nucleotide/JN604547.1

Zaragoza O, Rodrigues M, De Jesus M, Frases S, Dadachova E, Casadevall A (2009). The Capsule of the Fungal Pathogen Cryptococcus neoformans. Advances in Applied Microbiology 1(68).

Zhao P, Quan C, Wang Y, Wang J, Fan S (2014). Bacillus amyloliquefaciens Q-426 as a potential biocontrol agent against Fusarium oxysporum f. sp. spinaciae. Journal of Basic Microbiology 54:448-456.

Zilda D, Harmayani E, Widada J, Asmara W, Irianto H, Patantis G (2012). Screening of thermostable protease producning microrganisms isolated from Indonesian hotspring. Squalen 7:105-114.