

Molecular Identification and Phylogenetic Analysis of Fungi Contaminants Associated with *In Vitro* Cultured Banana Based on ITS Region Sequence

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ABSTRACT

This study characterized, identified and conducted phylogenetic analysis on fungi contaminants *in vitro* bananas based on the sequence of inter-space (ITS) regions. Genomic DNA was extracted from the pure culture of fungi contaminants, amplified and sequenced using ITS1 and ITS4 markers. Analysis of the sequences using MEGA 7 Software at higher similarity sequence identified five *Aspergillus* spp., three *Penicillium* spp., one each of *Fusarium*, *Trichoderma* and *Cladosporium* as the contaminants. The genetic distance between the fungi species was 0.205, which suggests a homogeneous substitution between the sequences, and thiamine was the most stable. The fungi clustered in three major groups at 0.10 genetic distance, subdivided into five clusters. A cluster and sub-cluster consisting of five *Aspergillus* strains; a major cluster of three *Penicillium* strains; a cluster comprising of *Fusarium chlamyosporum* and *Trichoderma viride*; and a sole fungi *Cladosporium tenuissimum*. The *Aspergillus* group were phylogenetically related to *A. flavus* and *A. parvissclerotigenus*, the identified *Penicillium* spp. were closely related to *Penicillium citrinum* while the detected *Cladosporium* aligned with *Cladosporium tenuissimum* and *Phoma multirostrata*. The information provided by this study could be utilized to develop a specific and compelling sterilization protocol to minimize the rate of contamination during *in vitro* culture procedures.

1. Introduction

Plant tissue culture is an agriculture biotechnological tool that allows rapid and economical production of genetically identical and healthy plants in relatively small space, with little supplies and a short period (Odotayo *et al.* 2004). Tissue culture technology is advantageous in producing disease-free and uniform planting materials, which can be made available throughout the year (Eziashi *et al.* 2014). Thus the technique is a veritable tool for the micropropagation of bananas to ensure the availability of genetically similar and disease-free plantlets for commercial production of the crop, to a great extent, will enhance food security and economic gain for the farmers. However, despite the numerous advantages of tissue culture technologies to banana cultivation

and agricultural development, contamination had been a significant constraint for its utilization for crops micropropagation (Enjalric *et al.* 1988; Goswami and Handique 2013; Rawal and Keharia 2019). Filamentous fungi, bacteria, yeast, viruses, and micro-arthropods such as mites and thrips have been implicated as contaminant agents in plant tissue culture (Cobrado and Fernandez 2016; Izarra *et al.* 2020), breaking through the barriers of sterilization methods to cause contamination.

The significant factors contributing to the high incidence of microbial contaminations are poorly prepared culture media, inadequate sterilization of explants, contaminated working tools and work-tops, and the culturist's body (Omamor *et al.* 2007). Microbial contamination could waste time, effort, and materials, contributing to severe economic losses (Abass 2013). Fungi and bacteria are the most reported frequent contaminants of the plant *in vitro* cultures (Cassells 1996; Altan *et al.* 2010;

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Cobrado and Fernandez 2016; Rawal and Keharia 2019), and their cosmopolitan nature can explain this; their ability to grow anywhere where moisture and food are available. *Bacillus subtilis*, *Bacillus licheniformis*, *Pseudomonas syringae*, *Erwinia* spp., and *Corynebacterium* sp. have been reported as the most common bacterial contamination agents *in vitro* plant cultures (Odutayo *et al.* 2004; Cassells 2012). At the same time, the most frequent fungal contaminants are *Aspergillus niger*, *Alternaria tenuis*, *Fusarium culmorum*, and *Aspergillus fumigatus*, among others (Odutayo *et al.* 2004; Odutayo *et al.* 2007; Msogoya *et al.* 2012; Cobrado and Fernandez 2016). According to Msogoya *et al.* (2012), endogenous microbial contaminants account for 40-60% loss of explant during banana tissue culture and micropropagation process.

Successful tissue culture protocols start with effective explants sterilization. Many sterilization techniques, which include the use of chemicals, antibiotics and fungicides and physical inactivation by heat and light solely or in combinations, have been employed in eliminating fungal and bacterial contaminations in plant cultures (Meghwal *et al.* 2000; Habiba *et al.* 2002; Maina *et al.* 2010; Venkatasalam *et al.* 2013; Moreno-Vazquez *et al.* 2014; Izarra *et al.* 2020). However, reintroducing contaminants such as endophytic microbes or microarthropods may occur through explant inoculation and culturing (Sharaf-eldin and Weathers 2006; Vichitra *et al.* 2014; Rahman *et al.* 2017). Meanwhile, some sterilants could be toxic to the plant tissues. Hence, optimum concentrations of sterilants, duration of exposure of explants, and the nature of the sterilants used need to be determined to minimize explants injury (CPRI 1992). The selection of an antibiotic or fungicide to be used as sterilant should depend on the type of the prevailing microbial contaminants. Therefore, proper identification of the common microbial contaminants is essential and should be followed by preliminary testing for antimicrobial activity of both antibiotic and fungicide at different concentrations to select the most appropriate (Abass 2013; Herman 2017).

Identification and classification of fungi based on the traditional analyses of macroscopic and microscopic structures (Larone 2002; Watanabe 2002) may be influenced by environmental conditions. Traditional techniques of identifying fungal contaminants in cultures include serological tests, culture ID, and morphological tests, and these tests are subjective and not always precise. More so, there is an infinite distribution of fungal species, and

only a few have been described (Pinto *et al.* 2012; Rawal and Keharia 2019). Therefore there is a need for a novel and rapid technique for detecting and identifying fungi contaminants in *in-vitro* cultures to explore the fungal diversity as a coherent whole (Saad *et al.* 2004). The use of molecular tools has helped detect the slightest variation within species and even within individual strains because different organisms have different genetic combinations (Adeyemo and Onilude 2014). Molecular techniques such as multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), ribotyping, repetitive sequence-based PCR (rep-PCR), and the use of 16S or 23S rDNA genes have been used for microbial identification (Ogier *et al.* 2002; Gomez *et al.* 2008; Paula *et al.* 2011; Nadha *et al.* 2012; Izarra *et al.* 2020).

Conventional microbial contaminants identification is usually less sensitive, time-consuming, and labour-intensive (Adeyemo and Onilude 2014; Hasan *et al.* 2016). The molecular technique, particularly the application of polymerase chain reaction, is a viable alternative as the technique is precise, reliable, and reproducible (Hasan *et al.* 2016). Furthermore, fungal strains and species have been identified and genotyped using ribosomal-DNA (rDNA) and inter-transcribed spacer (ITS) regions (Lord *et al.* 2002; Anderson *et al.* 2003; Junmyoung 2010; Cobrado and Fernandez 2016). The ITS region is conserved, consisting of high copy numbers in the fungal genome with a high variation in sequences of closely related species (Hasan *et al.* 2016). Given this, the International Sub-Commission of fungal barcoding proposed the ITS regions as the prime fungal bar code for species identification (Schoch *et al.* 2012). ITS region sequences are highly variable and could serve as potential markers for taxonomically more distant groups. Therefore, the present study attempted to identify and conduct phylogenetic analysis based on ITS region sequences of fungal contaminants associated with *in vitro* cultured banana, with the view of providing basal information for effective sterilization and fungal containment during micropropagation.

2. Materials and Methods

2.1. Glasswares, Plastic Wares, Chemicals and Reagents

All glassware was high quality from Pyrex (Corning, NY, USA), and plastics wares were standard pre-sterilized grades (Parsons Ltd, India). The glasswares were soaked in chromic acid solution, washed with Tween 20 in warm water, rinsed with double distilled water, and oven-dry at 120°C for

6 hours before use. The non-sterile plastic ware was thoroughly washed with detergent, dried, and autoclaved. All the chemicals and reagents used were of high analytical grade obtained from Sigma-Aldrich, Germany; BDH, Poole, UK and Merck, Germany.

2.2. Preparation and Disinfestation of the Explants

The banana mother plants (FIA 9) used for the micropropagation were obtained from the Genetic Resources Unit, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Meristematic condensed basal stems of banana measuring 2-3 cm in length prepared from matured mother plants were used as the explant source for initiating aseptic cultures. After preparation, the explants were thoroughly washed and rinsed in changes of double distilled water to remove dust particles, adhering microbes, and other dirt on the surface. The explants were surface sterilized for about 15 minutes in a mixture of Tween 20, 0.2% Bavistin, ten ml/L of Dettol, 70% isopropanol, 1.3 g/L of citric acid, one g/L of ascorbic acid, and 143 ml/L of sodium hypochlorite. For another 2 min, the explants were soaked in 70% ethanol (v/v) and rinsed in distilled water. The explants were further surface-sterilized in an aqueous solution of 0.05% HgCl₂ for one min with agitation and later transferred into a solution of 0.01% HgCl₂ for another one minute. The explants were removed and rinsed with double distilled water four times, kept in an autoclaved beaker, and covered with sterile aluminum foil in preparation for initiation.

2.3. Sterilization of the Materials and Media

Sterilization of glassware, culturing tools (scalpel, forceps, scissor blade holder), and media was carried out by moist heat autoclaving to decontaminate and kill the microorganisms by dehydrating the cell under severe heat and pressure conditions. The autoclaving was carried out at a high temperature (121°C) and 15 atmospheric pressure (15 psi) for 30 minutes.

2.4. Initiation of Aseptic Explants Into Cultures

The sterilized explants were removed from the beaker under sterile conditions in a laminar airflow hood with care to prevent contamination. The explants were carefully placed in a vertical position in culture bottles (about 250 ml capacity) containing 20 ml of MS (Murashige and Skoog's) basal medium (Murashige and Skoog 1962) fortified with two mg/L of 6-Benzylaminopurine (BAP), two mg/L of 3-Indole-acetic acid (IAA), sucrose (3%), and agar. Before autoclaving, the pH of the media was adjusted

to 5.7. The cultures bottles were well sealed with biofilm and transferred into the growth room. They are incubated to grow under a photosynthetic photon flux density of 70±5 µmol/m²/s from fluorescent lamps. The growth room condition was maintained at 16 hr in light and 8 hr in a dark regime, while the temperature was kept at 25±2°C. The cultures were observed for 30 days for the appearance of any contamination during shoot initiation.

2.4.1. Isolation and Preservation of Fungi Contaminants

Contaminated culture bottles were identified and observed for visible fungi growth (Figure 1). The fungi were extracted from the contaminated *in vitro* banana cultures with the aid of a hot flame sterilized wire loop and inoculated into sterile potato dextrose agar (PDA) plate, incubated at 28°C for 72 hrs. (Msogoya *et al.* 2012). The inoculation was carried out inside a biological safety cabinet (SterilGard Class II, Baker, Sanford, USA). After incubation, several colonies were observed growing on the plates (mixed culture), ten pure isolates (A, B, C, D, E, F, G, H, J, and K) were obtained from the mixed culture by repeated sub-culturing. These isolates were maintained and preserved by inoculating into agar slants in McCartney bottles, incubated at 24°C for 1-2 weeks, and subsequently refrigerated at 4°C till used.

2.4.2. Genomic DNA Isolation and Amplification

Genomic DNA (gDNA) was extracted from 25 mg pure culture tissue of morphologically similar fungi isolates. The isolates' gDNA was extracted individually using a commercial fungal extraction kit (Zymo Research, Fungi Mini-Prep; USA) following the manufacturer's instructions. The quality of the gDNA was checked on 0.8% agarose gel stained with Ethidium bromide. And the electrophoresis was run for 30 min at the constant current supply of 100 V and viewed under UV transilluminator (Sigma, USA). The amplification of the conserved internal transcribed spacer (ITS) gene regions of the fungal gDNA was performed with Polymerase Chain Reaction (PCR) using nuclear primer pair; ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) synthesized by Eurofins (Germany). The PCR reaction was carried out in 20 µl volume in 200 µl capacity thin-walled tube (Axigen, USA) containing 10 µl of Taq 2X PCR pre-mix (New England Biolabs, England), 1 µl each of ITS1 and ITS4 primer, and 1 µl gDNA. The total volume of the reaction mixture was made up to 20

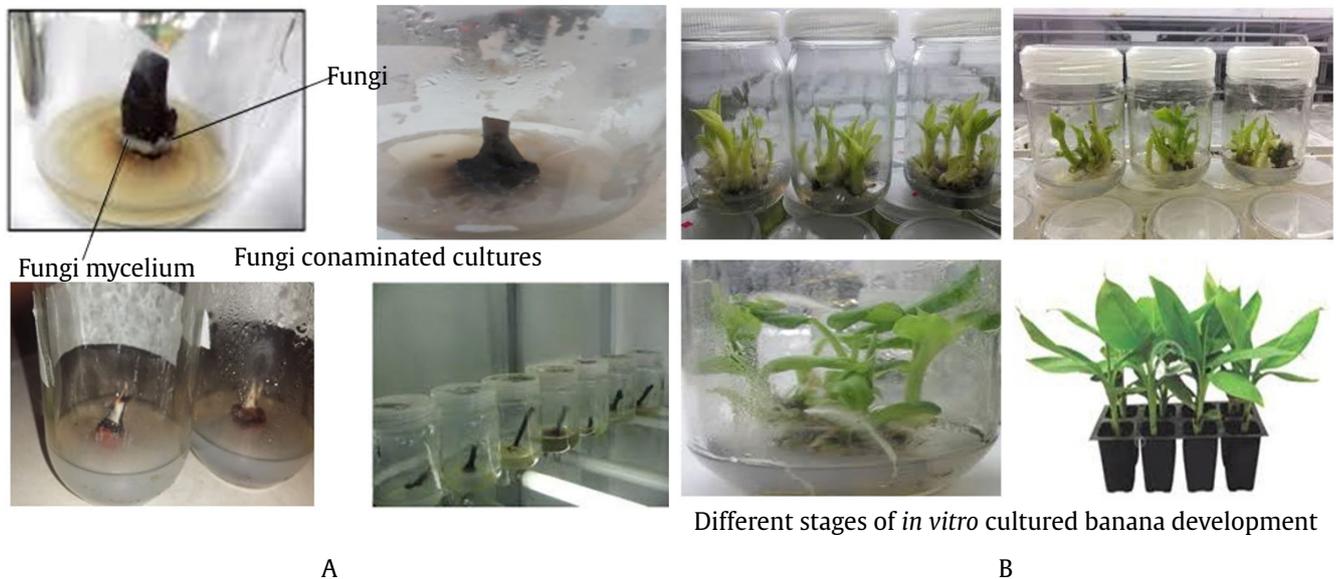


Figure 1. (A) Fungi contaminated *in vitro* cultures banana, (B) uncontaminated culture of banana at different stages of growth

μl with nuclease-free water. A control reaction was set up using double-distilled deionized water as the template instead of the gDNA. The amplification was performed on Eppendorf gradient Mastercycler (Eppendorf, USA). The PCR condition was set up at 95°C for 5 minutes for initial denaturation, followed by 35 cycles for denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and elongation at 72°C for 1 min with a final elongation at 72°C for 10 min. The reaction was put on hold for 10 min at 4°C.

The amplicon confirmation of PCR products was checked (Animasaun *et al.* 2015). 5 μl of the PCR product mixed with 1 μl of 6X loading dye (Bromophenol blue) were resolved on 1.5% agarose gel stained with 1% ethidium bromide in 1X TAE buffer, electrophoresed at a constant current of 100 V for 40 min with the aid of Biorad Midi Cast (Biorad, USA). Fragment sizes of the amplicons were anticipated by comparison with standard molecular weight marker ladder-low range DNA Ruler Plus (3000-100 bp; Thermofisher, USA). The product was visualized as a single compact band of expected size under UV light and documented by a gel documentation system (Z5, Bio-Rad, USA).

2.5. ITS Gene Sequence and Assembly

PCR products were cleaned using ExoSAP protocol. The Exo/SAP master mix was prepared by adding 50 μl of exonuclease I (NEB M0293) 20 U/ μl and 200 μl of shrimp alkaline phosphatase (NEB M0371) 1 U/ μl to 0.5 ml micro-centrifuge tube. The reaction mixture of 10 μl PCR and 2.5 μl Exo/SAP Mix was prepared. The reaction mixture was properly mixed and incubated at 37°C for 30 min. The reaction was stopped by

heating the mixture at 95°C for 5 min. Sequencing was done with the ABI V3.1 Big dye Terminator Cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions available at http://mvz.berkeley.edu/egl/inserts/Big_Dye_v3.1_Protocol_Manual.pdf. The labeled products were cleaned with the Zymo Seq clean-up kit. (<http://www.zymoresearch.com/downloads/dl/file/id/52/d4052i.pdf>). The cleaned products were injected on the ABI3500XL analyzers with a 50 cm array, using POP7. The Sanger sequencing of the amplicons was carried out on the ABI PRISM sequencer platform (Inqabba Biotec, Pretoria, South Africa) using the primers pair of ITS1 and ITS4.

2.5.1. DNA Sequence Assembly and Alignment

The sequences were downloaded and converted to a readable format using Finch TV, an online software. The consensus nucleotide sequences for both markers (ITS1 and ITS2 regions) were obtained using SeqTrace version 0.9.0. A sequence with minor allele frequency $\geq 30\%$ was further cleaned. Identification of the fungi was carried out based on sequence homology with the Gene Bank database maintained of the National Center for Biotechnology Information (NCBI) using the BLASTN analysis. (<http://www.ncbi.nlm.nih.gov/BLAST>). The genetic relationship among the isolates was determined, and the evolutionary divergence between the sequences was estimated. Also, the homogeneity of substitution patterns between sequences was evaluated.

2.6. Phylogenetic Analysis

Phylogeny analysis of the identified organisms was constructed using MEGA 7 software (Kumar *et*

al. 2016). To determine the percentage similarity and analyze the evolutionary relationship between the identified fungi and selected fungi strains based on the consensus sequence of ITS1 (5.8S) and ITS4 gene regions, sequences that are highly similar to those of the identified organisms were searched retrieved from the NCBI databank. ClusterW pairwise multiple alignments were used to align the nucleotide sequences, all sequences were equally weighted, and unread nucleotides (gaps) were assumed as missing data. The phylogenetic relationship of the organism was inferred based on the Maximum Composite Likelihood (MCL) method (Tamura and Nei 1993), a comparative matrix of nucleotide substitution was performed to compare the selected fungi strains, and the phylogenetic tree of the isolates was constructed (Kumar et al. 2016).

3. Results

Despite the use of various chemicals for the surface sterilization of the explants and proper sterilization of the media and culture tools by autoclaving and the aseptic inoculations of the explants under the laminar hood, fungal contamination was observed (Figure 1). The average fungi contamination per batch was 18%, with the highest contamination rate occurring under high humidity. On examining the contaminated cultures, eleven distinct colonies (designated as A-K) were sequenced using ITS1 and ITS4 markers. The results of the cluster sequence alignment (Supplementary Figure 1) showed a remarkable conserved region for the fungal contaminants for the ITS genes. Sequences with similar alignment were obtained based on max score, total score, %query cover, E-value, and percentage identity. Analysis of the eleven fungi isolates nucleotide sequences identified by highly similar sequence BLASTN parameter revealed five *Aspergillus* spp., three *Penicillium* spp., one each of *Fusarium*, *Trichoderma*, and *Cladosporium*

as the contaminants (Table 1). Isolate A had 94.30% identity similarity to *Aspergillus parvisclerotigenus* (Strain NR40). The other four *Aspergillus* species identified *Aspergillus flavus*, *Aspergillus niger* strain FF14, *Aspergillus niger* strain M2, and *Aspergillus awamori* strain ND6 had percentage similarity of 96.55, 96.99, 99.65, and 99.64% to the corresponding isolates C, H, J, and K, respectively.

Also, the detected *Penicillium citrinum* species (strains HNMF093, MSEF105, isolate PecGx21L02) were similar to their respective isolates with a percentage identification index above 97%. Isolates B, D and F were identified as *Fusarium chlamydosporum*, *Trichoderma viride* isolate P23-11, and *Cladosporium tenuissimum* strain 7P6. Generally, the %Query cover and identity of the isolates to the identified fungi were high. The corresponding Gene bank accession numbers of the identified fungi contaminants associated with *in vitro* banana culture are presented in Table 1. Figure 2 showed the frequency of occurrence of the contaminants. *Aspergillus flavus* isolate FMPV44 was the most frequent and prevailing fungi contaminant of the *in vitro* banana culture, followed by *Aspergillus niger* strain FF14. At the same time, the contamination caused by *Penicillium citrinum* isolate PecGx21L02 had a minor occurrence throughout culturing.

The genetic relationship between the identified fungi contaminants based on the pattern of nucleotide substitution was estimated using the Maximum Composite Likelihood (MCL) method. The model revealed that the rate of transitional substitution of one base to another was 18.24, 17.87, 13.92, and 14.51 for the nucleotide pairs A-G, T-C, C-T, and G-A, respectively (Table 2). Meanwhile, the highest transversional substitution of 5.09 occurred between C-A and C-G, while the least 3.85 was found in A-T and A-C. The nucleotide frequencies are 21.69% (A), 22.35% (T/U), 28.68% (C), and 27.27% (G). The genetic distance among the fungi species was 0.205,

Table 1. Summary of the isolates blast sequence on the NCBI nucleotide database and the fungi identified from the NCBI based on internal space region markers

Sample ID	*Seq accession No.	Matched organism	%Query cover	E-value	% identity	NCBI Accession N°
A	OM471852	<i>Aspergillus parvisclerotigenus</i> strain NR40	100	0.00	94.30	MH270568.1
B	OM471853	<i>Fusarium chlamydosporum</i>	99	0.00	98.23	KX7383374.1
C	OM471854	<i>Aspergillus flavus</i> isolate FMPV44	97	0.00	96.55	MH244383.1
D	OM471855	<i>Trichoderma viride</i> isolate P23-11	98	0.00	98.42	MH285092.1
E	OM471855	<i>Penicillium citrinum</i> isolate HNMF093	97	0.00	97.83	MH725581.1
F	OM471857	<i>Cladosporium tenuissimum</i> strain 7P6	99	0.00	98.83	KY400093.1
G	OM471858	<i>Penicillium citrinum</i> strain MSEF105	97	0.00	97.86	KT310999.1
H	OM471859	<i>Aspergillus niger</i> strain FF14	99	0.00	96.99	MH055397.1
I	OM471860	<i>Penicillium citrinum</i> isolate PecGx21L02	99	0.00	98.26	MH483870.1
J	OM471861	<i>Aspergillus niger</i> strain M2	100	0.00	99.65	MH622753.1
K	OM471862	<i>Aspergillus awamori</i> strain ND6	98	0.00	99.64	MG659600.1

*The sequence accession number was given by NCBI for the fungi ITS 1 and 4 submitted to the Genebank with the submission number SUB11026862

suggesting a homogenous substitution between the nucleotides (Supplementary Table 1). However, the estimation of average percentage evolutionary divergence of thiamine, cytosine, Adenine, and Guanine was 21.7%, 21.2%, 27.7%, and 29.4%, respectively. This signifies that cytosine accounted for the highest sequence pair divergence among the eleven identified fungi.

The genetic and evolutionary relationships between the isolated fungi contaminants of *in vitro* culture banana inferred using the UPGMA method revealed the optimal tree with the sum of branch length = 0.70393521 (Figure 3). The dendrogram partitioned the eleven fungi contaminants into two major groups (A and B) at an evolutionary scale of about 0.17. At a genetic distance of 0.05, the group A organisms were separated into two sub-groups, A1 and A2. The A1 consisted of five *Aspergillus* spp., (*Aspergillus parvissclerotigenus*, *Aspergillus flavus*, *Aspergillus awamori* and two strains of *Aspergillus niger*) in two sub-clusters (A1(i) and A1(ii)), while the A2 had three different strains of *Penicillium citrinum*. Group B members were segregated into two clusters; cluster B1 had only *Cladosporium tenuissimum* as the sole member. It was separated from the other cluster at an evolutionary index of 0.165, which shows the organism is a far relative of those in other clusters.

The cluster B2 had two related heterogeneous species (*Fusarium chlamydosporum* and *Trichoderma viride*).

The evolutionary history inferred by the Maximum Likelihood method showed the tree's highest log-likelihood was -1128.02 based on fifty-five nucleotide sequences. The tree revealed that the *Aspergillus* contaminants were phylogenetically related to *A. flavus* and *A. parvissclerotigenus*. All the isolates identified as *Aspergillus* species have a strong relationship with other known *Aspergillus* members. However, isolate K (*Aspergillus awamori* strain ND6) had the most substituted base pair within the ITS gene regions (Figure 4). The identified *Penicillium* species from isolates E, G, I was closely related to *Penicillium citrinum* with the strong evolutionary

Table 2. Estimation of the pattern of nucleotide substitution of the identified fungi contaminants of *in vitro* banana culture based on Maximum Composite Likelihood (MCL) method

	A	T	C	G
A	-	3.96	5.09	18.24
T	3.85	-	17.87	4.84
C	3.75	13.92	-	4.84
G	14.51	3.96	5.09	-

Each nucleotide shows the probability of substitution *r* from one base (row) to another base (column). The sum of the *r* values is made equal to 100

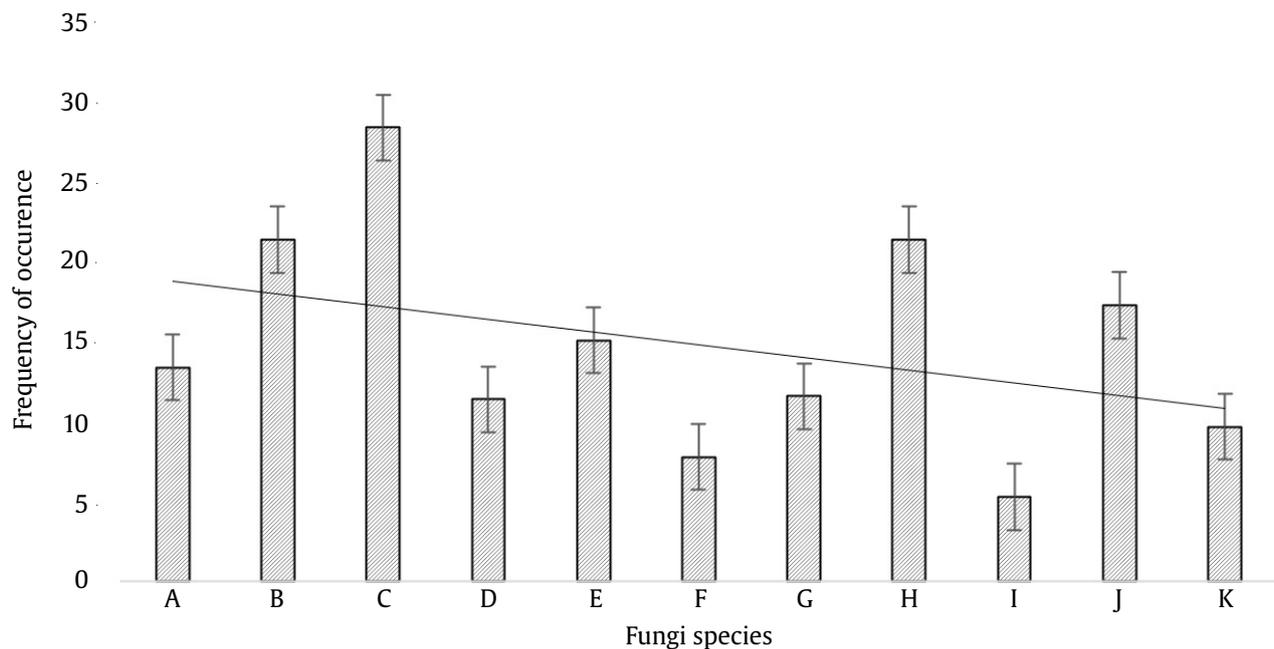


Figure 2. The prevalence and frequency of occurrence of the contaminant fungi associated with *in vitro* culture banana. Keys: A: *Aspergillus parvissclerotigenus* strain NR40, B: *Fusarium chlamydosporum*, C: *Aspergillus flavus* isolate FMPV44, D: *Trichoderma viride* isolate P23-11, E: *Penicillium citrinum* isolate HNMF093, F: *Cladosporium tenuissimum* strain 7P6, G: *Penicillium citrinum* strain MSEF105, H: *Aspergillus niger* strain FF14, I: *Penicillium citrinum* isolate PecGx21L02, J: *Aspergillus niger* strain M2, K: *Aspergillus awamori* strain ND6

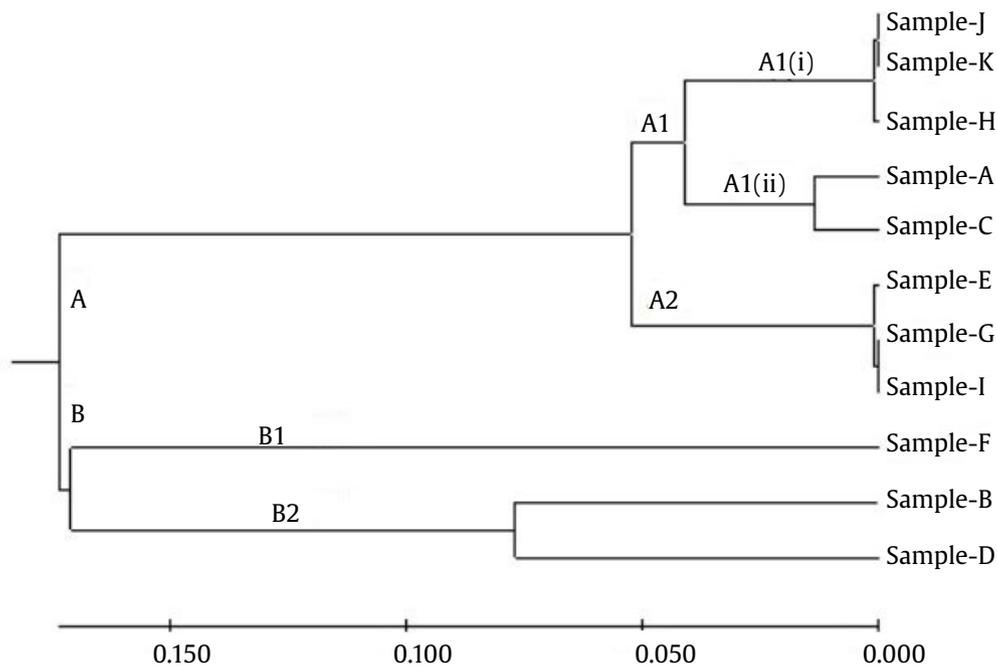


Figure 3. Genetic and evolutionary relationship between contaminant fungi of the *in vitro* culture banana. Keys: A: *Aspergillus parvisclerotigenus* strain NR40, B: *Fusarium chlamyosporum*, C: *Aspergillus flavus* isolate FMPV44, D: *Trichoderma viride* isolate P23-11, E: *Penicillium citrinum* isolate HNMF093, F: *Cladosporium tenuissimum* strain 7P6, G: *Penicillium citrinum* strain MSEF105, H: *Aspergillus niger* strain FF14, I: *Penicillium citrinum* isolate PecGx21L02, J: *Aspergillus niger* strain M2, K: *Aspergillus awamori* strain ND6

tide, which confirms that the organisms are different strains but are much closer than other strains of *Penicillium*.

Furthermore, the detected *Cladosporium* from isolate F aligned with *Cladosporium tenuissimum*. Sheared some molecular composition link with the *Trichoderma artroviride*, the group to which isolate D (*Trichoderma viride* isolate P23-11) belongs. As expected, isolate B (*Fusarium chlamyosporum*) is a member of the *Fusarium* group. Meanwhile, the phylogeny suggests the possible evolution of other strains from it.

4. Discussion

Fungal contaminants remain a significant challenge for plants' successful *in vitro* micropropagation. They pose a significant threat at every stage of the *in vitro* plant culture process, and their development and growth are faster than the growth of the plant culture (Mng'omba *et al.* 2012); this culminates into time, resources, and ultimately, economic loss (Cassells 1990; Guan *et al.* 2005; Abass 2013; Herman 2017). To effectively combat the menace of fungi contamination *in vitro* culture, accurate identification of the common contaminants is imperative. The present study isolated

and identified eleven fungi strains of five genera, *Aspergillus*, *Fusarium*, *Trichoderma*, *Cladosporium*, and *Penicillium* spp., as the significant contaminants of *in vitro* banana culture. In early studies, Odutayo *et al.* (2004, 2007) identified four and five genera of fungi contaminants in tissue culture materials, respectively. Also, Rahman *et al.* (2017) reported that *Aspergillus*, *Penicillium*, *Fusarium*, and *Rhizopus* species are the primary contaminants of potato *in vitro* culture under Temporary Immersion Bioreactor System (TIBs).

Although the species and strains of the fungi contaminants may differ from one location to another, the genera and species identified in this study conformed to the earlier studies (Odutayo *et al.* 2007; Junmyoung 2010; Msogoya *et al.* 2012; Benishelkh *et al.* 2015; Cobrado and Fernandez 2016; Rahman 2017). This study documented *Aspergillus*, *Fusarium*, *Trichoderma*, *Cladosporium*, *Alternaria*, and *Penicillium* species as the most typical and most reported tissue culture contaminants. The arrays of species and strains of these fungi implicated in plant culture contamination showed the extent of diversity and their adaptability to varying environmental conditions. Consequently, the species and strain's availability and prevalence in a tissue culture laboratory may depend on various environmental factors and practices of

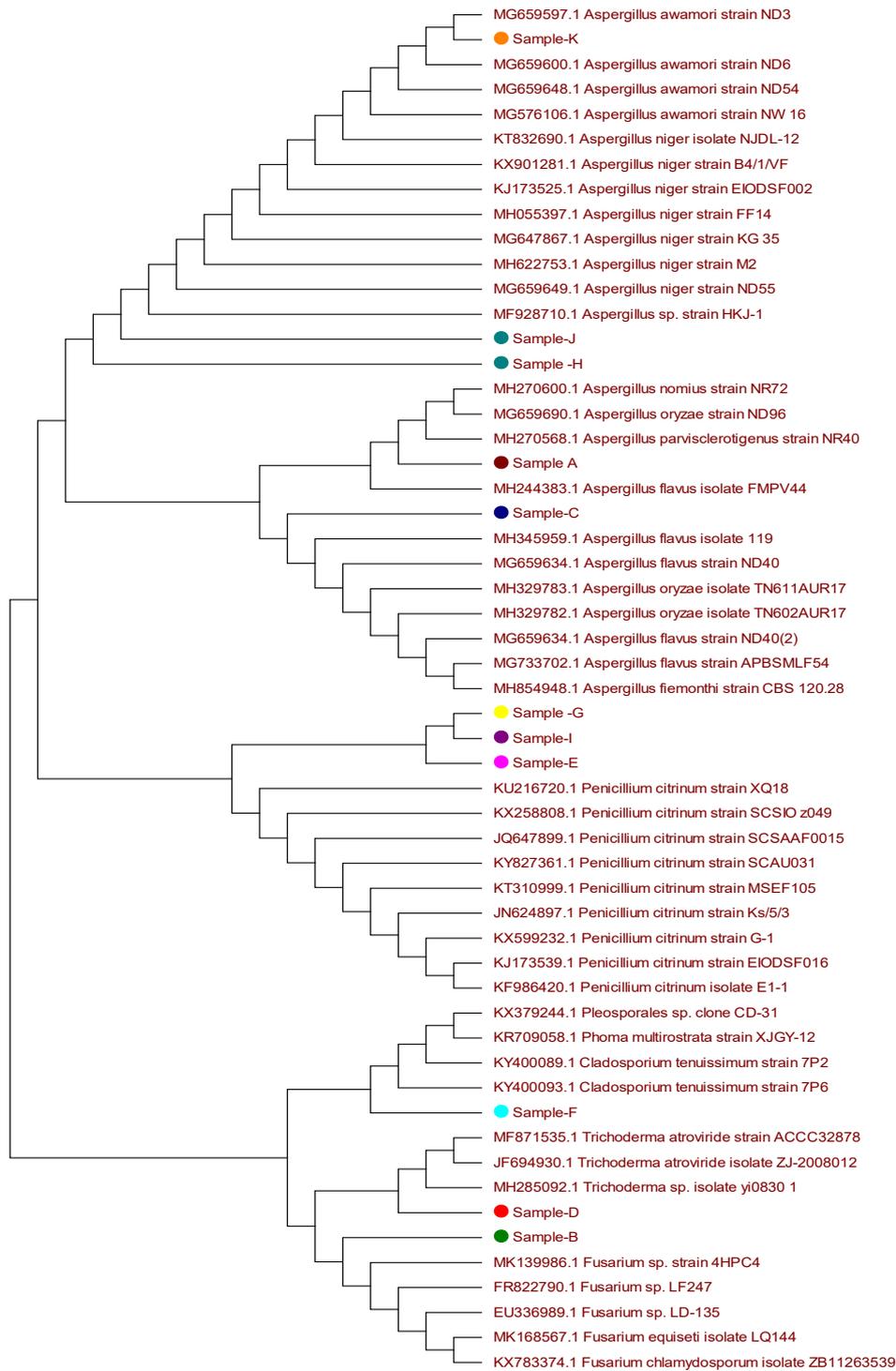


Figure 4. Phenogram generated using the ITS1 and ITS4 gene regions of 11 fungi isolates and 44 homologous sequences from Gene bank of NCBI with different similarity level. The phylogenetic relationship was inferred by Maximum Likelihood method of Tamura-Nei Model (Tamura and Nei 1993). Keys: A: *Aspergillus parvisclerotigenus* strain NR40, B: *Fusarium chlamyosporum*, C: *Aspergillus flavus* isolate FMPV44, D: *Trichoderma viride* isolate P23-11, E: *Penicillium citrinum* isolate HNMFO93, F: *Cladosporium tenuissimum* strain 7P6, G: *Penicillium citrinum* strain MSEF105, H: *Aspergillus niger* strain FF14, I: *Penicillium citrinum* isolate PecGx21L02, J: *Aspergillus niger* strain M2, K: *Aspergillus awamori* strain ND6

the laboratory. The factors may include temperature, moisture, light intensity, the chemical used for explant decontamination, surface sterilization, and the aseptic nature of the culture area, among others. Some organisms may sometimes develop resistance to a specific sterilant or undergo mutation to become a serious contaminant of an economic burden during plant micropropagation (Danso *et al.* 2011).

The high incidence of contamination by *Aspergillus flavus*, *Aspergillus niger*, and *Fusarium chlamydosporum* suggests that they are the most common contaminants in the plant tissue culture laboratory. They might have evolved mechanisms to survive the routinely used surface sterilization chemicals. *Aspergillus flavus* has been identified as the leading cause of contamination of an *in vitro* cultured plant (Benishelkh *et al.* 2015); likewise, most of the contaminations to a tissue culture of *Musa textiles* were reportedly caused by *Aspergillus* spp. and *Chrysosporium* sp. (Cobrado and Fernandez 2016). Furthermore, Odutayo *et al.* (2007) discovered that fungi contaminants in tissue culture laboratories in southwestern Nigeria varied for different locations. However, *Aspergillus* and *Fusarium* species are the most encountered contaminants irrespective of the locations. Some authors have implicated the *Aspergillus* and *Fusarium* species as one of the primary causative organisms of food spoilage (Watanabe 2002; Fiori *et al.* 2008; Alwakeel 2013); this may be due to their cosmopolitan nature, ability to survive under critical conditions, and continuous evolution of adaptive strains.

The ability of the PCR using the pair of primers ITS1 and ITS4 to identify the fungal isolates to species and strain level showed that the technique is efficient and the primers are reliable as molecular markers for fungi identification. Unlike the conventional methods such as the morphological and biochemical assays, markers delimited the fungi without ambiguity, even at the strain level. Since such identification is based on nucleotide sequences, it is not subjective or misleading. The use of ITS1 and ITS4 has been demonstrated by Abd-Elsalam *et al.* (2003); the authors used the primers to amplify the entire 5.8S rDNA gene, both ITS1 and 2 regions, and a portion of the 18S nuclear small-subunit rDNA gene of *Fusarium* spp. It was demonstrated that ITS markers could detect many fungi species than selective plating (Mansfield and Kuldau 2007). Peterson and Horn (2009) isolated and identified two new *Penicillium* species from groundnut field soil based on ITS sequences to corroborate this. In the present

study, although there are five strains of *Aspergillus*, three strains of *Penicillium citrinum*, ITS region sequences primers segregated the species to different strains. In an earlier study, Sanjaya *et al.* (2016) used ITS markers to identify novel fungal contaminants of *Tetranychus kanzawai*. Thus, molecular identification conveniently overcomes the setback and bottleneck usually associated with macroscopic and microscopic examination and identification of fungi (Watanabe 2002; Pinto *et al.* 2012) by effectively delimiting the organisms at inter- and intraspecific variation levels. The precise characterization and identification of the fungi contaminants will enable the choice of an effective fungicide and surface sterilant.

Meanwhile, Msogoya *et al.* (2012) reported that 200 mg/liter fluconazole effectively suppressed *Penicillium* spp. and *Aspergillus* spp. but was unable to suppress *Fusarium* spp. They also documented that ketoconazole at a 200 mg/liter concentration was effective against *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. isolated from banana *in vitro* cultures.

The knowledge of the genetic and evolutionary relationship among microbial contaminants of plant tissue culture will to a greater extent, help formulate effective broad-based sterilization procedures to minimize time and material loss in the micropropagation process (Msogoya *et al.* 2012; Abass 2013). The clustering of all the contaminant *Aspergillus* species identified in this study at a genetic distance of less than 1 showed the organisms are genetically related. The different species arise due to some variation in the nucleotide sequence (Castañeda-Ramírez *et al.* 2016). For instance, the two *Aspergillus niger* strains (M2 and FF14) could have arisen from a few nucleotide base substitutions. The mutation that gave birth to the strains may be induced by environmental condition fluctuations or an adaptive response to the routinely used sterilants (Vichitra *et al.* 2014). Therefore, it is essential to understand the genetic diversity of the microbial community in a tissue culture laboratory to develop a holistic asepsis culturing condition, as opined by Nadha *et al.* (2012). The slight genetic variations in the fungal population have to be considered in sterilization and decontamination protocols.

Meanwhile, genetically similar organisms as found among the *Aspergillus* and the *Penicillium* species identified in this study are likely from a common progenitor. This information explains the clustering pattern of the species with genetically related species

of many strains despite the evolutionary dynamics. Since organisms clustered together may be similar (Animasaun *et al.* 2021), the different species of the same genera identified in this study are products of evolution, as evident in the phylogenetic tree. Still, the strains may be separated by nucleotide base substitutions induced by the environment (Pinto *et al.* 2012). The existing evolutionary relationship between genera and species could be explored to choose an effective sterilant during tissue culture.

In conclusion, the present study utilized ITS region markers (ITS1 and ITS4) to identify eleven fungal isolates from contaminated *in vitro* banana culture. The effectiveness of the markers to precisely identify the contaminant fungi to the strain level alluded to their utility in the molecular identification of the organisms. The knowledge of the existing genetic and evolutionary relationship among the fungi species in the current study provides basal information that could be explored in developing broad-based sterilant and sterilization protocol to combat the challenge of fungi contamination of plant tissues culture. This information will reduce the loss of time and reagents and ultimately help achieve optimum economy during plant tissue culture.

References

- Abass, M.H., 2013. Microbial contaminants of date palm (*Phoenix dactylifera* L.) in Iraqi tissue culture laboratories. *Emirates J. Food Agric.* 25, 875-882. <https://doi.org/10.9755/ejfa.v25i11.15351>
- Abd-El salam, K.A., Aly, I.N., Abdel-Satar, M.A., Khalil, M.S., Verreet, J.A., 2003. PCR identification of *Fusarium* genus based on nuclear ribosomal-DNA sequence data. *Afri. J. Biotechnol.* 2, 82-85. <https://doi.org/10.5897/AJB2003.000-1016>
- Adeyemo, S.M., Onilude, A.A., 2014. Molecular identification of *Lactobacillus plantarum* isolated from fermenting cereals. *Inter. J. Biotechnol. Molec. Biol. Res.* 5, 59-67.
- Altan, F., Burun, B., Sahin, N., 2010. Fungal contaminants observed during micropropagation of *Lilium candidum* L. and the effect of chemotherapeutic substances applied after sterilization. *Afric. J. Biotechnol.* 9, 991-995. <https://doi.org/10.5897/AJB08.090>
- Alwakeel, S.S., 2013. Molecular identification of isolated fungi from stored apples in Riyadh, Saudi Arabia. *Saudi J. Biol. Sci.* 20, 311-317. <https://doi.org/10.1016/j.sjbs.2013.05.002>
- Anderson, I.C., Campbell, C.D., Prosser, J.I., 2003. Potential bias of fungal 18S rDNA and internal transcribed spacer polymerase chain reaction primers for estimating fungal biodiversity in soil. *Appl. Environ. Microbiol.* 5, 36-47. <https://doi.org/10.1046/j.1462-2920.2003.00383.x>
- Animasaun, D.A., Morakinyo, J.A., Mustapha, O.T., Krishnamurthy, R., 2015. Assessment of genetic diversity in accessions of pearl millet (*Pennisetum glaucum*) and napier grass (*Pennisetum purpureum*) using microsatellite (ISSR) markers. *Iranian J. Genet. Plant Breed.* 4, 25-35.
- Animasaun, D.A., Adikwu, V.O., Alex, G.A., Akinsunlola, T.P., Adekola, F.O., Krishnamurthy, R., 2021. Morpho-agronomic traits variability, allelic polymorphism and diversity analysis of African yam bean: towards improving utilization and germplasm conservation. *Plant Genetic Resources: Characterization and Utilization.* 19, 216-228. <https://doi.org/10.1017/S1479262121000253>
- Benishelkh, A.A.G., Tijani, Y., Askira, N.K., Waziri, M., Awana, A.U., 2015. Prevalence and management of laboratory microbial contaminants in plant tissue culture. *Inter. J. Res.* 2, 625-630.
- Cassells, A.C., 1990. Problems in tissue culture: culture contamination, in: Debergh, P.C., Zimmerman, R.H. (Eds.), *Micropropagation Technology and Application*. Kluwer Academic Publishers, Dordrecht, Netherlands. pp. 31-44. https://doi.org/10.1007/978-94-009-2075-0_3
- Cassells, A.C., 1996. Production of healthy plants, in: Alerson, P.G., Dullforce, W.M. (Eds.), *Micropropagation in culture*. University of Nottingham Trent Print Unit, Nottingham. pp. 53-71.
- Cassells, A.C., 2012. Pathogen and biological contamination management in plant tissue culture: phytopathogens, vitro pathogens, and vitro pests. *Methods Mol. Biol.* 877, 57-80. https://doi.org/10.1007/978-1-61779-818-4_6
- Castañeda-Ramírez, G.S., Mendoza-de-Gives, P., Aguilar-Marcelino, L., López-Arellano, M.E., Hernández-Romano, J., 2016. Phylogenetic analysis of nucleotide sequences from the ITS region and biological characterization of nematophagous fungi from Morelos, Mexico. *Journal of Mycology.* 2016, 8502629. <https://doi.org/10.1155/2016/8502629>
- Cobrado, J.S., Fernandez, A.M., 2016. Common fungi contamination affecting tissue-cultured Abaca (*Musa textiles* Nee) during initial stage of micropropagation. *Asian Res. J. Agric.* 1, 1-7. <https://doi.org/10.9734/ARJA/2016/28353>
- [CPRI] Central Potato Research Institute., 1992. *Tissue Culture Technique for Potato Health, Conservation, Micropropagation and Improvement*. CPRI, Shimla.
- Danso, K.E., Azu, E., Elegba, W., Asumeng, A., Amoatey, H.M., Klu, G.Y.P., 2011. Effective decontamination and subsequent plantlet regeneration of sugarcane (*Saccharum officinarum* L.) *in vitro*. *Inter. J. Integra. Biol.* 11, 90-96.
- Enjalric, F., Carron, M.P., Lardet, L., 1988. Contamination of primary cultures in tropical areas: the case of Hevea brasiliensis, in: Cassells, A.C. (Eds.), *Bacterial and Bacteria-like Contaminants of Plant Tissue Cultures*. ISHS, Wageningen, Netherlands. <https://doi.org/10.17660/ActaHortic.1988.225.6>
- Eziashi, E.I., Asemota, O., Okwuagwu, C.O., Eke, C.R., Chidi N.I., Oruade-Dimaro, E.A., 2014. Screening sterilizing agents and antibiotics for the elimination of bacterial contaminants from oil palm explants for plant tissue culture. *European J. Exp. Biol.* 4 111-115.
- Fiori, S., Fadda, A., Giobbe, S., Berardi, E., Migheli, Q., 2008. *Pichia angusta* is an effective biocontrol yeast against postharvest decay of apple fruit caused by *Botrytis cinerea* and *Monilia fructicola*. *FEMS Yeast Research.* 8, 961-963. <https://doi.org/10.1111/j.1567-1364.2008.00424.x>
- Gomez, B.C., Esteves, C.T., Palazzo, I.C., Darini, A.L., Felis, G.E., Sechi, L.A., Francho, B.D., de Martinis, E.C., 2008. Prevalence and characterization of *Enterococcus* spp. isolated from Brazilian foods. *Food Microbiol.* 25, 668-675. <https://doi.org/10.1016/j.fm.2008.03.008>

- Goswami, N., Handique, P., 2013. *In vitro* sterilization protocol for micropropagation of Musa (AAA group) 'Amritsagar' Musa (AAB group) 'Malbhog' and Musa (AAB group) 'Chenichampa' Banana. *Indian J. Appl. Res.* 3, 51. <https://doi.org/10.15373/2249555X/JUNE2013/18>
- Guan, S.H., Sattler, I., Lin, W.H., Guo, D.A., Grabley, S., 2005. P-Aminoacetophenonic acids produced by a mangrove endophyte: *Streptomyces griseus* subspecies. *Journal of Natural Products.* 68, 1198-1200. <https://doi.org/10.1021/np0500777>
- Habiba, U., Reja, S., Saha, M.L., Khan, M.R., 2002. Endogenous bacterial contamination during *in vitro* culture of table banana: identification and prevention. *Plant Tissue Culture*, 12, 117-124.
- Hasan, N.A., Rafii, M.Y., Rahim, H.A., Ali, N.S., Mazlan, N., Abdullah, S., 2016. Morphological and molecular characterization of fungal pathogen, Magnaphorthe oryzae. *AIP Conference Proceedings*, 1705, 020015. <https://doi.org/10.1063/1.4940263>
- Herman, E.B., 2017. Plant tissue culture contamination: challenges and opportunities. *Acta Hort.* 1155, 231-238. <https://doi.org/10.17660/ActaHortic.2017.1155.33>
- Izarra, M.L., Panta, A.L., Maza, C.R., Zea, B.C., Cruzado J, Gutarra L.R., Rivera C.R., Ellis, D., Kreuze, J.F., 2020. Identification and control of latent bacteria in *in vitro* cultures of sweet potato [*Ipomoea batatas* (L.) Lam]. *Front. Plant Sci.* 11, 903. <https://doi.org/10.3389/fpls.2020.00903>
- Junmyoung, Y.U., 2010. Identification of Fungi and Bacteria Associated with Internally Discolored Horseradish Roots [Thesis]. University of Illinois, Urbana, Illinois.
- Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870-1874. <https://doi.org/10.1093/molbev/msw054>
- Larone, D.H., 2002. Medically Important Aungi-A Guide to Identification. fourth ed. American Society for Microbiology Press, Washington.
- Lord, N.S., Kaplan, C.W., Shank, P., Kitts, C.L., Elrod, S.L., 2002. Assessment of fungal diversity using terminal restriction fragment pattern analysis: comparison of 18S and ITS ribosomal regions. *FEMS Microbiol. Ecol.* 42, 327-337. <https://doi.org/10.1111/j.1574-6941.2002.tb01022.x>
- Maina, S.M., Emongor, Q., Sharma, K.K., Gichuki, S.T., Gathaara, M., de Villiers, S.M., 2010. Surface sterilant effect on the regeneration efficiency from cotyledon explants of groundnut (*Arachis hypogea* L.) varieties adapted to eastern and southern Africa. *Afri. J. Biotechnol.* 9, 2866-2871.
- Mansfield, M.A., Kuldau, G.A., 2007. Microbiological and molecular determination of mycobiota in fresh and ensiled maize silage. *Mycologia* 99, 269-278. <https://doi.org/10.1080/15572536.2007.11832586>
- Meghwal, P.R., Sharma, H.C., Singh, S.K., 2000. Effect of surface sterilizing agents on *in vitro* culture establishment of guava (*Psidium guajava* L.). *J. Applied Hort.* 2, 94-95. <https://doi.org/10.37855/jah.2000.v02i02.28>
- Mng'omba, S.A., Sileshi, G., du Toit, E.S., Akinnifesi, F.K., 2012. Efficacy and utilization of fungicides and other antibiotics for aseptic plant cultures, in: Dhanasekaran, D., Thajuddin N., Panneerselvam, A. (Eds.), Fungicides for Plant and Animal Diseases. InTech, Rijeka, Croatia. pp. 246.
- Moreno-Vázquez, S., Larrañaga, N., Uberhuaga, E.C., Braga, E.J.B., Perez-Ruiz, C., 2014. Bacterial contamination of *in vitro* plant cultures: confounding effects on somaclonal variation and detection of contamination in plant tissues. *Plant Cell Tiss Organ Cult.* 119, 533-541 <https://doi.org/10.1007/s11240-014-0553-x>
- Msoyoya, T., Kanyagha, H., Mutigitu, J., Kulebelwa, M., Mamiro, D., 2012. Identification and management of microbial contaminants of banana *in vitro* cultures. *J. Appl. Biosci.* 55, 3987-3994.
- Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant.* 15, 473-497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Nadha, H.K., Salwan, R., Kasana, R.C., Anand, M., Sood, A., 2012. Identification and elimination of bacterial contamination during *in vitro* propagation of *Guadua angustifolia* Kunth. *Pharmacogn Mag.* 8, 93-97. <https://doi.org/10.4103/0973-1296.96547>
- Odutayo, O.I., Oso, R.T., Akinyemi, B.O. and Amusa, N.A. 2004. Microbial contaminants of cultured *Hibiscus cannabinus* and *Telfaria occidentalis* tissues. *Afri. J. Biotechnol.* 3, 473-476. <https://doi.org/10.5897/AJB2004.000-2092>
- Odutayo, O.I, Amusa, N.A., Okutade, O.O., Ogunsanwo, Y.R., 2007. Sources of microbial contamination in tissue culture laboratories in southwestern Nigeria. *Afri. J. Agric. Res.* 2, 67-72.
- Ogier, J.C., Son, O., Gruss, A., Tailliez, P., Delacriox-Buchet, A., 2002. Identification of the bacterial microflora in diary products by temporal temperature gradient gel electrophoresis. *Appl. Environ. Microbiol.* 68, 3691-3701. <https://doi.org/10.1128/AEM.68.8.3691-3701.2002>
- Omamor, I.B., Asemota, A.O., Eke, C.R., Eziashi, E.I., 2007. Fungal contaminants of the oil palm tissue culture in Nigerian Institute for Oil Palm Research (NIFOR). *Afri. J. Agric. Res.* 2, 534-537.
- Paula, M.M., Luana, M.P., Abelardo, S.J., Luis, A.N., 2011. Comparison of phenotypic and molecular tests to identify lactic acid bacteria. *Braz. J. Microbiol.* 44, 109-112. <https://doi.org/10.1590/S1517-83822013000100015>
- Peterson, S.W., Horn, B.W., 2009. *Penicillium parvulum* and *Penicillium georgiense*, sp. nov., isolated from the conidial heads of *Aspergillus* species. *Mycologia*, 101, 71-83. <https://doi.org/10.3852/08-036>
- Pinto, F.C.J., de Lima, D.B., Agustini, B.C., Dallagassa, C.B., Shimabukuro, M.F., Chimelli, M., Brand, D., Fadel-Picheth C.M.T. Bonfim, T.M.B., 2012. Morphological and molecular identification of filamentous fungi isolated from cosmetic powders. *Braz. Arch. Biol. Technol.* 55, 897-901. <https://doi.org/10.1590/S1516-89132012000600013>
- Rahman, M.d.Z., Shahinul Islam, S.M., Chowdhury, A.N., Subramaniam, S., 2017. Identification and prevention of microbial contaminants of potato culture in temporary immersion bioreactor (TIB) system. *Malaysian J. Microbiol.* 13, 289-297. <https://doi.org/10.21161/mjm.103217>
- Rawal, K., Kihara, H., 2019. Prevention of fungal contamination in plant tissue culture using cyclic lipopeptides secreted by *Bacillus amyloliquefaciens* AB30a. *Plant Tissue Cult. and Biotech.* 29, 111-119. <https://doi.org/10.3329/ptcb.v29i1.41983>
- Saad, D.S., Kinsey, G.C., Kim, S., Gaylarde, C.C., 2004. Extraction of genomic DNA from filamentous fungi in biofilms on water-based paint coatings. *Inter. Biodeterio. Biodegra.* 54, 99-103. <https://doi.org/10.1016/j.ibiod.2004.05.003>
- Sanjaya, Y., Ocampo, V.R., Caoili, B.L., 2016. Identification of potential entomopathogenic fungi of *Tetranychus kanzawai* kishida (tetranychidae: acarina) using its-5.8s rdna region as molecular marker. *AGRIVITA J. Agric. Sci.* 38, 186-192. <https://doi.org/10.17503/agrivita.v38i2.560>

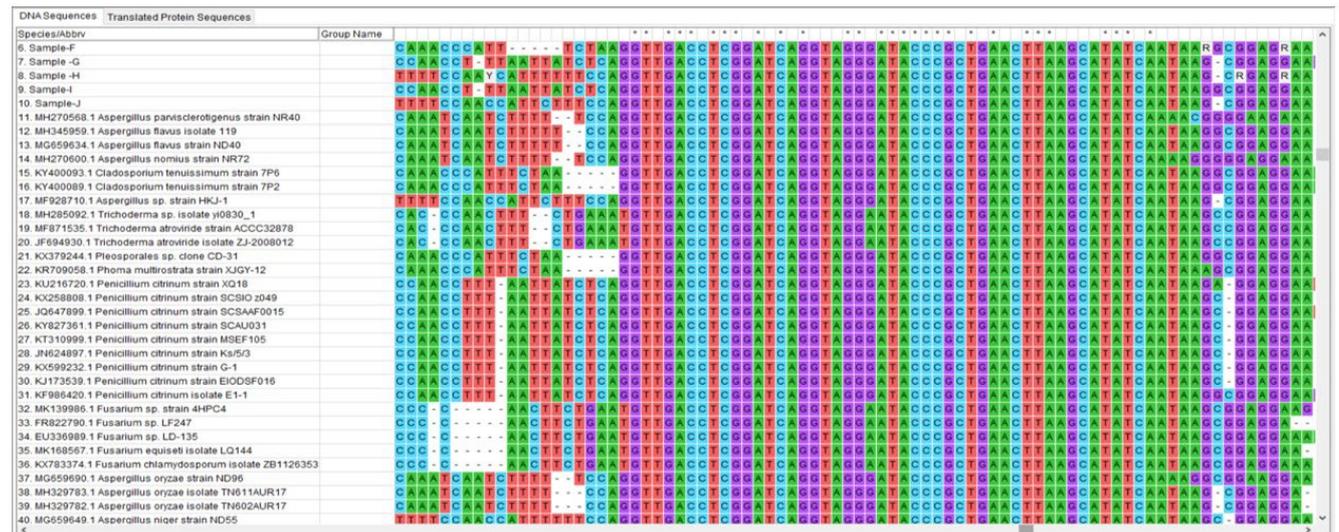
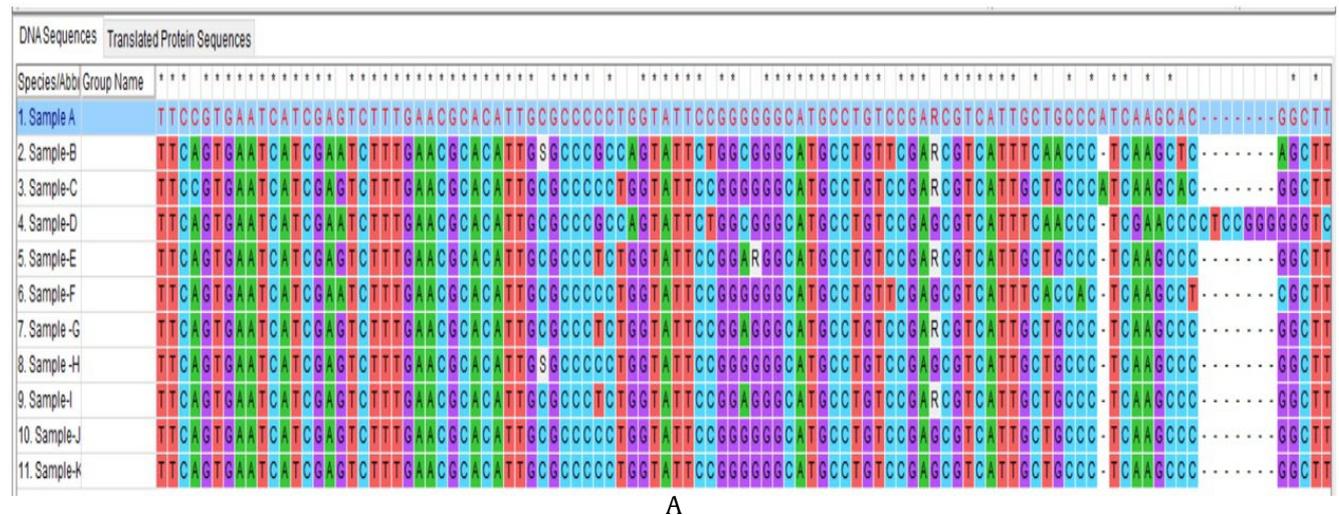
- Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Bolchacova, E., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. *PNAS*, 109, 6241-6246. <https://doi.org/10.1073/pnas.1207508109>
- Sharaf-eldin, M., Weathers, P.J., 2006. Movement and containment of microbial contamination in the nutrient mist bioreactor. *In Vitro Cellular and Developmental Biology- Plant*. 42, 553-557. <https://doi.org/10.1079/IVP2006817>
- Tamura K., Nei M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*. 10, 512-526.
- Venkatasalam, E.P., Pandey, K.K., Singh, B.P., Vandana, T., Sharma, S., Sood, R., Sharma, A.K., 2013. Efficacy of antimicrobial agents on *in vitro* micropropagation potential of potato. *Potato Journal* 40, 45-54.
- Vichitra, A., Bakshi, M., Harsh, N.S.K., 2014. Identification and bioassay of fungal contaminants observed during *in vitro* propagation of *Saraca asoka* (Roxb.) De wilde. *Biotech International* 7, 35-42.
- Watanabe, T., 2002. Pictorial atlas of soil and seed fungi-morphologies of cultured fungi and key to species, second ed. CRC Press, Boca Ratón. <https://doi.org/10.1201/9781420040821>

Supplementary Table 1. Test of homogeneity of substitution patterns between nucleotide sequences of fungi contaminants of *in vitro* culture banana

Isolates	1	2	3	4	5	6	7	8	9	10	11
A											
B	0.000*										
C	0.426	0.000*									
D	0.026*	0.030*	0.010*								
E	0.094	0.000*	0.294	0.014*							
F	0.008*	0.160	0.000*	0.002*	0.000*						
G	0.088	0.000*	0.230	0.016*	1.000	0.000*					
H	1.000	0.000*	1.000	0.016*	0.060	0.002*	0.042*				
I	0.060	0.000*	0.254	0.008*	1.000	0.000*	1.000	0.038*			
J	1.000	0.000*	1.000	0.004*	0.038*	0.000*	0.026*	1.000	0.034*		
K	1.000	0.000*	1.000	0.004*	0.052	0.002*	0.018*	1.000	0.044*	1.000	

*The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences (Disparity Index test). p-values smaller than 0.05 are considered significant

Keys: A: *Aspergillus parvisclerotigenus* strain NR40, B: *Fusarium chlamydosporum*, C: *Aspergillus flavus* isolate FMPV44, D: *Trichoderma viride* isolate P23-11, E: *Penicillium citrinum* isolate HNMF093, F: *Cladosporium tenuissimum* strain 7P6, G: *Penicillium citrinum* strain MSEF105, H: *Aspergillus niger* strain FF14, I: *Penicillium citrinum* Isolate PecGx21L02, J: *Aspergillus niger* strain M2; K: *Aspergillus awamori* strain ND6



Supplementary Figure 1. Conserved region of the (A) identified fungi isolates (B) fungi sequences used for the phylogenetic analysis of fungi contaminants associated with *in vitro* banana culture. The multiple alignments were conducted using ClusterW in MEGA7 Software