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African Journal of Microbiology Research

Full Length Research Paper

GC-MS analysis of bioactive compounds and evaluation of antimicrobial activity of the extracts of *Daedalea elegans*: A Nigerian mushroom

Grace Oluwatoyin Mensah-Agyei*, Kolawole IfeOluwa Ayeni and Cajethan Onyebuchi Ezeamagu

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Phytochemicals are naturally occurring compounds present in varying levels in plants and help significantly in protecting them against pathogens. The chemical composition of acetone and ethanolic extracts of *Daedalea elegans* were determined using Gas Chromatography-Mass Spectrometry. Twenty-eight and fourty-four compounds were identified in the acetone and ethanolic extracts respectively. Only four compounds were found to dominate the acetone extract accounting for 83.58% [9,12-octadecadienoic acid (44.64%), n-hexadecanoic acid (23.59%), 9(11)-dehydroergosteryl benzoate (8.37%), octadecanoic acid (6.98%)] and six compounds for the ethanolic extract accounting for (70.04%) [cis-9-hexadecenal (23.44%), n-hexadecanoic acid (14.13%), n-propyl 9, 12-octadecadienoate (12.78%), 9(11)-dehydroergosteryl benzoate (8.88%), hexadecanoic acid, ethyl ester (5.78%), and octadecanoic acid (5.03%)]. The extracts were tested against several bacteria and fungi by the agar well diffusion technique to ascertain their antimicrobial properties. No zone of inhibition was recorded for the extracts at both concentrations tested. Of the compounds identified in the extracts, only hexadecanoic acid has previously been reported to show antibacterial and antifungal potencies. This compound was present in the ethanolic extract of *Daedalea elegans*, albeit at a very low concentration (5.78%).

Key words: Antimicrobial, cis-9-hexadecenal, *Daedalea elegans,* 9(11)-dehydroergosteryl benzoate, hexadecanoic acid ethyl ester, n-propyl 9, octadecanoic acid, 9, 12-octadecadienoic acid, 12-octadecadienoate.

INTRODUCTION

Mushrooms are macrofungi with distinctive fruiting bodies and reproductive structures (Pilz et al., 2003). Higher fungi have been identified as a major source of biologically active natural products that provide varieties of active secondary metabolites (Jonathan and Fasidi, 2003). Many people use them as food, but few use them as medicine (Aina et al., 2012; Jonathan et al. 2012). Several researchers have reported the antimicrobial potentials of many macrofungi (Jonathan and Fasidi, 2003; Jonathan et al., 2008; Olawuyi et al., 2010).

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The medicinal values associated with mushrooms arose from superstitious beliefs and myths and these have provided information and curiosity for research studies. Research has shown that some of these claims are not mere myth but are authentic (Mau et al., 1998; Jonathan and Fasidi, 2003). Plants and macrofungi are large sources of bioactive compounds which can be exploited for the development of new novel drugs and due to the minimal side effects and low cost, about 80% of the world's population now depend on natural products for the maintenance of their health (Jagtap et al., 2009). *Daedalea elegans* is a Nigerian wild mushroom and have been reported to have antibacterial and antifungal properties (Aina et al., 2012; Jonathan and Fasidi, 2003). Other Nigerian wild mushrooms have also been reported to have antimicrobial potentials, but little/no study has reported the bioactive compounds conferring these antimicrobial properties. Therefore, it is important to determine bioactive compounds present in the extracts which are responsible for the bioactivity of the mushroom and their medicinal values making the GC-MS analysis inevitable. GC-MS is the combination of separation (GC) and identification (MS) techniques making it an ideal technique for both quantitative and qualitative analysis of volatile and semi-volatile compounds. This study was aimed at screening for the presence of phytochemicals in *D. elegans,* identify the compounds present in the acetone and ethanol extracts of *D. elegans* and determine the therapeutic properties of the extracts.

METHODOLOGY

Sample collection and identification: *D. elegans* used in this study was collected from different areas (University Botanical Gardens and Nursery Section of Botany and Microbiology Department) within the University of Ibadan campus between September and November 2016 in a sterile polyethylene bag and transported to the Botany Laboratory of the University of Ibadan. The mushroom was immediately identified in the laboratory by their spore prints and by comparing their morphological, anatomical and physiological characteristics with the standard descriptions of Zoberi (1978) and that of Alexopolous et al. (1996). This identification was authenticated by Professor S. G. Jonathan, Department of Botany, University of Ibadan, Nigeria. After proper identification was done, the sample was then transported to Babcock University where the experiment was carried out.

Microorganisms

The stored culture of *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* were collected from the Medical Laboratory Science Department while fungi strains (*Aspergillus niger, Aspergillus flavus and Fusarium chlamydosporum*) were obtained from store culture from the Department of Microbiology both of Babcock University, Ilishan-Remo, Ogun State.

Preparation of crude extract

The fruiting body of the test mushroom was allowed to air dry at room temperature. The dried carpophore was divided into bits and pulverized with a grinding machine. Eighty grams of the powdered sample was soaked separately in 700 ml of 99.7 - 100% (absolute) ethanol and acetone in an Erlenmeyer flask. The flasks were covered with cotton wool and aluminum foil and allowed to stand for 48 h with intermittent shaking. It was filtered by use of Whatman filter paper no 1 and the sample was re-suspended in solvent and allowed to stand for 7 days for another extraction. This was also filtered through Whatman filter paper no 1 and the filtrate obtained from both extractions was concentrated using rotary evaporator at 40 and 60^{0}C for acetone and ethanol respectively. The solvents were recovered and the extract was collected and was further concentrated using hot air oven at 45°C for 19 and 28 h for acetone and ethanol respectively and stored in the refrigerator prior to further analysis.

Assay for antimicrobial activities

The antimicrobial activities of both acetone and ethanol extracts were determined using agar well diffusion method of Stoke and Ridgway (1980). Using a 5mm sterile cork-borer, holes were bored in plates containing already prepared Mueller Hinton Agar (MHA) and Potato Dextrose Agar (PDA). Extracts were reconstituted in Dimethyl sulfoxide (DMSO) to make a concentration of 20.0 and 40 mg/ml for both fractions. 100 µl of the reconstituted extracts was then transferred to the holes. The DMSO used in the dilution of solid mushroom extracts was used as a control. Plates were then incubated at 37 and 28°C for 24 and 72 h for bacteria and fungi respectively and observed for zones of inhibition. All the tests were carried out in duplicates.

GC-MS analysis

GC-MS analysis was conducted at Shimadzu Training Center for Analytical Instruments (STC) Lagos. The extracts were analyzed by GCMS-QP2010SE (SHIMADZU, JAPAN) equipped with DB-5 MS (0.25 µm × 30 m × 0.25 mm). Helium was used as the carrier gas at a flow rate of 0.9ml/min. 1.0µl injection volume, injector temperature was 250°C; ion source temperature was 200°C. Interface temperature was 250°C. Oven temperature was 60°C held for 2 min with an increase of 15°C/min to 120°C, ending with 300°C (15°C/min). Mass spectrometer was set to operate in electron ionization mode with an ionizing energy of 70eV as acquisition mass range from 45-700 a.m.u. Total running time was about 30 min. Further identification was made by comparison of their mass spectra with those stored in the National Institute of Standards and Technology (NIST) database.

RESULTS

Forty-four and twenty-eight compounds were identified in the ethanol and acetone extracts respectively. The dry weight of the yield was 1.2 and 0.58 g for ethanol and acetone fractions respectively which can be said to be directly proportional to the number of compounds present in the extracts. Compounds were identified from NIST database Library of GC-MS instrument. Identified compounds with their name, retention time and peak (area) percentage are given in Tables 1 and 2, while the GC-MS chromatogram is shown in Figures 1 and 2.

Only four compounds were found to dominate the acetone extract accounting for 83.58% [9,12-

Table 1. GC-MS profiling of ethanol extract of *D. elegans*.

Peak#	R. Time	Name	Area%
1	5.957	2,5 Furandione, 3,4-dimethyl-	0.46
2	7.167	Benzaldehyde, 4-benzyloxy-2-fluoro-5-hyd	0.70
3	7.225	1-Benzoyl-3-(tetrahydro-furan-2-ylmethyl)-	0.73
4	7.298	Chloroacetic acid, 6-ethyl-3-octyl ester	0.54
5	7.803	2-Thiophenecarboxylic acid, 4-methylpenty	0.29
6	8.065	Butanoic acid, 3-methyl-, octyl ester	0.35
7	8.175	Pyrimidine, 2,4,5-triamino-	0.63
8	8.232	Acetamide, N-methyl-N-(2-propynyl)-	0.54
9	8.670	Propane, 1,1,3-triethoxy-	0.21
10	8.722	4-Methyl-2-pentyl acetate	0.58
11	8.936	2-Cyclopenten-1-one, 3-ethyl-2-hydroxy-	0.54
12	9.058	Cyclopentanone, dimethylhydrazone	0.55
13	9.418	2-Methoxydecanoic acid	0.48
14	9.526	Chloroacetic acid, 4-pentadecyl ester	0.15
15	9.745	Octadecanoic acid	0.23
16	10.111	Decanoic acid, ethyl ester	3.04
17	10.499	1-Decanol, 2-hexyl-	0.79
18	10.728	Solasonine	0.27
19	11.298	Guanethidine	0.53
20	12.266	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-	1.34
21	12.930	10-Nonadecanone	0.36
22	13.224	Dodecanoic acid, ethyl ester	2.82
23	13.670	Pentatriacontane	0.46
24	15.872	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	0.74
25	15.984	Ethyl 14-methyl-hexadecanoate	0.37
26	16.472	Eicosanoic acid	0.77
27	16.781	Ethyl 14-methyl-hexadecanoate	0.39
28	16.856	Pentadecanoic acid	0.65
29	17.490	Cyclopentanetridecanoic acid, methyl ester	0.39
30	17.771	9-Tetradecenal, (Z)-	1.32
31	18.038	n-Hexadecanoic acid	14.13
32	18.222	Hexadecanoic acid, ethyl ester	5.78
33	18.976	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dion	0.62
34	19.168	Lidocaine	0.86
35	19.226	Z,Z-3,13-Octadecadien-1-ol	1.16
36	19.796	cis-9-Hexadecenal	23.44
37	19.867	n-Propyl 9,12-octadecadienoate	12.78
38	19.957	Octadecanoic acid	5.03
39	20.112	Ethyl 14-methyl-hexadecanoate	2.74
40	20.554	Isopropyl linoleate	1.12
41	20.874	Ethyl 9-hexadecenoate	0.92
42	25.492	Spiro[androst-5-ene-17,1'-cyclobutan]-2'-on	1.12
43	26.420	9(11)-Dehydroergosteryl benzoate	8.88
44	26.943	4,6-Decadienal, 8-ethyl-10-[4-hydroxy-8-(2	0.18
			100.00

octadecadienoic acid (44.64%), n-hexadecanoic acid (23.59%), 9(11)-dehydroergosteryl benzoate (8.37%), octadecanoic acid (6.98%)] while six compounds were dominated in the ethanolic extract accounting for (70.04%) [cis-9-hexadecenal (23.44), n-hexadecanoic acid (14.13%), n-propyl 9, 12- octadecadienoate

Table 2. GC-MS profiling of acetone extract of *D. elegans*.

Peak#	R.Time	Name	Area%
1	7.154	Benzoic acid	0.40
2	8.449	Nonanoic acid	0.14
3	8.734	Oxetane, 2,2,4-trimethyl-	0.28
4	9.714	n-Decanoic acid	0.09
5	12.551	Phthalimide	0.44
6	12.720	Dodecanoic acid	0.24
7	14.057	E-2-Hexenyl benzoate	0.21
8	14.267	2,4-Difluorobenzene, 1-benzyloxy-	0.16
9	15.067	Tetratetracontane	0.55
10	15.141	Isopropylphosphonic acid, fluoroanhydryde	0.28
11	15.453	Benzene, (1-methylundecyl)-	0.21
12	15.628	Tetradecanoic acid	0.76
13	15.868	Cyclohexanepropanol, .alpha.,2,2,6-tetrame	0.56
14	16.464	Pentadecanoic acid	0.71
15	16.703	E-2-Hexenyl benzoate	0.32
16	16.845	Pentadecanoic acid	0.97
17	17.435	1-Decanol, 2-hexyl-	0.46
18	17.764	9-Tetradecenal, (Z)-	1.67
19	18.045	n-Hexadecanoic acid	23.59
20	18.837	Phthalic acid, butyl undecyl ester	1.08
21	18.972	Eicosanoic acid	0.79
22	19.801	9,12-Octadecadienoic acid (Z,Z)-	44.64
23	19.970	Octadecanoic acid	6.98
24	23.641	Bis(2-ethylhexyl) phthalate	2.64
25	24.302	2,2,4-Trimethyl-3-(3,8,12,16-tetramethyl-he	1.95
26	26.423	9(11)-Dehydroergosterylbenzoate	8.37
27	26.694	9(11)-Dehydroergosteroltosylate	1.28
28	26.948	4,6-Decadienal,8-ethyl-10-[4-hydroxy-8-(2	0.22
			100.00

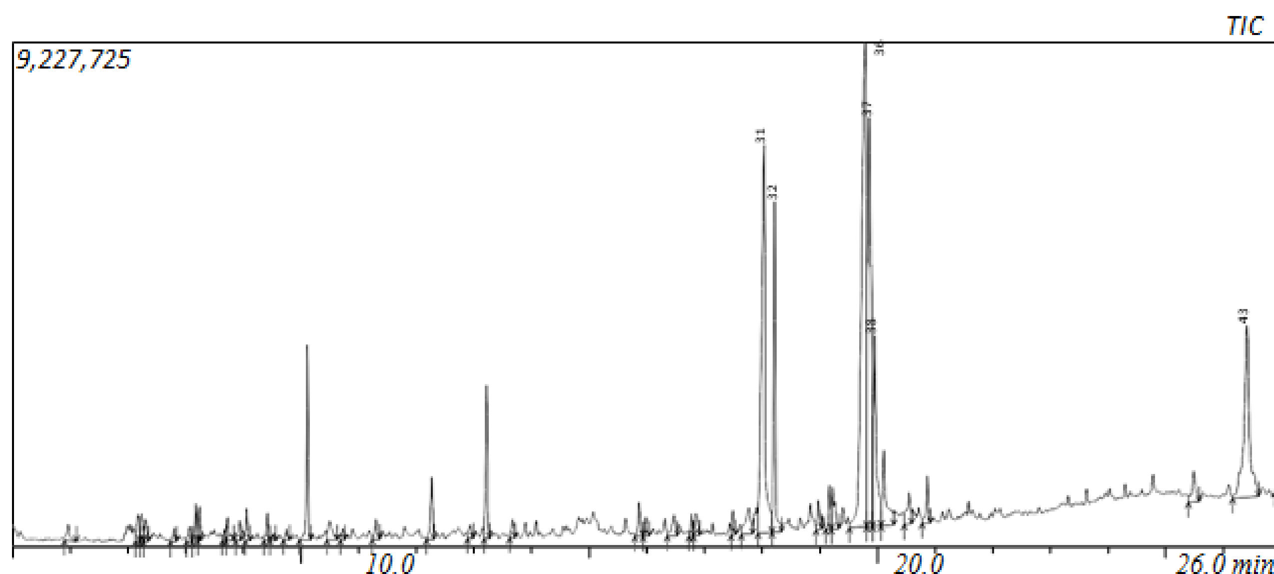

Figure 1. GC-MS chromatogram of ethanol extract of *D. elegans.*

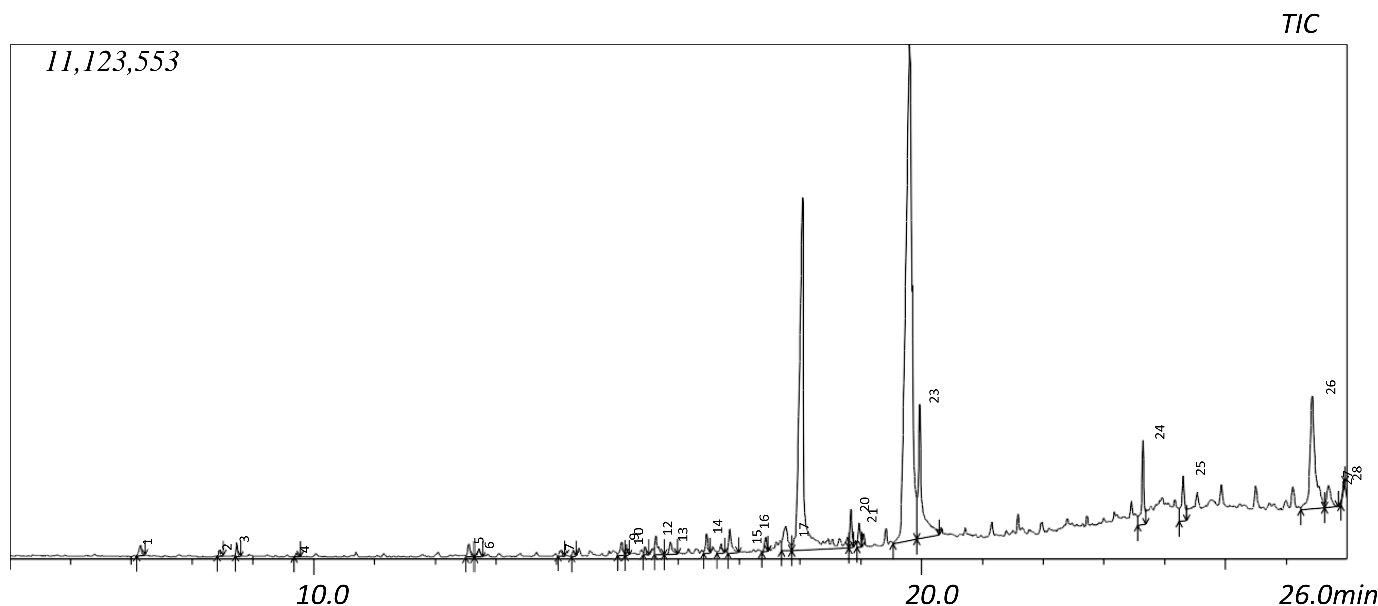

Figure 2. GC-MS chromatogram of acetone extract of *D. elegans.*

(12.78%), 9(11)-dehydroergosteryl benzoate (8.88%), hexadecanoic acid, ethyl ester (5.78%), octadecanoic acid (5.03%)]. The extracts (20 and 40 mg/ml) were tested against several bacteria (*E. coli, P. aeruginosa, K. pneumoniae, S. aureus*) and fungi (*A. niger, A. flavus and F. chlamydosporum*) by the agar well diffusion technique in order to ascertain their antimicrobial properties. No zone of inhibition was recorded for the extracts at the concentrations tested for both bacteria and fungi.

DISCUSSION

This study is the first GC-MS analysis on *D. elegans*; however, the result obtained from this study is similar to other GC-MS studies done on other mushroom types as some of the compounds identified here are the same (Parmar and Kumar, 2015) as well as those obtained from other plant sources (Kriti et al., 2014; Shah et al., 2015).

Mushrooms are rich sources of natural antibiotics and therefore mushrooms extracts have been investigated for their antimicrobial activity. Mushrooms are known to produce various compounds having antiviral, antibacterial and antifungal properties. Antifungal activity was reported from *Sparasis ramosus* as early as 1923 by Falck (Brian, 1951) but the first investigations on the potential of mushrooms as sources of antibiotics were performed by Anchel et al. (1941) when they examined extracts of fruiting bodies and mycelia culture from over 2000 species. Wilkins and Harris (1946) observed antibacterial activity in *Fomes, Polyporus* and *Tramates.* Bohus (1969) reported the antifungal activity of *Coprinus comatus*. Musilek and Sasek (1967) reported the antimicrobial activity of *Flummulina velutipes*. Benedict and Braddy (1972) tested the activities of selected mushroom metabolites on some bacteria and they reported that the best inhibitory responses were against Gram positive bacteria including acid fast bacterium (*Mycobacterium smegmatis*) and pathogenic strains of yeast (*Candida albicans*). They also reported that antibacterial property of the mushrooms is due to the presence of polyacetylene. Vogel et al. (1974) reported that antibacterial activity of *Agaricus bisporus* is due to the quinoid and Phenolic derivatives. Many of the external secondary metabolites (extracellular secretions by the mycelium) of mushrooms are known to combat bacteria (Benedict and Braddy, 1972; Kupra et al., 1979) viruses (Eo et al., 1999; Brandt and Piraino, 2000) and protozoa (Lovy et al., 1999).

In the present study, no zone of fungal and bacterial inhibition was found at concentrations of 20 and 40 mg/ml for both fractions. This is in contrast to the studies of Jonathan and Fasidi (2003) and Aina et al. (2016) which reported that the ethanol extract of *D. elegans* had an antagonistic effect on the tested organisms. The antimicrobial result obtained from the current study can be substantiated by the result obtained from the GC-MS analysis of the extract as the most abundant compounds identified were anti-inflammatory. Of all the 44 and 28 compounds of the ethanol and acetone extracts, only one (hexadecanoic acid) has been previously reported by Henry et al. (2002) and Praveen et al. (2010) to have antimicrobial property, however the concentration of this compound in *D. elegans* is very low (5.78%) and may explain the inactivity observed in the sensitivity tests.

The most abundant compounds of *D. elegans* as observed by the GC-MS profiling in this study is n-hexadecanoic acid (14.13 and 23.59% for ethanol and acetone respectively) which has been previously proved to have anti-inflammatory, antioxidant, hypocholesterolemic, flavour, nematicide, pesticide, anti-androgenic activities (Henry et al., 2002; Praveen et al., 2010). Hexadecanoic acid (5.78%) found only in the ethanolic extract is used in the production of soap, cosmetics and industrial mold release agents, anti-inflammatory, antibacterial and antifungi (Henry et al., 2002; Praveen et al., 2010). 9, 12-octadecadienoic acid (Z,Z) at a concentration of 12.78 and 44.64% for ethanol and acetone respectively is a polyunsaturated essential fatty acid in mammalian nutrition and is used in biosynthesis of prostaglandins and cell membranes (O'Neil, 2013; Adeoye-Isijola et al., 2018), anti-inflammatory, hepatoprotective, anti-arthritic, anti-histamine (Henry et al., 2002). Octadecanoic acid with concentration of 5.03 and 6.98% for ethanolic and acetone extracts respectively was reported by Duke (2013) as emulsifier for food and no bioactivity. Hexadecenoic acid ethyl ester is a flavouring agent used in foods including condiments and seasonings, hair and skin conditioning agent, anti-inflammatory, antioxidant, hypocholesterolemic, nematicide, pesticide, lubricant, antiandrogenic, hemolytic, 5-alpha reductase inhibitor (Shah et al., 2015). Cis-9-hexadecenal is an agrochemical of the attractant category. It is a pesticide used for preventing, destroying or mitigating pests. 9(11)-dehydroergosteryl benzoate is used in preserving foods, fats, fruit juices, alkaloidal solutions; manufacture of benzoates and benzoyl compounds, dyes; in calico printing; for curing tobacco; as standard in volumetric and calorimetric analysis (O'Neil, 2013); Plasticizers, benzoyl chloride; alkyd resins; food preservative; seasoning tobacco; flavors; perfumes; dentifrices; standard in analytical chemistry (Lewis, 2007).

CONCLUSION AND RECOMMENDATION

Among all the compounds obtained from the GC-MS analysis of the extracts, only hexadecanoic acid has previously been reported to show antibacterial and antifungal potencies. This compound was present in the ethanolic extract of *D. elegans*, albeit at low concentration (5.78%) which may explain the reason for its inactivity on the microorganisms tested.

The many chemical components found in the Nigerian wild mushroom as seen from the GC-MS analysis of acetone and ethanol extracts of *D. elegans* indicates the beneficial role it can contribute to human beings and can be considered as a source of different supplements and curative for many illnesses without any fear of pathogens developing resistance due to its natural origin. The mushroom exhibits great potentials in the pharmaceutical industry as it is a great source of anti-inflammatory agent and antioxidant. *D. elegans* can also be exploited in other areas of life such as nutraceutical, cosmeceutical, agriculture, food, textile and paint industries. However, the medicinal potential of these compounds needs further studies on the toxicity level to be able to develop safe drug(s) from the mushroom sample.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Diversity of arbuscular mycorrhizal fungi (AMF) associated with cotton (*Gossypium hirsutum* L.) growing in the Far-North region of Cameroon

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The present study was carried out with the aim of highlighting the diversity of arbuscular mycorrhizal fungi (AMF) associated with the rhizosphere of cotton grown in the Far-North region of Cameroon. To achieve this, composite soil and root samples were taken in six fields, chosen according to edapho-climatic conditions and types of cultural practices. After entrapping the glomales in the greenhouse, the roots of the trap plants were thinned and stained to assess colonization. The spores were isolated by wet sieving and their identification was made after analysis of their morpho-anatomical structures; then the diversity was evaluated through the calculated index. The results obtained have revealed the presence of AMF in all sites surveyed with a maximum abundance of 432 spores per 100 g of soil. Despite the low diversity of AMF that exist, four genera have been identified: *Acaulospora*, *Gigaspora*, *Glomus* and *Scutellospora*. A significant dominance of *Glomus* spp. (42%) was found. A strong correlation was found between soil physico-chemical parameters and abundance, as well as between species richness. This study confirms the presence of AMF strains in the cotton rhizosphere cultivated in this zone. The exploitation of this AMF could lead to a controlled production of local fungal inoculum, adapted to the edaphic and climatic conditions of the region, for a sustainable agriculture in Cameroon.

Key words: Cotton, rhizosphere, arbuscular mycorrhizal fungi (AMF), diversity, Far-North, Cameroun.

INTRODUCTION

Microorganisms represent the majority of living organisms in the soil compartment, thus constituting an important part of the planet's genetic diversity (Leake et al., 2004). Among these microorganisms, arbuscular mycorrhizal fungi (AMF) establish symbiosis with approximately 80% of the vascular plant species in all terrestrial biomes (Smith et al., 2010). These are considered to be the key elements in the functioning of terrestrial ecosystems,

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particularly for their ability to promote plant development in degraded environments through the establishment of a symbiotic association with their roots called mycorrhizae (Abbas, 2014). This key role attributed to them is due to the influences they may have in the biological and geochemical processes that govern ecosystems, namely the acquisition of nutrients for the plant (Smith et al., 2010), the improvement of plant growth (Megueni et al., 2011), the improvement of soil quality (Caravaca et al., 2002) and increasing plants ability to resist to soil pathogens, as well as their tolerance to biotic and abiotic stresses (Dalpé, 2005). Also mycorrhizal symbiosis increases plants tolerance to salinity, heavy metals, drought stress and low pH (Hassan et al., 2011). Despite their multiple benefits, AMF are untapped and almost ignored by Cameroonian farmers. In the Far-North region of Cameroon, cotton growing is the main cash crop for many populations and even contributes to food security (Folefack et al., 2014); but it is subject to several soil constraints (Olina et al., 2008). Indeed, this region located in a Sudano-Sahelian agro-ecological zone of Cameroon is characterized by a harsh and unstable climate; but also, by very low level of soil fertility (Tsozue et al., 2015). This constitutes the main constraint of declining agricultural production in this zone. In addition, factors that favor the decline of soil fertility in this area are, anthropogenic pressure on, cultural practices characterized by chemical fertilizer inputs below or above recommended doses and without restitution residues (Olina et al., 2008). Thus, under these conditions, the variability of the endomycorrhizal potential of soils in this region should be exploited to optimize agricultural production in general and cotton production in particular. In addition, to better control inoculation trials and make them accessible to the public, it is important to identify native AMF to estimate their competitiveness in the soil. Furthermore, with the exception of the work done by Tobolbai et al. (2018) on maize and Ngonkeu et al. (2013), the current data on the identity of glomales in this area are few. It is in this context that it has seemed interesting to evaluate the specific diversity of arbuscular mycorrhizal fungi associated with cotton (*Gossypium hirsutum* L.) grown in the Far-North region of Cameroon. Specifically, it involved (a) the identifications and quantification of different AMF species associated to cotton, (b) assessment of their distribution and diversity on cotton rhizosphere in the studied area.

MATERIALS AND METHODS

Physical description of study sites and soil sampling

Based on their edapho-climatic conditions and types of cultural practices, six sites (Dargala, Dogba, Doukoula, Laf, Tokombere and Zidim) were selected in the Far-North region Cameroon. Located in the northern cotton zone, they are characterized by a dry tropical Sudano-Sahelian climate, with an annual temperature and rainfall of 27°C and 800 mm, respectively.

The samples were taken between November 2017 and January 2018, a transition period between the rainy and dry seasons. The choice of sampling plots (Table 1) mainly concerned the type of crop (monoculture), the cropping system (non-use of chemicals input), the appearance of the plot (plot slightly degraded by erosion and not on a slope), the history of these over the last two years and the separation with neighboring fields (preferably nearest the organic production lands or enough distance end from conventional lands). The samples were taken according to the zigzag method described by Barker (1985). At each selected site, 5 to 10 kg of soil was collected at 10-20 cm depth, near the root rhizosphere of cotton, at different points of the site. Then, the soil samples were sieved (sieve 2 mm mesh size) in order to remove the large size particles and homogenize to obtain representative 10 kg of composite soil sample. These samples were packaged in plastic bags and stored at room temperature until use.

Determination of the physicochemical characteristics of soils

Physical and chemical properties were analyzed in the Faculty of Agronomy and Agricultural Science of the University of Dschang (Cameroon). Soil sample was air-dried and ground to pass through a 2-mm sieve. Soil pH was determined according to Pansu and Gautheyrou (2003). Organic carbon was assessed after the method by Walkley and Black (1934) and soil nitrogen (N) by the Kjeldahl method (MacDonald, 1977). Soil cationic exchange capacity (CEC) and total phosphorus (P) were determined using the method by Duchaufour (1977) and Pansu and Gautheyrou (2003) respectively. Soil available phosphorus was determined after Olsen (1952) and soil textural classes using French standards of soil classification (ISO, 1991). The exchangeable bases (Ca^{2+}, Mg^{2+}, K^{+}) were extracted using the ammonium acetate (NH4OAC, pH: 7) and determined by flame atomic absorption spectrophotometry.

Trapping of endomycorrhizal spores and assessment of root mycorrhization

The greenhouse trapping method of Morton (1992) was chosen using soils sampled as an inoculum to allow the eventual hatching of all spores and their development. A local variety (CMS variety) of corn (*Zea mays* L.) was used as a trap plant. Plastic pots of 10 L volume containing a mixture (v / v) of soil used as inoculum and sand previously sterilized with a dry heat at 120°C temperature for 5 h in an oven were used as a culture substrate. After eight weeks of greenhouse cultivation, the aerial plant of maize plants was dissociated and removed, underground part. Mycorhization criteria were evaluated, for each sample, on 30 fine root fragments stained according to the method (Philips and Hayman, 1970) and mounted in glycerol on three slides each. Under optical microscope, these root fragments were carefully examined along their entire length to record the mycorrhizal structures. The parameters noted were frequency (F), root mycorrhizal intensity (M) and arbuscular contents (Trouvelot et al., 1986).

Extraction and enumeration of spores

Spores were extracted according to the wet sieving method described by Gerdemann and Nicholson (1963). A 100 g sample of dry soil was suspended in 1 L of tap water. The suspension obtained was transferred to a series of five sieves with decreasing mesh size (1, 250, 100, 63 and 50 µm). The suspension of spores in the sieves was centrifuged for 3 min at 4000 rpm and at a temperature of 4°C with sucrose (60%). Then the suspensions were

Table 1. Geographical coordinates of the different study sites.

Sites	Latitude (°)	Longitude (°)	Altitude (m)
Dargala	10°31’57.65’’N	14°36’15.84’’E	351
Dogba	10°46’18.68 N	14°18’27.90’’E	402
Doukoula	10°11’49.17’’N	14°97’46.79’’E	357
Laf	10°25’47.53’’N	14°21’70.11’’E	480
Tokombéré	10°86’16.17’’N	14°14’63.94’’E	360
Zidim	10°29’18.72’’N	13°58’40.90’’E	539

placed in a Petri dish with grid bottom for observation and counting. This observation was made with the EUROMAX edublue brand binocular loupe, while separating the various morphotypes with a micropipette. Spores were considered viable if they had a clear content under an optical microscope, with an intact wall. They were classified according to their morphological characteristics (size, color, shape, consistency, wall structure and attachment of the hyphal suspensor), then quantified. The spores were mounted on glass slide in PVLG with Melzer and the identification of species were made using the identification keys of Schenk and Perez (1990); as well as the INVAM (International Culture Collection of Arbuscular and Vesicular Arbuscular Mycorrhizal Fungi), http://invam.caf.wvu.edu/fungi/taxonomy/species/id.htm.species were ranked according to Redecker et al. (2013).

The following parameter were evaluated

The density of spores, corresponding to the number of spores per 100 g of dry soil; the species richness (S) represents the total number of species present in a site; the Shannon index, which expresses diversity by taking into account the number of species and the abundance of individuals within each of these species:

H '= - [(ni / N) log2 (ni / N)];

With: ni = number of individuals of a given species (i) in a site; N = total number of individuals of all species of the site; Log2 = logarithm based on 2.

Piélou's equitability index (E), which is the regularity of the distribution of species:

EQ = H '/ Log2 S;

With: S = Shannon diversity index, S = total number of species. Simpson's diversity index (1-D), it measures the probability that two randomly selected individuals belong to the same species:

1-D = Ì {[ni (ni-1)] / [N (N-1)]

With: n-i = number of individuals in species i; N = total number of individuals.

Statistical analyzes

The values of the various parameters evaluated were calculated as an average of three repetitions using the EXCEL 2010 spreadsheet and the graphs were also made using the same spreadsheet. All data were processed by one-way analysis of variance using the Statgraphics plus version 5.0 software and the main component analysis was performed by the xl stat pro software. For multiple comparisons, the averages of the different variables were compared by the Duncan test (p <0.05).

RESULTS AND DISCUSSION

Physicochemical characteristics of cotton rhizosphere soil

The distribution of mineral fractions varied among different sites (Table 2); these soils are of lumpy nature, characterized by sandy-loamy structures with a large predominance of sand compared to clay and silt. Furthermore, in the Doukoula soil, clay is predominant (56%), unlike Dargala where silt is the most dominant (47%). The pH of soil samples from the different sites was essentially acid with average values between 5 and 6. Contents of mineral elements vary from one soil to another but remain, therefore quite weak. The Dogba site is moderately rich in assimilable phosphorus (71 ppm), unlike the other soil poor in this element and whose content was less than 45 ppm. The total nitrogen (0.12 to 0.48%) and organic matter (4.81 to 1.15) levels were significant in the sites surveyed, but the Dogba site remained poor in organic matter (1.15%). Concerning the exchangeable bases (Ca, Mg and K), data indicate a great variability of their contents in different soils.

Mycorrhization of trap plants

Microscopic examination of maize roots (*Zea mays* L.) was used as a trap plant, after eight weeks of greenhouse culture and no trace of mycorrhizal fungi was observed in the control pots. In addition, all the other root samples were densely colonized by fungi and the cytological organization of these mycorrhizae was essentially arbuscular. The average mycorrhizal frequency has always been very high, ranging from 90 to 100% depending on the sites surveyed; but no significant difference was revealed among them (Table 3). Despite some differences reported for mycorrhization intensity values, particularly for Laf (34.60 ± 4.08%), Doukoula (32.6 ± 8.07%), and Dargala (31.66 ± 5.6%), this difference also remained insignificant at the 5% threshold according to ANOVA. Similarly, for different arbuscular contents, the site factor showed no significant effect on the presence of arbuscular structures in the roots observed.

Table 2. Physical and chemical characteristics of soils collected from study sites.

Sites	pH-H_2O	pH-KCl	Sa (%)	Si (%)	C (%)	OM (%)	N (%)	Ca (ppm)	Mg ppm)	K (ppm)	P (ppm)
Dargala	6.6	5.8	44	47	12	2.81	0.4	135	167	32	45
Dogba	6.4	5.77	57	15	26	1.15	0.32	520	110	60	71
Doukoula	6.8	5.12	27	23	56	3.34	0.25	750	216	65	41
Laf	6.01	5.66	18	55	29	3.05	0.42	598	115	96	27
Tokombéré	6.5	5.24	57	37	10	2.98	0.48	101	95	27	15
Zidim	5.98	4.95	73	16	14	4.81	0.12	150	60	45	43

C: Clay; Si: Silt; OM: Organic matter; Sa : Sand; N: Total N; P: Phosphorus.

Table 3. Quantification of various root colonization parameters of greenhouse maize plants.

Sites	Frequency (%)	Intensity (%)	Arbucular content (%)
Dargala	90 ± 10.00	31.66 ± 5.60	18.69 ± 3.15
Dogba	100 ± 0.00	26.96 ± 4.64	18.42 ± 3.87
Doukoula	100 ± 0.00	32.6 ± 8.07	21.76 ± 8.7
Laf	93.33 ± 5.77	34.26 ± 3.13	16.42 ± 5.3
Tokombéré	96.66 ± 5.77	26.3 ± 4.9	12.87 ± 5.19
Zidim	93.33 ± 5.77	28.07 ± 1.95	20.6 ± 4.33
Moyenne	95.55 ± 4.03	29.97 ± 3.29	18.13 ± 3.17
P-Value	0.271 ^{Ns}	0.649 ^{Ns}	0.440 ^{Ns}

Ns: not significant at the 5% threshold.

Table 4. Composition of arbuscular mycorrhizal fungi according to the taxonomic classification of Redecker et al. (2013).

Family	Genrera	Species	Occurence (%)
Glomaceae	*Glomus*	*Glomus aggregatum* Schenck and Smith emend. Koske *Glomus hoi* Berch and Trappe *Glomus manihotis* Howeler, Sieverd and Schenck *Glomus* sp.	42
Gigasporaceae	*Scutellospora*	*Scutellospora cerradensis* Spain and Miranda *Scutellospora gregaria* Schenck and Nicolson emend. Walker and Sanders *Scutellospora nigra* Redhead, Walker and Sander	26
	Gigaspora	*Gigaspora margarita* Gerd and Trappe	2
Acaulosporaceae	*Acaulospora*	*Acaulospora* sp.1 *Acaulospora* sp.2	19
Unknow	Myco Brun-orange		11

Composition of the AMF of the prospected soils

On the basis of the morphological features and on some identification keys of reference works, 10 AMF have been described and one morphotype has not been identified (Myc Brown-orange). According to the taxonomic classification of Redecker et al. (2013), the most isolated spore species belong to Glomaceae family (4 species), Gigasporaceae (4 species) and Acaulosporaceae (2 species) (Table 4). On the four isolated genera (Acaulospora, Gigaspora, Glomus and Scutellospora), only seven species were identified with specific taxa and the identification of the other four was restricted to the genus.

The *Glomus* genus has been represented by four species (Figure 2), such as *Glomus aggregatum*

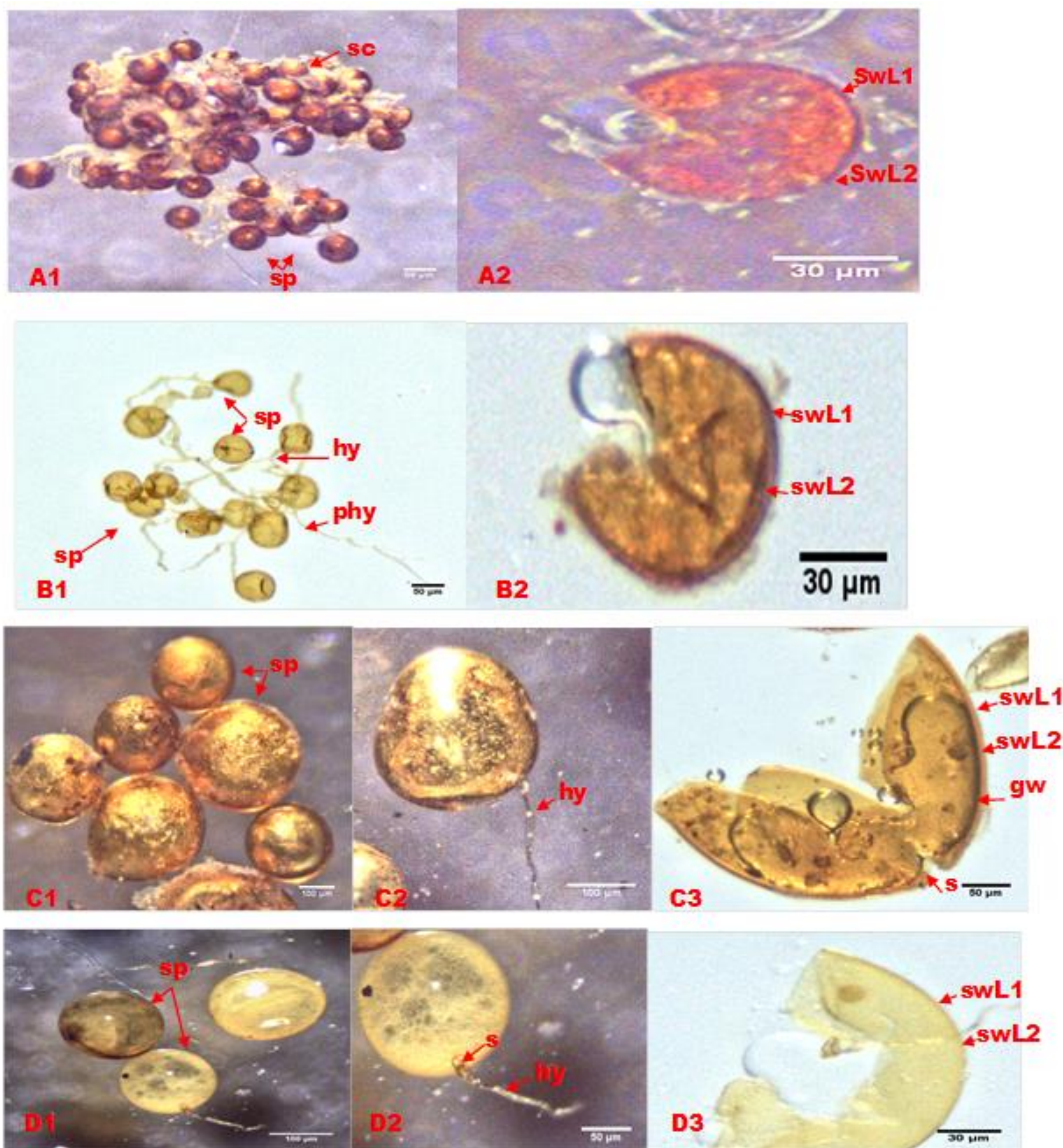

Figure 1. Morphological and anatomical characteristics of *Glomus* genus with; A: *Glomus aggregatum* B : *Glomus hoi*; C: *Glomus manihotis* ; D: *Glomus* sp.: A1 B1: sporocarp; C1 and D1: isolate spores C2, D2: hyphal structure on single spore ; A2, B2, C3, D3: wall structure of spore in PVLG with Melzer ; (hy) subtending hypha; (phy) central hyphal plexus; (sc) sporocarp; (s) septum; (sp) whole spore ; (sw) spore wall ; (swL1) outer layer of spore wall; (swL2) inner layer of spore wall.

[Schenck & Smith emend. Koske (Figure 2A), *G. hoi* [Berch & Trap (Figure 2B)], *G. manihotis* [Howeler, Sieverd & Schenck (Figure 2C) and *Glomus* sp. (Figure 1D). This were the most abundant in the region with nearly 42% of isolated spores (Table 4). These fungi consist of aggregates of several compact spores (Figure 1A1) or not, called sporocarp. Otherwise some species like *G. hoi* were characterized by a free sporocarp with constituted spores connected each to other by a more or less extensive hyphal plexus (Figure 2B1). Of a shiny and more or less rough appearance, *G. Manihotis* spores on the other hand, are rather solitary in the soil and can

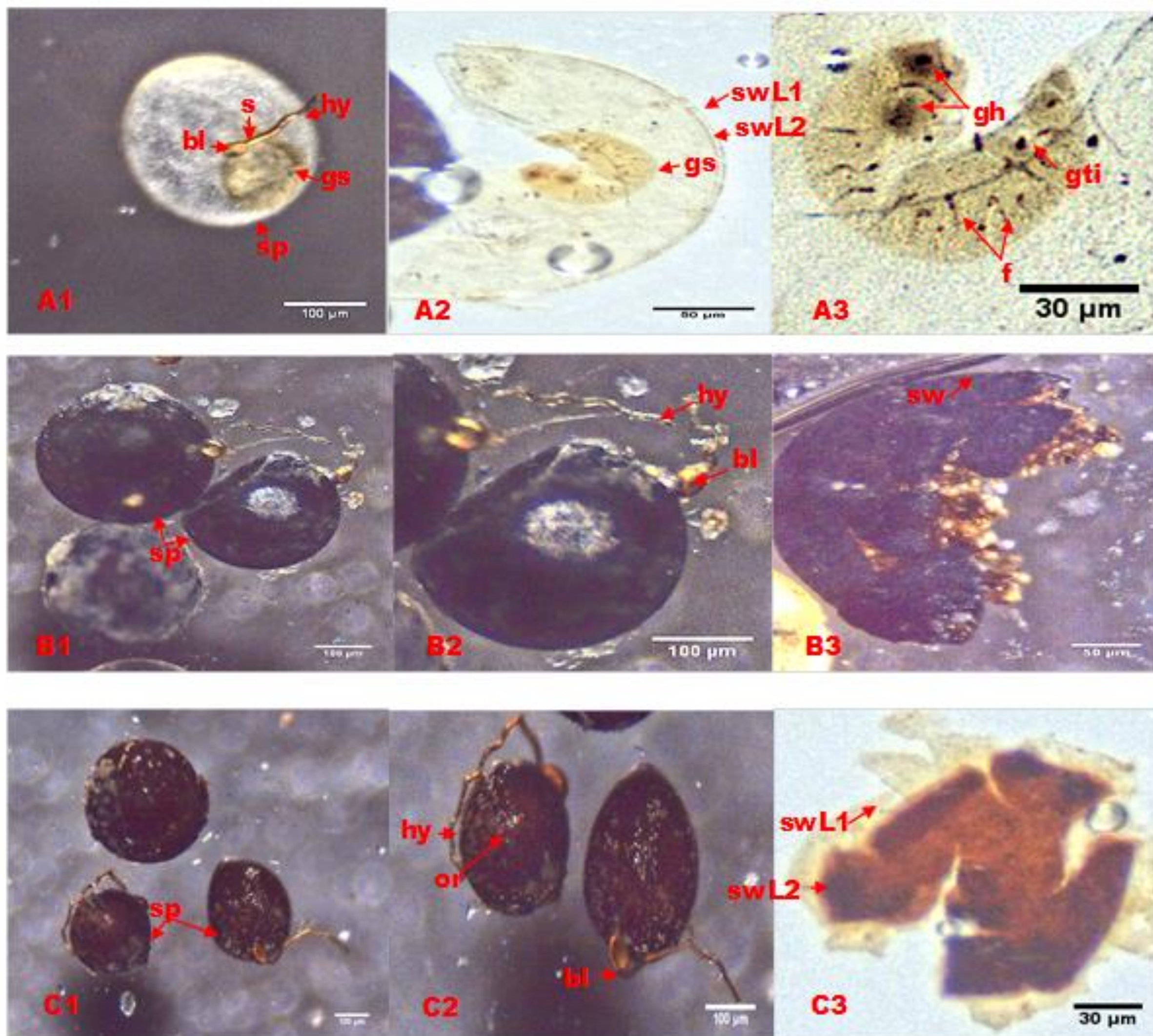

Figure 2. Morphological and anatomical characteristics of *Scutellospora* genus with; **A:** *Scutellospora cerradensis*; **B:** *Scutellospora gregaria*; **C:** *Scutellospora nigra* : **B1, C1:** isolate spores; **A1, B2, C2:** hyphal strurture; **A3:** germination shield; **B2, C3:** wall structure of spore in PVLG with Melzer. (bl) bulbous suspensor; (f) folds; (gh) germ hole; (gs) germination shield; (gti) germ tube initiation; (hy) subtending hypha; (or)ornament; (s) septum; (sp) whole spore ; (swL1) outer layer of spore wall; (swL2) inner layer of spore wall.

reach up to 250 µm in diameter (Figure 2-C1), with narrow and long hyphae (Figure 2C2). Unlike the latter, *Glomus* sp has a thin, cylindrical suspensory hypha, but it slightly thickens at the point of contact with the spore (Figure 2D2).

The *Scutellospora* genus has been represented by three species such as *Scutellospora cerradensis* [Spain and Miranda (Figure 3A)], *Scutellospora gregaria* [Schenck & Nicolson emend. Walker & Sanders (Figure 3B)] and *Scutellospora nigra* [Redhead emend. Walker & Sander (Figure 3C)]. Representing 26% of isolated spores (Table 4), these species are characterized by large spores with diameters ranged from 250 to 400 µm and by bulbous hyphae (Fig 3B2 and C2). Moreover, the main criterion of distinction of the different species is essentially based on the presence of germination shield (Figure 3A3) and whose structure is specific to each taxon.

Gigaspora margarita (Figure 3D) was the only species of the genus *Gigaspora*, isolated from the prospected soils. This is characterized by the presence of a bulbous suspensory hypha whose point of attachment to the spore opening out from inside the spore wall (Figure 3A2). The inner wall, also called the germinal wall (Figure 3A2) is thinner, smooth, flexible and less thick compared to the previous one; it surrounds a spore content rich in hyaline lipid elements more or less dense and rather concentrated after bursting of the spore.

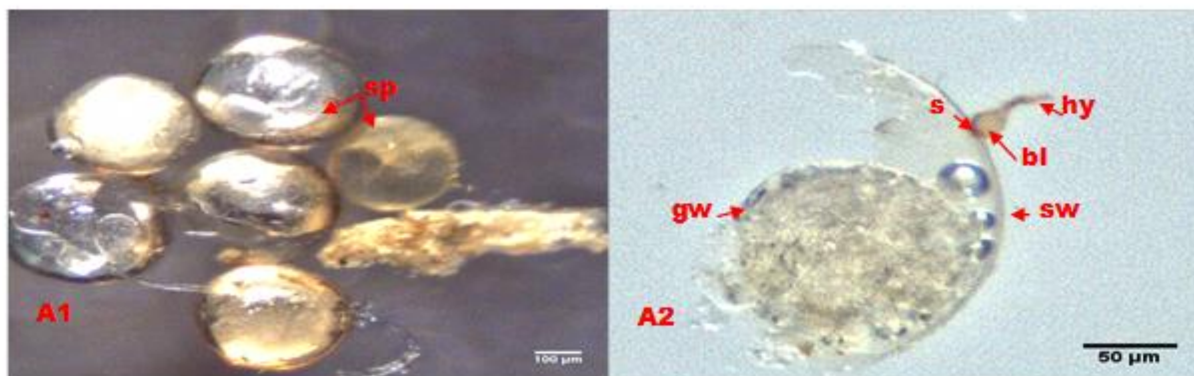

Figure 3. *Gigaspora margarita*: **A1:** isolate spores; **A2:** spore structure in PVLG with Melzer. (bl) bulbous suspensor; (gw) germination wall; (hy) subtending hypha; (s) septum; (sp) whole spore; (sw) spore wall.

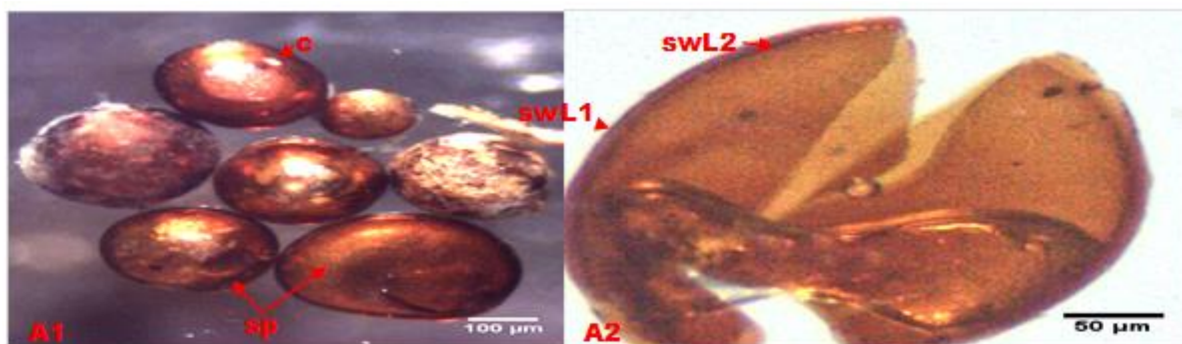

Figure 4. Morphological and anatomical characteristics of "Myco Brun-orange"spores, with: **A1:** isolated spores; **A2:** wall structure of spore in PVLG with Melzer ; (sp) whole spore ; (swL1) outer layer of spore wall; (swL2) inner layer of spore wall.

The *Acaulospora* genus constitutes 19% of isolated spores (Figure 5) and is represented by only two species whose identification is limited to the genus (*Acaulospora* sp.1 (Figure 5A) and *Acaulospora* sp.2 (Figure 5B)). Their spores appear sessile and the presence of a sporiferous saccule is one of their main characteristics (Figure 4A and B), as well as one or more germinal walls distinct from the spore walls were generally pore-dotted.

Despite the distinctive characteristics of "Myco Brown-orange" spores comparatively to other isolated spores, no correspondence with the strains described in the literature was found (Figure 4C). Representing 11% of isolated spores, with a shiny appearance; these spores were ovoid and sessile because they bore no attached hyphae.

Abundance and distribution of AMF

Whatever the study site, the number of spores present in 100 g of each soil prospected varied significantly ($P < 0.05$) according to the identified species (Table 5). Most of them have been found in almost all the sites sampled, but their numbers remain variable. *Acaulospora* sp.2 was the most abundant (144 spores / 100 g sol), but was only found in Dogba site; unlike *Glomus* sp. which was present in almost all the sites sampled, but in very low numbers (less than 20 spores / 100 g of soil). *G. hoi, S. gregaria, S. Nigra* and "Myco Brown-orange" spores were the most distributed species (100%), with populations of up to 60 to 80 spores / 100 g of soil. As for other species, they are unequally distributed in half of sampled sites and their numbers are between 4 to 50 spores / 100 g of soil.

Diversity of AMF communities

The diversity of isolated AMF in the study area varied from one site to another, but this remains low. Eleven species of AMC from a total of 1491 spores, unequally distributed in the prospected soils were identified (Table 6). The largest number of species was obtained in the

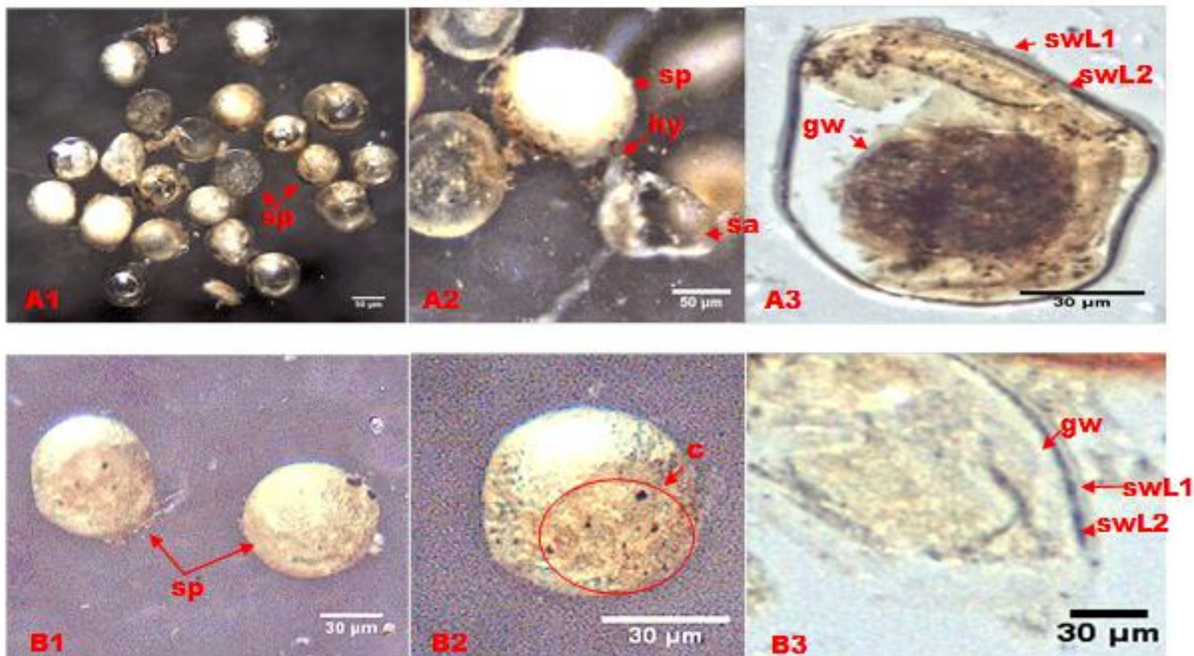

Figure 5. Morphological and anatomical characteristics of *Acaulospora* genus **A:** *Acaulospora* sp.1and **B :** *Acaulospora* sp.2 with: **A1, B1:** isolate spores; **A2, B2:** saccule structure on spore; **A3, B3:** wall structure of spore in PVLG with Melzer. (c) cicatrix; (hy) subtending hypha; (gw) germination wall; (sa) saccule; (sp) whole spore ; (swL1) outer layer of spore wall; (swL2) inner layer of spore wall.

Table 5. Average count of spores present in the soils sampled.

Species	Dargala	Dogba	Doukoula	Laf	Tokombéré	Zidim	Frequence of occurrence
Acaulospora sp.1	0	36±9^{bc}	30±3^{ab}	56±8e	26±4^{b}	0	66
Acaulospora sp.2	0	144±66d	0	0	0	0	16
Gigaspora margarita	0	16±4^{abc}	14±2^{ab}	16±2^{bc}d	0	0	50
Glomus aggregatum	42±6^{a}	15±2^{abc}	0	15±6^{bc}	0	25±10^{a}	66
G. hoi	50±44^{a}	66±2^{abc}	36±29^{b}	48±22e	52±30^{b}	27±12^{a}	100
G. manihotis	58±7^{a}	0	0	107±30f	0	0	50
Glomus sp.	0	8±7^{a}	9±3^{a}	8±5^{a}	8±1^{a}	18±5^{a}	83
Scutellospora cerradensis	27±4^{a}	0	0	13±3^{b}	0	0	50
S. gregaria	13±2^{a}	12±3^{ab}	25±8^{ab}	21±11^{c}d	12±5^{a}	30±5^{a}	100
S. nigra	29±8^{a}	52±8^{c}d	62±20^{c}	23±4d	31±22^{b}	41±22^{a}	100
Myco Brun-orange	7±4^{a}	83±77d	34±27^{b}	4±3^{a}	25±17^{b}	23±12^{a}	100
Total	226±75	432±178	210±92	310±94	154±79	164±66	
P-value	0.1163^{Ns}	0.0008***	0.0013**	<0.0001***	0.0009***	0.4122^{Ns}	

^{Ns}: not significant; *: significant ($P < 0.05$); **: very significant ($P < 0.01$); ***: very highly significant ($P < 0.001$). Values followed by the same letter on the same column are not significantly different according to the Duncan test at the 5% sill.

soil of Laf (10 species), followed by Dogba (9 species), Dargala (7) and Doukoula (7). In addition, the smallest number of species obtained came from the soils of Tokombere and Zidim which contained 6 and 5 species respectively. The values of the Shannon-Wiener index obtained according to the soils sampled were substantially equal, but low (not exceeding 0.82) with a very small variation. In addition, the highest value was obtained at Laf (0.82) and the lowest at Tokombere (0.7). As for the values of the Pielou Equitability Index, they are

Table 6. AMF diversity in sampled sites.

Sites	Rw	N	H'	EQ	S'
Dargala	7	225	0.77	0.74	0.81
Dogba	9	432	0.80	0.76	0.80
Doukoula	7	210	0.78	0.75	0.86
Laf	10	310	0.82	0.79	0.80
Tokombere	6	150	0.71	0.68	0.78
Zidim	5	164	0.76	0.73	0.82
Total	11	1491	/	/	/
Moyenne			0,77	0,74	0,81

Rw: Specific wealth; N: Total number of spores; H ': Shannon-Wiener index; EQ: Pielou Equitability Index; S: Simpson diversity index (1-D).

greater than 0.7 in all the prospected soils. This value is close to 1, indicating an almost equitable distribution of isolated species in the region. Nevertheless, the probability that two randomly selected individuals belong to different species varied from one site to another, but remained close to 1. The value of the lowest Simpson's index is obtained at Tokombere (0.78) and the largest in Doukoula (0.86).

Correlation between AMF parameters and physico-chemical properties of soils

Principal component analysis (PCA) was used to verify whether there is a relationship between species richness, total spore count and soil physico-chemical parameters, as well as root mycorrhizal parameters of trapping plants (Figure 6). In this model, the axes 1 and 2 describe respective variations of 32.21 and 30.14%, for a total variation of 62.35%. The first axis expressed the highest percentage variation and was positively correlated with the total number of spores and species richness. This correlation was also positive for the pH_{-H2O}, the silt (L), the organic matter (O.M), the total nitrogen (N), the assimilable potassium (K), as well as the intensity of mycorrhization (M). In relation to species richness, there was a significant correlation and a positive correlation with the total number of spores (r = 0.82), silt (r = 0.81) and total nitrogen (r = 0.82). Moreover, for the number of spores, this correlation was significantly negative ($P < 0.05$) with assimilable phosphorus (r = -0.88) and clay (r = -0.88).

DISCUSSION

Soils are usually complex and particular environments; the loss of one or more of their properties degrades their ability to produce biomass (Ouallal et al., 2018). In fact, the soils of the Far North region are generally acidic (Olina et al., 2008; Abakar et al., 2019) and the analysis of soil samples carried out in this study confirms this acidity status (pH = 4.98 to 6.5) according to the INRA (1995) pH interpretation standards. They are sandy-loamy in nature with an average predominance of sand. Furthermore, the detailed analysis of the distribution of mineral fractions reveals that the Doukoula soil is relatively rich in clay (56%), unlike the Dargala and Laf soil which are richer in silt (47 and 50% respectively). In fact, soils rich in colloids would be favorable for the formation of mycorrhizae (Ouallal et al., 2018), since they are generally more porous and less fertile, thus giving them good aeration; optimal condition of development of mycorrhizal fungi. However, the chemical parameters of the soils analyzed show that they are moderately rich in organic matter and nitrogen, but their phosphorus levels are still rather low according to the interpretation standards of the Calvet and Villemin (1986) soil analyzes. Since the plots sampled are agricultural areas, this wealth in organo-mineral element could be explained by the continual supply of organic amendment by the farmars. Moreover, the phosphorus level found in the Dogba soil could negatively influence the presence of arbuscular mycorrhizal fungi (AMF). According to Gosling et al. (2006), increased phosphorus significantly reduces fungal colonization, density and spore diversity in the soil.

In the directly sampled soils samples, not all spores are always represented (Zézé et al., 2007). Most of the AMF isolated directly from the sampled soils are poorly diversified and represent only those whose root colonization activity is sufficiently important for sporulation and to produce biomass especially in arid and semi-arid condition (Morton et al., 1993). Therefore, trapping is undertaken to create favorable conditions for the sporulation of all native species found there and to obtain viable and healthy spores for better observation (Straker et al. 2010). Thus, microscopic examination of maize roots (*Z. mays* L.) used as a trap plant revealed the presence of mycorrhizal structures, reflecting the mycotrophic nature of this species on the one hand, and

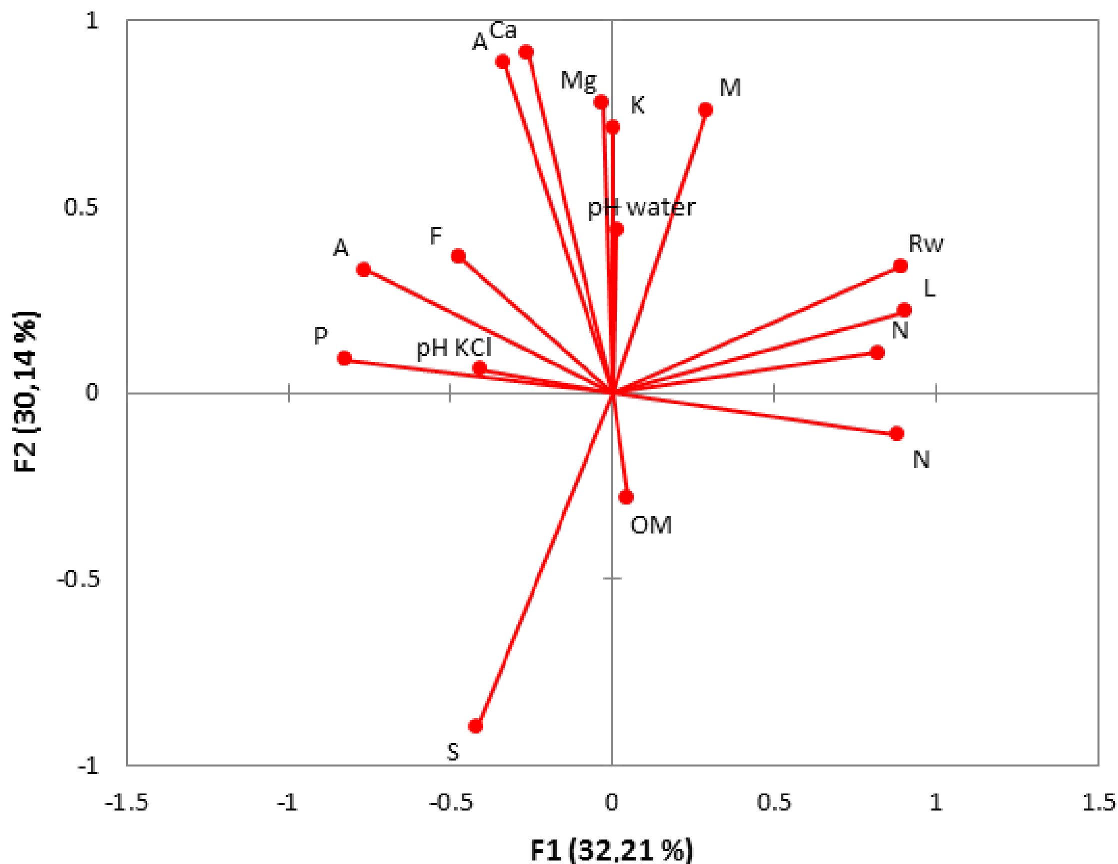

Figure 6. Graphical correlation between AMF parameters and physico-chimical properties of investigated soils; with: A: argile; L: slit; OM: organic matter; S: Sand; Rw: Specific wealth; N: Total number of spores.(axis F1 and F2: 62.35%)

the effective presence of AMF in the different soils. The main mycorrhizal structure observed in the totality of the root samples was of the type "arum" according to Gallaud's classification (1905) cited by Sidhoum (2011). In fact, this arbuscular mycorrhiza is dominant in most cultivated plants and is characterized by a rapid expansion of the fungus, facilitating the transfer of nutrients into the plant (Dickson et al., 2007). Despite the fact that the quantified mycorrhizal frequencies were very high, the ANOVA found no change in soil factor for all evaluated mycorrhizal parameters. Although mycorrhizal intensities and arbuscular levels are moderately low, they largely exceed those obtained by Tobolbai et al. (2018), who obtained mycorrhization intensities ranging from 1 to 14% only in maize cultivation in Diamaré (Far North Cameroon), as well as those obtained by Bossou et al. (2019) on maize cultivation in the Benin cotton zone (around 2.47%).

The abundance of isolated AMF spores showed variability among the sampled sites. This abundance was higher in the Dogba site (432 spores per 100 g soil) and lower in the Tokombere site (154 spores per 100 g soil). These numbers are comparable to those obtained by Tobolbai et al. (2018) on maize cultivation in the Far-North Cameroon region; as well as those obtained by Begoude et al. (2016) in the southern and eastern regions of Cameroon and by Haougui et al. (2013) in Niger's market gardening zone. But these remained very low compared to those obtained by Bossou et al. (2019) in the cotton zone of Benin with 12501 spores per 100 g of soil; as well as those obtained by Temegne et al. (2017) on the rhizosphere of peanut in the region of Central Cameroon. Indeed, all the sites sampled were characterized by a low level of soil fertility and the cultural practices were essentially based on a strong use of chemical amendments which leads to a strong reduction in root colonization, as well as the density of spores in the soil. In addition, the variation in AMF spore abundance among different sites could be due to vegetation, host specificity between AMF and plants, and the sporulation ability that is specific to each species from AMF (Husband et al., 2002).

Moreover, the analysis of morpho-anatomical characteristics of isolated spores in our study revealed

the presence of ten species belonging to three families Acaulosporaceae (2), Gigasporaceae (4) and Glomaceae (4), unequally distributed in two orders, that of Diversisporales and that of Glomerales (Redecker et al., 2013). This species richness is much higher than that obtained for maize cultivation in the same study area by Tobolbai et al. (2018), but remained comparable to that obtained by Bossou *et al.* (2019) in the Benin cotton zone. On the other hand, it remained low compared to those obtained in natural environments, such as in the forest zone of Cameroon (Musoko et al., 1994; Onguéné et al., 2002, Ngonkeu et al., 2013; Tchinmegni et al., 2016). Indeed, Tchabi et al. (2008) found that the species richness of AMF in natural forests is higher than in agricultural plots. This low abundance and specific wealth obtained may be due to the different agricultural practices carried out in the study area, because the cotton growing in this part of Cameroon is characterized by a strong use of mineral fertilizers, as well as by a continual turnover of soil by animal traction. Peyret-Guzzon (2014) has shown that cultural practices such as soil turnaround and especially chemical fertilization cause significant and considerable changes in the composition of AMF communities in soils.

Of the four genera identified, the description of *Glomus* species was facilitated by their cosmopolitan character, but also by their mode of anchoring in the soil. In this taxonomic genus, most species have been isolated in the form of compact (*G. aggregatum*) and free (*G. hoi*) sporocarps. This predominance has been reported in several studies in plots grown in Cameroon (Mbogne et al., 2015; Begoude et al., 2016; Temegne et al., 2017; Tobolbaï et al., 2018), in West Africa (Tchabi et al., 2008; Haougui et al., 2013; Johnson et al., 2013; Voko et al., 2013; Nandjui, 2015; Bossou et al., 2019), and in Eastern and Southern Africa (Jefwa et al., 2009; Straker et al., 2010). Indeed, several authors have associated the dominance of *Glomus* spp. by their rapid multiplication capacity and their better ability to adapt to the most hostile environmental conditions such as drought, extreme pH and other environmental stresses (Blaszkowski et al., 2002). Among the species identified in this genus, *Glomus manihotis*, also known as *Rhizophagus manihotis* (Schüßler and Walker, 2010) or *Rhizoglomus manihotis* (Sieverding et al., 2014), was the most abundant species in the study area despite its low distribution (50%). In sub-Saharan Africa, very few authors have reported its presence in the rhizosphere of cultivated plants, but the work carried out by Tobolbaï et al. (2018) have revealed its presence in the far north of Cameroon. Unlike the latter, *G. hoi*, still known under the name of *Simiglomus hoi* (Oehl et al., 2011), was encountered in all the sites surveyed. Like the previous species, its presence in the cultivated soils was only very little reported, because it is morphologically similar to *Glomus deserticola* present in many ecosystems (Talbi et al., 2014). In addition, the genera *Acaulospsora*, *Gigaspora* and *Scutellospora* have also been identified in culture medium in Cameroon by Ngonkeu et al. (2013), Mbogne et al. (2015) and Temegne et al. (2017). Three species were identified in the *Scutellospora* genus, with *Sctellospora ceradensis* comparable to those isolated by Straker et al. (2010) in the rhizosphere soils of cassava cultivation in South Africa; *S. gregaria* by Diallo (1998) and Belay et al. (2013) in Sudano-Sahelian area of Senegal and Ethiopia, respectively and *S. nigra* which has been identified in the soil of some plantations in Kenya by Jefwa et al. (2009). Concerning *Gigaspora*, only a poorly distributed species (50%) was isolated from the sites surveyed (*Gigaspora margarita*). Indeed, this genus is strongly represented in arid or semi-arid tropical zones (Diallo, 1998) and *G. margarita* for its part is a species highly represented in acid soils in Cameroon; it would promote the tolerance of plants sensitive to soil acidity (Ngonkeu et al., 2013). In addition, previous work on maize cultivation in the same study area by Tobolbaï et al. (2018) mentioned the absence of species belonging to *G. margarita*, but it has been identified in the cassava rhizosphere cultivated in the eastern and southern regions of Cameroon and peanuts grown in the Central Cameroon region by Temegne et al. (2017) then joining the assertion of Bossou et al. (2019) that the species richness of AMF soil varied according to the type of crop.

However, the identification and interpretation of the structural composition of spores has not been easy for some AMF, like *Acaulospora* spp. and *Glomus* sp. The observation and structural description of their spores was not sufficient to match those described in the literature. These could constitute fungal isolates specific to the study area, especially for *Acaulospora* sp.2 which is only found in one site and at high density.

The analysis of the diversity of isolated AMF in the study area, varied from one site to another, but this remained low throughout the region. Although the Shonnon-Wiener index evaluated in this study (0.77) was larger than that obtained by Tobolbai et al. (2018) in the Far-North Cameroon (0.45); these remained very low compared to that obtained by Bossou et al. (2019) in the Benin cotton zone (2.12); as well as compared to that obtained by Temegne et al. (2017) in humid zone in Cameroon (1.94). This low diversity of AMF in this zone could be due mainly to different cultural practices such as mineral fertilization or fungicide input, as well as to the harsh climate. Several studies have shown that intensive use of arable land, as well as cropping systems, strongly influenced soil AMF diversity and presence (Oehl et al., 2003). Indeed, Marschner et al. (2003), Vestberg et al. (2005), Gosling et al. (2006) and Borriello et al. (2012) have shown that these cultural practices led to significant changes in the composition of AMF communities. The principal component analysis conducted in this study confirmed these results, while showing how these factors influenced the presence and activity of AMF in the soil.

A significant correlation among species richness, total

number of spores, silt, total nitrogen assimilable phosphorus and clay. But this was negative among the total number of spores, the assimilable phosphorus and the rate of clay; as well as between the total nitrogen and the arbuscular content of the roots. Similar results have also been reported by Mohammad et al. (2003) and Nehila (2016). In the cultivated fields, the number of spores seems to reach a maximum under conditions where the phosphate applications, necessary for the maximum growth of plants were the least (Mekahlia, 2014). According to Juniper and Abbott (1993), high levels of phosphorus in soil prevent some AMF from providing substantial benefits to host plants and may affect the distribution and density of these fungi. This has also been confirmed by Balzergue et al. (2011) who showed that phosphorus is able to almost completely inhibit mycorhization at a very early stage, even before attachment of the fungus to the surface of the root epidermis.

Conclusion

The present study was carried out with the aim of highlighting the diversity of arbuscular mycorrhizal fungi (AMF) associated with the rhizosphere of cotton grown in the Far North Cameroon region. Through the results obtained, it appears that this rhizosphere has a specific diversity of native AMF; but it remains weak. Analysis of corn root fragments used here as trap plant was densely mycorrhized, but with low arbuscular content. Of the 11 spores species isolated from the different sites surveyed (6 sites), 10 of them were associated with the genera *Acaulospora, Gigaspora, Glomus* and *Scutéllospora*, with a dominant species of the genus *Glomus*. One type of spore was not identified in this study; this could thus show that there may be strains of some fungal specific to this area. These results could thus induce a controlled production of local fungal inoculum, adapted to the edapho-climatic conditions of the region and indirectly boosted the agricultural production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Phenotypic detection of extended spectrum β-lactamase and Metallo β-lactamase production by Gram negative uropathogens after exposure to gamma radiation

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Eighty-five uropathogen isolates were collected and differentiated on the basis of their Gram stain reaction. Among the collected isolates, 25 (29.4%) were Gram positive cocci and 60 (70.6%) were Gram negative bacilli. Antibiotic susceptibility profile towards 15 different antibiotics concluded that impenime (IMP), amikacin (AK) and Pipperacillin/tazobactam (TZP) were the most effective against Gram negative; while linezolid (LZD), and vancomycin (VA) were the most potent against Gram positive. The highly drug resistant isolates were identified as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* using 16srRNA; the two strains were subjected to different doses of gamma-radiation and the sub lethal dose of both strains was 5.0 kGy. The D_{10} values were recorded (1.2 and 1.1 kGy) for *P. aeruginosa* and *K. pneumoniae),* respectively. In addition, gamma-irradiation technique decreases the resistance of *K. pneumoniae* towards IMP and the resistance of *P. aeruginosa* towards more than one antibiotic (TZP, AZM and CIP). The results also, revealed that both of the tested strains had the ability to produce Extended Spectrum β-lactamase (ESBL) before and after gamma-radiation but only *P. aeruginosa* had the ability to produce Metallo β-lactamase (MBL), that is, ESBL and MBL co-production was detected in *P. aeruginosa.* So, these findings must be supported by other studies on the level of genes to prove the possibility for using gamma-irradiation technique to overcome microbial drug resistance problem.

Key words: Urinary tract infection, antibiotic resistance, β-lactamases, gamma radiation.

INTRODUCTION

Urinary tract infection (UTI) is one of the most common human bacterial infections both in the community and hospital setting (the health care settings) and accounts for one-third of all nosocomial infections (Dougnon et al., 2020). Infection of urinary tract marked a range of severity that spans from mild self-limiting infection to life threatening systemic disease (Lee and Kim, 2015; Klein and Hultgren, 2020). UTI causes many urinary disorders as urosepsis, renal scarring and progressive kidney damage that lead to a high health risk with high mortality, morbidity and cost a significant financial burden (Gashti et al., 2020).

Emergence of multidrug-resistance bacteria has become a major problem (Woc-Colburn and Godinez, 2020). Resistance rates vary across countries because of differences in antimicrobial agent usage and systems for the prevention of antimicrobial resistant bacteria. In addition to resistance rates, modes of resistance also differ among countries and even among cities within the same country (Kim et al., 2017).

In developing countries, the increase in resistance including third- and fourth-generation drugs like cephalosporins, penicillins and fluoroquinolones may be due to their extended use and purchase directly from the pharmacies without doctors' prescription as self-medication is a common practice (Mohammed et al., 2016).

The World Health Organization has categorized antibiotic resistance as one of the three most significant severe public health problem of the 21st century (WHO, 2014). The infections which are caused by multidrug-resistant Gram negative bacilli that produce various β-lactamase enzymes have been reported with an increasing frequency and are associated with a significant morbidity and mortality (Deshmukh et al., 2011).

Extended spectrum beta-lactamase (ESBL) producing organisms are those that hydrolyse the oxyimino beta-lactams and monobactams, but have no effect on the cephamycins and carbapenems (Ghafourian et al., 2014). Detection of ESBL producers from samples such as urine may be of highest importance because this represents an epidemiologic sign of colonization and therefore there is potential for transfer of such organisms to other patients (Aggarwal et al., 2008).

The production of hydrolytic β-lactamase enzymes is the most prevalent resistance mechanism towards β-lactam antibiotics. Metallo-β-lactamases constitute a troublesome group of enzymes, since they present a broad-spectrum profile, hydrolyse penicillins, cephalosporins and carbapenems, but not monobactams e.g.: aztreonam. Carbapenem antibiotics are currently used as the last choice for treatment of the infections caused by multidrug-resistant Gram-negative bacteria. The mortality rate associated with MBL producers is reported to be from 18 to 67% (Adam and Elhag, 2018).

Hence, this study aimed to: (1) determine the antibiotic resistance pattern of the uropathogen collected isolates (2) investigate the sensitivity of the most resistant isolates to gamma radiation. (3) Evaluate the effect of gamma radiation on the antibiotic susceptibility, and finally 4) investigate the prevalence of ESBLs and MBL among the highly drug resistant isolates by phenotypic method before and after gamma irradiation treatment.

MATERIALS AND METHODS

Bacterial isolates and cultivation media

Eighty- five isolates including 25 isolates Gram positive cocci and 60 Gram negative bacilli were obtained over 8-month period from different specialized hospitals in Great Cairo, Egypt, from UTIs samples. The tested isolates were cultivated in a standard laboratory culture media. Tryptone Glucose Yeast Extract Agar (TGY), Nutrient agar (NA), Blood agar (BA) and MacConkey agar were purchased from Difco [Difco Labs, Detroit, Michigan (USA)]. Muller Hinton Agar (MHA) were obtained from Oxoid (Oxoid. comp., Basigstoke, Hants, UK)].

Purification of bacterial isolates

The bacterial isolates were streaked for several consecutive times on nutrient agar medium until pure single colonies were obtained. The colonies were then isolated and checked by microscopic examination using Gram's stain.

Antibiotic resistance profile

Antibiotic susceptibility testing was done using the Kirby-Bauer disc diffusion method (Bauer et al., 1966) on Mueller Hinton (Oxoid) agar according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2017). The following antimicrobial discs (µg) were used: ciprofloxacin (CIP5), aztreonam (ATM10), ceftriaxone (CRO 30), carbenicillin (PY100), vancomycin (VA30), Linezolid (LZD30), amikacin (AK30), sulphamethoxazole-trimethoprim (SXT25), tobramycin (TOB10), ceftazidime (CAZ 30), gentamicin (CN10), azithromycin (AZM15), cefepime (FEP 30), piperacillin tazobactam (TZP110) and imipenem (IPM10). Bacterial colonies from a pure overnight culture were suspended in 2 ml of 0.85% NaCl in order to maintain the bacterial strains in osmotic equilibrium and the bacterial suspension was standardized to 0.5 McFarland (10^7 CFU/ml). The suspension was inoculated on Mueller Hinton agar using a sterile swab and antimicrobial agents are placed onto the surface of the agar and incubated at 37°C for 24 h. A zone of inhibition was measured and the results were interpreted as sensitive, resistant, or intermediate based on resistance data interpreted according to Clinical and Laboratory Standards Institute (Franklin et al., 2012); thereafter, the experiment was repeated in triplicate. The tested isolates which revealed 100% resistance were selected for the following studies.

Molecular identification of the most MDR bacterial isolates

The most resistant bacterial isolates (two) were biochemically identified previously, and then the identification of these selected bacteria was confirmed using 16s rRNA sequencing according to a methodology published previously (James, 2010). Briefly, the total bacterial DNA was purified from each strain using Wizard genomic DNA purification system (#A1120, Promega Corporation, USA) according to manufacturer instructions. Universal 16s primers were used for PCR amplification of 16s rRNA of the purified bacterial genomes; the forward primer is 8f (5' AGA GTT TGA TCC TGG

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CTC AG -3'), and the reverse primer is U1492R (5' GGT TAC CTT GTT ACG ACT T -3'). PCR procedures were carried out using the instrumentations in the centre of Virology, Cairo University, Cairo, Egypt.

Irradiation process

The irradiation process was achieved by using Cobalt 60 (^{60}Co) Gamma Cell GC 220, product of Canada Co. Ltd. located at the National Centre for Radiation Research and Technology (NCRRT) Cairo, Egypt. Irradiation process was achieved at ambient temperature. The dose rate of this source was 1.538 (kGy/h) at the time of the experiment.

Effect of different doses of gamma-radiation on viability of the selected strains (D_{10} value determination)

The two selected multidrug resistant strains; *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were cultured and prepared in sterile normal saline solution. Five ml aliquots were distributed in sterile plug capped test tubes and exposed to gamma radiation at level doses of (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 kGy). After irradiation, appropriate dilution of 0.1 ml of the un-irradiated bacterial cells (control) and irradiated ones were serially diluted and plated onto nutrient agar plates. The plates were incubated for 24 h at 37°C. The radiation dose-response curves were graphically represented for irradiated and un–irradiated cells.

Radiation dose-response curves

Response to gamma-irradiation was expressed as the logarithm of the ratio of survivors (N/N0), where N represents the mean CFUml^{-1} of irradiated bacterial suspension and N0 the mean number of CFU ml^{-1} of un-irradiated control. D_{10} values, defined as the radiation dose (kGy) required to reduce the number of CFU ml^{-1} by one log_{10} were determined by calculating the negative reciprocal of the slope of the linear regression curve (Rajkowski et al., 2003).

Evaluation of the effect of gamma-radiation on the antibiotic susceptibility

Antibiogram test was performed to record the sensitivity or resistance of tested strains after gamma-irradiation treatment as mentioned above.

Phenotypic detection of β-lactamases

Detection of extended spectrum β- Lactamases (ESBL) production

The strains that showed resistance towards at least of the third generation cephalosporins (ceftazidime, cefotaxime and ceftriaxone) were tested for ESBL production by double disc synergy test and phenotypic confirmation test recommended by CLSI as reported by Jarlier et al. (1988).

Double disk synergy test (DDST)

Test inoculum was spread by lawn culture on Muller Hinton Agar (MHA). Antibiotic discs of amoxicillin clavulanate (20/10 µg) (augmentin) was placed at the centre and three different antibiotics including cefotaxime, ceftazidime, ceftriaxone and aztreonam were placed at different distances (10, 25 and 30 mm) (centre to centre). The plates were incubated overnight at 37°C. Distance was maintained properly in order to accurately detect the synergy. Any distortion or increase in the zone of inhibition of three antibiotic discs towards the augmentin disc was considered as positive for the ESBL production.

Phenotypic confirmatory disc diffusion test

Combined disc method

This method was designed by Jacoby and Han (1996). In this experiment, cefoperazone (CFP) (75 µg) and cefoperazone/sulbactam (SCF) (75 µg/30 µg) were used. An increase of 5 mm or more in the zone of inhibition in a disc containing sulbactam compared to the drug alone was considered as a positive test for the presence of β- lactamase enzyme.

Metallo β-lactamase production

This test was detected by Imipenem-EDTA combined disc test which was described by Behera et al. (2008). *P. aeruginosa* and *K. pneumoniae* were inoculated onto Mueller-Hinton agar as lawn culture. Two 10 µg IPM discs were placed at 20 mm centre to centre on the plate, and 10 µl of 0.5 M EDTA solution was added to one of them. After 18 h of incubation at 35°C, zone of inhibition of Imipenem + EDTA disc compared to that of Imipenem disc alone. If the increase in inhibition zone was ≥ 7 mm, then the tested organism was considered to be MBL producer.

Effect of gamma-radiation on the (ESBL) and (MBL) production

In this experiment, the tested strains which were considered as ESBL and/or MBL producers were subjected to different doses of gamma radiation, thereafter (ESBL) and (MBL) production was detected as mentioned above.

RESULTS AND DISCUSSION

The infections with MDR uropathogens have become significantly challenging due to their high resistance to commonly used antibiotics. In the present study, eighty-five uropathogenic isolates were collected and identified on the basis of their morphological characters and Gram stain reaction. The results revealed that among all the examined isolates, 25 isolates (29.4%) were Gram positive cocci and 60 (70.6%) were Gram negative bacilli or short rods.

Antibiotic resistance profile of the isolated bacteria towards 15 different standard antibiotics has been shown in Table 1. The results showed that out of eighty-five isolates, 63 and 65 isolates (74.1 and 65.9%) were resistant to (CRO and FEP) and 66 isolates (77.6%) were resistant to AZM, whereas 69 and 70 isolates (81.2 and 82.3%) were resistant to CAZ and SXT. Out of 60 Gram-negative uropathogens, 59 isolates showed extreme resistance rate (98.3%) towards PY while high degree of resistance was observed towards ATM (beta-lactam

Table 1. Antibiotic resistance profile of the collected clinical isolates.

Tested antibiotics	No. of resistant isolates (from 85 isolates)	Percentage of resistance
*ATM10	45 (from 60 isolates) G-ve	75.0
CRO30	63	74.1
TOB10	55	64.7
AK 30	30	35.8
CAZ30	69	81.2
CN 10	46	54.1
*PY100	59 (from 60 isolates) G-ve	98.3
FEP 30	65	65.9
IMP 10	21	24.7
**VA 30	6.0 (from 25 isolates) G+ve	24.0
AZM 15	66	77.6
CIP 5	41	48.2
SXT 25	70	82.3
TZP 110	42	49.4
**LZD 30	7.0 (from 25 isolates) G+ve	28.0

ATM10, Aztreonam; CRO 30, Ceftriaxone; TOB10, Tobramycin; AK30, Amikacin; CAZ 30, Ceftazidime; CN10, Gentamicin; PY100, Carbenicillin; FEP 30, Cefepime; IPM10, Imipenem; VA30, Vancomycin; AZM15, Azithromycin; CIP5, Ciprofloxacin; SXT25, Sulphamethoxazole-trimethoprim; TZP110, Piperacillin tazobactam; and LZD30, Linezolid. *ATM & PY, used only with G-ve isolates; **VA& LZD, used only with G+ve isolates.

agents) with resistance rate of 75%. Among Gram positive uropathogens (25 isolates), the isolates showed low resistance rate towards VA and LZD (24 and 28%).

In general, IMP, AK, CIP and TZP were the most effective drugs towards Gram negative isolates while, VA and LZD were most effective against Gram positive isolates.

The results in this study showed that the isolated uropathogens were mostly resistant to 3rd-generation cephalosporins including CAZ, CRO and FEP (which considered extended β-lactam agents) than to non-β-lactam agents such as CIP and CN. This result is in agreement with that of Bhatt et al. (2017) who reported that β-lactams, and fluoroquinolones antibiotics have limited value for the treatment of UTI infected by Gram negative bacteria and there was extreme resistance towards CAZ, FEP and CRO higher than CN.

Talbot (2013) stated that the resistance of bacteria against β-lactam agents may be a result of numerous mechanisms comprising modification of penicillin-binding proteins, loss of porins, overexpression of efflux pumps or production of β-lactamases.

The results in this study were in accordance with those of Salleh et al. (2017) who reported that, all the isolated uropathogens (Gram negative isolates) showed high sensitivity to AK, IMP and TZP.

The data of antimicrobial resistance profile of this study are also consistent with many previously reported studies (Joly-Guillou et al., 2010; Abujnah et al., 2015) who declared that AK, IMP were highly effective against Gram negative bacteria which are highly resistant to cephalosporins (first, second and somewhat third generations) and penicillins.

Regarding the tested Gram positive isolates, the data revealed that VA and LZD were the strongest agents and this agreed with the results of Bhatt et al. (2017) who reported that VA and LZD were found to be the most effective against Gram-positive isolates.

The antibiotic susceptibility profile of the eighty five isolates indicated that two isolates were 100% resistant against all the tested antibiotics (data not shown).

Molecular identification of the selected isolates using 16s rRNA:

Neighbour-joining phylogeny trees of the output result of BLAST indicated that the submitted gene corresponding to rRNA sequence is identical by 100% to *P. aeruginosa* strain 127 16s ribosomal RNA gene, partial sequence (Figure 1) and by 99% to *K. pneumoniae* strain MLST-15 16s ribosomal RNA gene, partial sequence (Figure 2).

Effect of gamma radiation on the viability of the tested strains

The relative sensitivity or resistance of different microorganisms to ionizing radiation is based on their respective D_{10}-value. D_{10}-value is the ionizing radiation dose required to kill 90% of the total viable number of microorganisms (Niemira et al., 2006; Aquino, 2012). Lower D_{10}-values indicate greater sensitivity of the organism to ionizing radiation.

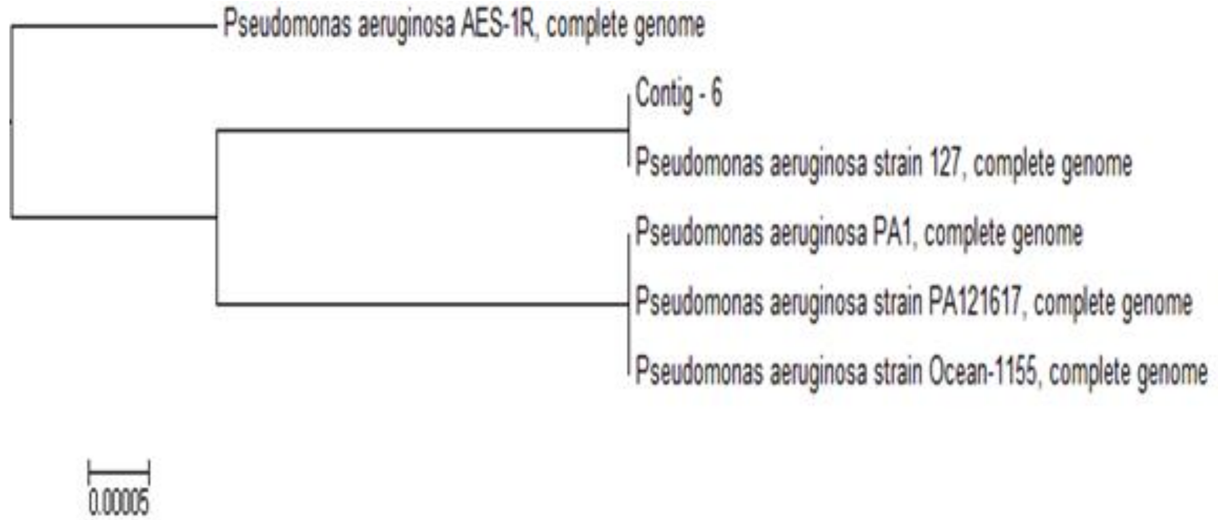

Figure 1. The phylogeny tree of. *P. aeruginosa* PCR molecular identification.

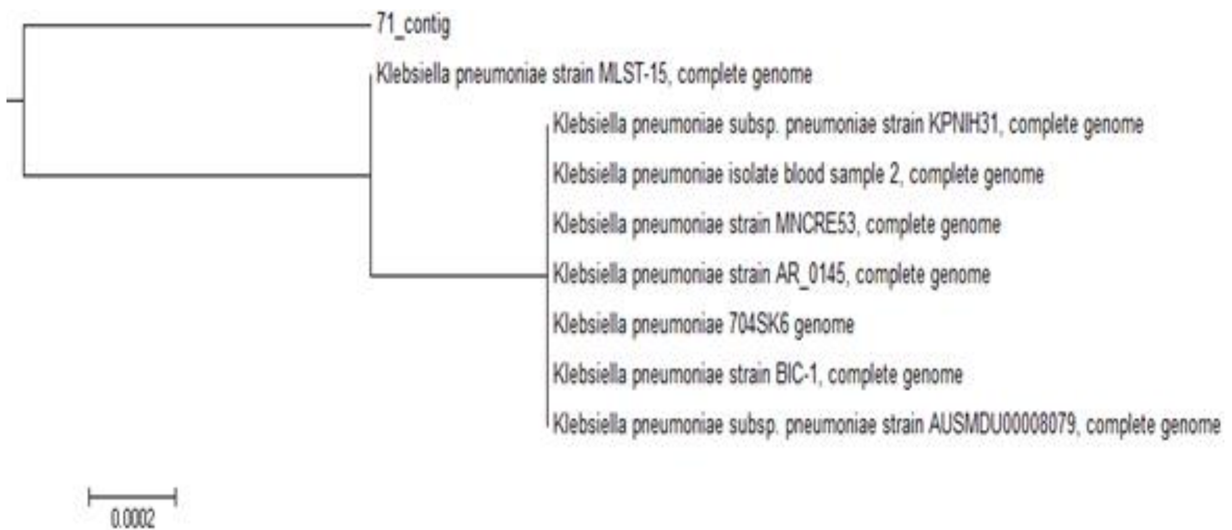

Figure 2. The phylogeny tree of *K. pneumoniae* PCR molecular identification.

In this experiment, the effects of gamma radiation at different exposure doses (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 kGy) on the viability of *P. aeruginosa* and *K. pneumoniae* was tested; thereafter the dose response curves were graphically represented as shown in Figures 3 and 4.

The survival number of irradiated cells at each irradiated dose after incubation for 18 h. at 35± 2°C was calculated. Curve was plotted between log survival numbers against different doses. The results for both strains showed that, by increasing the radiation doses, the total bacterial counts gradually decreased when compared with un-irradiated one (control). The lethal dose at which there was no growth of the tested strains was 6.0 kGy for both strains. The calculated D_{10} values were recorded (1.1 and 1.2 kGy) for *P. aeruginosa* and *K. pneuomoniae*, respectively.

Generally, the decrease in population of *P. aeruginosa* and *K. pneumoniae* in this study was probably due to the effect of energy produced from increasing doses of irradiation, which might have broken the bonds in the DNA molecules, leading to inability of microorganisms to replicate and reproduce resulting in bacterial death (Gillard et al., 2007).

Ionizing radiation induces damage to DNA by both direct energy deposition in DNA (direct effect) and by generating reactive species from the radiolysis of water

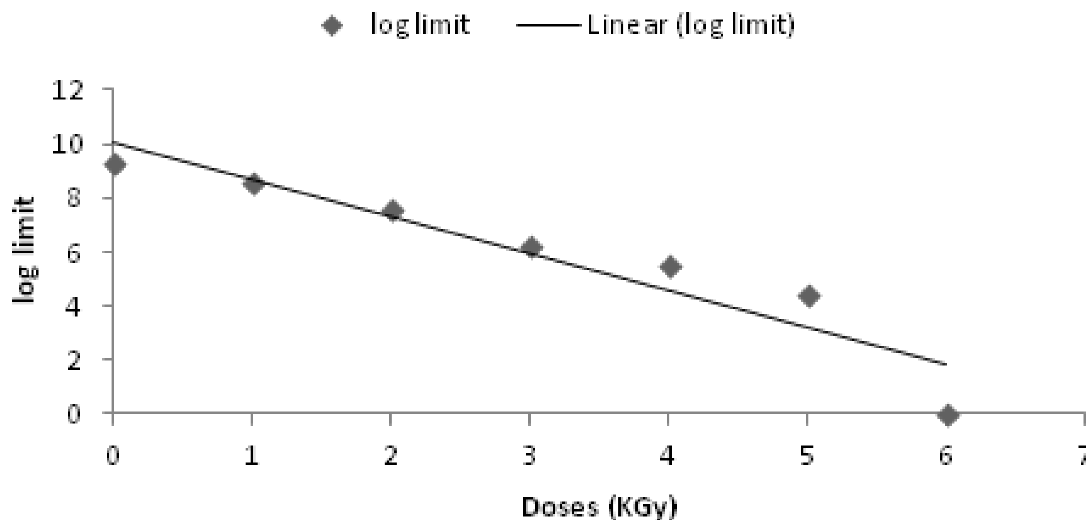

Figure 3. Effect of different doses of gamma-radiation on the viability of *P. aeruginosa*.

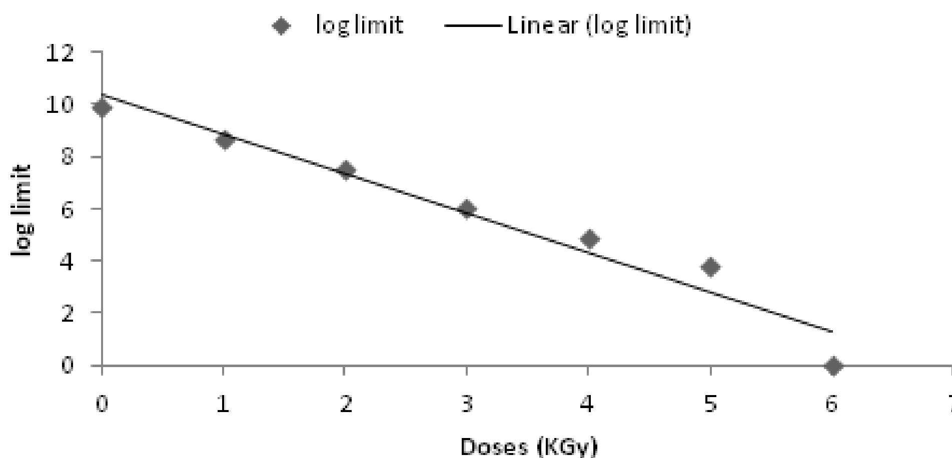

Figure 4. Effect of different doses of gamma-radiation on the viability of *K. pneumonia.*

and other biomolecules surrounding the DNA (indirect effect), which subsequently react with DNA (Kuefner et al., 2015; Sage and Shikazono, 2017). The lethal effect of the ionizing radiation was associated with other bacteria to an oxidizing stress due to the presence of reactive oxygen species (Pouget et al., 2002).

Regarding *K. pneumoniae*, it was observed that from Figure 4 which showed resistance towards gamma-radiation, this may be due to the wall composition of its capsule which consists mainly of a heavily packed accumulation of fine fibers, and represented a polymer of capsular polysaccharide with approximate layer thickness of 160 nm (Amako et al., 1988; Sridhar-Rao et al., 2008). Meanwhile, Atique et al. (2013) showed that, lower D_{10} (0.60 to 0.74 kGy) values were recorded for Gram negative *Pseudomonas* spp (associated with human amniotic membrane), their sublethal dose for *P. aeruginosa* was from 4-5 kGy and all Gram negative isolates were found to be killed after 5 kGy.

Effect of gamma-radiation on the antibiotic sensitivity

In order to determine the biological effects of gamma-radiation on the antibiotic susceptibility to the selected strains, a standardized bioassay using the disc diffusion method was applied. Bacterial cells in liquid culture were exposed to 1.0, 2.0, 3.0, 4.0 and 5.0 kGy of gamma-radiation. Antibiograms were performed and the diameter of inhibition zones of the exposed cells and un-irradiated

Table 2. Antibiotic susceptibility pattern for *P. aeruginosa* before and after treatment with different doses of gamma- radiation.

Dose (kGy)	ATM	CRO	TOB	AK	CAZ	CN	PY	FEP	IMP	AZM	CIP	SXT	TZP
C	R	R	R	R	R	R	R	R	R	R	R	R	R
1.0	R	R	R	R	R	R	R	R	R	R	R	R	30/S
2.0	R	R	R	R	R	R	R	R	R	R	21/S	R	S
3.0	R	R	R	R	R	R	R	R	R	30/S	S	R	S
4.0	R	R	R	R	R	R	R	R	R	S	S	R	S
5.0	R	R	R	R	R	R	R	R	R	S	S	R	S

C- Control (un-irradiated), R- resistant, I- intermediate, S- sensitive.

Table 3. Antibiotic susceptibility pattern for *K. pneumoniae* before and after treatment with different doses of gamma-radiation.

Dose (kGy)	ATM	CRO	TOB	AK	CAZ	CN	PY	FEP	IMP	AZM	CIP	SXT	TZP
C	R	R	R	R	R	R	R	R	R	R	R	R	R
1.0	R	R	R	R	R	R	R	R	26/S	15/I	R	12/I	R
2.0	R	R	R	R	R	R	R	R	34/S	I	R	I	R
3.0	R	R	R	R	R	R	R	R	S	I	R	I	R
4.0	R	R	R	R	R	R	R	R	S	I	R	I	R
5.0	R	R	R	R	R	R	R	R	S	I	R	I	R

C- Control (un-irradiated), R- resistant, I- intermediate, S- sensitive.

ones (control) for each dose was determined. The sensitivity test was carried out twice for each strain.

From Table 2, it is obvious that the sensitivity of the tested strains towards antibiotics depends on gamma-radiation dose and the type of antibiotic. The results also revealed that exposure of *P. aeruginosa* to gamma-radiation changed its sensitivity towards CIP, AZM and TZP from resistant to sensitive with inhibition zone of 21, 30 and 30 mm in diameter at dose levels of 2.0, 3.0 and 1.0 kGy respectively, whereas its resistance to all the remaining antibiotics was still constant without change after exposure to gamma-radiation.

Regarding *K. pneumoniae,* its resistance towards IMP changed to be sensitive at 1.0 kGy. Also, its susceptibility towards AZM and SXT changed to moderate at 1.0 kGy (Table 3), while, its resistance to all the remaining antibiotics was still constant without change after exposure to gamma-radiation. These data suggested a possible stress response to gamma-radiation. The variability of the radiation effect on susceptibility towards the different antibiotics could be explained by the nature, penetration mode inside the cell or by the mode of action of antibiotics.

The increase in sensitivity of irradiated *P. aeruginosa* and *K. pneumoniae* cells can be explained on the action of gamma irradiation on one or more mechanisms. Pouget et al. (2002) said that the increase of sensitivity may be related to membrane composition modifications following the irradiation. The main theories that try to explain the biological effects of oxidative stress are based on the possible effects on the permeability of ionic channels in the membrane (Berrier et al., 1993; Galvanoskis et al., 1999).

Another study conducted by Potron et al. (2015) documented that resistance of *P. aeruginosa* to antibiotics is the result of the production of enzymes that inactivate and degrade antibiotics, reducing the membrane permeability and the efflux system.

Dreier and Ruggerone (2015) obtained that MexAB-OprM is usually stated in wild type (WT) *P. aeruginosa* and participate in the passage of various antimicrobials such as fluoroquinolones, -lactams, macrolides and trimethoprim, sulfamides. Indeed, Nebras et al. (2016) confirmed that gamma irradiation make inhibition to gene expression of MexAB-OprM and MexXY efflux pumps of *P. aeruginosa*. As a result, it can be suggested that the tested strains showed alterations in the cell wall/cell membrane/porins which lead to change in the resistance of *K. pneumoniae* towards IMP and *P. aeruginosa* towards TZP.

Effect of gamma-radiation on extended spectrum β-lactamases and Metallo β-lactamases production

Seid and Asrat (2005) and Kumar et al. (2014) mentioned that inappropriate and incorrect use of third-generation cephalosporins in empirical therapies and lack of suitable

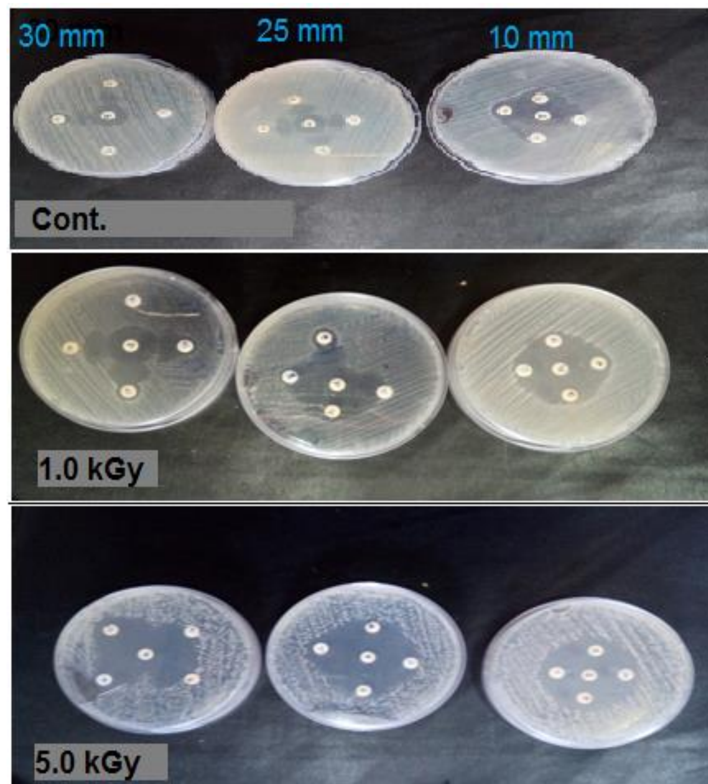

Figure 5. Expansion in the inhibition zone of CAZ, CRO, CTX and ATM towards (AMC) disc by *K. pneumoniae* before and after gamma-irradiation at different distances.

infection-control policies can be considered as the most important factors in the emergence of ESBL-producing strains.

According to double-disc diffusion synergy test (DDST), the ability of the choiced strains to produce ESBLs was tested. The results revealed that the two tested strains (*P. aeruginosa* and *K. pneumoniae*) had the ability to produce ESBLs; these results are in agreement with those of Vandana and Honnavar (2009) who reported that ESBLs are found in certain genera of the Enterobacteriaceae family including *P. aeruginosa*.

Figure 5 showed the ability of the tested *K. pneumoniae* to produce ESBLs before and after gamma-radiation at different distances. The results revealed that there was a gradual expansion in the inhibition zone of the disc containing CAZ, CRO, CTX and ATM towards AMC disc from control (un-irradiated sample) till sublethal dose. This indicated that ESBL production was not affected by gamma irradiation process and is confirmed by combined disc method which showed an increase in the diameter of inhibition zone of the disc containing Sulbactam (SCF) in comparing to the drug alone (CFP) by 5 mm which is considered as positive results for the production of (ESBLs) (data not shown).

From the previous data in this study, it was concluded that un-irradiated *K. pneumoniae* was resistant to IMP, but after exposure to gamma-radiation, its resistance was changed to be susceptible although its ability to produce ESBL was still found. These results were in agreement with those of Dutta et al. (2014) and Shaikh et al. (2015) who observed that all the ESBLs producing isolates were sensitive to IMP. So, it can be concluded that the presence of ESBL is not the reason for microbial resistance towards IMP in case of the control strain but may be due to the incidental presence of various other mechanisms of resistance and counter resistance in a given bacterium as loss of porins. In addition, increased use of carbapenems to treat ESBL-producing organisms has been involved in the emergence of carbapenem-resistant organisms.

Bhattacharjee et al. (2008) and Livermore (2009) reported that carbapenems are advocated for use in treatment of infections caused by ESBLs producing Enterobacteriacea, particularly *Escherichia coli* and *K. pneumonia,* and Giriyapur et al. (2011) stated that carbapenems are stable in the presence of hydrolytic effects of ESBLs, which may explain the consistent finding that 98% of ESBL-producing organisms retain

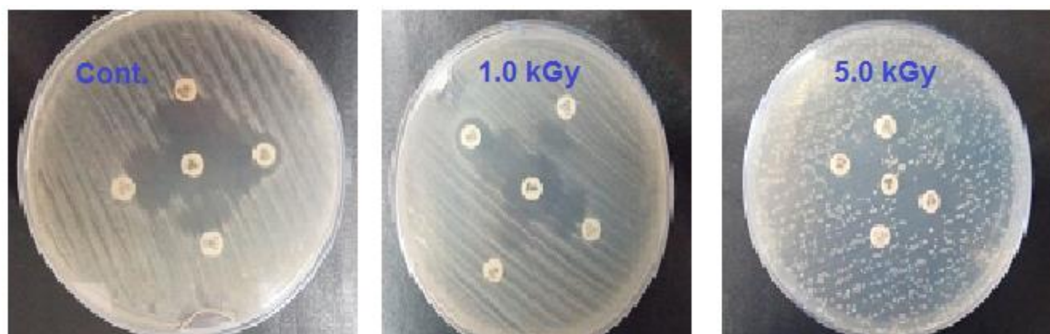

Figure 6. Expansion in the inhibition zone of CAZ, CRO, CTX and ATM towards (AMC) disc by *P. aeruginosa* before and after gamma- radiation at fixed distance.

susceptibility to either imipenem or meropenem.

Also, the results in this study revealed that irradiated and un-irradiated *K. pneumoniae* showed resistance towards all the tested third and fourth generations of cephalosporins antibiotics as well as aztreonam and this is owing to its ability to produce ESBL in both cases. Livermore (1995) discussed that ESBL producing strains show variable resistance against fourth generation cephalosporins. Giriyapur et al. (2011) documented that ESBLs are enzymes that offer resistance against wide spectrum third generation cephalosporins antibiotics along with monobactams (aztreonam).

As a result of the resistance of the tested *K. pneumoniae* towards IMP, its ability to produce Metallo β-lactamase (MBL) by combined disc test (IMP/EDTA) was screened, with the results showing its inability to produce MBL. This could be explained by the presence of other resistance mechanisms involved, such as permeability mutations via the loss of porins or the up-regulation of efflux systems (production of efflux pumps, β–lactamases enzymes and mutations) that alter the expression and/or function of PBPs and porins.

Regarding *P. aeruginosa*, by using (DDST) at a distance 25 cm, there was ghost zone of CAZ, CRO, CTX and ATM towards AMC disc (an evidence of the ability of *P. aeurginosa* to produce ESBL) before gamma radiation and there was no change in the synergy of third cephalosporine towards AMC after exposure to gamma-radiation (Figure 6).

Additionally, this result was confirmed by applying (SCF) test which showed an inhibition zone of 18 mm and 16 mm for un-irradiated and irradiated strain till sublethal dose, respectively towards Sulbactam (SCF) in comparing to the drug alone (CFP) (data not shown).

Arora and Bal (2005) reported that the number of infections caused by extended spectrum beta lactamases producing *P. aeruginosa* is on the rise and poses a threat to patients due to therapeutic failure if they remain undetected.

Mettalo Beta-Lactamases producing *P. aeruginosa* which exhibited multiple resistances to β-lactam antibiotics and multi drug-resistant has been reported to be responsible for severe problem of the management of nosocomial infections worldwide (Potron et al., 2015). The results in this study also revealed that, *P. aeruginosa* was resistant to IMP. Using combined disc test showed resistance towards IMP alone and appearance of inhibition zone in the presence of EDTA plus IMP before and after gamma-radiation at different doses. This result confirmed that *P. aeruginosa* can be considered as MBL producer (Figure 7).

Gupta (2008) concluded that MBL-producing *P. aeruginosa* have been reported to be responsible for serious nosocomial infections worldwide. In the present study, ESBL and MBL co-production was detected in *P. aeruginosa* which was in concordance with other studies (Salimi and Efterkhar, 2013) which found that among the 128 IMP resistant isolates, there were four isolates considered as co-producers of ESBL and MBL.

Thomson and Bonomo (2005) reported that IMP represents one of the last alternatives for the treatment of nosocomial infections caused by multidrug-resistant Gram-negative bacteria, particularly *P. aeruginosa,* but the use of IMP as the first choice of treatment for MDR *P. aeruginosa* in this unit provides a possible explanation for the presence of increasing imipenem-resistance (Nakamura et al., 2013). In addition, Ozyurt et al. (2008) and Hocquet et al. (2010) concluded that one of the most important ways for resistance towards IMP is the generation of MBL enzymes.

Results of this study also indicate that *P. aeruginosa* was resistant towards ATM before and after exposure to gamma- radiation at different doses. This is in accordance with Meini et al. (2014) who discussed that MBL enzymes hydrolyze all β-lactams except ATM. Also, Shahcheraghi et al. (2010) reported that all their metallo β-lactamase producing isolates were resistant to ATM. Lucena et al. (2014) concluded that MBL producing strains of *P. aeuroginosa* (clinical isolates) were 49% resistant for ATM, while Abd El-Baky et al. (2013) showed

Figure 7. Combined disc test (CDT) for *P. aeruginosa* before and after gamma- radiation.

that, MBL-producing *P. aeurginosa* of clinical isolates were 90.3% resistant for ATM. Sedighi et al. (2015) showed that MBL-producing strains of *P. aeuroginosa* isolated from hospitalized patients were 100% resistant against cefepime and this is accordance with the obtained data in this study. Lucena et al. (2014) showed that 93 MBL-producing clinical isolates of *P. aeuroginosa* were 100% resistant to ciprofloxacin and gentamicin.

Salimi and Eftekhar (2013) discussed that TZP was considered the most effective antimicrobial agent against MBL producers, but in this study, *P. aeruginosa* was resistant towards TZP, a resistance that was changed into susceptible after exposure to gamma-radiation.

From the previous discussion, this study suggests that use of cephalosporins and carbapenems should be restricted in treatment of uropathogens to minimize the development and spread of multidrug resistant problem treatment.

Conclusion

From the previously mentioned results, it can be concluded that the most resistant isolates (100% resistance) were identified as *P. aeruginosa* and *K. pneumoniae* using 16srRNA. The D_{10} values were recorded as (1.1 and 1.2 kGy for *P. aeruginosa* and *K. pneumoniae)* respectively, and both of the tested strains had the ability to produce Extended Spectrum β-lactamase (ESBL) before and after gamma-radiation. ESBL and MBL co-production was detected in *P. aeuroginosa,* finally, gamma- irradiation technique decreased the resistance of *K. pneumoniae* towards IMP and the resistance of *P. aeruginosa* towards more than one antibiotic (TZP, AZM and CIP). This therefore enables us to use gamma-ray in sterilization of implemented devices in hospitals to prevent emergence of these extremely hazardous strains before causing the infection. These findings must be supported by other studies on the level of genes to prove the possibility for using gamma- irradiation technique to overcome microbial drug resistance problem.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation of an emerging thermotolerant medically important Fungus, *Lichtheimia ramosa* from soil

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***Lichtheimia ramosa*, a ubiquitous clinically important mould was isolated during a screen for thermotolerant fungi obtained from soil on a freshly burnt field in Ikorodu, Lagos State. In the laboratory, as expected it grew more luxuriantly on Potato Dextrose Agar than on size limiting Rose Bengal Agar. The isolate had mycelia with a white cottony appearance on both media. It was then identified based on morphological appearance, microscopy and by fungal Internal Transcribed Spacer ITS-5.8S rDNA sequencing. This might be the first report of molecular identification of *L. ramosa* isolate from soil in Lagos, as previously documented information could not be obtained.**

Key words: Soil, thermotolerant, *Lichtheimia ramosa*, Internal Transcribed Spacer (ITS).

INTRODUCTION

Zygomycetes of the order Mucorales are thermotolerant moulds that are ubiquitous in nature (Nagao et al., 2005). The genus *Lichtheimia* (syn. Mycocladus, Absidia) belongs to the Zygomycete class and includes saprotrophic microorganisms that can be isolated from decomposing soil and plant material (Alastruey-Izquierdo et al., 2010a). The awareness of the thermotolerant genus *Lichtheimia* increased markedly since its separation from the mesophilic genus *Absidia* (Hoffmann et al., 2007) and its taxonomic revision (Alastruey-Izquierdo et al., 2010b). Members of the genus *Lichtheimia* are considered to constitute thermotolerant fungi, because they grow at a wide range of temperatures, from 20 to 53°C, with optimum temperature for growth being 37°C, where it exhibits rapid growth (André et al., 2014).

L. ramosa is abundant in soil, decaying plant debris and foodstuff and is one of the causative agents of mucormycosis in humans (Barret et al., 1999). Mucormycosis is an opportunistic invasive infection caused by *Lichtheimia*, *Mucor*, and *Rhizopus* of the order Mucorales. Soil serves as a habitat and spore reservoir for *Lichtheimia* species. Several cases are known where traumatic injuries contaminated with soil resulted in *Lichtheimia* infections in immunocompetent patients (Belfiori et al., 2007, Corti et al., 2009, Blazquez et al., 2010). Mucormycosis (both rhino-orbital-cerebral and pulmonary) are acquired by the inhalation of their spores. Despite their low virulence, *Lichtheimia* species are currently regarded as emerging pathogens among Mucoralean fungi (Kutlu et al., 2014). There has been an increase in reports of *L. ramosa* infections among

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immunocompromised patients (Schwartze and Jacobsen, 2014).

L. ramosa is a microbe of clinical importance. Reported cases include *L. ramosa* isolated from a young patient's infected wound after a road traffic accident (Neelaveni et al., 2017) and as a causal agent of primary cutaneous mucormycosis in a burn victim (Kaur et al., 2014). A fatal case of mucormycosis due to *L ramosa* affecting a 56-year-old male with diabetes mellitus and siderosis has also been reported (Mouronte-Roibás et al., 2016).

Genome sequence of *L. ramosa* and its close relative *L. corymbifera* have been published (Linde et al., 2014, Schwartze et al., 2014). Although *Lichtheimia* species tend to be morphologically and genetically distinct, they often share very similar antifungal drug susceptibilities.

The growth of the *L. ramosa* in media is rapid, with mycelia expanding to cover the entire plate in only a few (one to seven) days (Ziaee et al., 2016). Microscopically, *L. ramosa is* similar to *L. corymbifera* and differs in its ellipsoidal to cylindrical sporangiospores as compared to the subglobose to broadly ellipsoidal sporangiospores of *L. corymbifera* (Alastruey-Izquierdo et al., 2010a). In this study, a screen was undertaken to isolate and identify a thermotolerant fungus, *L. ramosa* from the environment by culture-based and molecular methods.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from a freshly burnt vegetation in Ikorodu Local Government, Lagos State with GPS coordinates 6°37'20.4"N 3°34'37.9"E. The farmland had been burnt following harvesting of farm produce. Samples were collected from the surface layer of the soil up to a depth of 7.5 to 10 cm.

Isolation of fungi

One gram of soil sample was placed in a test tube containing 9 ml of sterile distilled water and homogenized by shaking thoroughly. A ten-fold serial dilution scheme was made up to 10^{-5} dilutions. One milliliter aliquot of the homogenized solutions was taken from 10^{-3}, 10^{-4} and 10^{-5} dilutions and plated on Potato Dextrose Agar supplemented with chloramphenicol and Rose Bengal chloramphenicol agar using the pour plate technique. Plating was done in triplicates for both Potato Dextrose Agar and Rose Bengal Agar. The plates were incubated at 30°C and the growth observed for 7 days.

Isolation of pure cultures and microscopy

Pure cultures of the white mycelial fungus were selected from the PDA plates and sub-cultured onto fresh PDA plates. The strain was stored on PDA slants at -20ºC for future use. For microscopic identification, a mycelial mat of the fungus was placed on a grease-free microscope glass slide and a drop of lactophenol cotton blue was added to the mycelia mat. A coverslip was placed on it with the aid of sterile forceps and the microscope slide was viewed under the microscope. Micrographs were thereafter obtained.

Molecular identification of the isolate

Genomic DNA was extracted from a five-day-old fungi culture grown on PDA using ZR Quick-DNA Fungal/Bacterial Miniprep™ extraction kit (Zymo Research, CA, USA) according to the manufacturer's instructions. To check the DNA quality, it was run on a 1% ethidium bromide agarose gel (Figure 2A). Polymerase Chain Reaction (PCR) of the extracted genomic DNA was done in a GeneAmp PCR system 9700 PCR thermal cycler. ITS5F (GGAAGTAAAAGTCGTAACAAGG) and ITS4R (TCCTCCGCTTATTGATATG) primers were used for amplification. The PCR reaction mix (25 µl) contained 2.5 µl of 10x PCR buffer, 1 µl of 25 mM $MgCl_2$, 1 µl each of forward and reverse primers, 1 µl of DMSO, 2 µl of 2.5 mM dNTPs, 0.1 µl of 5µg/ µl Taq DNA polymerase, 3 µl of 10ng/µl DNA and 13.4 µl Nuclease free water. The thermal cycler program used was as follows: Initial denaturation at 94˚C for 5 min, followed by 36 cycles of denaturation at 94˚C for 30 s, annealing at 54˚C for 30 s, elongation at 72˚C for 45 s, a final elongation step at 72˚C for 7 min and hold temperature at 10˚C. The amplicons were electrophoresed on 1.5% agarose gels. The gel was prepared and run at 75 volts for 90 min and visualized with a UV Transilluminator (Figure 2B). The PCR product was Sanger sequenced using the BigDye 3.1 reaction protocol on 3130XL genetic analyzer (Applied Biosystems) at the Bioscience Center, IITA, Ibadan, Oyo. The sequences were checked for quality and assembled using BioEdit (version 7.2.5) Sequence Alignment Editor (Hall, 1999). The consensus sequence was compared to the GenBank nucleotide data library using the Basic Local Alignment Search Tool, BLAST software (Altschul et al., 1990) at the National Centre for Biotechnology Information (NCBI(http://www.ncbi.nlm.nih.gov). The sequences were submitted to the GenBank database and an accession number was assigned to the isolate.

RESULTS

Isolation of thermotolerant fungus from the soil sample

After 7 days of incubation, mixed culture PDA plates of few fungi were obtained (Figure 1A). From the 10^{-3} dilution plate, a dominant whitish fungus that appeared on all the other serial dilution plates was selected and sub-cultured. This selected strain (IYN69) had a more luxuriant growth on PDA plates than on Rose Bengal agar plate when grown in pure culture (Figures 1B and C). The strains grew at room temperature up to 37°C. The micrograph showed sporangiospores ellipsoidal to cylindrical in shape (Figure 1D).

Molecular identification of Isolate IYN69

In order to confirm the identity of the isolate IYN69 by molecular techniques, DNA product was visualized on 1% agarose gel stained with ethidium bromide (Figure 2A) and then the PCR product amplified by the pair of ITS4-R and ITS5-F primers were confirmed on a 1.5% agarose gel (Figure 2B).

The sequencing results from ITS5-ITS4 amplification of the ITS regions of isolate IYN69 was edited using BioEdit (version 7.2.5) Sequence Alignment Editor (Hall, 1999)

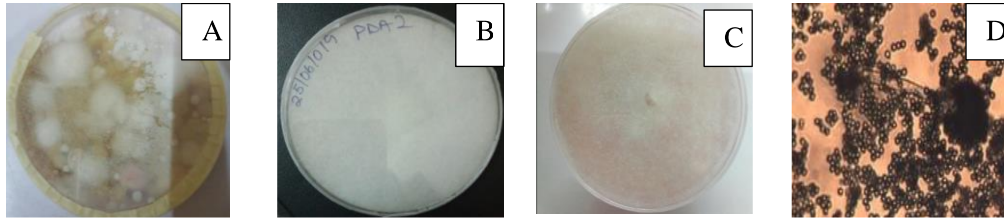

Figure 1. A. Mixed culture of 10^{-3} dilution PDA plate. Growth of strain (IYN69 on laboratory media **B.** PDA + Chloramphenicol. **C.** Rose Bengal Chloramphenicol agar. **D.** Micrograph of sporangiospores of the new isolate.

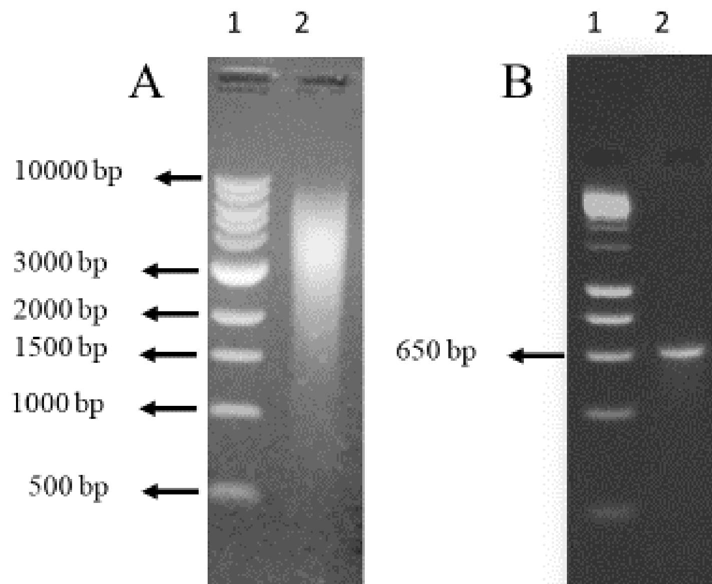

Figure 2. Agarose gel electrophoresis. **A.** 1% gel of genomic DNA from Isolate IYN69. Lane 1: 1 kb NEB ladder; lane 2: 5 µl of genomic DNA. **B.** 1.5% gel of the expected PCR amplicon size of 650 bp. Lane 1- Bioline Hyperladder 1 kb; lane 2- Isolate IYN69.

and compared with the GenBank database using NCBI Basic Local Alignment Search Tool (BLASTn). The mould was identified as *Lichtheimia ramosa* with 100% identity to *Lichtheimia ramosa* isolate PUR 3 internal transcribed spacer 1 on the NCBI platform.

Nucleotide sequence

The partial sequence of the internal transcribed spacer (ITS) region of this isolate has been submitted to GenBank and can be found under accession number MT373684.

DISCUSSION

L. ramosa was the dominant fungus among others from a screen of thermotolerant fungi from recently burnt vegetation. Its isolation under such conditions is an indication that it can withstand high temperatures. Its thermotolerance has been linked to its virulence (Schwartze et al., 2012). Morphological and microscopic characteristics and phylogenetic identification were sufficient to confirm that the isolate was *L. ramosa* strain. Its features are similar to *L. ramosa* strain H71D and mycelia were identical to the type species of *L. ramosa* described by Hoffmann (2010) and Alastruey-Izquierdo et al. (2010a).

It is likely that this report might be the first documenting soil isolation of *L. ramosa* from Lagos and its molecular identification as no existing records were found. The BLAST hits showed many *L. ramosa* species that had significant sequence alignments with the query sequence. Based on the available molecular data, *L. ramose* isolate IYN69 most closely matched another

isolate PUR 3 (GenBank Accession Number: MF033505) associated with a study on soil fungi and *L. ramosa* clone 7 (Alastruey-Izquierdo et al., 2010a). *L. ramosa* isolate IYN69 ITS 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and ITS 2, partial sequence can be found in GenBank under accession number MT373684.

Incidences of *L. ramosa* infection in patients have been linked to road traffic accidents (RTAs) suggesting contamination by this soil fungus (Neelaveni et al., 2017; Bibashi et al., 2012). This is of great significance because RTAs are a major public health concern and third leading cause of death in Nigeria (Dixie, 1999; Onyemachi and Ofoma, 2016). The next steps would be to do whole genome sequencing and compare its genome with the existing reference genome (Linde et al., 2014) especially regions responsible for sensitivity to antifungals. This strain would be tested for its sensitivity to existing antifungal drugs (Amphotericin B, posaconazole, itraconazole) that are known to be effective against *Lichtheimia* species. Although, there are not many cases of *L. ramosa* infections worldwide (Mouronte-Roibás et al., 2016); for an emerging pathogen, the information from such studies would help to put in place good treatment options should burn, accident victims or diabetics get infected with this strain from this locality. Since *L. ramosa* is also known for the production of enzymes like xylanase (Alvarez-Zuniga et al., 2017), and mannase (Xie et al., 2019), an assay for these and other thermophilic enzymes with biotechnological applications will be conducted on this new isolate.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Fungal diversity within organic and conventional farming systems in Central Highlands of Kenya

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Fungal diversity in agro-ecosystems is influenced by various factors related to soil and crop management practices. However, due to the complexity in fungal cultivation, only a limited number has been extensively studied. In this study, amplicon sequencing of the Internal Transcribed Spacer (ITS) region was used to explore their diversity and composition within long-term farming system comparison trials at Chuka and Thika in Kenya. Sequences were grouped into operational taxonomic units (OTUs) at 97% similarity and taxonomy assigned via BLASTn against UNITE ITS database and a curated database derived from GreenGenes, RDPII and NCBI. Statistical analyses were done using Vegan package in R. A total of 1,002,188 high quality sequences were obtained and assigned to 1,128 OTUs; they were further classified into eight phyla including *Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, Calcarisporiellomycota, Kickxellomycota*, *Mortierellomycota* and unassigned fungal phyla. *Ascomycota* was abundant in conventional systems at Chuka site while *Basidiomycota* and *Chytridiomycota* were dominant in conventional systems in both sites. *Kickxellomycota* and *Calcarisporiellomycota* phyla were present in all organic systems in both sites. Conventional farming systems showed a higher species abundance and diversity compared to organic farming systems due to integration of organic and inorganic inputs.

Key words: Long-term farming systems, fungi, internal transcribed spacer (ITS), diversity, Illumina sequencing.

INTRODUCTION

Fungal communities are an essential constituent of soil microbial biomass that is involved, and/or linked to processes of carbon and nitrogen cycles, organic matter decomposition, as well as nitrogen mineralization and immobilization (Bloem et al., 1995; Bååth and Anderson, 2003; Wall et al., 2012; Berthrong et al., 2013; Milner, 2014; Fierer, 2017). On the other hand, fungi which constitute one of the largest groups of eukaryotes, play a

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key role in nutrient and carbon cycling as mutualists, symbionts, pathogens and free-living saprotrophs (Barea et al., 2005; Gadd, 2007; Lindahl et al., 2007; McLaughlin and Spatafora, 2014). Fungi are also involved in formation of soil aggregates, elevated water holding capacity, plant growth promotion and suppression of phytopathogens (Sommermann et al., 2018). Mutualistic root endophytic fungi induce systemic resistance in host plants thereby increasing crops tolerance levels to biotic and abiotic stress factors (Lahlali et al., 2014). Therefore, they are a key component of sustainable soil-plant systems that govern major plant nutrient cycles hence sustaining the vegetation cover and ecosystem services (Schreiner and Bethlenfalvay, 1997; Dighton, 2003; Johansson et al., 2004).

Soil fungal community composition is influenced by soil physicochemical properties, plant populations and geo-climatic conditions (Tkacz et al., 2015). However, in agro-ecosystems, they are exposed to added influencing factors associated with soil and crop agronomic management practices. To date, only few studies have been keen to determine the impact of farming systems on microbial diversity. Little information is available concerning the effect of cultivation systems on fungal diversity and the level of fungal diversity between different crops in the same farm (Lentendu et al., 2014; Lopes et al., 2014; Kazeeroni and Al-Sadi, 2016). The fungal diversity ecosystem is still undefined; though, Wang et al. (2008) reported that about 5-13% of the total estimated global fungal species have been described. Since many fungi are unculturable and rarely produce visible sexual structures, molecular techniques have become widely used for taxonomic detection of species to understand shifts in their richness and composition along environmental gradients (Persˇoh, 2015; Balint et al., 2016; Tedersoo and Nilsson, 2016; Tedersoo et al., 2018).

It is still not understood how fungal communities respond to different inputs within organic and conventional farming systems (Hartmann et al., 2015; Wang et al., 2017). In this study, conventional farming systems received inorganic and organic inputs whilst organic systems received organic inputs only. High throughput sequencing of the ITS gene amplicons was used to explore the population diversity and composition of fungal communities within conventional and organic farming systems in system comparison trials within central highlands of Kenya. The study sites were initiated to compare the performance of organic and conventional farming systems in the tropics on farm productivity, profitability and sustainability.

MATERIALS AND METHODS

Study sites characteristics

Samples were collected from the on-going long-term farming systems comparison (SysCom; www.system-comparison.fibl.org) trials in Kenya (Adamtey et al., 2016). The trials were established in 2007 at two locations: Chuka (Tharaka Nithi County) and Thika (Murang'a County) in the Central Highlands of Kenya. These sites are 125 km apart and they have a bimodal rainfall pattern with long rains occurring between March and June and short rains occurring between October and December. The site at Chuka is located at 1458 m above sea level (Longitude 037° 38.792' and Latitude 00° 20.864'), with an annual mean temperature of 20°C and mean annual rainfall of 1500 to 2400 mm. This site is situated in the upper midland 2 agro ecological zone, also referred to as the coffee zone (Jaetzold et al., 2006a). The site at Thika is located at 1500 m above sea level (Longitude 037° 04.747' and Latitude 01° 00.231'), with an annual mean temperature of about 20°C and mean annual rainfall of 900 - 1100 mm. This site is situated in the upper midland agro ecological zone 3, also referred to as the sunflower maize zone (Jaetzold et al., 2006b). The soils at Chuka site are classified as Humic Nitisols and those at Thika as Rhodic Nitisols (Adamtey et al., 2016) in the Food and Agricultural Organization (FAO) World reference base for soil resources (IUSS Working Group WRB. 2006).

Farming systems

Food and Agricultural Organization (FAO) defines farming systems as a set of population of individual farm systems that have broadly similar resource bases, enterprise patterns, household livelihoods and constraints and for which similar development strategies and interventions would be appropriate (Dixon et al., 2001). This was adopted in this study and at each site, conventional (Conv) and organic (Org) systems were compared at low input levels (Conv-Low and Org-Low), where the N and P application rates and management practices mimicked small-scale farmers' practices in the region (Muriuki and Qureshi, 2001). Conventional (Conv) and organic (Org) systems at high input levels (Conv-High and Org-High) represented recommended N and P application rates and other management practices embraced by market-oriented and large-scale production systems farmers (Musyoka et al., 2017). The high input systems received supplementary irrigation during the dry period and management of pests and diseases was guided by scouting reports (Adamtey et al., 2016). The four farming systems in each site were arranged in a randomized complete block design with plot sizes of 8 m × 8 m replicated 4 times. The type of inputs and their application rates in each farming system are indicated in Supplementary Table 1.

Soil sampling

Soil sampling was done before land preparation in March 2015. Surface organic materials were removed and a homogenized composite soil sample collected from 12 single cores within top soil (0-20 cm depth) which is the root zone of majority crops grown in the trial sites. Two batches of sixteen (16) composite samples from each site were packed in sterile 500 g containers. One batch of the soil samples for molecular analysis was preserved on dry ice and transported to the laboratory at International Centre for Insect Physiology and Ecology for preservation at -80°C. The other batch of soil samples was used for soil physicochemical analysis (using methods summarized in Table 1) at Crop Nutrition Laboratory Services, Nairobi in Kenya.

Fungal community analysis

DNA was extracted from 0.25 g of the soil samples in triplicates as described by Sambrook et al. (1989). The DNA from triplicate samples was pooled at precipitation stage, pellets were air dried

Table 1. Soil physicochemical parameters analyzed and their respective methods.

Parameter	Method
pH and Electrical conductivity (EC)	Potentiometric (Okalebo et al., 2002)
Cation exchange capacity (CEC), Potassium (K), Calcium (Ca), Magnesium (Mg), Sulphur (S), Sodium (Na), Copper (Cu), Boron (B), Zinc (Zn) and Iron (Fe)	Mehlich 3 (Mehlich, 1984)
Exchangeable Aluminium (Exch. Al)	Spectrophotometry (Kennedy and Powell, 1986)
Organic Carbon (OC)	Wet oxidation (Anderson and Ingram, 1993)
Total Nitrogen (N)	Kjeldahl acid digestion (Gupta, 1999)
Total Phosphorous (P),	Olsen (Okalebo et al., 2002)
Soil moisture and Temperature	Soil Moisture Meter (IMKO GmbH – Germany)
Aggregate size separation (Small macro-aggregates and micro-aggregates)	Wet sieving (Six et al., 1998)

and sent to Molecular Research DNA Laboratory (www.mrdnalab.com, Shallowater, TX, USA) for amplicon library preparation and sequencing.

Amplicon DNA library preparation and sequencing

Polymerase Chain Reaction (PCR) amplification of ITS region was done using ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) barcode primers (White et al., 1990; Ihrmark et al., 2012). Amplification proceeded in a 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) with initial heating at 94°C for 3 min, followed by 28 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 40 s and extension at 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. PCR products were visualized on 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together (e.g., 100 samples) in equal proportions based on their molecular weight and DNA concentrations during sequencing. The pooled samples were purified using calibrated Ampure XP beads (Agencourt Bioscience Corporation, MA, USA) and used to prepare DNA library by following Illumina sequencing protocol (Yu and Zhang, 2012). Sequencing was performed at Molecular Research DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq 2x300 bp Version 3 platform following the manufacturer's guidelines.

Sequence analysis

Sequences were analyzed using QIIME2 pipeline (Bolyen et al., 2018) whereby the input file was created using "convert_fastaqual_fastq.py" script on Qiime v1.9 (Caporaso et al., 2010). Sequences were demultiplexed using barcode information; and quality control and construction of feature tables was done using dada2 software in QIIME2 (Callahan et al., 2016). The pipeline denoises sequences, removes chimeras, creates OTU table, picks representative sequences and calculates denoising statistics. Sequences which were < 200 base pairs after phred20-base quality trimming, with ambiguous base calls, and those with homopolymer runs exceeding 6bp were removed (Callahan et al., 2016). Taxonomic classification of representative sequences obtained from the OTU clustering was done using QIIME feature-classifier classify-sklearn based on UNITE ITS Reference Database (Kojalg et al., 2005; Koljalg et al., 2013) and a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov; http://rdp.cme.msu.edu) at 97% level of similarity using default settings as implemented in QIIME2 (Bolyen et al., 2018). The sequence reads have been deposited at NCBI Sequence Read Archive with SRA accession: PRJNA532741 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA532741).

Diversity analysis was carried out using Vegan Community Ecology Package version 2.5.2 (Oksanen et al., 2012). In order to support OTU-based analysis, taxonomic groups were derived from the number of reads assigned to each taxon at all ranks from domain to species output from QIIME2. Alpha diversity indices (Richness - S and Shannon - H') were used to test statistically significant differences between high and low input farming systems. Rarefaction curves were generated, plotted and customized using Vegan Community Ecology Package. Community and environmental distances were compared using analysis of similarity (ANOSIM) while significance was determined at 95% confidence interval ($P<0.05$). Calculation of Bray-Curtis dissimilarities between datasets and hierarchical clustering were carried out using Vegan package in R (Oksanen et al., 2012). To estimate diversity between samples (β Diversity), Principal Component Analysis of soil physicochemical characteristics and prokaryotic taxa was done using R programming language (R Development Core Team., 2012). Besides Principal Component Analysis, Non-metric multidimensional scaling (NMDS) and hierarchal clustering were also performed for beta diversity. In order to understand the influence of farming systems on soil physicochemical characteristics, analysis of variance was performed at $P<0.05$, 0.01 and 0.001 using a linear mixed-effect model with *lmer* function from lme4 package in R software (Bates et al., 2013) with system and site as fixed effects; while replication was used as random effect. In order to delineate the farming systems within sites, computation of least mean squares was done using *lsmeans* package in R software. Means were separated with Tukey's method implemented using *cld* function from *multicomp* package as developed by Piepho (2004) in R software version Ri386 3.1.1 (R Development Core Team, 2014).

RESULTS

Soil physicochemical properties of the long-term system comparison trials

In this study we assessed the fungal community composition in 32 soil samples collected from long-term farming system comparison trials at Chuka and Thika in Kenya. The physicochemical characteristics of soils are as shown in Table 2. Tukey's separation of means

Table 2. Soil physicochemical characteristics of the long-term system comparison trial sites at Chuka and Thika.

Soil property	Farming systems				System x Site								Source of variation	
					Chuka				Thika					
	Conv-High	Org-High	Conv-Low	Org-Low	Conv-High	Org-High	Conv-Low	Org-Low	Conv-High	Org-High	Conv-Low	Org-Low	System	System x Site
pH	5.68^{a}	6.61^{ab}	5.43^{a}	5.87^{a}	5.64^{ab}	6.50^{bc}	5.58^{ab}	5.75^{ab}	5.72^{ab}	6.71^{c}	5.23^{a}	5.98^{abc}	***	ns
EC.S (uS/cm)	85.75^{a}	113.75^{a}	60.13^{a}	75.50^{a}	48.50^{a}	74.00^{ab}	46.50^{a}	48.50^{a}	123.00^{bc}	153.50^{c}	73.75^{ab}	102.50^{abc}	ns	ns
OC (%)	2.29^{a}	2.52^{a}	2.29^{a}	2.34^{a}	2.60^{cd}	2.89^{d}	2.78^{d}	2.51^{bcd}	1.97^{ab}	2.16^{abc}	1.79^{a}	2.16^{abc}	ns	ns
N (%)	0.19^{a}	0.205^{a}	0.185^{a}	0.196^{a}	0.208^{cde}	0.223^{e}	0.203^{bcde}	0.215^{de}	0.173^{ab}	0.188^{abcd}	0.168^{a}	0.178^{abc}	ns	ns
S (mg/kg)	16.37^{a}	8.00^{a}	15.59^{a}	14.04^{a}	10.09^{ab}	1.22^{a}	9.80^{ab}	8.10^{ab}	22.65^{b}	14.78^{ab}	21.39^{b}	19.97^{b}	ns	ns
P (mg/kg)	30.80^{ab}	42.31^{b}	16.97^{a}	20.18^{a}	35.75^{a}	39.08^{a}	14.55^{a}	19.23^{a}	25.86^{a}	45.55^{a}	19.38^{a}	21.14^{a}	**	ns
K (mg/kg)	472.63^{a}	1077.25^{b}	453.13^{a}	541.63^{a}	339.00^{a}	994.25^{bc}	334.75^{a}	366.00^{a}	606.25^{ab}	1160.25^{c}	571.50^{a}	717.25^{ab}	***	ns
Ca (mg/kg)	1462^{a}	2086^{b}	1438^{a}	1539^{a}	1765^{ab}	2315^{b}	1598^{ab}	1695^{ab}	1159^{a}	1858^{ab}	1279^{a}	1384^{a}	**	ns
Mg (mg/kg)	248^{a}	342^{b}	260^{a}	245^{a}	250^{ab}	344^{c}	237^{a}	235^{a}	246^{a}	340^{bc}	283^{abc}	256^{abc}	***	ns
Na (mg/kg)	21.63^{a}	32.73^{a}	18.03^{a}	18.34^{a}	7.17^{ab}	9.29^{ab}	4.48^{a}	5.70^{ab}	36.10^{bc}	56.18^{c}	31.58^{abc}	30.98^{abc}	ns	ns
Exch. Al (meq/ 100g)	0.07^{a}	0.04^{a}	0.19^{a}	0.11^{a}	0.78^{ab}	0.12^{a}	0.53^{ab}	0.04^{a}	0.06^{ab}	0.07^{ab}	0.33^{b}	0.17^{ab}	ns	ns
B (mg/kg)	0.58^{a}	0.96^{b}	0.55^{a}	0.68^{a}	0.54^{a}	0.93^{ab}	0.53^{a}	0.58^{a}	0.63^{ab}	0.99^{b}	0.58^{a}	0.78^{ab}	***	ns
Mn (mg/kg)	434^{a}	443^{a}	446^{a}	429^{a}	567.50^{b}	533.50^{b}	575.75^{b}	553.75^{b}	300.50^{a}	353.25^{a}	315.25^{a}	303.75^{a}	ns	*
Fe (mg/kg)	89.25^{b}	70.19^{a}	83.70^{b}	77.33^{ab}	97.93^{c}	72.76^{ab}	89.63^{bc}	83.78^{abc}	80.58^{ab}	67.60^{a}	77.75^{ab}	70.88^{a}	**	ns
Zn (mg/kg)	8.89^{a}	10.51^{a}	7.19^{a}	8.06^{a}	12.23^{de}	12.80^{e}	9.55^{cd}	10.80^{cde}	5.49^{ab}	8.23^{bc}	4.82^{a}	5.32^{ab}	ns	ns
Small Macro-aggregate (g)	48.11^{ab}	52.15^{b}	42.17^{a}	42.28^{a}	46.09^{b}	48.56^{bc}	36.53^{a}	36.76^{a}	50.15^{bc}	55.75^{c}	47.82^{bc}	47.80^{bc}	**	ns
Micro-aggregate (g)	21.15^{ab}	17.43^{a}	28.66^{b}	27.13^{b}	25.58b^{c}	22.29^{b}	34.22^{c}	33.81^{c}	16.72^{ab}	12.58^{a}	23.10^{b}	20.46^{ab}	*	ns

Letters designate significant differences at P ≤ 0.05. Means followed by the same letter are not significantly different; ns= not significant; *P ≤ 0.05; ** P ≤ 0.01 and *** P ≤ 0.001.

revealed a trend in the means of soil pH, P, K, Ca, Mg, B and small macro-aggregates that were found to be significantly high (P<0.05) in organic farming systems. Fe and micro-aggregates were high in conventional farming systems (Table 2). Soils from Chuka contained 59.4% primary clay minerals and 40.6% secondary clay minerals, while soils from Thika were characterized by high primary minerals (78.3%) and low secondary clay minerals (21.7%) (Adamtey et al., unpublished results). Congruently, the rate of formation and stabilization of small macro aggregates was found to be higher at Thika than Chuka site.

Fungal sequence coverage analysis within farming systems

After denoising and demultiplexing, a total of 556,135 and 466,053 high quality sequences were obtained from Chuka and Thika sites respectively. Rarefaction analysis of the extent of diversity captured in each farming system and the level of sequence coverage visualized using rarefaction curves showed a steep slope that plateaued to the right in some of the replications within farming systems (Figure 1a and b). This indicated that a good proportion of the fungal diversity had been captured within the represented farming systems and an increase in the number of sequences extracted would only marginally increase the number of OTUs obtained. However, rarefaction curves of some replications within farming systems displayed a steep slope, denoting that more intensive sampling within the replicate plots was likely to

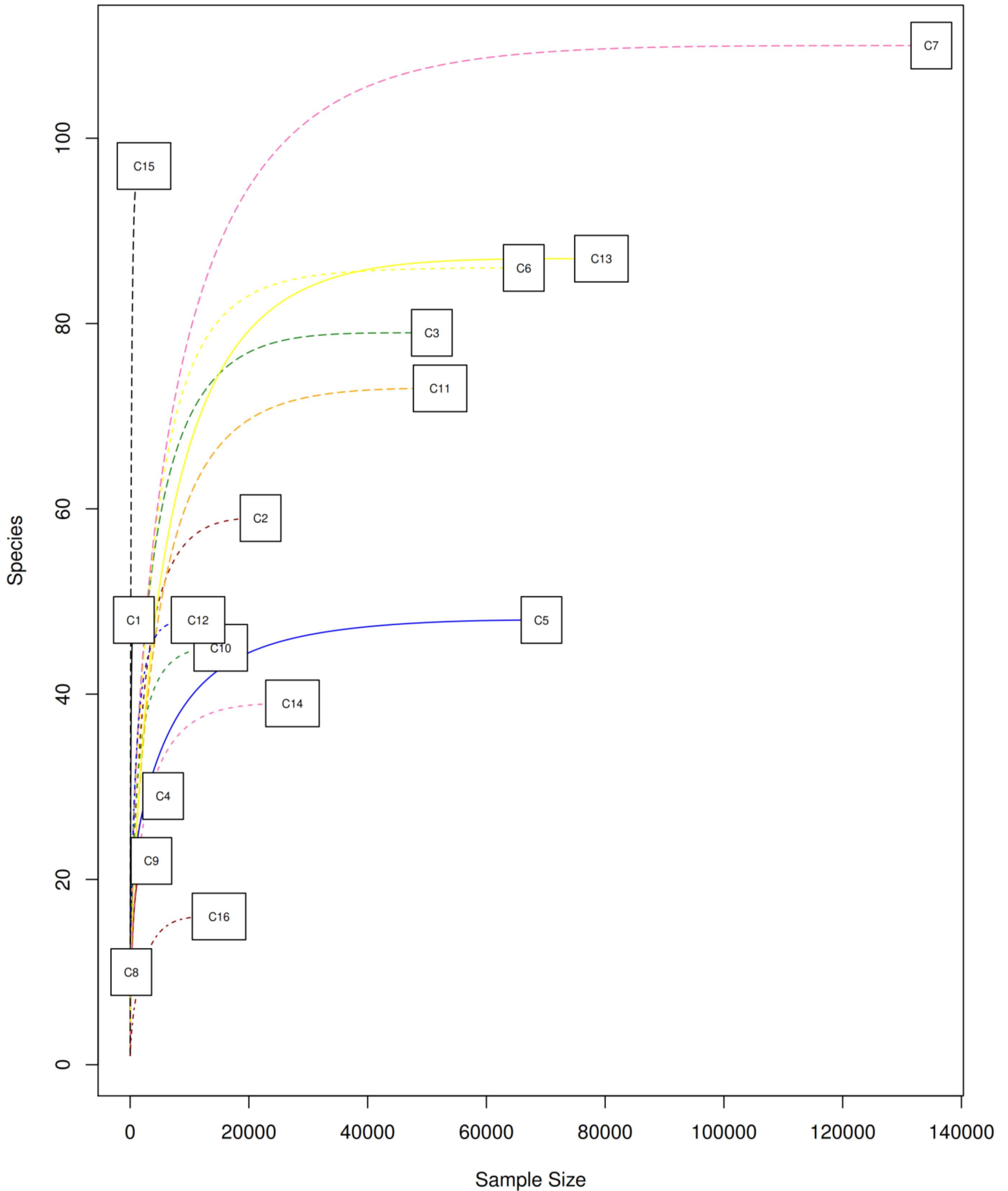

Figure 1a. Rarefaction curves of each farming system replication indicating the level of fungal ITS sequence coverage at Chuka site. C3, C6, C12 and C14 represents Conv-High; C2, C7, C11 and C16 represents Conv-Low; C4, C8, C9 and C15 represents Org-High; C1, C5, C10 and C13 represents Org-Low.

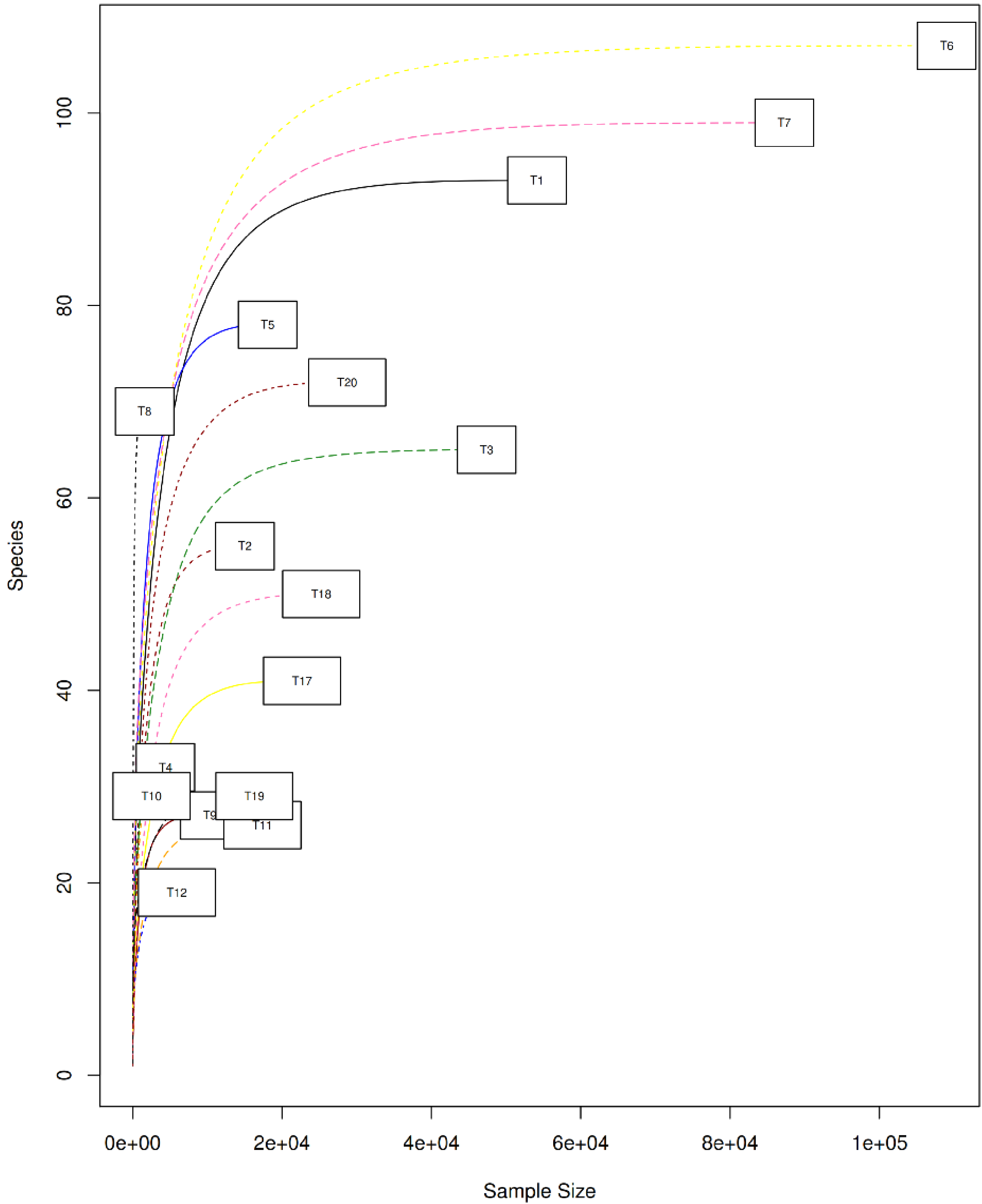

Figure 1b. Rarefaction curves of each farming system replication indicating the level of fungal ITS sequence coverage at Thika site. T2, T7, T9 and T20 represents Conv-High; T1, T6, T12 and T18 represents Conv-Low; T3, T8, T11 and T17 represents Org-High; C1, C5, C10 and C13 represents Org-Low.

Table 3. Distribution of high-quality sequences, Operational Taxonomic Units (OTUs), diversity indices and fungal taxa at Chuka and Thika sites (The farming systems have been sorted as per total number of OTUs in each site).

Site	System	High quality sequences	OTUs	Unique OTUs	Richness (S)	Shannon index (H')	Number of phyla	Number of classes	Number of orders	Number of families	Number of genera	Number of species
Chuka	Conv-Low	224,073	161	82	64.5	1.15	8	18	21	103	134	204
	Org-Low	164,528	155	76	57.0	1.53	8	19	21	103	131	201
	Conv-High	155,879	143	65	63.0	1.53	8	18	21	96	129	196
	Org-High	11,655	113	35	39.5	2.05	8	16	19	92	124	185
Thika	Conv-Low	194,317	168	98	67.3	1.43	8	19	24	101	147	224
	Conv-High	141,355	144	72	63.3	1.44	8	21	24	101	141	213
	Org-High	89,075	128	56	50.3	2.00	8	20	24	101	134	200
	Org-Low	41,306	116	46	42.0	1.49	8	17	20	94	124	189

yield more fungal communities for further classification (Figure 1a and b).

Effect of the farming systems on operational taxonomic units (OTUs)

The high-quality sequences obtained were assigned to 1,128 OTUs at 97% genetic distance. Conventional systems were found to harbor more (both total and unique) OTUs as compared to organic farming systems (Table 3). Taxonomic classification of final OTUs based on UNITE ITS Reference Database and a curated database derived from GreenGenes, RDPII and NCBI grouped the OTUs into a total of eight phyla. Farming systems were dominated by *unassigned fungal* phyla with low input farming systems in both sites scoring the highest relative abundance. Notably, known fungal taxa revealed included *Ascomycota*, *Basidiomycota*, *Chytridiomycota, Glomeromycota*, *Calcarisporiellomycota*, *Kickxellomycota* and *Mortierellomycota*. *Ascomycota* was most abundant in organic systems in both sites while *Chytridiomycota* was dominant in conventional systems in both sites. *Basidiomycota* was dominant in conventional systems at Chuka site whilst *Kickxellomycota* and *Calcarisporiellomycota* phyla were present in all organic systems in both sites but relative abundances were too low to allow their view in Figure 2.

High input systems: unknown fungi, *Basidiomycota* and *Chytridiomycota* were the fungal groups that showed the greatest relative abundance in conventional systems, whereas in the organic systems *Ascomycota* and *Glomeromycota* were the prevalent groups, in both sites. The *Kickxellomycota* phyla occurred more strongly in the Org-High system in Thika site; the same occurred for *Mortierellomycota* phyla in Org-High system in Chuka site (Figure 2).

Low input systems: Unknown fungi and *Chytridiomycota* were more abundant in conventional systems in both sites. In organic systems, unknown fungi, *Basidiomycota* and *Ascomycota* were the most abundant groups, in both sites. In addition, *Chytridiomycota, Glomeromycota* and *Calcarisporiellomycota* phyla were abundant in Org-Low system at Thika site (Figure 2).

Taxonomy assignment at genus level revealed the most abundant genera within farming systems to include; at Chuka site, *Gnomonia*, *Sporobolomyces*, *Saccharomyces* and *Exophiala* in Conv-Low; *Minimedusa*, *Pluteus*, *Macrophomina*, *Leucoagaricus* in Org-Low; *Penicillium*, *Malassezia*, *Aspergillus* and *Marasmius* in Conv-High; and *Alternaria*, *Marasmius*, *Harknessia* and *Laetisaria* in Org-High farming systems. At Thika site, the most abundant genera within farming systems included *Alternaria*, *Spizellomyces*, *Rhizophlyctis* and *Conocybe* in Conv-Low, *Leucoagaricus*, *Marasmius*, *Rhizophagus* and *Mortierella* in Org-Low; *Lepiota*, *Penicillium*, *Phialemonium* and *Conocybe* in Conv-High; and *Racocetra*, *Tomentella*, *Spizellomyces* and *Ramicandelaber* in Org-High farming systems (Figure 3). The

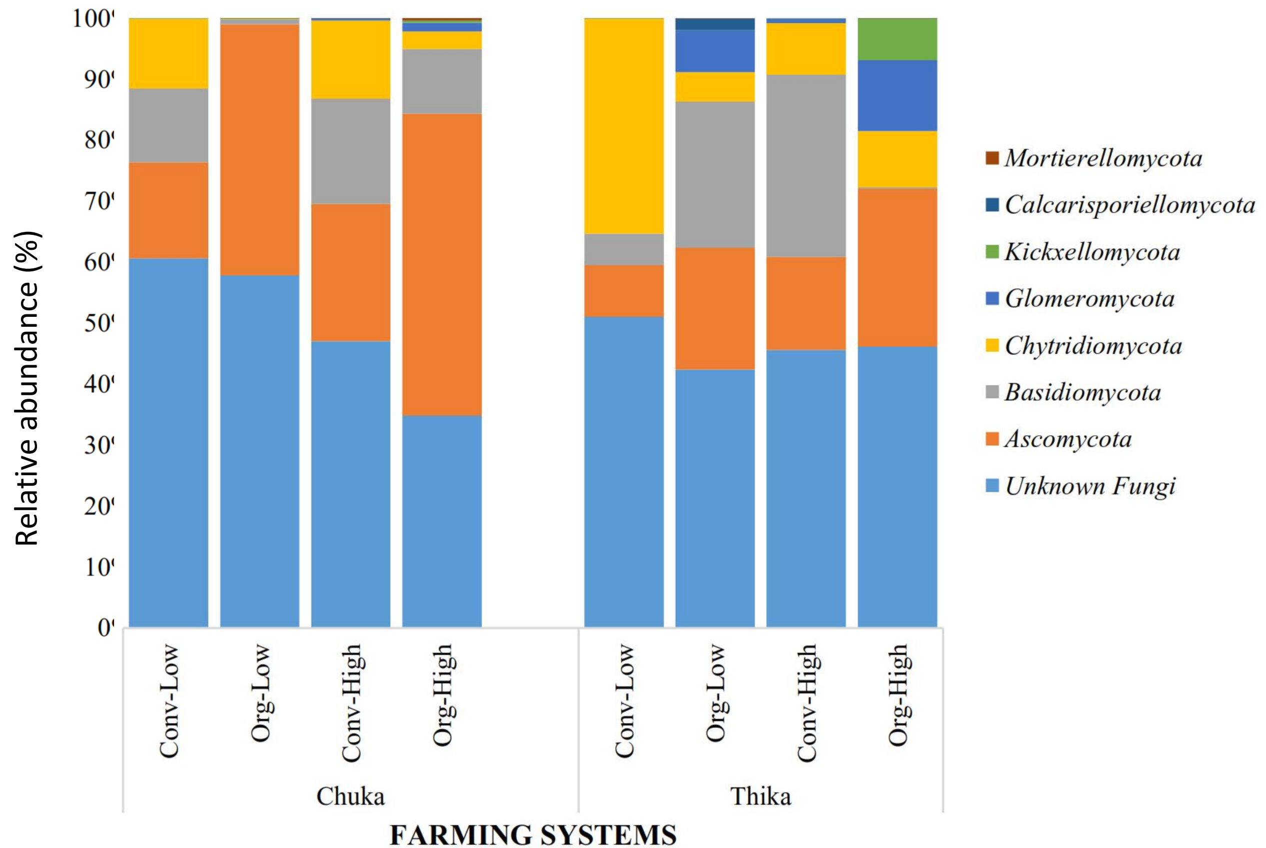

Figure 2. Relative abundance of fungal taxa at phylum level as revealed in the long-term comparison trials at Chuka and Thika sites.

distribution of various fungal OTUs and taxonomic groups within farming systems in both sites are as shown in Table 3.

Diversity indices of soil fungal communities as influenced by farming systems

Alpha diversity was applied to analyze species' diversity in each farming system through Richness (S) and Shannon index (H'). In both sites, there was a higher species richness in conventional farming systems. For instance, at Chuka site, species richness was: Conv-High = 63, Org-High = 39.5, Conv-Low = 64.5 and Org-Low = 57; while at Thika site, the species richness was Conv-High = 63.3, Org-High = 50.3 Conv-Low = 67.3 and Org-Low = 42. However, fungal communities within organic farming systems were more diverse (H) as compared to conventional farming systems (Table 3). At Chuka site, Analysis of Similarity (ANOSIM) of fungal diversity within farming systems indicated significant differences between fungal community OTUs within high and low input farming systems at 95 % level of confidence (P value = 0.05 and R = 0.115). However, there were no significant differences observed at Thika site (P value = 0.17 and R=0.066).

Effect of farming systems on beta diversity of fungal communities

Beta diversity analysis was used to evaluate differences in species complexity among the farming systems. Beta diversities were based on non-metric multidimensional scaling and hierarchical clustering. β-diversity analyzed by community comparison of the Non-metric multidimensional scaling plot indicated the four different ellipses formed by each farming system. There was an overlap of ellipses between farming systems indicating that some fungal taxa were shared across farming systems; while numerous taxa appeared outside the ellipses, signifying that the fungal taxa revealed were highly diverse (Figure 4). At Chuka site, diversity was higher in Org-High system while at Thika, Org-Low system revealed the highest diversity of fungal communities as shown by Shannon index (H') (Table 3).

Hierarchical clustering analysis was done to compare the similarity and dissimilarity of most abundant fungal

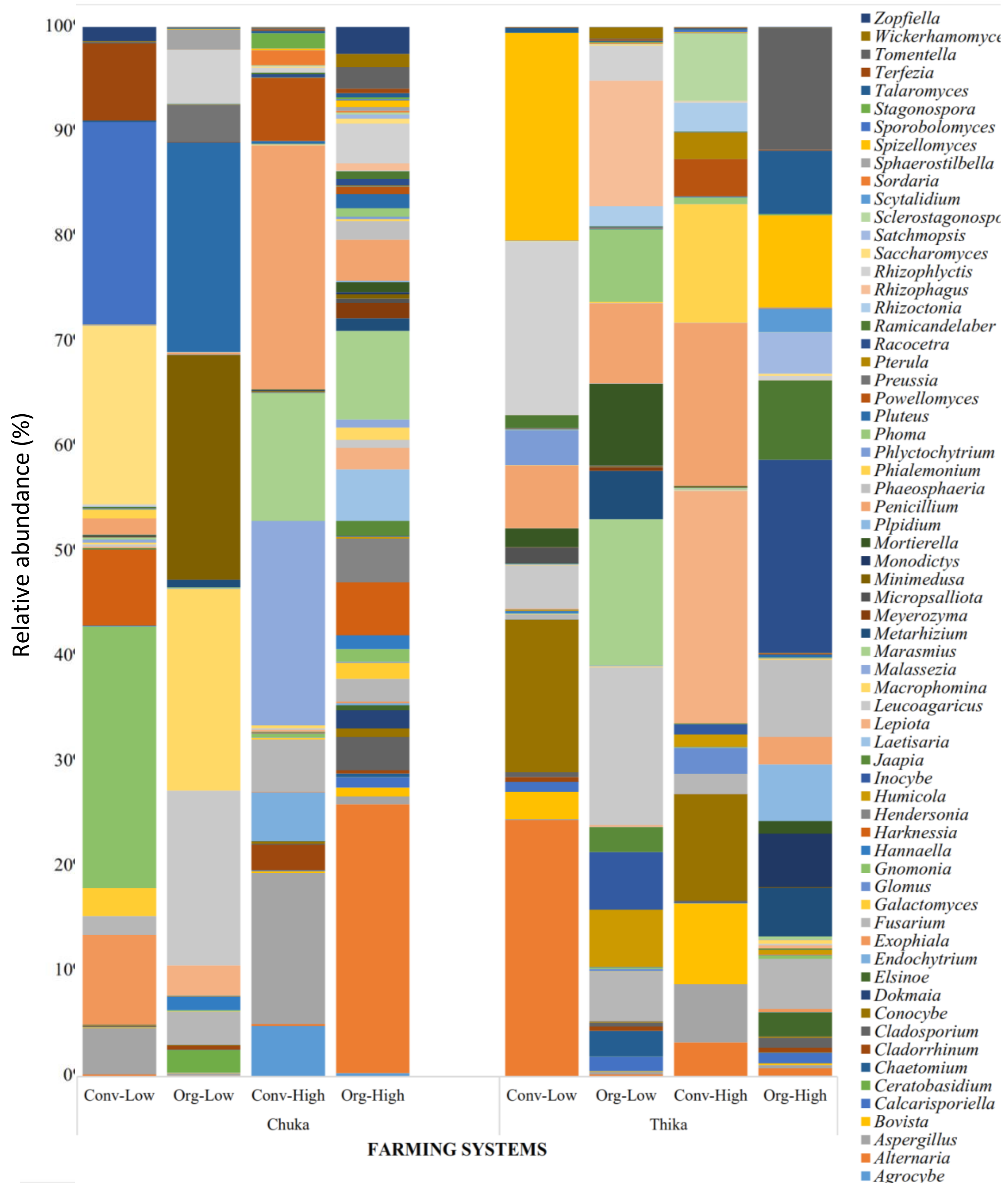

Figure 3. Relative abundance of the most predominant fungal taxa at genus level as revealed in the long-term comparison trials at Chuka and Thika sites.

taxa at family level as well as clustering of the four farming systems in each site. The hierarchical heatmap of fungal community was generated based on bray–curtis distance indices, displaying the relative abundances of fungal communities across farming systems. The dendrogram revealed two main groups within farming systems; the first group consisting of Org-High systems in both sites. Within the second group, Conv-Low systems in both sites and; Chuka Conv-High and Thika Org-Low systems were shown to cluster together. Thika

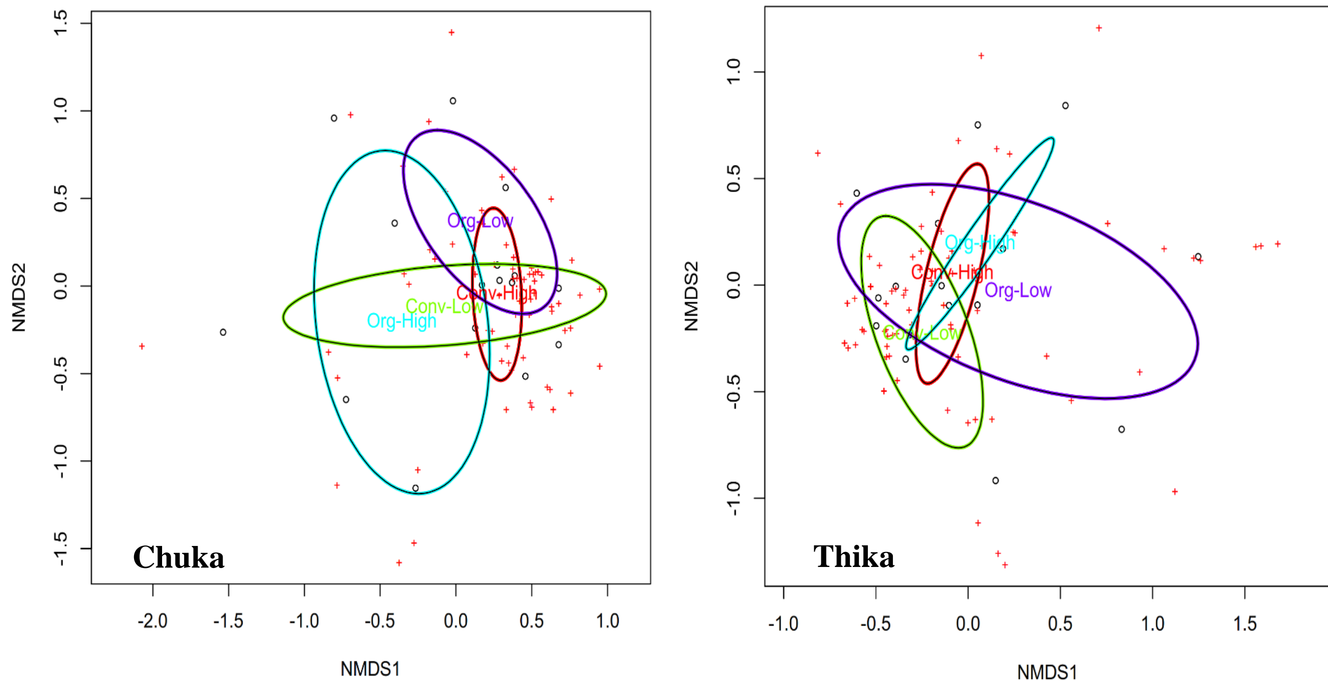

Figure 4. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities between fungal taxa at species level grouped according to farming systems.

Conv-High and Chuka Org-Low systems were outliers within the second group on the dendrogram as shown in Figure 5. Although some farming systems were shown to cluster together, they harbored different fungal taxa, an indication that soil ecosystem supports a diverse group of microorganisms.

Key environmental drivers of fungal community diversity and structure

In order to assess how environmental variables shaped soil fungal community composition, Principal Component Analysis (PCA) was performed on soil physicochemical characteristics within farming systems and fungal taxa at species level. Each characteristic was assessed on its ability to positively or negatively influence diversity within sites and farming systems. At Chuka, pH, C, N, Zn, Fe and Al were designated as major drivers of fungal diversity within farming systems while at Thika, key properties displayed were pH, EC, C, N, K, Fe, Zn, B and micro-aggregate (MA) as shown in Figure 6. Aluminum (Al) was shown to have a negative influence on fungal diversity at Chuka site.

DISCUSSION

This study combined high-resolution power of Illumina sequencing technology and analysis of fungal ITS amplicon sequences to assess the effects of organic and conventional farming systems on the diversity and composition of fungi and generate a taxonomic profile within long-term experiment trial sites in the central highlands of Kenya. The number of OTUs and alpha diversity analysis gives a glimpse of the resident fungal diversity. Eight fungal phyla (*Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Glomeromycota*, *Calcarisporiellomycota*, *Kickxellomycota*, *Mortierellomycota* and *unknown fungal phyla*) were identified at Thika and Chuka sites. Taxonomic composition analysis indicated *unknown fungal phyla, Ascomycota*, *Basidiomycota*, *Chytridiomycota* and *Glomeromycota* as the most predominant phyla within both sites and farming systems. *Ascomycota* and *Basidiomycota* are important decomposers in carbon cycle. They break down organic substances such as cellulose, lignocellulose, and lignin within plant residues into micro-molecules hence, promoting the carbon cycle in soil (Purahong et al., 2016). At family level, unique families to Chuka site included; *Unknown Pleosporales*, *Lentitheciaceae, Unknown Eurotiales* and *Unknown Cystobasidiomycetes* while at Thika site, unique families included *Didymellaceae*, *Periconiaceae*, *Phaeosphaeriaceae*, *Thyridariaceae*, *Chaetosphaeriaceae*, *Plectosphaerellaceae*, *Clavicipitaceae*, *Ophiocordycipitaceae*, *Unknown Sordariomycetes*, *Unknown Xylariales*, *Lentinaceae*,

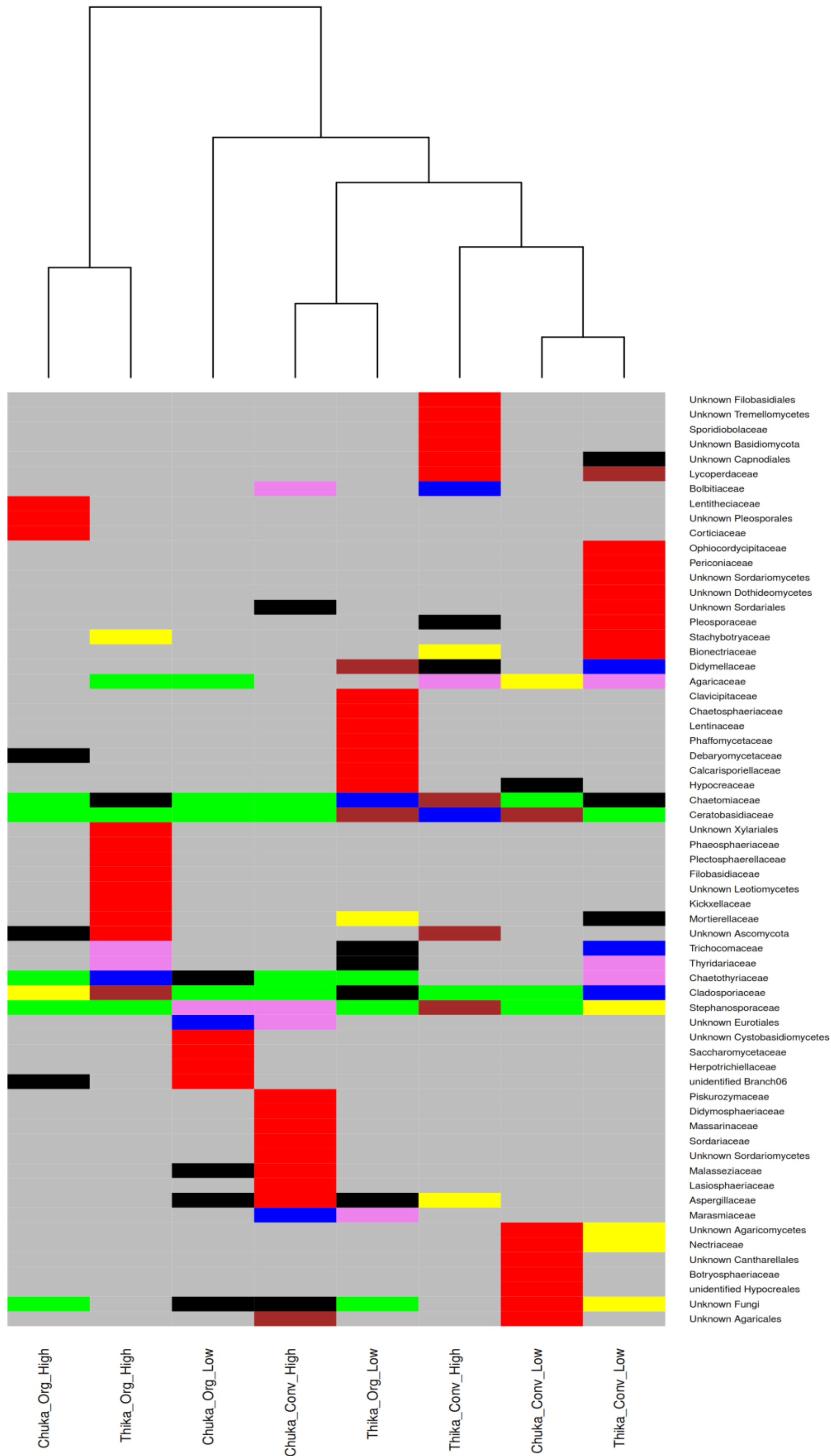

Figure 5. Hierarchical clustering of most predominant fungal taxa at family level in both sites. X-axis indicates the farming systems at Chuka and Thika.

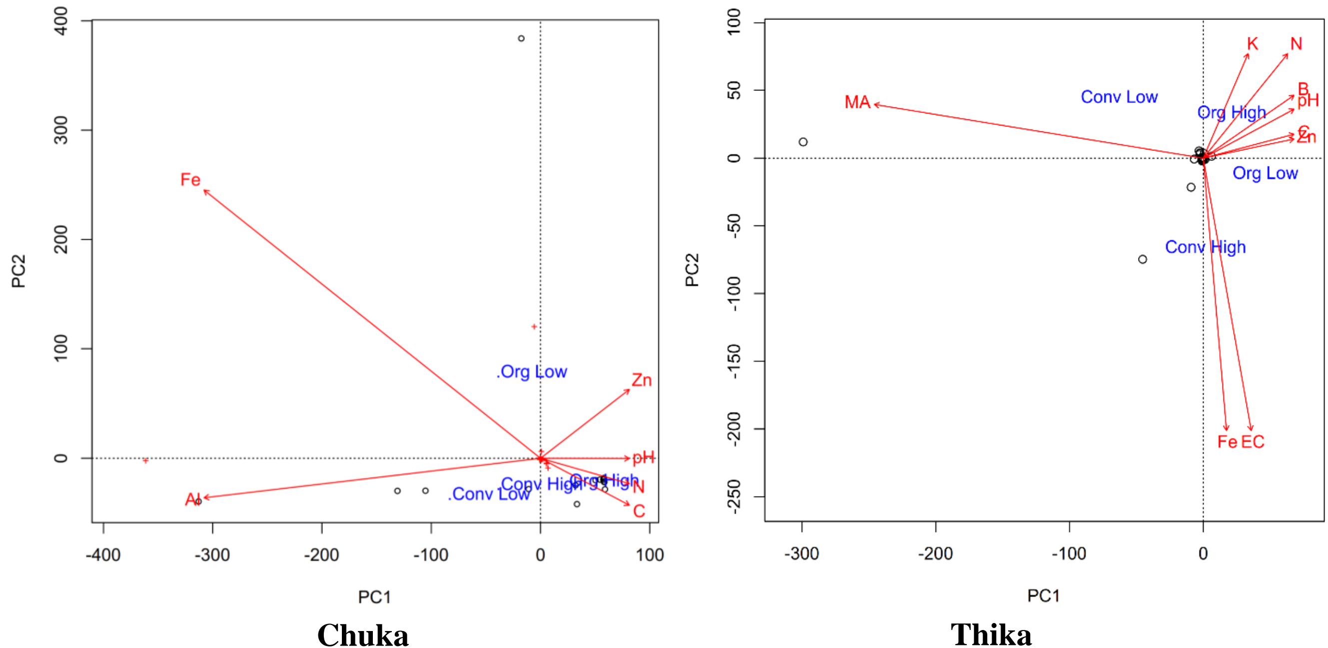

Figure 6. Principal component analysis of soil physicochemical characteristics that drive diversity within farming systems.

Filobasidiaceae, *Unknown Filobasidiales*, *Unknown Tremellomycetes* and *Mortierellaceae*. At genus level, potentially phytopathogenic genera (Sharma-Poudyal et al., 2017; Fraç et al., 2018) were revealed and they included *Alternaria* (scored up to 92% relative abundance at Chuka Org-High and 87% at Thika Conv-Low), *Epicoccum (*1.4% relative abundance at Chuka Org-High and 0.1% at Thika Org-Low), *Fusarium* (17% relative abundance at Chuka Conv-High and 17% at Thika Org-High and Org-Low), *Olpidium* (0.4% relative abundance at Chuka Org-High and 20% at Thika Org-High), *Phoma* (2.3% relative abundance at Chuka Org-High and 26.3% at Thika Org-Low), *Rhizoctonia* (0.2% relative abundance at Chuka Org-High and 10.7% at Thika Conv-High), and *Stagonospora* (5.4% relative abundance at Chuka Conv-High and 0.4% at Thika Org-High). Other major putative plant pathogenic groups revealed included members of *Nectriaceae*, *Ceratobasidiaceae, Bionectriaceae, Phaeosphaeriaceae* and *Mycosphaerellaceae* families.

Some potentially plant beneficial fungal genera (Madi et al., 1997; Harman et al., 2004; Fraç et al., 2018) were revealed within farming systems. They included; *Glomus* (scored up to 0.2% relative abundance at Chuka Org-High and 9.7% at Thika Conv-High), *Trichoderma* (0.5% relative abundance at Chuka Org-High and 0.3% at Thika Org-Low) and *Talaromyces* (1.5% relative abundance at Chuka Org-High and 22.1% at Thika Org-High). *Glomus species* have plant endosymbiotic properties especially arbuscular mycorrhizal fungi which form symbiotic relationships with plant roots (Harman et al., 2004). The species within *Glomus* genus consisted of *Glomus cerebriform*, *Rhizophagus intraradices*, *Rhizophagus diaphanum* and unknown *Glomus species*. *Trichoderma* and *Talaromyces* are prominent biocontrol agents with antagonistic potential and mycoparasitic life-style (Harman et al., 2004). *Trichoderma* genus included *Hypocrea lixii*, *Hypocrea koningii*; while *Talaromyces* genus included *Talaromyces islandicum*, *Talaromyces rotundus* and unknown *Talaromyces* species. Plant inoculation with *Epicoccum nigrum* and *Trichoderma atroviride* has been reported to protect potato against *Rhizoctonia solani* (Lahlali and Hijri, 2010). In this study, *Epicoccum nigrum* and *Epicoccum sorghi* were among the fungal species found within farming systems. The presence of potential phytopathogens, recognized plant beneficial fungi, biocontrol agents, mycoparasites and plant endosymbiont fungal groups within farming systems was similar to a previous study carried out to analyze the fungal community profiles in agricultural soils of a long-term field trial under different tillage, fertilization and crop rotation conditions (Sommermann et al., 2018). The study revealed eight potentially phytopathogenic genera, namely *Alternaria*, *Bionectria*, *Epicoccum*, *Fusarium*, *Olpidium*, *Phoma, Rhizoctonia, Stagonospora, Ophiosphaerella* and *Verticillium*. Among the biocontrol agents identified were *Trichoderma sp.*, *Coniothyrium minitans* and *Talaromyces* some of which have designated efficacy against phytopathogens (Sommermann et al., 2018).

A few groups of fast-growing soil-inhabiting saprophytic fungi and root colonizers such as *Humicola* (Family *Chaetomiaceae*), *Mortierella* (Family *Mortierellaceae*) and

Exophiala (Family *Herpotrichiellaceae*) were revealed. Some species within these genera are potential pathogens while others are considered potential biocontrol agents and may benefit plant health (Sommermann et al., 2018). Also common within the farming systems were *Penicillium* and *Aspergillus* (Family *Trichocomaceae*), common cellulolytic colonizers of soil and plant residues (Sharma-Poudyal et al., 2017).

Fungal diversity in all farming systems was majorly dependent on the flow of nutrients within the soil. Composition and diversity assessment of fungal communities within sites and farming systems displayed Thika site to harbor more OTUs as compared to Chuka site. This could be attributed to the presence of high small macro-aggregates that provided unique environmental habitats for soil fungi. Macro-aggregates have been considered as massively concurrent incubators that allow enclosed microbial communities to pursue their own independent progression (Rillig et al., 2017), hence creating more unique habitats for microbial colonization within these farming systems. Chuka soils contained high primary and secondary clay minerals, while Thika soils were characterized by high primary minerals and low secondary clay minerals. Clay minerals and oxides of Fe and Al have been exhibited to play important roles in adsorbing dissolved organic carbon (Singh et al., 2016, 2017b). Since Thika soils contained high Fe levels coupled with high primary clay minerals, this may have created a stable atmosphere for fungal groups to thrive. At Chuka site, fungal diversity was also negatively influenced by high Al levels, hence low OTU numbers obtained. However, in both sites, Conv-Low had the highest number of OTUs (161 and 168 OTUs at Chuka and Thika respectively) compared to other farming systems. This could be attributed to the application of undecomposed farmyard manure as input component in the system during planting. The fungal diversity within farming systems is influenced by complex interactions between a wide range of soil properties and agronomic inputs, thus signifying that fungi within the soils are exceptionally diverse. These inputs change soil properties and microbial diversity, and the microbial community in turn manipulates nutrient cycling processes altering soil fertility, plant productivity and environmental sustainability.

Conclusion

This study revealed that farming systems have a profound impact on soil fungal communities. Conventional farming systems were shown to support diverse fungal communities compared to organic farming systems. This was possibly due to the integration of organic and inorganic inputs into conventional farming systems which enhanced nutrient availability for fungal proliferation, thus increasing their diversity. The results of this study provide a foundation for further studies on the regulation of quality and quantity of farming inputs and could provide guidance for selecting the best farming system model to protect soil ecology.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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ABBREVIATIONS

ITS, Internal Transcribed Spacer; **OTUs,** Operational Taxonomic Units; **DNA,** Deoxyribonucleic Acid; **QIIME,** Quantitative Insights into Microbial Ecology.

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Table S1. SysCom trials soil fertility managment plan; crop rotation cycle, inputs types used, amount of nitrogen and phosphorous contained in the inputs and amounts applied per hectare.

Year	Season		CONV LOW		CONV HIGH		ORG LOW		ORG HIGH	
1	Long	**Crop**	**Maize**		**Maize**		**Maize**		**Maize**	
		Starter	5 t fresh FYM 50 kg DAP	22 kg N; 6 kg P; 9 kg N; 12 kg P;	Approx. 5 t rotten FYM (start with 7.5 t) 200 kg DAP	34kg N; 9 kg P; 36kg N; 46 kg P;	Rotten FYM (started with 5 t fresh FYM) 1.36 t Tithonia mulch (FW) 100 kg Phosphate rock	22 kg N; 6 kg P; 9 kg N; 1 kg P; 11 kg P;	Compost (start with 7.5 t fresh FYM) 5.4 t Tithonia mulch (FW) 364 kg Phosphate rock	34 kg N; 9 kg P; 36 kg N; 3 kg P; 40 kg P;
		Top dressing	No		100 kg CAN	26 kg N;	No	No	3.9 t Tithonia (FW) as mulch or liquid manure	26 kg N; 2 kg P;
		Lime	No		Based on pH monitoring		No		Based on pH monitoring	
		Micro-nutrients	No		Based on soil analysis and crop requirements		No		Based on soil analysis and crop requirements	
		Green manures	No		No		No		Mucuna	
1	Short	**Crop**	**Kales&Swiss chard ("spinach")**		**Cabbage**		**Kales&Swiss chard ("spinach")**		**Cabbage**	
		Starter	1 t fresh FYM 50 kg TSP	4 kg N; 1 kg P; 12 kg P;	Approx. 10 t rotten FYM (start with 15 t) 200 kg TSP	67 kg N; 18 kg P; 46 kg P;	Rotten FYM (started with 1 t fresh FYM) 1.2 t Tithonia mulch (FW) 90 kg Phosphate rock	4 kg N; 1 kg P; 8 kg N; 1 kg P; 10 kg P;	Compost (start with 15 t fresh FYM) 6 t Tithonia as mulch 400 kg Phosphate rock	67 kg N; 18 kg P; 40 kg N; 4 kg P; 44 kg P;
		Top dressing	60 kg CAN	16 kg N	300 kg CAN	78 kg N	1.2 t Tithonia FW as liquid manure	8 kg N; 1 kg P	6 t Tithonia FW as liquid manure	40 kg N; 4 kg P;
		Lime	No		Based on pH monitoring		No		Based on pH monitoring	
		Micro-nutrients	No		Based on soil analysis and crop requirements		No		Based on soil analysis and crop requirements	
		Green manures	No		No		No		Mucuna	
2	Long	**Crop**	**Maize**		**Baby corn**		**Maize**		**Baby corn**	
		Starter	5 t fresh FYM 50 kg DAP	22 kg N; 6 kg P; 9 kg N; 12 kg P;	Approx. 7.5 t rotten FYM (start with 11.3 t) 200 kg DAP	51 kg N; 14 kg P; 36 kg N; 46 kg P;	Rotten FYM (started with. 5 t fresh FYM) 1.36 t Tithonia mulch (FW) 100 kg Phosphate rock	22 kg N; 6 kg P; 9 kg N; 1 kg P; 11 kg P;	Compost (start with 11.3 t fresh FYM) 5.4 t Tithonia mulch (FW) 364 kg Phosphate rock	51 kg N; 14 kg P; 36 kg N; 3 kg P; 40 kg P;
		Top dressing	No		100 kg CAN	26 kg N;	No	No	3.9 t Tithonia (FW) as mulch or liquid manure	26 kg N; 2 kg P;
		Lime	No		Based on pH monitoring		No		Based on pH monitoring	
		Micro-nutrients	No		Based on soil analysis and crop requirements		No		Based on soil analysis and crop requirements	
		Green manures	No		No		No		Mucuna	
2	Short	**Crop**	**Grain legumes & ...**		**French beans**		**Grain legumes & ...**		**French beans**	
		Starter	No		Approx. 7.5 t rotten FYM (start with 11.3 t) 200 kg DAP	51 kg N; 14 kg P; 36 kg N; 46 kg P;	No		Compost (start with 11.3 t fresh FYM) 5.4 t Tithonia mulch (FW) 364 kg Phosphate rock	51 kg N; 14 kg P; 36 kg N; 3 kg P; 40 kg P;
		Top dressing	No		100 kg CAN;	26 kg N;	No		3.9t Tithonia (FW) as liquid manure	26 kg N; 2 kg P;
		Lime	No		Based on pH monitoring		No		Based on pH monitoring	
		Micro-nutrients	No		Based on soil analysis and crop requirements		No		Based on soil analysis and crop requirements	
		Green manures	No		No		No		Mucuna	
3	Long	**Crop**	**Maize**		**Baby corn**		**Maize**		**Baby corn**	
		Starter	5 t fresh FYM 50 kg DAP	22 kg N; 6 kg P; 9 kg N; 12 kg P;	Approx. 7.5 t rotten FYM (start with 11.3 t) 200 kg DAP	51 kg N; 14 kg P; 36 kg N; 46 kg P;	Rotten FYM (started with 5t fresh FYM) 1.36 t Tithonia mulch (FW) 100 kg Phosphate rock	22 kg N; 6 kg P; 9 kg N; 1 kg P; 11 kg P;	Compost (start with 11.3 t fresh FYM) 5.4 t Tithonia mulch (FW) 364 kg Phosphate rock	51 kg N; 14 kg P; 36 kg N; 3 kg P; 40 kg P;

Table S1. Contd.

		Top dressing	No		100 kg CAN	26 kg N;	No		3.9 t Tithonia (FW) as mulch or liquid manure	26 kg N; 2 kg P;
		Lime	No		Based on pH monitoring		No		Based on pH monitoring	
		Micro-nutrients	No		Based on soil analysis and crop requirements		No		Based on soil analysis and crop requirements	
		Green manures	No		No		No		Mucuna	
3	Short	**Crop**	**Irish potatoes&local vegetables**		**Irish potatoes**		**Irish potatoes&local vegetables**		**Irish potatoes**	
		Starter	2 t fresh FYM 100 kg DAP	9 kg N; 2 kg P; 18 kg N; 23 kg P;	Approx. 7.5 t rotten FYM (start with 11.3 t) 300 kg TSP 200 kg CAN	51 kg N; 14 kg P; 69 kg P; 52 kg N;	Rotten FYM (started with 2 t fresh FYM) 2.72 t Tithonia (FW) 200 kg Phosphate rock	9 kg N; 2 kg P; 18 kg N; 2 kg P; 22 kg P;	Compost (start with 11.3 t fresh FYM) 8.2 t Tithonia mulch (FW) 581 kg Phosphate Rock	51 kg N; 14 kg P; 54 kg N; 5 kg P; 64 kg P;
		Top dressing	Nothing		Nothing		Nothing		Nothing	
		Lime	No		Based on pH monitoring		No		Based on pH monitoring	
		Micro-nutrients	No		Based on soil analysis and crop requirements		No		Based on soil analysis and crop requirements	
		Green manures	No		No		No		Mucuna	
		TOTAL	18 t FYM 250 kg DAP 50 kg TSP 60 kg CAN	79 kg N; 21 kg P 45 kg N; 59 kg P; 12 kg P; 16 kg N	Approx. 45 t rotten FYM (start with 68 t) 800 kg DAP 500 kg TSP 900 kg CAN	305 kg N; 83 kg P; 144 kg N; 184 kg P; 115 kg P; 234 kg N;	Rotten FYM (started with 18 t fresh FYM) 9.2 t Tithonia 590 kg Phosphate rock	79 kg N; 21 kg P; 61 kg N; 6 kg P; 65 kg P;	Compost (start with 68 t FYM FW) 65 t Tithonia 2392 kg Phosphate Rock	305 kg N; 83 kg P; 382 kg N; 33 kg P; 268 kg P;
				140 kg N; 92 kg P		**683 kg N; 382 kg P**		**140 kg N; 92 kg P**		**683 kg N; 382 kg P**

Nutrient contents: FYM/compost (DW): 1.12% total N and 0.3% P (Lekasi et al., 2003); DM of FYM is assumed to be 40%; Tithonia diversifolia (DW): 3.3% N; 0.31% P; 3.1% K (Nziguheba et al. 2004); DM of Tithonia = 20%; Phosphate rock from West Africa (Finck): 11 - 13% P; DAP: 18% N; 23% P; TSP: 23% P; CAN: 26% N.

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Presence of phytopathogenic fungi and oomycetes on rice and avocado crops in Tolima (Colombia)

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Tolima is one of the largest zones in Colombia where avocado and rice are produced. The disease management programs for these crops are mainly chemical-dependent and tend to have variable results since the diagnosis of some diseases is based on unspecific symptoms. In this context, the present study aims to bring information about the phytopathogens affecting Tolima´s rice and avocado crops. From foliar tissue, culm, and roots of rice plants, 18 isolates closely related to *Gaeumannomyces graminis var. graminis/G. oryzae* and 06 *Magnaporthe oryzae* were obtained. The isolation of these fungi was concordant with the crown sheath rot and rice blast symptoms observed. Also, 13 different fungi belonging to the *C. acutatum* and *C. gloeosporioides* complex were isolated from avocado fruits with symptoms of anthracnose. Interestingly, only two *Phytophthora* sp. isolates were purified from necrotic roots of avocado trees. However, several fungi belonging to the *Cylindrocarpon/Ilyonectria* genera and one strain identified as *Bjerkandera adusta* were isolated from these samples. Symptoms caused by these fungi are similar making it necessary to do a differential diagnosis of the actual pathogen causing avocado root necrosis. The data obtained constitute the first report of the above-mentioned phytopathogens in Tolima avocado crops and established the basis for an epidemiologic study of its distribution in Colombia.

Key words: Rice, avocado, *Gaeumannomyces, Magnaporthe, Phytophthora, Cylindrocarpon, Ilyonectria, Colletotrichum.*

INTRODUCTION

Rice and avocado are two of the most relevant crops in Colombia either for internal consumption (rice) or as an exportation product (avocado). In 2019, 1.904.819 tons of rice and 210.280 tons of avocado were produced (Dane-

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Abbreviations: Anam, Anamorph; **ITS,** Internal Transcriber Spacer; **Ggg,** Gaeumannomyces graminis (Sacc.) von Arx & D. Olivier var graminis; **cv** – Cultivar; **Ha,** Hectares.

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Fedearroz, 2020; DANE, 2020). Tolima has the first and second-largest area cultivated with these crops in Colombia (51.189 ha. for rice and 21.115 ha for avocado). In the first six months of 2019, Tolima contributed 18.19 and 21.6% of the production of avocado and rice, respectively. However, in the case of avocado crops, its yield (2.27 tons/ha) was lower than other departments with smaller production areas such as Antioquia (3.98 tons/ha) and Valle del Cauca (9.6 ton/ha) (DANE, 2020) leading to a less participation in avocado exportation.

On the other hand, lack of crop rotation, exclusive use of moist conditions for rice grown, and environmental conditions such as high humidity and temperature characteristics of Tolima favor the development of several phytopathogenic fungi being one of the reasons related to the rice yield reduction in Tolima (Echeverri-Rico, 2016). In rice crops, more than 70 different pathogens have been reported including several soil-borne fungi (Ke et al., 2016). Two of these fungi are wide distributed and have a significant impact on rice production: *Gaeumannomyces graminis* var *graminis*, the causal agent of crown sheath rot (or sheath blight) and *Magnaporthe oryzae* (teleomorph) (Herbert) Barr (anamorph: *Pyricularia oryzae*) the causal agent of rice blast (Zhang et al., 2016).

Gaeumannomyces graminis (Sacc.) von Arx & D. Olivier var *graminis* (Ggg) is a soil-borne phytopathogen that colonizes the roots and culms of rice (Hawerroth et al., 2017). This pathogen generates typical dark brown and black lesion on rice sheath and culms and necrosis on rice roots which could lead to early grain maturation and reduce the number of grains per panicle (Peixoto et al., 2013). Even though it has been reported that this pathogen causes minor loses in production (Hernández-Restrepo et al., 2016), its incidence in crops is high in several regions including Brazil (Peixoto et al., 2013), Texas, Florida (USA) (Datnoff et al., 1997) and Colombia where 34 to 99% incidence is reported (Echeverri-Rico, 2016).

Magnaporthe oryzae (previously classified as *M. grisea*) is a hemibiotrophic fungus described as the most devasting fungal pathogens in rice crops, causing annual production losses of 10 - 30% (Ebbole, 2007; Zhang et al., 2016). This pathogen was first reported in China (1637) and Japan (1704) (Kumar and Ashraf, 2019) and has caused several outbreaks in rice cropping regions of Italy (Kunova et al., 2014; Titone et al., 2015), Kenya (Kihoro et al., 2013), Bangladesh (Callaway, 2016) and Australia (Fang et al., 2017); it is used as a model for the study of plant-pathogen interaction and development of plant resistance.

Rice blast is characterized by the production of diamond-shaped lesions on leaves and, to a lesser extent on leaf collars, necks, panicles, pedicels, and seeds (TeBeest et al., 2007). The symptoms on leaves usually appear during tillering and could be followed by neck blast lesions that are correlated with higher yield losses (Ghataka et al.,2013). Because of that, an early diagnosis is necessary to prevent the negative impacts of this fungus on rice production.

Avocado crops are also affected by a large number of microbial phytopathogens. However, the higher reductions in avocado yields are probably caused by the oomycete *Phytophthora cinnamomi var. cinnamomi* Rand (Hardham and Blackman, 2018). Root rot, a disease caused by *P. cinnamomi*, has been reported in more than 56 countries and was described since 1927 (Tucker, 1929). Root rot affected 60-75% of avocado orchards in California (USA) and had presented serious outbreaks that caused economical losses of almost USD$40 million in 1989 (Belisle et al., 2019; Pagliaccia et al., 2013; Ploetz, 2013). In Australia, *P. cinnamomi* caused production losses up to 50% in 1974 and is to date, one of the most limiting factors of avocado production in this country (Ploetz, 2013; Salgadoe et al., 2018). On Antioquia (Colombia) fields, root rot incidence could cause economical losses of 356 USD/nursery and 2340 USD/ha showing the impact that this pathogen can cause on this crop (Ramírez-Gil et al., 2017).

During the fruit development and postharvest time, avocados are also susceptible to the attack of different fungi including those belonging to the *Colletotrichum* genus. This Ascomycota phytopathogen is classified as a hemibiotrophic fungus and is the causal agent of anthracnose disease in a wide range of host plants (Phoulivong, 2011). Several species of *C. gloeosporioides, C. boninense* and *C. acutatum* complexes have been related to avocado anthracnose (Jayawardena et al., 2016) and are reported as the main production constraint in the bigger avocado producer countries including Mexico (Silva-Rojas and Ávila-Quezada, 2011; Velázquez-del Valle et al., 2016) and Colombia (Gil and Morales-Osorio, 2019). *Colletotrichum* isolates are able to colonize inflorescences, leaves, stems, and fruits of avocado and typically generate semicircular or angular necrotic, sunken and wet-looking lesions (Cannon et al., 2012; Pérez-Jiménez, 2008). These injuries cause the fruits to be unsuitable for export, thus losing their commercial value.

In Colombia, the information regarding Ggg, *M. oryzae, P. cinnamomi,* and *Colletotrichum* distribution in Tolima for the past years is insufficient, even when previous reports from this and other zones in the country showed the high incidence of these pathogens in rice or avocado crops (Echeverri-Rico, 2016; Grisales et al., 2016; Prado-Patiño, 2016; Ramírez and Morales-Osorio, 2013). Moreover, in the case of Ggg, *Colletotrichum* and *P. cinnamomi* the diagnosis is currently done based on symptoms that could be confused with other diseases. For example, some symptoms caused by *P. cinnamomi* on root trees could be confused with the symptoms caused by *Cylindrocarpon destructans* infection (Ramírez-Gil, 2018), root asphyxiation caused by water

and temperature stress (Nakova, 2010; Ramírez-Gil, 2018) or tree poor nutrition (Akinsanmi et al., 2016). The misdiagnosis could lead to the implementation of inefficient management strategies increasing the negative effects on crop productivity.

In this context, the differential diagnosis of the diseases caused by these phytopathogens should rely on pathogen isolation and identification. For this reason, the present research aims to do an exploratory study of the presence of these four microorganisms in rice and avocado crops on the centrum of Tolima department. This information is going to be used as initial data for a second phase study that seeks to obtain epidemiological information about the prevalence of Ggg, *M. oryzae*, *P. cinnamomi* and *Colletotrichum* in Colombia.

MATERIALS AND METHODS

Rice and avocado sampling

In order to isolate and identify the fungal phytopathogen present in symptomatic rice and avocado plants with a preliminary diagnosis of crown sheath rot, rice blast, anthracnose and root rot were obtained from 9 and 21 different rice and avocado crops respectively located at the centrum zone of Tolima department (Supplementary Table 1). For rice samples, a presumptive diagnosis was done evaluating the symptoms and severity of crown sheath rot (Supplementary Table 2) and rice blast (Supplementary Table 3) (Ghazanfar et al., 2009). Plants with severity index higher than 2 for Ggg isolation or 4 for *M. oryzae* isolation were taken out of the field and transported at 4°C to the laboratory for pathogen isolation.

For avocado samples, necrotic roots of trees with symptoms of root rot were obtained. For this purpose, at least 10 trees per location were evaluated and classified according to the scale proposed by Ramirez-Gil (Ramírez-Gil, 2018) (Supplementary Table 3). Trees with symptoms recorded in the scale as 3 to 5 were used for roots sampling. Only secondary roots with initial symptoms of necrosis were selected and transferred at 4°C for pathogen isolation. On the other hand, 5 Avocado fruits (cv Hass) with initial anthracnose symptoms (lesion of 1 - 6 cm diameter), advance necrosis (lesions with > 6 cm diameter), modified fruits and healthy fruits were randomly selected from each evaluated location. The fruits were put into plastic bags and maintained refrigerated (4°C) until processing.

Isolation and purification of rice pathogens

Rice samples included sheath, culms and leaves of three different cultivars (F67, F68 and F2000). The sheath and culm samples were microscopically inspected to identify the presence of black-brown mycelium, perithecium and or hyphopodia suggestive of Ggg. Positive samples were cut into pieces of 5 cm^2 and transferred to moist chambers. To favour mycelium growth and perithecium opening for discharge of ascospores, the moist chambers were incubated at 25°C for 7 days. Then, the growing mycelium was aseptically transferred to PDA medium supplemented with chloramphenicol (50 µg/ml). Additionally, the remnant plant tissue was surface sterilized in sodium hypochlorite (3%) and ethanol (70%) for 1 min and washed with sterile water (3 min). Once sterilized, the treated plant tissue was transferred to PDA medium and incubated at 25°C for seven days.

For *M. oryzae* or *Magnaporthe*-like fungi isolation, a similar approach was followed using rice leaves samples. The material was inspected looking for diamond-shape lesions. Positive samples were cut in pieces (5 cm^2) and transferred aseptically to moist chambers. As with Ggg samples, the moist chambers were incubated for seven days at 25°C and the growing mycelium was then transferred to Chloramphenicol supplemented PDA and further incubated at 25°C.

Individual colonies were purified on PDA for Ggg like isolates or Tomato-oatmeal medium (Oatmeal 20 g/L, tomato paste 20 g/L, bacteriological agar 15 g/L pH 7.0) for *M. oryzae* like isolates (Supplementary Table 4). Macroscopic and microscopic characteristics of pure colonies were observed and only isolates with phenotypic characteristics similar to those of the fungi of interest were preserved for molecular identification.

Isolation and purification of avocado pathogens

Necrotic secondary roots of avocado for the isolation of *P. cinnamomi* were initially washed with tap water for 5 min. Then the roots were dried at room temperature and the external cover of the root was carefully removed. All the roots were inspected to identify zones with partial necrosis. Once identified, the roots were superficially sterilized following the same protocol as mentioned for rice foliar tissue. The surface-sterilized roots were then transferred to PDA and V8-ACBPHR medium (Ramírez and Morales-Osorio, 2013) and incubated at 22°C in darkness for 7 days. All the growing fungi were further purified and preserved for phenotypic and molecular characterization. For *Colletotrichum* isolation, avocado fruits were inspected and the tissue with necrotic lessons was cut in pieces of 0.25 cm^2. These pieces were surface-sterilized (same protocol as described above) and transferred to PDA supplement with chloramphenicol (50 µg/ml). The agar plates were incubated at 25°C for five days. The fungi growth was verified, and the individual colonies were purified on new PDA medium. Only those colonies with growth, pigmentation and microscopic characteristics typical of *Colletotrichum* species were preserved for molecular characterization.

DNA extraction and molecular identification

For molecular characterization, Genomic DNA of each fungal strain was obtained using a modified protocol of CTAB (Cetyltrimethyl Ammonium Bromide) / Chloroform-isoamyl alcohol extraction described by Clarke (Clarke, 2009). Molecular characterization was done through ITS region sequencing using the universal primers ITS1 (5´- TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (White et al., 1990). Polymerase chain reaction (PCR) amplification was carried out as described by Mosca et al. (2014). The thermal cycling profile of initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 45 s were used (Mosca et al., 2014). The obtained amplimer was sequenced (Macrogen, Korea) and the ITS sequence obtained was compared with the corresponding reference data retrieved from GenBank database.

Sequence alignment was carried out using MUSCLE algorithm and adjusted manually. Phylogenetic analysis of the partial ITS sequences was carried out using the Maximum likelihood method or Neighbor-joining method with MEGA7 software (Kumar et al., 2016). Evolutionary distance matrices were generated by either Juker-Cantor, Tamura-Nei or Kimura 2-parameter method. The stability of tree topologies was accessed by bootstrap analysis based on 1000 resampling.

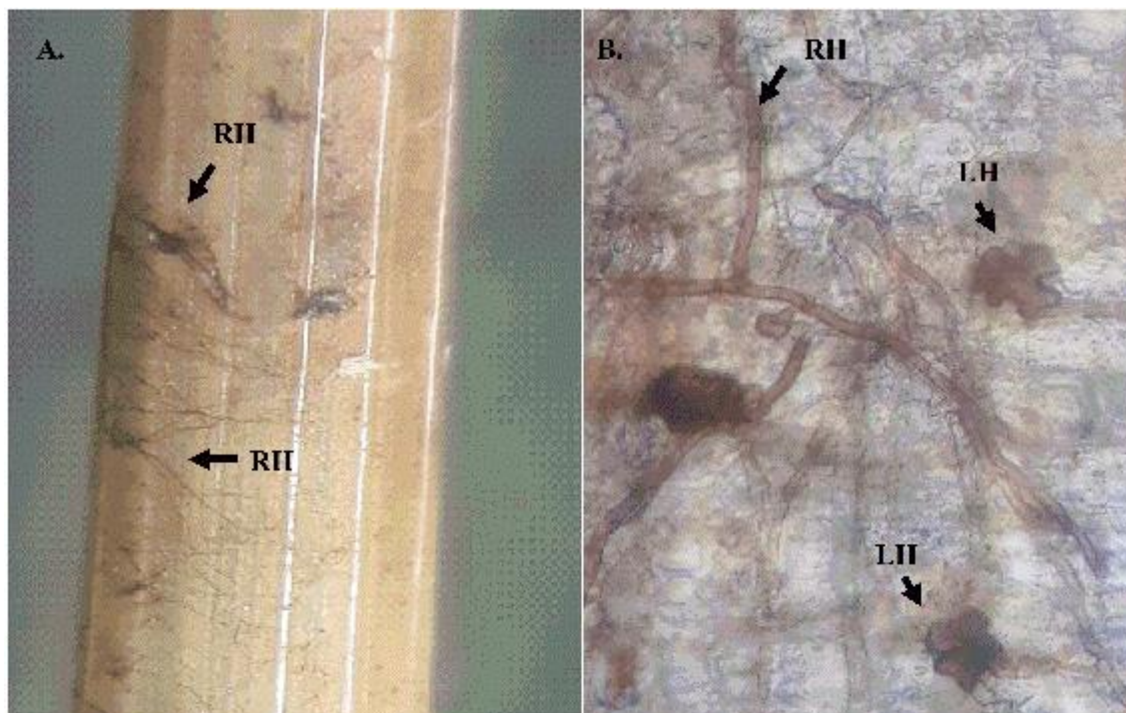

Figure 1. Symptoms of crown sheath rot and presumptive isolation of Ggg and other *Gaeumannomyces* isolates from rice samples.

RESULTS AND DISCUSSION

Phytopathogenic fungi obtained from rice samples

In order to contribute to the diagnosis of rice diseases in Tolima, in the present research, 53 samples of rice plants from 12 different crops located in the centrum and west part of the department (Supplementary Table 1) were evaluated and used for fungal pathogen isolation. As shown in Figure 1, the studied samples present brown septate hyphae and lobulated hyphopodia. Runner hyphae are evident macroscopically as black filaments growing on the surface of rice culms and are used as a preliminary diagnostic symptom of crown sheath rot. In sheath and culm tissues, the presence of these structures can be a sign of advanced Ggg colonization (Hawerroth et al., 2017; Hernández-Restrepo et al., 2016).

Figure 1 shows the runner hyphae (A) and lobulated hyphopodia (B) present on rice sheaths. Previous reports from FEDEARROZ diagnostic group have indicated the presence of Ggg in the south of Tolima and other departments of Colombia (Echeverri-Rico, 2016). However, other zones of Tolima have not been studied so far and are having problems of misdiagnosis due to the presence of unspecific symptoms and co-occurrence of fundal diseases in the same phenological stage. In the present study, 18 different isolates were obtained from the evaluated samples and presented the typical grey-black mycelium on PDA medium characteristic of *Gaeumannomyces* isolates. Most of them were recovered from Ambalema (13 isolates), Espinal (2 isolates), Piedras (2 isolates) and Ibague (single isolate). The ITS sequences of these fungi allowed us to classify them into the *Gaeumannomyces genus.* Moreover, as seen in Figure 2, most of the isolates were closely related to *G. oryzinus* ang *G. graminis var. graminis* with percentages of similarity higher than 98 % (Supplementary Table 5).

The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0701)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree was rooted with *M. oryzae* (CBS 255.38 and CBS 128304).

Originally, *G. oryzae* and Ggg were described as the same microorganism since there are able to infect rice plants; they do not have morphological differences and the ITS sequence by itself is not strong enough to separate them into different clades (Walker, 1972). However, recent phylogenetic studies done using the sequences of several housekeeping genes such as ITS, LSU, *rpb1* and *tef1* demonstrated that both microorganisms correspond to different clades within the

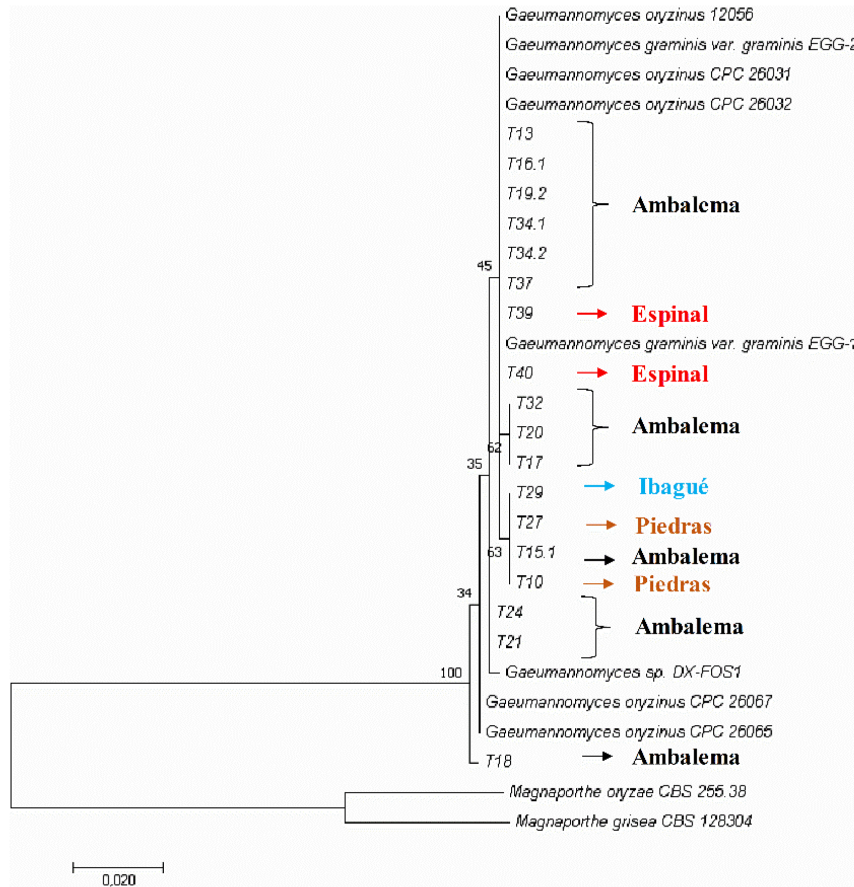

Figure 2. Molecular phylogenetic analysis by maximum likelihood method of partial ITS1 sequences of *Gaeumannomyces* isolates.

Gaeumannomyces genera (Hernández-Restrepo et al., 2016). Ggg and *G. oryzae* are commonly isolated from rice cultivars but only Ggg has been recognized as the causal agent of crown sheath rot. Further information is needed in order to confirm the pathogenesis of *G. oryzae* and its relationship with crown sheath rot.

On the other hand, as can be seen in Figure 3 six isolates of rice blast lesions were purified. All of them correspond to fungi of the *Magnaporthe* genera. Based on the ITS sequences, it can be suggested that they belong to the *M. oryzae* species. These fungi were isolated from samples coming from Espinal, Piedras and Ibague. In other locations, isolates of the *Curvularia* and *Fusarium* genera were identified based on phenotypic characteristics, but they were not identified by ITS sequencing (Data not shown).

The evolutionary history was inferred by using the Neighbor–joining method based on the Tamura-nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree was rooted with *Barretomyces calatheae* (CBS 129274). The similarity of *M. oryzae* with other species such as *M.* grisea is explained by the close relationship of both species. *M. grisea* and *M. oryzae* belong to the *M grisea complex* (Zhang et al., 2016). They are morphologically indistinct but could be differentiated from each other based on its host plant (rice for *M. oryzae* and grasses for *M.* grisea) and multi Locus Phylogenetic analysis using conserved genes like actin, beta-tubulin, and calmodulin (Couch and Kohn, 2002; Klaubauf et al., 2014). Although *M. oryzae* is the currently accepted causal agent of rice blast, some reports indicate cross-infection in rice with *M grisea* isolates (Choi et al., 2013). Therefore, the presence of both pathogens should be monitored to prevent rice blast outbreaks and cross-infection with grasses.

Colletotrichum sp. isolates as the causal agent of anthracnose disease on avocado Hass fruits in Tolima

In the present research, 21 different avocado crops in Tolima were sampled in order to determine the presence

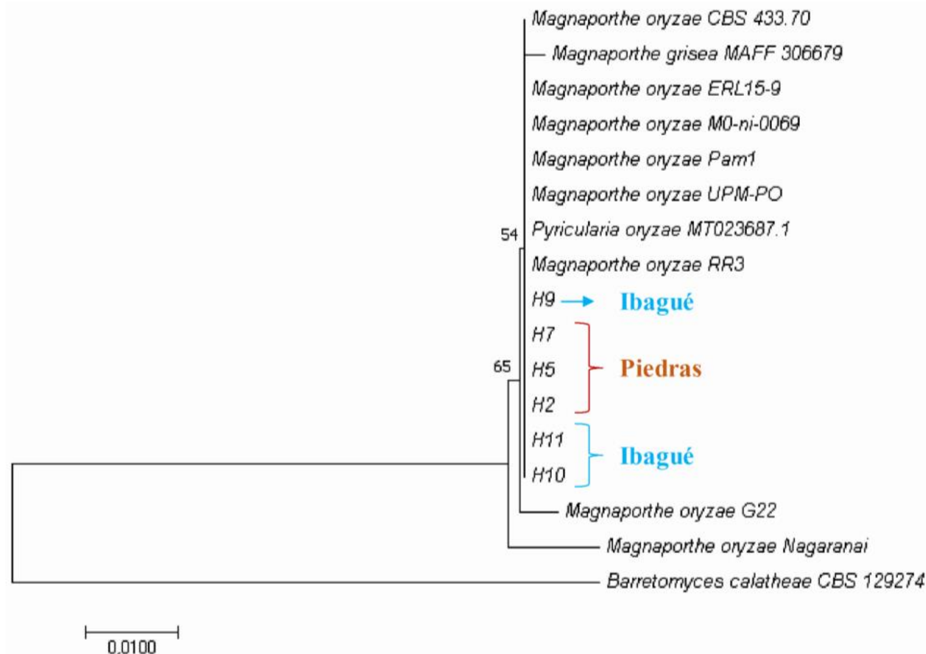

Figure 3. Molecular Phylogenetic analysis by Neighbor-joining method of partial ITS1 sequences of *Magnaporthe* isolates.

of *Colletotrichum* as causal agent of anthracnose symptoms observed on fruits in the evaluated crops. 13 *Colletotrichum*-like fungi were isolated from avocado fruits (Hass cv.) with necrotic lesion (Figure 4A). From them, only those isolates that presented orange-black pigmentation and abundant production of hyaline septate ovoid conidium were preserved for identification (Figure 4B-C). Figure 4 shows the typical circular necrotic wound in Avocado cv Hass (A) and the macroscopic (B) and microscopic (C) characteristics of isolate F27 isolated from this kind of lesion.

Colletotrichum is a widespread Ascomycota fungus that has been reported as plant pathogen in economically important fruits crops such as avocado. Due to its distribution and incidence this fungus is classified as one of the ten most important fungal phytopathogens of the world causing losses in production up to 50% in several fruits and vegetable crops (Dean et al., 2012). Related to its impact on crops, the isolation and identification of *Colletotrichum* species have been a major field of research for the last decades. The taxonomy of *Colletotrichum* is complex. Currently, 11 fugal complexes have been reported for this genus (Jayawardena et al., 2016). From them, species belonging to the *C. gloeosporioides, C. acutatum* and *C. boninense* complexes are the most associated with anthracnose on avocado fruits.

In Colombia, some efforts have been done in order to identify the prevalence and distribution of these pathogens in avocado (Cobo-Nuñez, 2017; Grisales et al., 2016) and others crops such as tomato and mango (Cabrera et al., 2018). As expected, different members of the *C. gloeosporioides* and *C. acutatum* were reported for avocado crops in Antioquia. According to their ITS sequences, two of the 13 isolates recovered in the present study, F3 and F28, were closely related to *C. acutatum* complex and were isolated from two different locations, “La Luisa” and “Pan de azúcar” respectively. The rest of the purified *Colletotrichum* fungi belong to the *C. gloeosporioides* complex and were related with several species including *C. gloeosporioides, C. siamense* and *C. fruticola* (Figure 5, Supplementary Table 6). *C. siamense* and *C. fruticola* have been previously reported as the causal agent of Anthracnose and Soft Rot in Avocado Fruits cv. Hass in Mexico and Australia (Fuentes-Aragón et al., 2018; Giblin et al., 2018) but to date, they have not been reported in Colombia. The isolates F27, F31 and F33 were isolated from the same location (La Luisa) but in different farms and were closely related to *C. gloeosporioides* and *C. boninense* another *Colletotrichum* species associated with anthracnose on avocado.

Other fungi obtained were *Pestalotiopsis sp.* F36 and *Alternaria* sp. F36.2 both of them from samples collected at “La cerrajosa”. *Pestalotiopsis microspora* has been previously isolated from anthracnose lesion in avocado in Kenya (Kimaru et al., 2018) and *P. clavispora* in Chile (Valencia et al., 2011). In this context, and since no *Colletotrichum* fungi were obtained from this location, it is possible to suggest that *Pestalotiopsis* sp. F36 is the

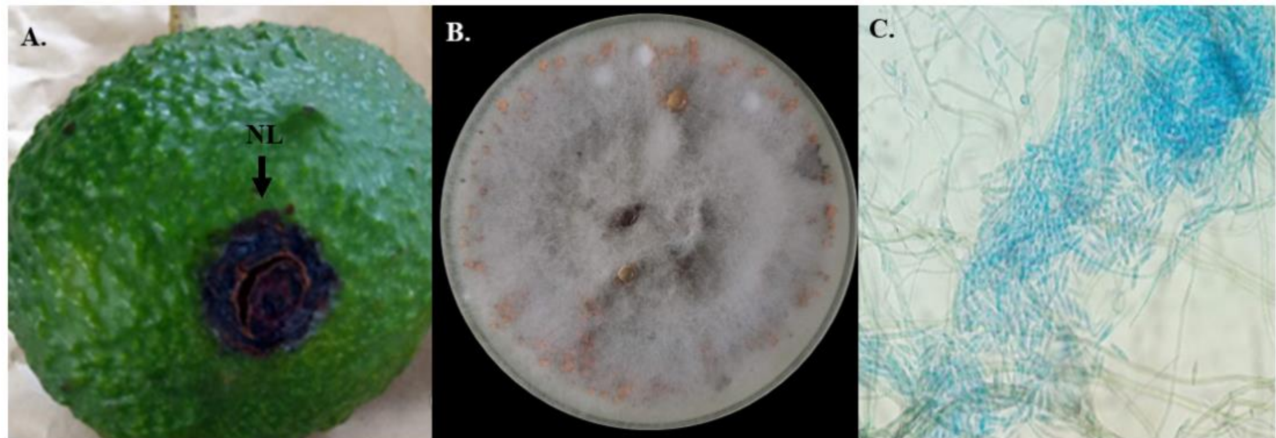

Figure 4. Phenotypic characteristics of *Colletotrichum* sp. F27.

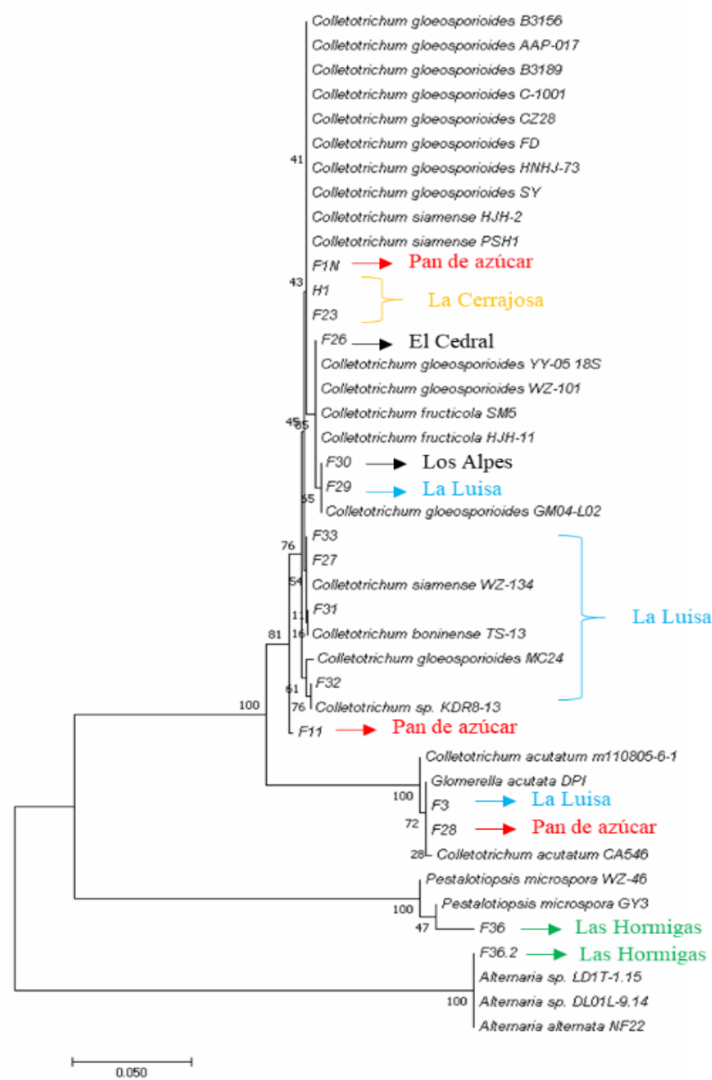

Figure 5. Molecular Phylogenetic analysis by Neighbor-joining method of partial ITS1 sequences of *Colletotrichum* and other isolates obtained from avocado fruits.

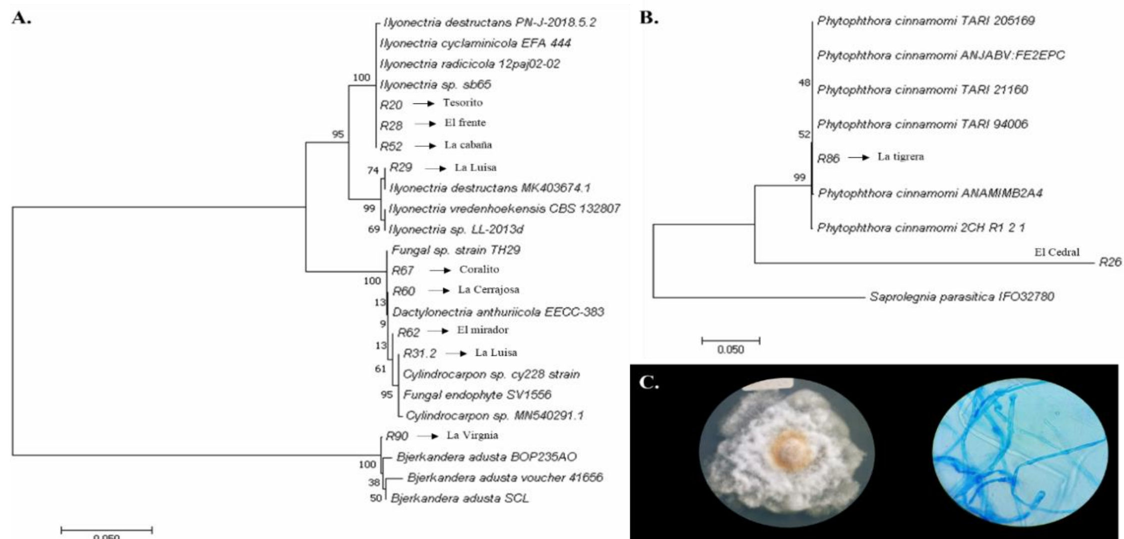

Figure 6. Molecular phylogenetic analysis by Neighbor joining method of partial ITS1 sequences of *Ilyonectria sp., Phytophthora cinnamomi* and related fungi obtained from avocado roots. (A) *Ilyonectria* destructans and related fungi. (B) *Phytophthora cinnamomi* isolates.

causal agent of the anthracnose symptoms observed in that location.

The evolutionary history was inferred by using the Neighbor–joining method based on the Tamura-nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. As previously mentioned, ITS alone sequence is not enough to differentiate *Colletotrichum* species (Damm et al., 2012). For that reason, it is necessary to complement the data obtained with a more complete genetic study in order to bring a clear conclusion about the possible new reports of *C. fruticola* and *C. siamense* affecting avocado crops in Colombia. With the obtained data, it is only possible to conclude the presence of several isolates belonging to the *C. gloeosporioides* and *C. acutatum* complex in Tolima.

Phytopathogenic fungi isolated from necrotic avocado roots

Root rot is one of the most devastating diseases in avocado crops around the world. This disease is generated by the oomycete *P. cinnamomi* (Hardham and Blackman, 2018). The infection with this phytopathogen causes root necrosis, wilting, leaf loss and reductions in fruit development and production (Granada et al., 2020). In Colombia, the incidence of *P. cinnamomi* has been documented in specific zones such as Antioquia (Ramírez-Gil, 2018, 2017), Bolivar and Sucre (ICA, 2012). In order to complement the available information about the distribution of this pathogen, a targeted isolation procedure was developed using samples obtained from trees with root rot symptoms in Tolima.

103 samples were processed from 21 avocado crops located at 15 different points of the central zone of Tolima. From them, eleven phytopathogens were recovered (Figure 6). Eight isolates correspond to fungi belonging to the genera *Ilyonectria* (anamorph. *Cylindrocarpon*) and were closely related to known phytopathogens of avocado like *I. destructans* that were previously isolated in Colombia as the causal agent of black root rot disease in Antioquia avocado plantations (Ramírez and Morales, 2013).

For A and B trees, the evolutionary history was inferred by using the Neighbor–joining method based on the Tamura-nei model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. In B, the tree was rooted with *Saprolegnia parasitica* IFO32780. (C) Macroscopic and microscopic characteristics of *P. cinnamomi* R86.

Only two isolates of *Phytophthora* genera, R26 and R86, were isolated from secondary roots with low necrosis degree (Figure 6B and C, Supplementary Table 6). Both microorganisms are closely related to *P. cinnamomi* and were obtained from “El cedral” and “La tigrera” avocado plantations respectively that have early symptoms of the disease. One of the main difficulties in the isolation of *P. cinnamomi* is its low growth rate under *in vitro* conditions. This causes that other saprophytic microorganisms with higher growth rates reduce or inhibit *P. cinnamomi* growth. The use of culture media with complex mixtures of antibiotics does not always work as it could be seen in this work. Here, from the presumptive

P. cinnamomi samples, a high number of saprophytic microorganisms were isolated including several species of *Fusarium, Acremonium, Verticillium* and *Phytium*. The presence of these fungi was also related to the necrotic state of some samples which had evident secondary infection with bacterial and fungi that prevented *P. cinnamomi* isolation. A more careful selection of the samples should be done in future works in order to improve the recovery rate of *P. cinnamomi*.

It is important to emphasize that despite the low isolation rate of pathogens recovered from avocado roots in this work, *Ilyonectria* and *Phytophthora* present similar symptoms. For this reason, both diseases can be diagnosed incorrectly, which can affect the crop phytosanitary management. To avoid this, it is important to establish programs that include follow-up processes for the phytopathogens present in susceptible crops so that an appropriate and early diagnosis can be made to improve crop productivity.

Conclusion

Major diseases of Tolima's rice crops are related to the rice blast and crown sheath rot diseases. In this study the presence of different pathogens related to these diseases, *G. graminis, G. oryzinus* and *M. oryzae* (sin= *P. oryzae*) was confirmed, being the first formal report of these fungi in different regions of Tolima. Similarly, the presence of isolates belonging to *C. gloeosporioides* and *C. acutatum* complex on avocado fruits supports the hypothesis of these fungi being the causal agent of anthracnose in the region. More accurate molecular identification and field re-inoculation studies should be done to unequivocally establish the role of these pathogens on rice and avocado fruit diseases. Finally, since more than one possible phytopathogen was isolated from root tissues, further studies are needed to confirm the role of both Ilyonectria (Anam= Cylindrocarpon) and *Phytophthora* isolates on the root rot syndrome observed on avocado trees. This information should be used in order to improve the management strategies for these pathogens on fields aiming to increase avocado production in Tolima. Currently, the microorganisms obtained from this study are being used for biocontrol and chemical tests to find an adequate method for their control on the field and are going to be identified to species level using a MLST approach.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY TABLES

Supplementary Table 1. Severity scale used for rice plants with crown sheath symptoms.

Severity	Symptoms
0	No lesion observed
1	Incipient lesions of dark color in the leaf sheath only. Slightly diseased material.
2	Significant necrosis of sheath with presence of perithecia
3	Presence of dark mycelium accompanied by hyphopodia, generally between the outer side of the stem and the leaf sheath.
4	Wilting of the stem due to mycelial growth of the fungus
5	Generalized drying of the stem and leaves. Tissue death

Supplementary Table 2. Severity scale used for rice plants with rice blast symptoms.

Severity	Foliar (aerial) symptoms
0	No lesion observed
1	Small brown specks of pin-point size
2	Small roundish to slightly elongated, necrotic gray spots, about 1-2 mm in diameter, with a distinct brown margin. Lesions are mostly found on the lower leaves
3	Lesion type same as in 2, but significant number of lesions on the upper leaves
4	Typical susceptible blast lesions, 3 mm or longer infecting less than 4% of leaf area
5	Typical susceptible blast lesions of 3mm or longer infecting 4-10% of the leaf area
6	Typical susceptible blast lesions of 3 mm or longer infecting 11-25% of the leaf area
7	Typical susceptible blast lesions of 3 mm or longer infecting 26-50% of the leaf area
8	Typical susceptible blast lesions of 3 mm or longer infecting 51-75% of the leaf area many leaves are dead
9	Typical susceptible blast lesions of 3 mm or longer infecting more than 75% leaf area affected

Source: Ghazanfar et al. (2009).

Supplementary Table 3. Severity scale used for avocado plants with root knot symptoms.

Severity	Foliar (aerial) symptoms	Root symptoms
0	Healthy plants with abundant dark green foliage and actively growing leaf buds. No symptoms of illness.	>90% of viable secondary roots
1	Slight yellowing of the leaves and lack of active shoots leading to stunned growth.	Symptomatic (necrotic) roots 10-15%
2	Pronounced chlorosis on leaves and growth arrest.	Symptomatic (necrotic) roots 16-25%
3	Generalized chlorosis leaf, wilt and slight defoliation <35%.	Symptomatic (necrotic) roots 26-50%
4	Generalized chlorosis of leaves, wilting and defoliation between 35.1-90%.	Symptomatic (necrotic) roots 50-90%
5	Descendant death and severe defoliation> 90%.	Symptomatic (necrotic) roots >90%

Source: Ramírez-Gil (2018).

Supplementary Table 4. Closest neighbors of rice isolates.

Isolate	T10	T13	T15.1	T16.1	T17	T18	T19.2	T20	T21	T24	T27	T29	T32	T34.1	T34.2	T37	T39	T40
ITS sequence length (pb)	532	559	486	558	495	554	495	554	497	547	556	546	571	556	526	571	528	516
Closest neighbor (% similarity)																		
G. oryzinus CPC26067	99.25	98.57		98.75	99.19	99.1		98.82	98.79	98.9	98.74	98.9	98.72	98.5	98.5		98.72	
G. oryzinus CPC26032	99.81	98.57	99.59	99.44		99.26	99.39		98.79	98.7	99.44	99.62	99.26	99.06	99.06	98.81	99.26	99.19
G. oryzinus CPC12056	99.81		99.59		99.79		99.39	99.44			99.44	99.62	99.26	99.06	99.06	98.81	99.26	99.19
G. oryzinus CPC26065				98.75		99.1		98.92		98.9		98.9						
G. oryzinus CPC26031			99.59															99.19
Gaeumannomyces sp. DX-FOS1									99.0									
G. graminis var graminis EGG-1							99.6									99.01		
G. graminis var graminis EGG-2		99.81		99.81	99.4						99.44	99.44						

The closest microorganisms were retrieved from the Genbank database.

Supplementary Table 5. Closest neighbors of rice isolate %similarity.

Isolate	H2	H5	H7	H9	H10	H11
ITS sequence length (pb)	543	544	544	544	466	487
Closest neighbor (% similarity)						
M. oryzae CBS433.70	99.45	99.63	99.81	99.25		
M. oryzae ERL 15-9	99.45		99.81			99.17
M. oryzae UPM-PO	99.63	99.63	99.81	99.43		
M. oryzae Nararanai		99.62		99.07		
M. oryzae G22		99.26				
M. oryzae Mo-ni-0669					100	99.79
M. oryzae Pam 1					99.57	99.39
M. oryzae RR3						
M. grisea MAFF306679		99.63	99.45			
M. grisea 70-15				99.07	99.57	

Supplementary Table 6. Closest neighbors of avocado isolates.

Isolate	H1	F1N	F3	F11	F23	F26	F27	F28	F29	F30	F31	F32	F33	F36.2
ITS sequence length (pb)	511	554	576	491	506	578	551	587	554	515	507	565	564	551
Closest neighbor (% similarity)														
Glomerella sp. ERS054	100				100									
Glomerella sp. ERS043					100									
Glomerella acutata DPI			99.65					99.83						
Colletotrichum sp. KDRS-13												99.11		
Colletotrichum gloeosporioides AAP-17	100				100									
Colletotrichum gloeosporioides B3189	100	100												
Colletotrichum gloeosporioides B3186		100												
Colletotrichum gloeosporioides SY		100		98.27										
Colletotrichum gloeosporioides FD				98.27										
Colletotrichum gloeosporioides HNHJ-73				98.27										
Colletotrichum gloeosporioides LCM 984.01							100							
Colletotrichum gloeosporioides cb-2							100							
Colletotrichum gloeosporioides CZ28												98.76	99.12	
Colletotrichum gloeosporioides MC24												98.76		
Colletotrichum gloeosporioides B3156											99.4			
Colletotrichum gloeosporioides C1 001											99.4			
Colletotrichum gloeosporioides GM04-L02										100				
Colletotrichum gloeosporioides MKC5										99.81				
Colletotrichum gloeosporioides YY-05						99.86			99.82	99.81				
Colletotrichum gloeosporioides WZ-101						98.96			99.82					
Colletotrichum acutatum A3						99.14			99.82					
Colletotrichum acutatum ml 10805-6			99.48					99.66						
Colletotrichum acutatum CA546			99.48					99.66						
Colletotrichum fructicola HJH-11									99.82					
Colletotrichum fructicola CBS 125390									99.82					
Colletotrichum fructicola SM40						98.97								
Colletotrichum fructicola 5						98.97								
Colletotrichum siamense WZ-134													99.29	
Colletotrichum siamense HJ-2													99.29	
Colletotrichum siamense PSH1											99.4			
Colletotrichum boninense TS13							100							
Alternaria sp. DL01L-9														100
Alternaria sp. LDL1T-1.15														100
Alternaria alternata NF22														100
ISOLATE	R20	R26	R28	R29	R52	R31.2	R60	R62	R67	R86	R90			
ITS sequence length (pb)	522	882	545	539	519	569	572	478	523	890	632			

Supplementary Table 6. Contd.

Closest neighbor (% similarity)											
Phytophthora cinnamomi TARi 94006										100	
Phytophthora cinnamomi TARi 205169										100	
Phytophthora cinnamomi TARi 21160										100	
Phytophthora cinnamomi ANAMIMB 2A4		93.96									
Phytophthora cinnamomi ANJABV_FE2EPC		93.96									
Phytophthora cinnamomi 2CH_R1_2_1		94.23									
Ilyonectria sp. sb65	100		99.81		100						
Ilyonectria sp. LL-2013d				99.25							
Ilyonectria cyclaminicola EFA 444	100		99.08								
Ilyonectria destructans PN-J-2018.5.2	99.81		99.26		99.81						
Ilyonectria vredenhoekensis CBS132807				99.25							
Ilyonectria radicola 12paj02-02					100						
Cylindrocarpon sp.						98.72					
Cylindrocarpon cy228						99.08	98.88	99.16	99.04		
Cylindrocarpon RGO_LsH_C2								99.58			
SV1556						99.81	98.5				
TH29							99.27	100	100		
Dactylonectria anthuriicola EECC-383									100		
Bkerkandera adusta SCL											99.37
Bkerkandera adusta BOP235AO											99.34
Bkerkandera adusta 06101_7A											99.66

The closest microorganisms were retrieved from the Genbank database.

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Evaluating the efficacy of household filters used for the removal of bacterial contaminants from drinking water

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Due to the erratic nature of microbial contaminants in drinking water, private and municipality water supply systems failed to deliver safe drinking water to households. In Ethiopia, there is lack of data and knowledge on the effectiveness of filter devices used to treat drinking water at household. This study aims to evaluate efficiency of household point of use filter devices (membrane filter, membrane with activated carbon, ceramic candle type filter and hybrid (multistage)) in reducing bacterial contaminants from drinking water. Percent reduction efficiency model was employed in evaluating bacterial removal efficiency. Membrane filter and membrane with activated carbon filter devices had good total coliform removal efficiency on the 1st and 2nd days than hybrid filter device which showed low removal efficiency. Similarly, all filter devices showed better fecal coliform removal efficiency on the 1st day compared to 2nd day but had low heterotrophic bacteria removal efficiency during the three days filtration. Fecal *Streptococcus* removal efficiency on the 2nd and 3rd days by all filter devices was low except the first day. The result in general showed that using of point of use filter devices for prolonged time could not guarantee in providing risk free drinking water at household level.

Key words: Coliform, drinking water, household filters, point of use, removal efficiency.

INTRODUCTION

Contamination of drinking water by waterborne pathogens in piped water distribution systems, at storage facilities and at point of use is the most serious human health risk, causing outbreaks of different diseases. In Ethiopia, 56% of the urban population had access to piped water through centralized water treatment and piped distribution networks but majority of the rural population used untreated water from surface water sources (Usman et al., 2016). But quality of drinking water gets poorer in water distribution systems due to leakage through corrosion of pipes, intrusion of microbial contaminants and other physicochemical pollutants that causes diarrheal and other diseases (Dawit, 2015; Adane et al., 2017).

To avoid the risk of poor-quality water consumption, different point of use water treatment and filtration technologies with variable microbial and other contaminant removal effectiveness have been developed and introduced to users. In many low-income nations, point of use filter devices made from locally available materials and/or available with inexpensive prices from venders used commonly as an intervention for household water treatment solutions (Angela, 2011). World Health

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Organization (WHO) and United Nations Children's Fund (UNICEF) recommend and promote these filter devices for removing microbial and physicochemical contaminants and as general strategy for preventing water-borne diarrheal diseases in low income countries (WHO/UNICEF, 2017).

Household water treatment and safe storage (HWTS) technologies provide moderate levels of safe water supply at point of use. Household water treatment interventions are the most effective alternative at household level than at community level to reduce various contaminants at point of consumption since treated water delivered through distribution systems gets contamination between point of treatment and consumption in low-income nations (Anke et al., 2018).

Filtration mechanisms such as bio sand filter, solar disinfection, ceramic filtration, chlorination at point of use and combined flocculation/disinfection are the most practiced systems in Ethiopia. The most used point of use filtration systems at household level are mesh like clothing in rural areas, membrane, and ceramic filter devices in urban areas (Abrham et al., 2018b). Ceramic filter devices can be made from locally available materials, affordable and used by individuals for household point of use (Enyew and Tesfaye, 2017).

A case study in Eastern Ethiopia showed that point of use drinking water filtration devices are the most effective and recommended alternatives in removing several pollutants and water-borne pathogens and makes water safe for household consumption under proper usage (Abrham et al., 2018a). In Addis Ababa, membrane filtration devices, hybrid filter devices and in some cases ceramic filters devices are the usual point-of-use household water filtration options people use for safe water consumption. Although point of use water treatment devices have a significant contribution in removing microbial contaminants, physical and chemical pollutants and improve water quality and safety, there is limitation in knowing their efficiency in removing such contaminants at longer time usage. Despite point of use filtration devices have limitations, their popularity as interventions in removing waterborne microbes and undesirable pollutants from water are increasing in many countries where absence of treatment facilities and inefficient disinfection risks people's health (Jerome et al., 2018). Therefore, the main objective of this study was to assess the effectiveness of different point of use water filtration devices used at household in reducing bacteria from the influent and effluent water samples.

MATERIALS AND METHODS

Experimental design and sampling

The study employed experimental approach to evaluate the bacterial removal efficacy of point of use filter devices from water samples supplied via distribution systems at Institute of Biotechnology laboratory, Addis Ababa University. In this study, four point of use filter devices; membrane filter, membrane with activated carbon filter, ceramic candle type filter and hybrid (multistage) filters were set in the laboratory as depicted by the schematic diagram as shown in Figure 1 and evaluated for their bacterial removal effectiveness from treated water used at household. The filter devices were obtained from the local market in Addis Ababa.

Pyrex glass sampling bottles, plastic bucket (5-L each) and Petri-plates were aseptically prepared for sample analysis. Distilled water and 80% ethanol were used for disinfecting the buckets prior to putting the filter devices (ceramic candle type and membrane filter devices) and pouring the sample water into the containers. The membrane and the hybrid filtration devices had their own containers and were set independently on the lab bench.

The influent water sample was taken from faucet mounted tap located outside the laboratory at College of Natural and Computational Sciences, Addis Ababa University. A vacuum pump filtration apparatus (Rotary Vane Vacuum Pump, Tanker 150-220V/50H,187130-22, Taiwan) was set in the laboratory for sample filtration. Petri-dishes, sampling bottles, filtration apparatus (filter funnel, clamps) and flasks were pre-sterilized before sample processing. Absorbent pads, cellulose acetate membrane filter (pore size of 0.45 μm) and growth media were prepared for culturing bacteria following standard methods for water quality analysis (APHA, 2017).

Sample filtration and analysis

Membrane Lauryl Sulphate Broth (MLSB) was used for culturing of total coliforms (TC) and fecal coliforms (FC). Slantez and Bartley agar media and R2A agar were prepared for fecal *Streptococcus* (FS) and for heterotrophic bacteria plate counting (HPC), respectively. About 100 mL water sample as influent from faucet mounted tap and filtered using 0.45 μm filter paper for TC, FC, and FS analysis. For HPC analysis, 100 ml of diluted sample (1 mL sample water diluted with 99 mL buffered with distilled water) was filtered on to a 0.45 μm pore size sterile membrane filter and the filter paper was placed on to a 50 × 9 mm Petri-dish where 15 mL of the liquified R2A agar was dispensed on to it. The incubation temperature for plates containing total and fecal coliforms respectively was 37°C for 24 hours and 45°C for about 24 h. All yellow colonies were counted using digital colony counter and recorded as CFU/100 mL. Plates containing faecal *Streptococcus* were incubated at 44°C for 48 h and reddish-purple coloured was counted whereas HPC plates were incubated at 28°C for 5 days and all cream-colored colonies were counted and recorded in CFU/mL.

To analyze TC, FC, FS and HPC from effluent water from each point of use filter devices set in the lab, 5-L plastic bucket was prepared and disinfected with 80% ethanol after washing with distilled water. The filtration apparatus was set with the same procedure as influent sample analysis. About 5 L of water from faucet mounted tap was poured at each point of use filter devices set in the laboratory for filtration. Then, from each of the four point of use filter devices, separate sampling bottles were prepared and used for taking 300 mL filtrated water sample for further culturing of indicator organisms. For each sample again, a separate filter paper was aseptically prepared and 100 mL sample water put on to filter funnel and then filtrated using vacuum pump pressure. Filtration process, culturing, and incubation of organisms employed the same procedure as influent sample analysis.

Data analysis

All the data were analyzed using IBM SPSS v.23. The bacterial removal efficiency of the filter devices were computed based on percent reduction efficiency using the following equation.

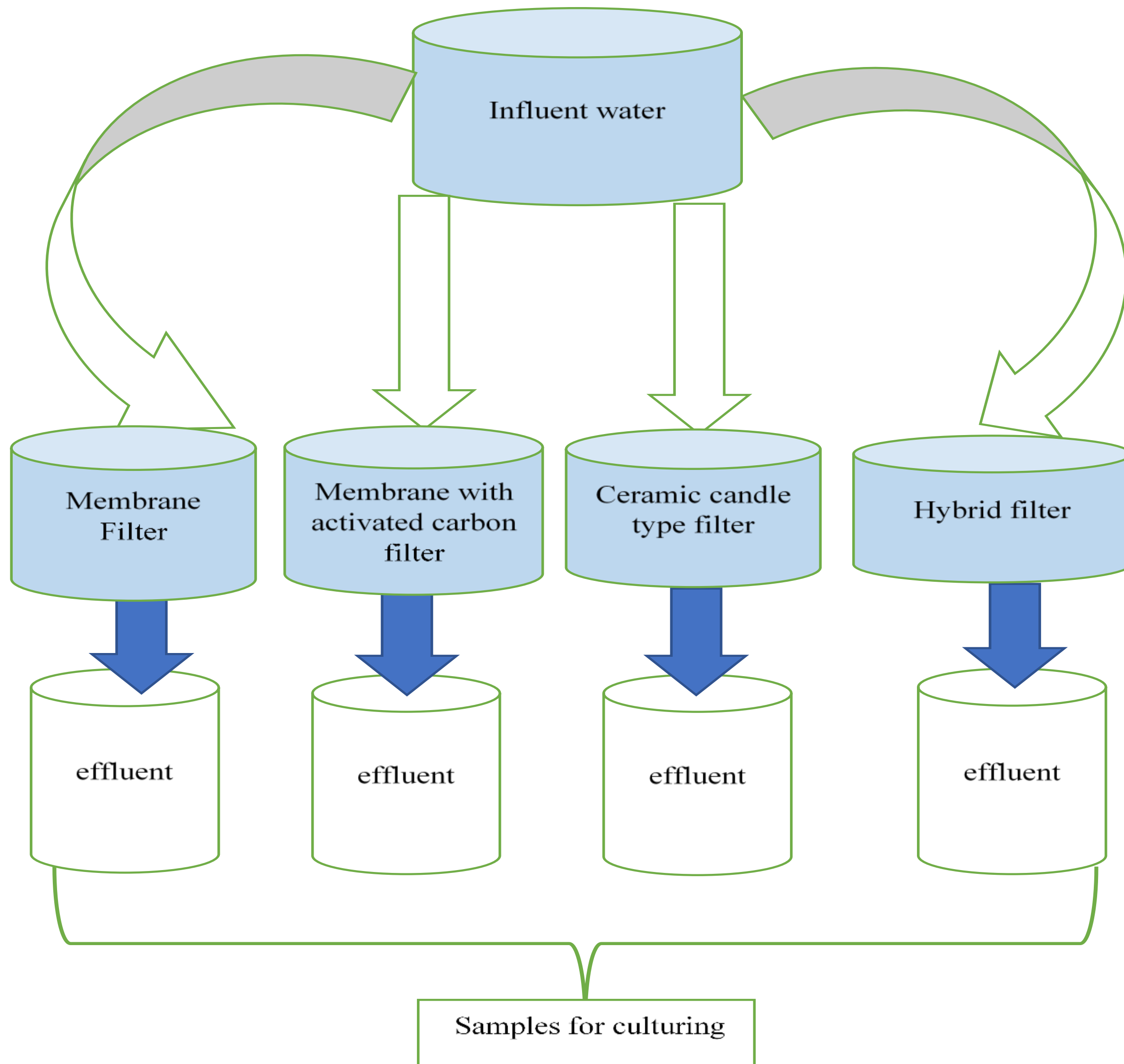

Figure 1. Schematic diagram representing set up of point of use filter devices in the laboratory.

%Reduction = (Influent - Effluent / Influent) × 100

A two tailed test at 5% significance (P<0. 05) was used for establishing the difference between the removal efficiency of filter devices during the three consecutive days.

RESULTS AND DISCUSSION

The microbial removal efficiency of four filter devices used at household level was evaluated using water quality indicator organisms, total coliform (TC), fecal coliform (FC), heterotrophic bacteria (HPC) and fecal *Streptococcus* (FS) (Figures 2 to 5). The results revealed that membrane and membrane with activated carbon filter devices showed good total coliform removal efficiency of 51.4 and 58.2% on the first day. These devices, respectively, achieved better removal efficiency of 78 and 74.5% on the second day. On the other hand, hybrid filter device had lower total coliform removal efficiency (42 and 25.5%) on the first and second days of filtration compared to the other filter devices. The flow rates measured for each filter devices had a decreasing trend after the first day filtration shown in Table 1 which indicated that efficiency of filtration of each filter device over consecutive days of usage decreased, may be due to clogging of organic matter and debris on the surface of the filters and hence be conducive platform for bacteria growth and multiplication.

Hence, membrane and membrane with activated carbon filter devices were better in removing microbial contaminants compared to the other filter devices on the first and second days. Similarly, Mark et al. (2016) also

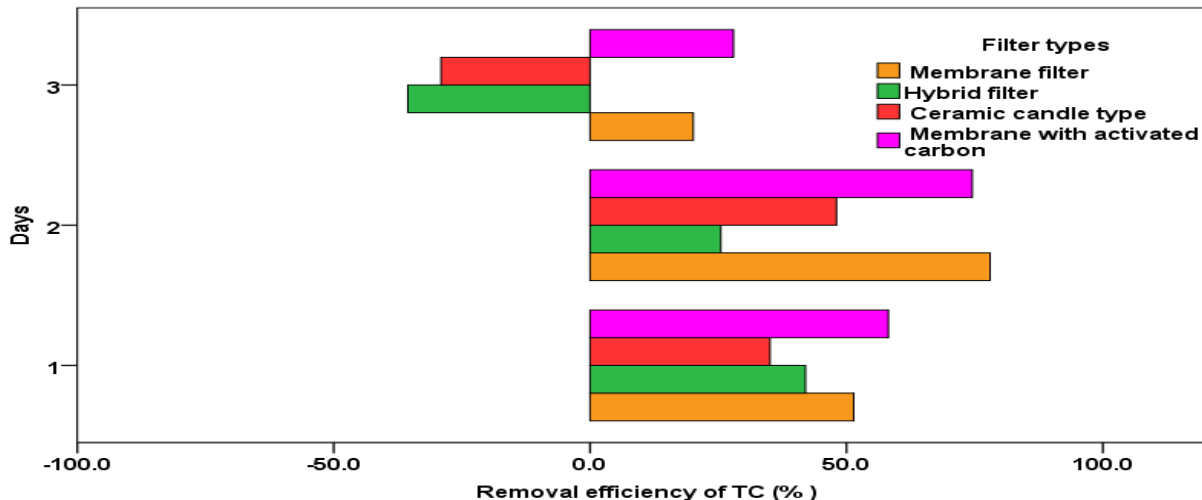

Figure 2. Removal efficiency of total coliform among the four point of use filters over the three days.

Table 1. Flow rates of the filter types measured at each filtration day.

Filter type	Flow rate/minute		
	Day 1	Day 2	Day 3
Membrane filter (ml)	120	110	100
Membrane with activated carbon (ml)	117	105	100
Ceramic candle type (ml)	110	95	90
Hybrid filter (ml)	125	115	100

showed drastic reduction in total and fecal coliforms from 65% in influent water samples to 3% in post filtered water samples by using membrane filter devices.

As observed from Figure 2, on the 2nd day of testing, there were better total coliform removal efficiency of membrane filter (78%), ceramic candle type (48%) and membrane with activated carbon (74.5%), except the hybrid filter devices which had removal efficiency of 25.5%. But on the 3rd day, there were significant number of total coliform bacterial counts measured from the filtered water indicating all filter devices were not that efficient in removing bacteria where membrane filter decreased from 78 to 20%, membrane with activated carbon filter decreased from 74.5 to 27% but hybrid and ceramic candle type, respectively had negative removal efficiency (-35.5 and -29%) of post filtered sample where the number of bacteria count of each respective filter were 167 and 160 CFU/100 mL compared to influent sample each having 123 CFU/100 mL bacterial count. This, on the other hand, indicates the failures of such filter devices in effectively removing biological contaminants as the lifetime of the filter devices longer. Despite the fact that the filter devices were expected to improve water quality by reducing bacterial load from the effluent water, this intervention did not achieve a satisfactory improvement in drinking water quality at point of use, probably, because of poor filter handling practices and its use in an environment with low hygiene and high loads of fecal bacteria in the households.

Even though parasite and viral removal efficiency of filter devices were not tested, the removal efficiency of ceramic and membrane point of use filters in this study was much lower than 99.9% efficiency of removing different bacteria, viruses, and parasites (Kathleen et al., 2017). However, this finding is consistent with a research which indicated that filtered water had higher bacterial count than the influent water on the third life time of point of use filter devices (Su et al., 2009). The results also conform with a research which indicated low bacterial removal efficiency where filtered water had higher bacterial count than influent water on the third life time of membrane and ceramic point of use filter devices. This was due to organic matter retained inside the filter devices which supported the growth of different bacteria and hence lead to the development of biofilms which in turn causes filtered bacteria to increase (Clark and Elmore, 2011).

The other suggestion might be point of use filter devices may create favorable conditions inside where coliform organisms may regrow and hence detected in

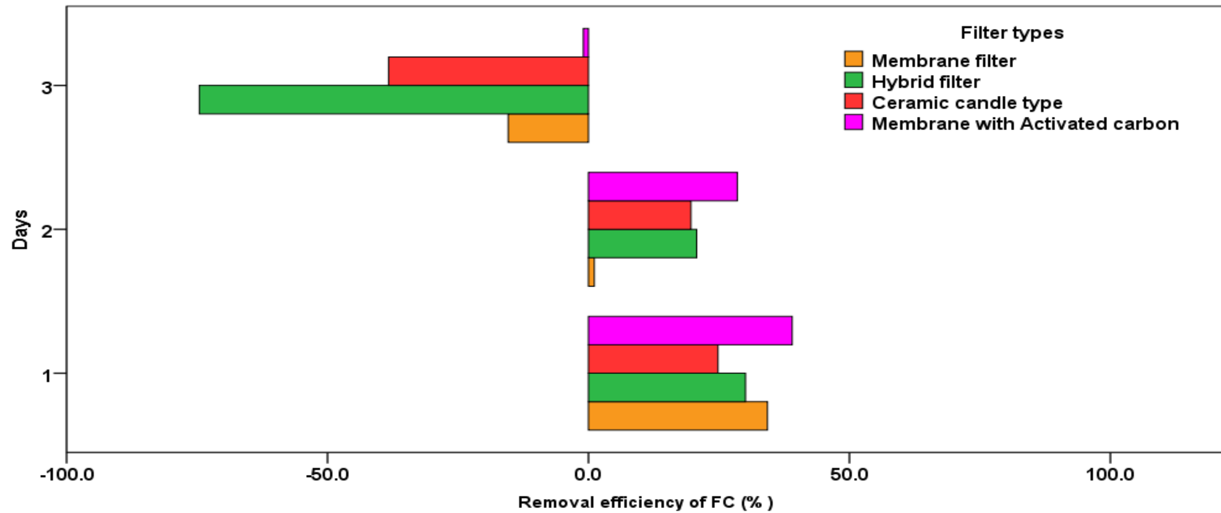

Figure 3. The removal efficiency of fecal coliform among the four filters over three days.

significant number in water coming out from the filter devices (Filipe et al., 2018). Moreover, particulate matter collected inside filter devices reduces flow rate of the filter devices which then contributes strong adhesion intensity of bacteria inside the filter devices and hence this would help to form biofilm and a high number of bacteria measurements in the effluent water (Su et al., 2009). Despite the use of the filter devices, the filtration did not achieve a satisfactory improvement in drinking water quality at the point of use, probably, because of poor filter handling practices and use in an environment with low hygiene and high loads of fecal bacteria in the households when people manage using contaminated hand.

The fecal coliform removal efficiency of the four filter devices is as shown in Figure 3 and in the first day of filtration, membrane filter, hybrid, ceramic candle type and membrane with activated carbon had removal efficiency of 34, 30, 24 and 39%, respectively which is then lowered to 1.1, 20.7, 19.6, and 28.5%, respectively on the second day. On the 3rd day, the removal efficiency of the filter devices was negative which means the number of bacteria counted from the effluent water were significantly greater than the influent water samples ($P>0.05$).

Similarly, the removal inefficiency of ceramic and membrane filter devices of fecal coliform and *Escherichia coli* over time was due to concentration of bacteria inside the pore spaces and when filtration process proceeds in the next day, bacteria can simply move out into the effluent water (Clark and Elmore, 2011).

Another study also showed that the decrease in bacterial removal efficiency of these point of use filter devices used at household may also be due to the lack of performance enhancing chemicals that could remove organic matter inside pore spaces and also the absence of elements like silver impregnation on the filter devices (Jocelyne et al., 2013).

The HPC removal efficiency of the devices is as shown in Figure 4 and on the first and second day of filtration, except membrane with activated carbon filter device; hybrid, membrane and ceramic with candle type filter devices had better removal efficiency (37, 32 and 26% on the first day whereas 42, 36, and 24% on the second day) but on the third day of filtration, hybrid and membrane filter had each 50% HPC removal efficiency and the other two, membrane with activated carbon and ceramic candle type filter showed 25 and 29% of HPC removal efficiency, respectively.

The reduced HPC removal efficiency of ceramic candle type filter on the first and second days of filtration (-12 and -4%, respectively) in this study may be associated with accumulated organic impurities inside filter pores which helped HPC bacteria to attach themselves and form gradually biofilms so that the bacteria remain there unlike the total coliforms, fecal coliforms and fecal *Streptococcus* (Syreeta et al., 2009).

Regarding the removal efficiency of fecal *Streptococcus* (Figure 5), all filter devices showed declining efficiency on the 2nd and 3rd days (membrane, hybrid, ceramic candle and membrane with activated carbon filter devices declined by 32, 87, 60 and 15% removal efficiency on the second day and 30, 14, 26 and 6% on the 3rd day, respectively) unlike on the first day of filtration (which had removal efficiency of 33, 27 and 17% except hybrid filter device). This was supported by a study done on a batch tested research on the efficacy of ceramic siphon household water purification device that showed the decrease in the removal of pathogenic organisms including *E. coli* decrease over time (Amanda et al., 2011).

The declining problem in performance of ceramic and

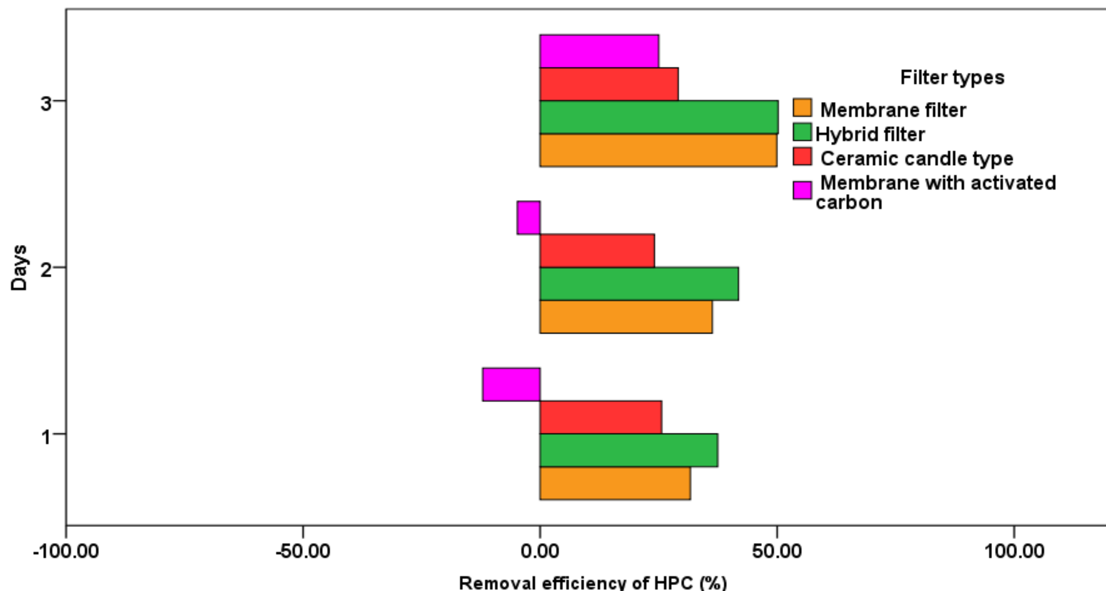

Figure 4. Removal efficiency of heterotrophic bacteria (HPC) among the four filters over three days.

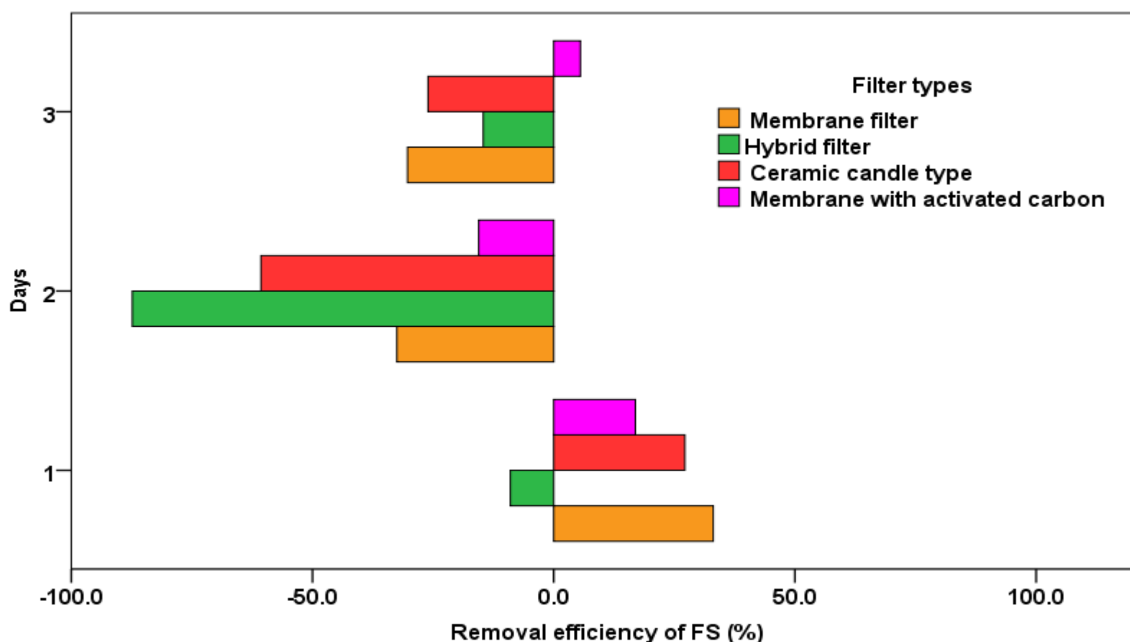

Figure 5. Removal efficiency of fecal *Streptococcus* among the four filters over three days.

membrane filter devices in bacterial removal when the life span is extended can be solved with maintaining regular cleaning practices which further improves flow rate although such practice is reported to decrease the life span of ceramic and membrane filters devise used at household (Jacqueline et al., 2010). Other possibility which is indicated to enhance bacterial removal performance of household point of water purification filter devices is the incorporation of hydroxyapatite into ceramic water filters which increases the percentage of porosity which intern has greater efficiency in removing bacteria than conventional filters (Mark et al., 2008).

The bacterial removal performance of the filter devices used in this study was irregular at each testing day. Such

ineffective performance of the filter devices over the three consecutive days may be due to the presence of organic matter and other contaminants in which intern leads to change in the quality of water tasted on the three days (Md Rezaul et al., 2016).

Conclusions

The use of filter devices to further treat water at point of use showed that on the first day of testing, membrane and membrane with activated carbon filter devices only were more efficient in removing total and fecal coliforms. However, they showed a decrease in their efficiency in removing bacteria from tested water as the life span of usage is longer. The number of HPC, FS and the coliforms were high in the effluent water on the second and third day of testing. Therefore, point of use filter devices used for household water treatment in reducing microbial and other physicochemical contaminants should be cleaned regularly and replacing non efficient devices after longer time usage with new one so that health risks caused by such contaminants can be avoided.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

First novel phages from rodents with lytic activity on clinical *Enterobacteriaceae* strains: Initiation for phage therapy in West Africa

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Bacteriophages (phages) are viruses that infect bacteria. The emergence of resistant bacteria has been reported worldwide. The use of phages to treat bacterial infection or to reduce bacteria load has become a new area of interest. The viral colonization of rodents represents an important source for a specific biotope. The objective of this study is to characterize the effect of novel phages isolated from rodents (*Mastomys*) using clinical *Enterobacteria* strains. Three novel phages were isolated from the intestinal tract of rodents. The phages belong to Myoviridae family. By using three Random Polymerase Chain Reactions (PCR), genomic characterization of the phages shows a high diversity that reveals several DNA bands. 3 virulent phages exhibited different patterns, indicating that the rodents' phages are genetically unique and could be distinguished by typing-PCR. The virulence spectrum of the phages shows phages Mα3 and Mα7 have 50% (7/15) lytic activity on enteropathogenic and enterohemorrhagic *Escherichia coli* strains while phage Mα11 has 26.6% (4/15) lytic activity. These phages have no lytic activity on *Salmonella* strains. This study is the first to isolate novel phages from rodents with lytic activity. The candidate rodents' phages (Mα3, Mα7, Mα11) can be used to control virulent *E. coli* in West Africa.

Keys words: Lytic phages, rodents, *Escherichia coli* strains, West Africa, enteropathogenic, enterohemorrhagic.

INTRODUCTION

Bacterial infections are mostly involved in causing diseases in sub-Saharan countries. Goualié et al. (2019) reported the high incidence of multidrug resistant bacteria in chickens in Cote d'Ivoire. This problem is emergent because of the misuse of drugs and the free accessibility to drugs in human or in animal trials. Several studies have reported the distribution of multi-drug resistant strains isolated from humans, animals and the environment (Guessennd et al., 2013). Fagmabila et al. (2017) reported the distribution of Salmonella serovars in

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commercial poultry farms in West Africa. Bacteriophages, the most ubiquitous organisms on Earth, are viruses that infect bacteria (Rohwer, 2003). These have been employed since their discovery in the development of therapeutics against infections. They are highly specific, very safe, and effective against their target pathogenic bacteria; they are easily modifiable to address new threats (Golkar et al., 2014). Regardless of the increase in antibiotics resistance worldwide, the use of phages is an alternative for the treatment of bacterial infections (Nagel et al., 2016). Many studies have reported the efficacy of phage therapy in humans, animals and the environment (Biswas et al., 2002; Wang et al., 2006; Watanabe et al., 2007; Wright et al., 2009; Abedon et al., 2011; Nale et al., 2016; Lin et al., 2017).

Phages were discovered before the development of antibiotics and their administration as pharmaceutical agents was a common practice in the pre-antibiotic era (Golkar et al., 2014). On the contrary, phage therapy is not known in sub-Saharan countries' medical care systems (Ehui et al., 2017). Some studies have reported the abundance and isolation of lytic phages in West Africa (Essoh et al., 2015; Kakou-Ngazoa et al., 2017; Ngazoa-Kakou et al., 2018, 2019), but the applications of phage therapy are not reported. Nagel et al. (2016) suggested the need to implement phage therapy in low-income countries because it is cheap to produce and is available everywhere in rural regions. Because of the renewed interest in lytic phages as biocontrol agents, new phages are sought after as they offer the possibility of increasing bacterial strain coverage in the design and development of phage cocktails (Golkar et al., 2014). Most of the studies on phages focus on Enterobacteria phages. Enterobacteria colonize major biotopes, intestine, sewage, water bodies (Kumari et al., 2009; Park et al., 2012; Maal et al., 2015; Hamdi et al., 2017) and are abundant in sub-tropical countries (Essoh et al., 2015). Rodents are an important reservoirs of pathogens involved in zoonosis. Lozano et al. (2015) isolated multi-drug resistant *Enterococcus faecalis* strains in *Rattus*. To propose that phages are effective against several bacteria strains, we investigated the phages that infect the *Enterobacteriaceae* family. The main aim of this study is to evaluate the lytic activity of novel phages isolated from rodents on clinical virulent bacteria strains.

MATERIALS AND METHODS

Isolation of rodent phages

Fifty samples of rodent (*Mastomys*) intestines were obtained from Pasteur Institute Project for the Surveillance of Zoonosis in Côte d'Ivoire (West Africa). 1-2 cm of the intestines was incubated in 1 ml PBS buffer (1X) and mixed hard for 15 min. For the amplification of the phages, the solution was passed through a filter of 0.45 µm. One ml of the filtrate was added to 3ml Luria Bethani broth containing 100 µl of bacteria host (*Escherichia coli*). The solution was incubated at 37°C overnight and centrifuged at 8000 rpm, for 10 min. The supernatant was filtered at 0.45 µm and the filtrate was incubated in 3 ml LB broth containing bacteria host (*E. coli*). The step was repeated twice for high viral load (Maal et al., 2015). Chloroform or 1% NaN_3 was added to the bacteria in filtrate samples. The isolated phages were named as Mα3, Mα7 and Mα11 and stored at 4°C.

Morphology identification

Twenty five microliters of each concentrated bacteriophage suspended (minimum 10^7 PFU/ml) in SM buffer was spotted on top of a Formvar-carbon-coated copper grid (Ted Pella, Inc, USA). The bacteriophages were allowed to adsorb for 2 min and were stained by adding 8 µl of 2% sodium phosphotungstate (pH 7.6) (Kumari et al., 2009). The grids were observed with a Zeiss EM 900 transmission electron microscope (TEM) at 80 kV.

Virulence of phages on bacteria strains

E. coli and *Salmonella* strains were obtained from Institut Armand Frappier, Microbiology Unit (Laval, Canada), with some virulence factors. The bacteria were incubated in LB broth overnight at 37°C. Fresh broths of bacteria were inoculated on fresh LB-Agar. Rodents' phages MαA3, MαA7 were propagated on *E. coli* DH5α and rodents' phage Mα11 on *E. coli* QT22 at 37°C, for 1 h (Maal et al., 2015).

Lytic activity of phages

5-10 µl of the phage solution was inoculated on LB-Agar (0.5%) containing fresh bacteria host by OD <0.3. The plate was incubated at 37°C, overnight. The plates were sectioned in 24 quadrats. The lytic activity of the phages was evaluated by the formation of clear plaques. The bacterial strains used were provided from the Biocollection of Institut Armand Frappier, Quebec, Canada (Microbiology Unit, Dr Dozois C.).

Phage DNA Isolation

8.6 µl of the phage solution was added to DNase-mix containing Dnase I buffer 1X, 20 Unit of DNaseI (Thermo Fisher, USA) to obtain a final volume of 10 µl. The solution was incubated at 37°C and 65°C, for 30 min. After the digestion, 0.5 µl of Proteinase K (20 mg/ml) was added and incubated at 37°C for 30 min and 95°C for 15 min. The DNA was stored at -20°C.

Random PCR

Three random primer PCRs were used for the genomic analysis of the isolated phages, according to the protocol described by Gutierrez et al. (2011) and Comeau et al. (2004). Briefly, 2 µl of the phages' DNA were added to 23 µl PCR Mix containing 12.5 µl Taq-Frogga Mix, 2 µl of 100 µM DEG Primer (R10D) or P2, or OPL5 and 8.5 µl Dnase-free water. The PCR conditions for R10D were: 95°C, 5 min; 95°C, 45 s; 40°C, 3 min; 72°C, 1 min (40 cycles); and 72°C, 10 min. The PCR conditions for Primers P2 and OPL5 were 95°C, 2 min; 95°C, 45 s; 30°C, 2 min; 72°C, 1 min (40 cycles), 26 cycles at 94°C, 45 s; 36°C, 30 s; 72°C, 60 s; and final extension of 10 min at 72°C. PCR products were separated in 1% agarose gel and the TIF image was created from UV Gel Doc system (Biorad, USA).

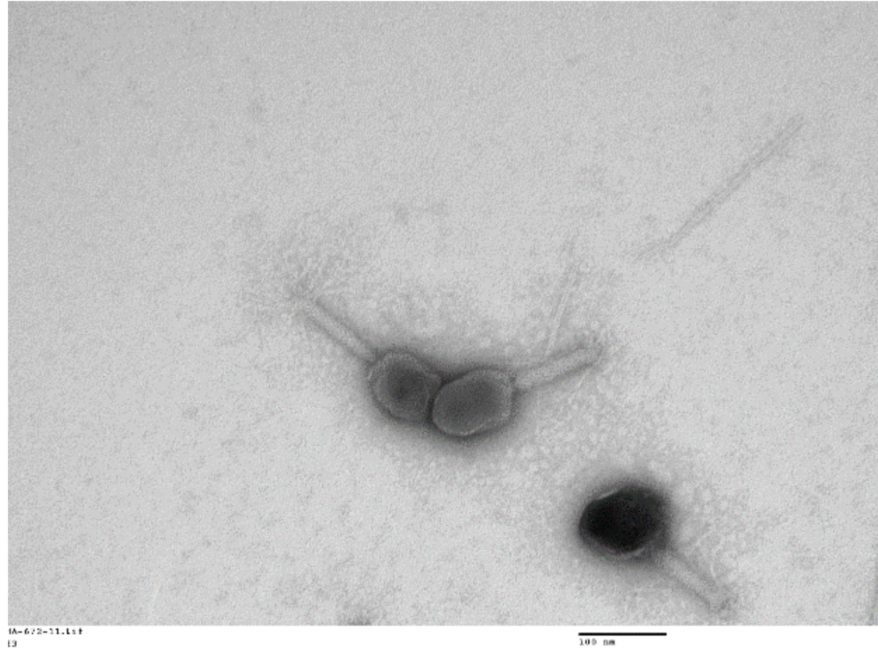

Figure 1. Morphology of rodent' phage Mα3 by TEM. The scale bar indicates 100 nm.

RESULTS

Morphology of rodents' phages by electronic microscopy

The transmission electron microscopy (TEM) classified the morphology of the virions of Mα3, Mα7 and Mα11 as *Myoviridae* family. The rodents' phages have elongated head (195 nm) and contractile tail (110-114 nm) (Figure 1).

Host range of rodents' phages on clinical strains

The host range of rodents' phages Mα3, Mα7 and Mα11 was tested on 20 bacterial strains by spot test of diluted phage lysate. Among the 20 strains tested, phages Mα3 and Mα7 were able to infect 7 bacterial strains while phage Mα11 infected 4 strains (Table 1). The virulence spectrum shows that phages Mα3 and Mα7 have 50% (7/15) lytic activity against *E. coli strains* and phage Mα11 has 26.6% (4/15) lytic activity. Out of 6 *EPEC/ETEC/EHEC* strains, rodent phages Mα3 and Mα7 were able to lyse 4 strains (Table 1 and Figure 2). Among three *APEC* strains, two were sensitive to the isolated rodent phages. These phages have no lytic activity against *Salmonella* strains (Table 1).

Rodents' phages typing by PCR

Random Amplified Polymorphism DNA (RAPD)-bases methods were used to establish a quick typing of the isolated phages. The results show the diversity of the isolated phages in 3 typing PCRs. With R10D primer, 3 different profiles were obtained. M3α reveals 3 bands (0.4, 0.6, 1.1 kb); 7 bands for Mα7 and 3 bands ranging from 0.2-1.100 kb for Mα11 (Figure 3). By using PCR for P2 Primer, the phages generated DNA band patterns with amplicons size ranging from 0.7-0.8 kb. Phage Mα11 shows two bands of amplification (Figure 3). The PCR used for OPL5 primer reveals a profile of 3 bands, 2 bands and one band for M3α, M7α and Mα11 respectively (Figure 3). Each phage showed a different profile regardless of the generated bands by RAPD-PCRs. All phages exhibited different patterns showing that all phages are genetically unique and could be distinguished by RAPD-PCR.

DISCUSSION

In this study, we isolated and characterized three virulent phages Mα3, Mα7 and Mα11 from intestinal tract of *Mastomys*. Our study reveals a new biotope for phage isolation. Several studies have isolated most *Enterobacteriaceae* phages from sewages, lakes and aquatic sources (Essoh et al., 2015; Maal et al., 2015; Hamdi et al., 2017). Morphological and genomic analyses revealed that all phages belong to the Myoviridae. The isolated phages have broad host ranges and were coliphages. Other studies have shown similar results with broad host range from phages isolated in sewage (Hamdi

Table 1. Ability of rodent' phages to lyse clinical strains of *E. coli* and *Salmonella*.

Bacteria strains tested	Phages		
***APEC* strains (n=3)**	**Mα3**	**Mα7**	**Mα11**
4812	+	-	-
4825	+	+	+
4832	-	+	+
***E. coli* Guadeloupe * strains (n=6)**			
1786	-	-	-
3725	-	-	-
3748	+	+	+
3719	+	-	-
3782	+	+	-
3829	+	-	+
***EPEC/ETEC/EHEC* strains (n=6)**			
3337	+	+	-
44	-	+	-
27	-	-	-
28	-	-	-
34	-	-	-
***Salmonella* strains (n=5)**			
St 1707	-	-	-
St 1716	-	-	-
Se 1738	-	-	-
Se 1741	-	-	-
Sty 1785	-	-	-

+: Clear lysis zone; −: no clear lysis zone; St: *Salmonella typhimurium;* Se*: Salmonella entereditis; Sty: Salmonella. Typhi; EPEC: Enteropathogenic E.coli; ETEC: Enterotoxigenic E.coli; EHEC: Enterohemorrhagic E.coli; *: virulent E. coli strains.*

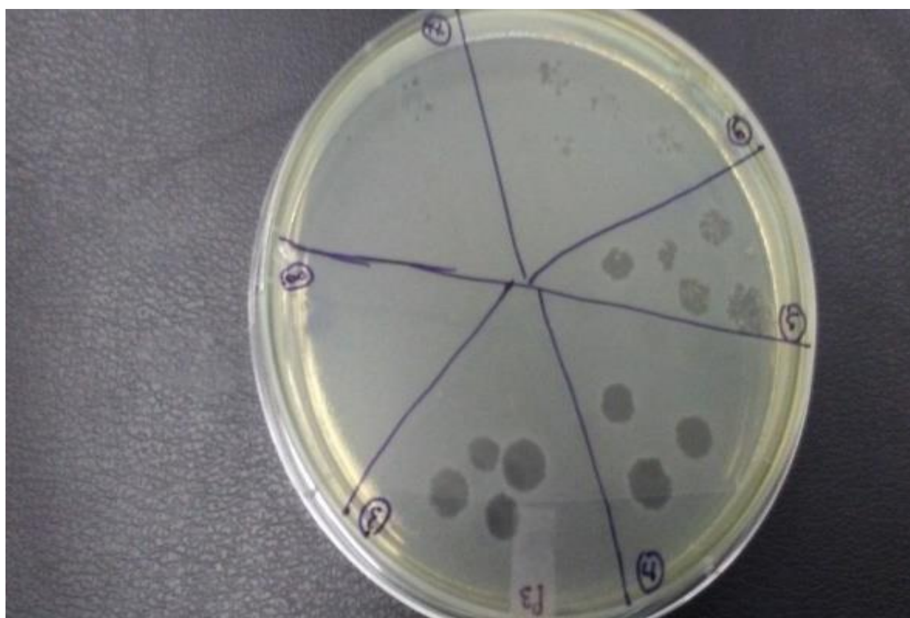

Figure 2. Phage activity on bacterial lawn serial dilution of phage Mα3 on *E. coli*.

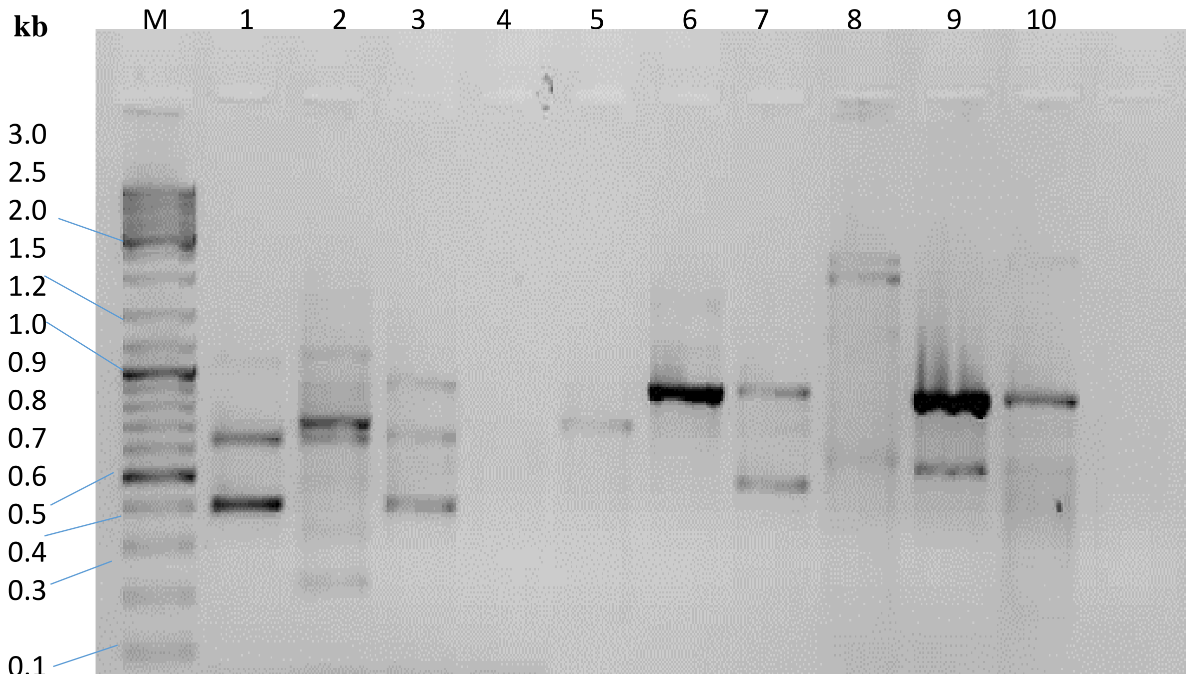

Figure 3. RAPD band patterns obtained from rodent'phages. M: Kb ladder; lane 1-3: Mα3, Mα7, Mα11 with primer R10D; lane 5-7: Mα3, Mα7, Mα11 with primer P2; lane 8-10: Mα3, Mα7, Mα11with primer OPL5.

et al., 2017). Jothikumar et al. (2000) have first reported ten phages specific to *Enterotoxigenic E. coli* (ETEC), and this study has revealed two phages Mα3, Mα7 against *ETEC* strains and all tree phages Mα3, Mα7, Mα11 against *APEC* strains.

Several studies have reported coliphages belong to *Myoviridae* and *Siphoviridae* families. The extended host range of phages correlates with the acquisition of new fiber genes. In this study, we cannot demonstrate the phylogeny of the rodent phages, because of the lack of NGS analysis. The genomic diversity of phages by using RAPD-PCR revealed the existence of different clusters for the rodent's phages. Previous studies have similar results to differentiate different phages families isolated from various sources by RAPD-PCR (Comeau et al., 2004; Gutierrez et al., 2011; Jothikumar et al., 2000). The lytic rodent's phages, individually or a cocktail, may be useful for reducing contamination in food processing, or to control bacterial infections in humans. Also, our results suggest the exploration of new microbiomes from rodents to identify phages of interest in biotechnological applications and the perspectives for genomics tools.

Conclusion

This study describes the first isolation of rodent's phages with lytic activity on clinical virulent bacterial strains. The rodent's phages Mα3, Mα7 and Mα11 have *E. coli* as host bacteria, suggesting they belong to the family of coliphages. 96% of coliphages belong to *Caudavirales*. Most isolated phages were in the family of Enterobacteriaceae phages. Genomic typing by RAPD shows the divergence of isolated rodent's phages. The effect of rodent's phages on hemorrhagic strains was to reduce bacterial infection. The candidate rodent phages can be applied in phage therapy in West Africa to fight multi-drug strains bacteria. Analysis of morphology and Next Generation Sequencing (NGS) method should be done in the future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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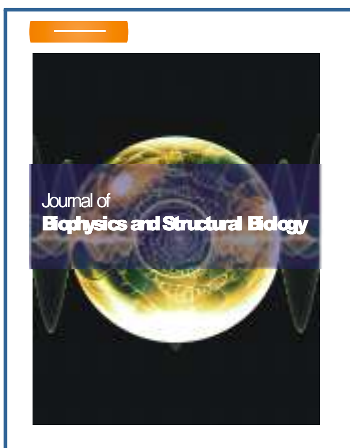

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