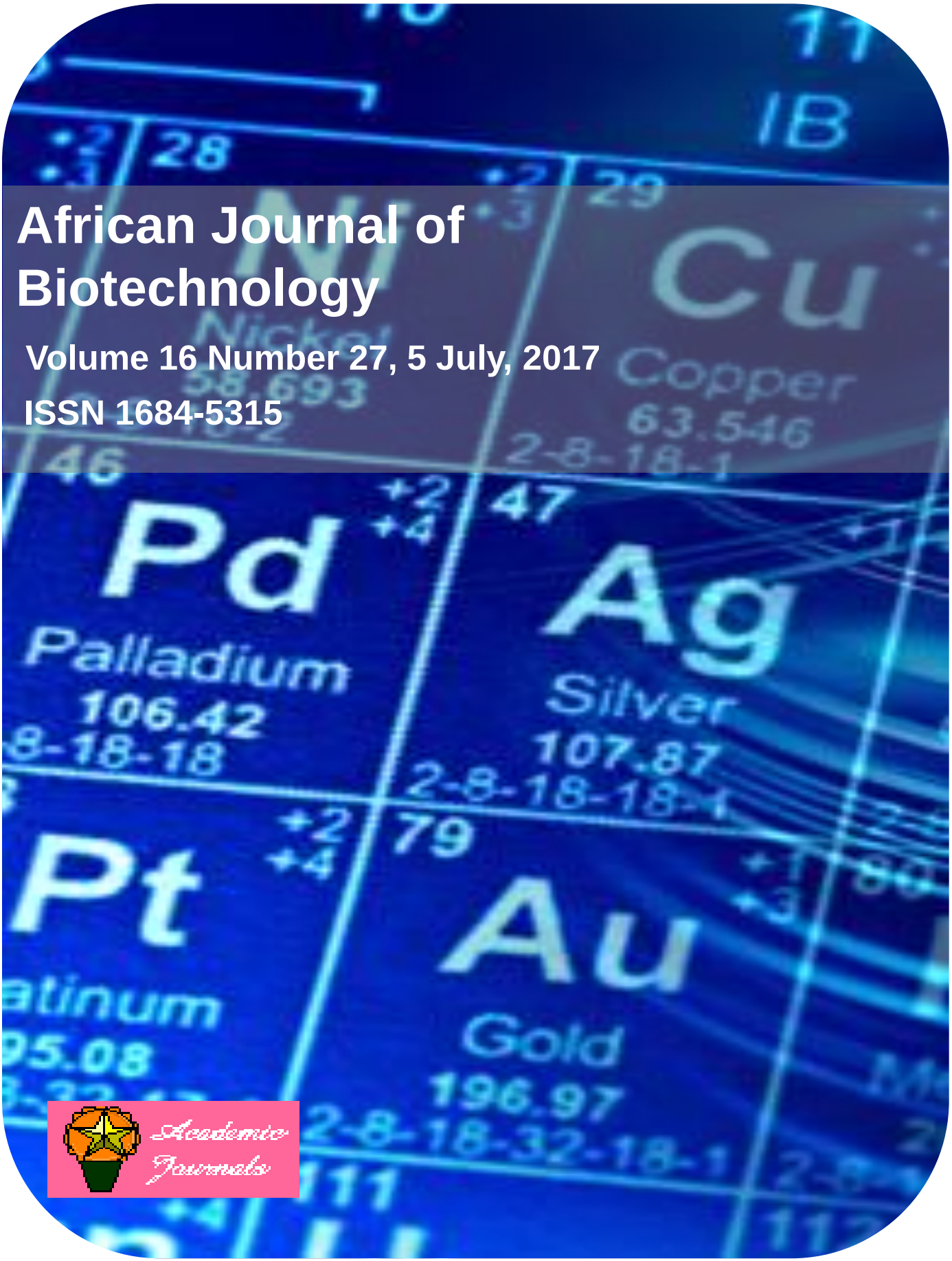

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Full Length Research Paper

Silver nanoparticles biosynthesis and impregnation in cellulose acetate membrane for anti-yeast therapy

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Silver nanoparticles (AgNPs) are nanoforms that express higher antimicrobial potential due to their shape and reduced size. The use of fungi to mediate the synthesis of AgNPs has increased the interest of scientists because of their rapid growth, large-scale cultivation and secretion of non-toxic molecules. The aim of this study was to synthesize AgNPs mediated by *Aspergillus oryzae* DPUA 1624 and evaluate the antimicrobial activity of these molecules incorporated in cellulose acetate nanomembranes (NanoMAC). The synthesis of AgNPs was confirmed by UV-visible spectroscopy and the characterization was performed by dynamic light scattering, transmission electron microscopy, X-ray diffraction, X-ray dispersive energy and advanced spectroscopy and spectroscopy methods. The synthesis of the membrane was done by electro-spinning and its thickness was analyzed in scanning electron microscope. The AgNPs were added to NanoMAC and the antimicrobial effect was evaluated by agar diffusion method against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*. The aqueous extract of *A. oryzae* mediated the synthesis of AgNPs with rounded and some triangular shapes. The diameter and zeta potential of these particles were 61.15±11.45 nm and -28.7 mv, respectively. The NanoMAC with AgNPs showed an increase in antifungal activity of 24.22% when tested against *C. albicans*. This study demonstrated that *A. oryzae* is able to mediate AgNP synthesis with anti-yeast action and the impregnation of AgNPs in acetate cellulose nanomembranes resulted in the development of a more efficient antimicrobial nanocomposite.

Key words: Silver nanoparticles, *Aspergillus oryzae*, cellulose nanofibers, anti-yeast.

INTRODUCTION

Silver nanoparticles (AgNPs) are nanoforms that express higher antimicrobial potential due to their shape and reduced size. The characters of AgNPs promote a better interaction with biological receptors, they intensify the interaction with cell surface and, consequently, they increase the bioactive efficiency (Tarafdar and Raliya, 2013; Pereira et al., 2014). Due to their excellent antimicrobial action, AgNPs are widely used in medical, technological and agricultural areas (Ingale and Chaudhari, 2013; Pereira et al., 2014; Moghaddam et al.,

2015; Siddiqi and Husen, 2016).

The world market of nanotechnological products have been in evolution. The annual arrecadation is around 25% and the prevision is an increase to 3 billions until 2020. Among these products, the ones from AgNPs stand out. The antimicrobial properties of AgNPs make possible their use in a wide diversity of products as plastics, soaps, pastes, food and textile products (Tran et al., 2013).

The colloidal solution of AgNPs can be combined with many polymeric matrices to extend the release of antimicrobial and increase its biocompatibility. The electrospinning nanofibers, for example, are polymers that have high potential of application in pharmaceutical and cosmetic industries for regeneration of skin and organs, therapies of drugs administration and others (Nista et al., 2015; Segala et al., 2015).

The search for nanostructured polymers with antimicrobial action are increasing due to their great potential of use in devices that require antiseptic characters as applications in skin wounds (Abdelgawad et al., 2013; Segala et al., 2015). For that reason, AgNPs are the nanoforms most used in polymers due to their electronic, optical, catalytic and antimicrobial properties (Segala et al., 2015).

The use of nanomembranes for the treatment of skin wounds provides better comfort to the patient because it reduces the number of antibiotic applications and doses, decreasing the side effects. In these kinds of infections, the nanomembranes also can act like a sponge absorbing all the fluids from the lesion and insure the oxygenation through the porous net of nanofibers (Nista et al., 2013).

The cellulose acetate is a polymer that presents good tenacity, high biocompatibility and relative low cost, besides, easily forms pellicles with porous structure, the characters that make it advantageous to build an antiseptic nanocomposite to wounds treatment (Nista et al., 2012; Segala et al., 2015).

The combination of AgNPs with polymers for application in many areas is very versatile. However, the method of chemical synthesis generates toxic residues that contaminate the environment and affect the ecosystem (Zhang et al., 2011; Dhanasekaran et al., 2011; Durán and Marcato, 2011; Kulkarni and Muddapur, 2014; Meva et al., 2016). In the last years, there is an increasing demand to for the development of processes mediated by microbial sources due to their low cost and the production of nontoxic molecules and for being ecofriendly (Mishra and Singh, 2015; Moghaddam et al., 2015; Siddiqi and Husen, 2016).

Among the microbial sources, the filamentous fungi are potential biologic factories to the production of metallic nanoparticles due to their fast growth, large scale cultivation, secretion of proteins with higher reducer properties and the production of molecules that stabilize nanoparticles (Durán et al., 2011; Tarafdar and Raliya, 2013; Phanjom and Ahmed, 2015).

Some species of fungi are alternative safe systems as intermediate for the AgNPs synthesis in function of their high tolerance and capacity of metallic absorption. *Aspergillus, Fusarium, Trichoderma, Cladosporium, Pleurotus, Penicillium* and *Verticillium* are examples of fungi that mediate the extracellular synthesis of AgNPs. The extracellular process is more desirable than the intracellular because is less expensive and difficult (Sastry et al., 2003; Roy et al., 2013; Siddiqi and Husen, 2016).

There are many reports in the literature showing the synthesis of AgNPs mediated by *Aspergillus* species but are rare studies showing the potential of amazonic fungi in this process. The interaction of biological AgNPs with cellulose acetate nanomembranes is also an inedited investigation because most of the researches present the development of antiseptic polymers using AgNPs obtained from chemical synthesis.

This investigation shows the capacity of *Aspergillus oryzae* DPUA 1624 to mediate AgNPs synthesis with antimicrobial potential and the anti-yeast action with the impregnation of these particles in cellulose acetate nanomembranes (NanoMAC).

MATERIALS AND METHODS

Morphological and molecular authentication of *A. oryzae*

A. oryzae DPUA 1624 (Culture Collection DPUA/UFAM) was maintained on CYA agar surface [Czapek dox agar + 0.5% (w/v) yeast extract] in plates under refrigeration (4°C) (Klich and Pitt, 1988). The macro and micro morphological identification were carried out according to Raper and Fennel (1977).

The molecular authentication was made from partial sequence of rDNA. The colony mycelium (200 mg) was used for DNA extraction by phenol chloroform method. The Internal Transcribed Spacer Region (ITS2) and part of 5.8S and 28S subunits from rDNA were amplified by PCR using ITS-3 (5´-GCA TCG ATG AAG AAC GCA GC-3') developed by White et al. (1990) and UNI-R (5'-GGT CCG TGT TTC AAG ACG-3') developed by Haynes et al. (1995). The rDNA sequences were obtained by automated sequencing (3500 ABI Applied Biosystem) and the species confirmation by comparison of DNA sequences from GeneBank using Basic Local Alignment Search Tool (BLASTn) from National Center for Biotechnology Information (NCBI).

Biomass production

A. oryzae was inoculated, by a spore suspension of 10^6 spores/ml, in 200 ml of MGYP [0.3% (w/v) malt extract; 0.1% (w/v) glucose;

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0.3% (w/v) yeast extract; and 0.5% (w/v) peptone]. Fermentation was conducted at 25°C, 180 rpm, for 96 h. The biomass was separated by filtration using Whatman paper no. 1, washed three times with ultrapure water, transferred to Erlenmayer flasks containing 200 ml of ultrapure water and maintained at 28°C, 200 rpm, for 96 h. The biomass was filtrated again and the aqueous extract recovered was used for AgNPs biosynthesis (Sastry et al., 2003; Durán et al., 2007; Vigneshwaran et al., 2007).

Silver nanoparticles biosynthesis

The aqueous extract (200 ml) was mixed with a silver nitrate solution (1 mol L^{-1}) until final concentration of 1 mmol L^{-1} and incubated at 28°C, 200 rpm for 96 h, in the absence of light. Simultaneously, two controls: one of the aqueous extract and other of nitrate solution were maintained under same conditions. The synthesis of silver nanoparticles was confirmed by the color change of the solution and the presence of plasmonic band in 400 nm (Vigneshwaran et al., 2007; Zomorodian et al., 2016). Ultraviolet-visible (UV-Vis) spectral analysis was done by using Cary 60 Agilent Spectrophotometer in the range of 200 to 800 nm.

Silver nanoparticles characterization

Zeta potential and size of AgNPs (DLS)

The AgNPs diameter, size distribution and superficial charge obtained were measured in Nano Zetasizer (ZS) (Malvern Instruments, Worcestershire, UK), at 25°C.

Transmission electron microscopy (TEM)

Size distribution and morphological variation of AgNPs were determined from high resolution images in TEM (Carl Zeiss CEM 902) operating at 80 KeV. A drop of nanoparticles solution (diluted ten times) was deposited on carbon-coated copper grid and the images were processed in *image J* software and the equation used to calculate de AgNPs diameter from the obtained area was $D = \sqrt{(\frac{A}{\pi})}$. The statistical analysis was carried out using the software origin (version 8.5.1.315).

X-Ray diffraction (XRD)

XRD patterns were performed on a Shimadzu XRD7000 with a high-power CuKα radioactive source at 40 kV/30 mA, 2° min^{-1} acanning speed, angles of 2θ from 5 to 90°.

Fourier-transform infrared spectroscopy (FTIR)

The data of IR spectra were acquired using an Agilent cary 630 FTIR spectrometer, with diamond attenuated total reflectance (ATR) attachment. In ATR mode, the spectral variation was from 400 to 4000 cm^{-1}. The AgNPs were analyzed depositing a drop of the colloidal suspension on ZnSe (zinc selenide) window.

Energy dispersive X-ray (EDX) and inductively coupled plasma optical emission spectrometry (ICP-OES)

The EDX was carried out in a Micro fluorescence x-ray spectrometer (microEDX1300, Shimadzu) in five points of the sample using spectrums of 15 and 50 kV and courting time of 200 s.

The inductively coupled plasma optical emission spectrometry (ICP-OES) was used for quantification of silver in the solution of AgNPs. The analysis was carried out in plasma emission spectrometer (PerkinElmer, Optima 8000). The calibration curve was constructed using 9 silver nitrate solutions with concentrations from 0.1 to 50 mg/L. The AgNPs solution was diluted 10 times for reading in the equipment.

Synthesis of cellulose acetate nanomembrane (NanoMAC)

The cellulose acetate (CA) polymer solution was prepared by mixing 15% (w/v) cellulose acetate into dimethylacetamida (DMAc)/Acetone (1:2). This solution was stirred for approximately 2 h to ensure its complete homogenization (Nista et al., 2012).

CA membranes were prepared by electrospinning the cellulose acetate solution at room temperature (25°C) and 50% humidity, using a 20 ml glass syringe fitted with a 4 cm long, 0.8 mm diameter metallic needle. The positive pole of a high voltage power supply was connected to the metallic needle of the syringe, while the ground electrode was used to ground the copper plate collector, which had dimensions of 30 × 40 cm. The feed stream was controlled by a KD Scientific pump, Model 100 (Campinas, Brazil) connected to a syringe. The distance from the needle to the collector was 10 cm; the applied voltage was 15 kV, and the flow rate was 1 ml h^{-1}. Membrane samples were collected in aluminium foil used to coat the copper plate during the experiments (Nista et al., 2012; Segala et al., 2015).

The morphological structure and uniformity of nanoMAC were observed in scanning electron microscope (JEOL JSM-6360LV), by vacuum, operated at 10 kV. The images were obtained after sample metallization with a layer of gold/palladium and the diameter was calculated in Image J software.

The nanoMAC coating was performed by slow dripping of the AgNPs solution in discs (5 mm) of the nanostructured membrane until the final concentration of 6.73 µg Ag on each nanoMAC disc.

Antimicrobial activity

AgNPs suspension

The agar diffusion method was used for the antimicrobial activity against *Escherichia coli* CBAM 0001, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* DPUA 1706 (Ostrosky et al., 2008). The bacterial cultures (10^8 CFU/ml) were inoculated on Müller Hinton agar (MHA) and the yeast culture (10^6 CFU/ml) on Sabouraud agar (SAB) and incubated at 37°C. In each plate, 100 µl of biosynthesized AgNPs (134.5 mg/L) were transferred to wells (8 mm) made on agar surface. The plates were incubated at 37°C for 24 h. The positive controls [streptomycin (50 µg/ml) to bacteria and itraconazole (50 µg/ml) to the yeast] were maintained at the same conditions. After incubation, the plates were examined for the presence of an inhibition zone.

NanoMAC impregnated with AgNPs

The antimicrobial activity of nanoMAC impregnated with AgNPs was carried out against *C. albicans* DPUA 1706 (10^6 CFU/ml) by agar diffusion method (Ostrosky et al., 2008) in Petri dishes containing Sabouraud agar. Discs of nanoMAC (5 mm diameter) impregnated with AgNPs (6.73 µg) were compared to suspension of AgNPs (6.73 µg). The plates were incubated at 37°C for 48 h. The positive control [itraconazole (6.73 µg/ml) and itraconazole (6.73 µg/ml) + AgNPs (6.73 µg/ml)] was maintained at the same conditions.

RESULTS AND DISCUSSION

Morphological and molecular authentication of *A. oryzae*

The macro and microscopic characteristics of the culture confirmed that the fungus is *A. oryzae* (Raper and Fennel, 1977; Klick and Pitt, 1988). For the molecular authentication, the rDNA sequences presented 99% of similarity (Score, 1554) (Evalue 0) with sequences at the same region from *A. oryzae* (Access number KT964480.1). The amplified sequence of *A. oryzae* DPUA 1624 was deposited at GenBank (Access number KY655350).

Silver nanoparticles biosynthesis

The biosynthesis of silver nanoparticles (AgNPs) was confirmed by the color change of the mycelial aqueous extract from yellow to brown (Figure 1A). There was no formation of aggregate particles and the extract did not present color intensification after 96 h. These results indicate that the nanoparticles were dispersed in the solution.

The color change occurs due to the effect of superficial plasmon resonance (SPR) and the reduction of $AgNO_3$ (Li et al., 2012). SPR refers to the collective oscillation of free electrons in the metallic nanoparticles when they interact with electromagnetic radiation (Cao et al., 2011; González et al., 2014; Al Juraifani and Ghazwani, 2015).

The absorption spectrum of the AgNPs solution (Figure 1B) shows a SPR band with peak of absorbance in 446 nm confirming the presence of AgNPs after 96 h of incubation. Similar results were reported for AgNPs from *Aspergillus tubingensis* in the same wavelength (Rodrigues, 2013).

Silver nanoparticles characterization

Zeta potential and size of AgNPs

Zeta potential (ζ) indicates the repulsion between the disperse particles with the same charge by electrophoretic mobility. Its value can be related to the stability of colloidal dispersions (Phanjom and Ahmed, 2015). In this study, the zeta potential of AgNPs mediated by *A. oryzae* was 28.7 mv. This result suggests that the AgNPs have an elevated electrostatic repulsion which contributes to their stabilization. According to Roy et al. (2013), particles in suspension with high negative or positive zeta potential tend to repel themselves and do not form aggregates.

Dynamic light analysis (DLS) is a technique that determines particles sizes by measurement of aleatory changes in the intensity of light from a solution or suspension (Fatima et al., 2015). The average hydrodynamic diameter of *A. oryzae* AgNPs by DLS was 57.88 nm (Figure 2) and the polydispersity index was 0.217, confirming the formation of particles in nano scale dimension (1-100 nm) and the definition of the solution as monodisperse due to the interval defined for this type of particles from 0.01 to 0.7 (Honary et al., 2013; Tarafdar and Raliya, 2013).

Transmission eletron microscopy (TEM)

The results of TEM images (Figure 3A) show that AgNPs are predominately rounded and, in less quantity, some are triangle with average size of 61.15±11.45 nm. AgNPs with variable shapes and sizes are common in the biological systems; however, the ones with triangle shapes have more antimicrobial efficiency than the rounded AgNPs (Pal et al., 2007; Rai et al., 2009; Roy et al., 2013).

The size distribution of AgNPs is as shown in Figure 3B. Most of the AgNPs are from 50 to 60 nm in size. Some studies report nanoparticles from *Aspergillus* with sizes from 1 to 140 nm (Sagar and Ashok, 2012; Khalil, 2013; Roy and Das, 2015). Binupriya et al. (2010) and Pereira et al. (2014) reported AgNPs synthesis by *A. oryzae* species with sizes of 5 to 50 nm and 25 to 76 nm, respectively.

X-Ray diffraction (XRD)

The XRD patterns of the AgNPs showed the crystalline nature of the sample and confirmed the presence of pure silver in the nanoparticles solution analyzed. Figure 4 shows that it is possible to observe five distinct bands in 37,97°, 44,29°, 64,66°, 77,46° and 81,44° corresponding to (111), (200), (220), (311) and (222) planes. These planes correspond to the Ag° nanoparticles face-centered cubic (fcc) (Crystal Structure Database-ICSD/Code 64,994) with a lattice parameter of a = 4.077 Å. They confirm the formation of metallic silver in the suspension of AgNPs.

The absence of the bands face-centered cubic 27.9°, 32.3°, 46.3°, 55.0°, 57.6°, 67.6°, 74.6°, 76.9°, and 85.7° corresponding to the indexed (111), (200), (220), (311), (222), (400), (331), (420), and (422) facets that are typical of XRD pattern of AgCl nanoparticles (Crystal Structure Database-ICSD/Code 64734) show that there was no formation of hybrid Ag/AgCl nanoparticles as final product of the reaction (Durán et al., 2016). The unassigned peaks at 2θ = 28°, 32°, and 36° in Figure 4 are thought to be related to crystalline and amorphous organic phases (Awwad et al., 2013). These results confirm that the molecules from *A. oryzae* are efficient reducers of silver ions.

Fourier-transform infrared spectroscopy (FTIR)

The spectra presented four distinct bands: 3288, 2101, 1994 and 1633 cm^{-1} (Figure 5). The 3288 cm^{-1} band is

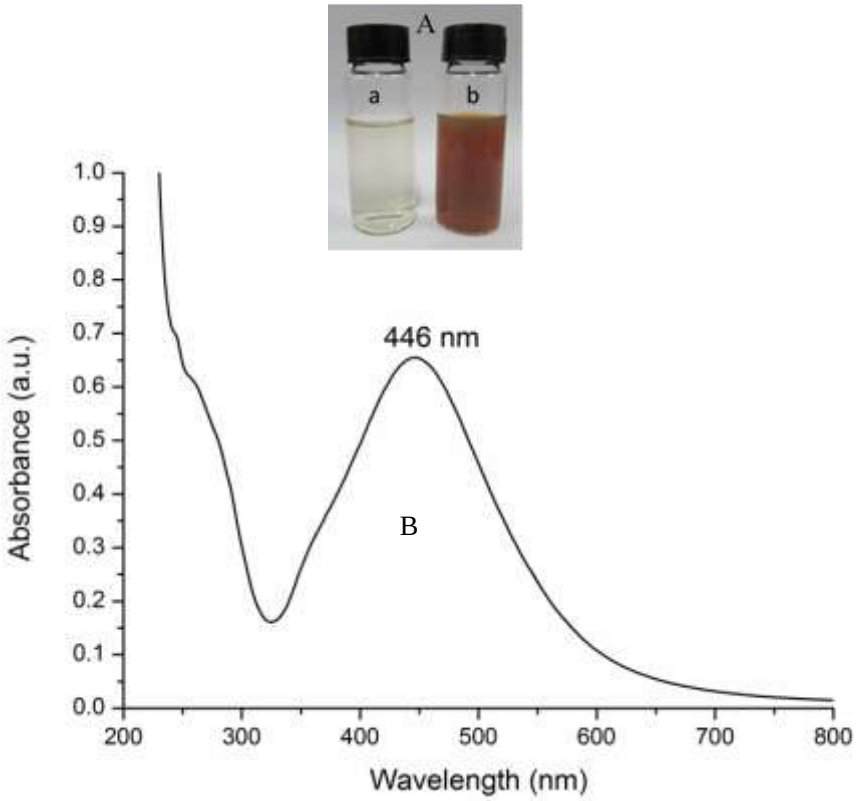

Figure 1. (A) Mycelial aqueous extract from *A. oryzae* DPUA 1624: (a) without $AgNO_3$; (b) with $AgNO_3$ (1 mol.L^{-1}); **(B)** UV-vis absorption spectrum of AgNPs after 96 h of reaction.

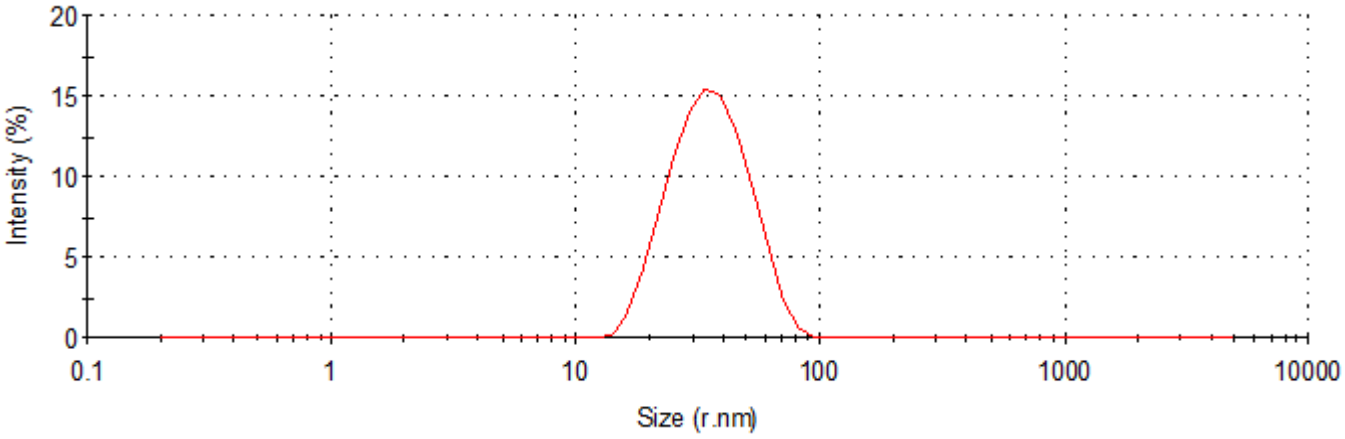

Figure 2. Hydrodinamic diameter of AgNPs biosynthetized by *A. oryzae* mycelial extract.

related to the vibration energy of primary and secondary amides from proteins and the 1633 cm^{-1} band is related to the vibration energy of carbonyl group and is assigned to the primary amide bond of the protein (El-Aziz et al., 2012). The presence of carbonyl and primary amide groups indicate that the proteins present in the aqueous extract of *A. oryzae* biomass are connected to the AgNPs. Many reports testify that proteins can connect to silver nanoparticles by the free amine groups turn them more stable particles (Phanjom and Ahmed, 2015).

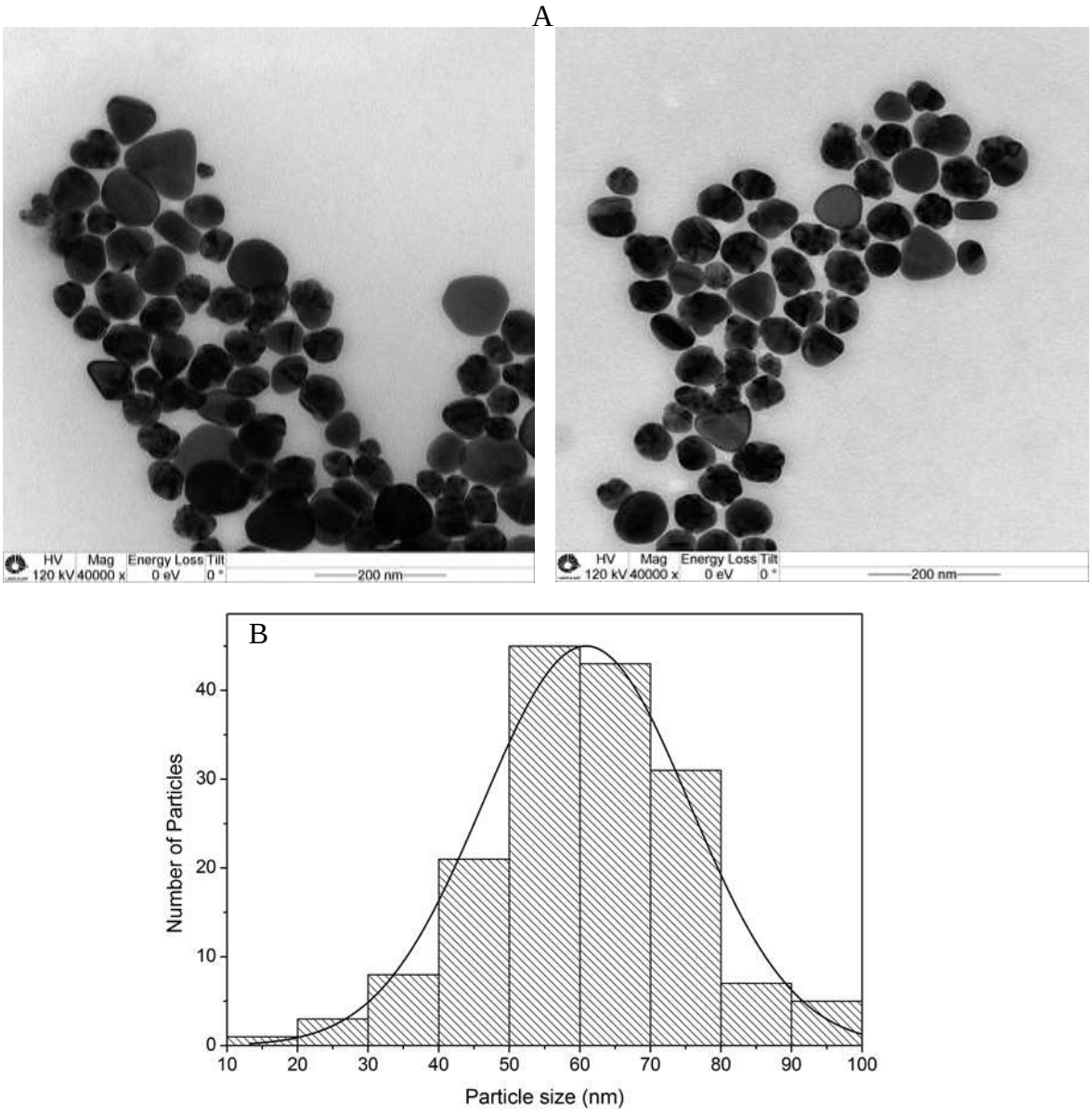

Figure 3. (A) Transmission electron microscopy images of AgNPs biosynthetized by *A. oryzae* mycelial extract. **(B)** Distribution histogram of AgNPs sizes.

Energy dispersive X-ray spectroscopy (EDX) and inductively coupled plasma optical emission spectrometry (ICP-OES)

The energy dispersive X-ray spectroscopy (EDX) revealed strong sign in the region next to 3 KeV that correspond to the peak of silver (Pereira et al., 2014; Elgorban et al., 2016). In EDX spectra, a peak was observed in 2.98 keV and others in 3.17 keV that correspond to bonds energy of AgLa and AgLb1, respectively (Figure 6). Both peaks composed of the optical absorption profile of silver element. These results indicate that the AgNP mediated by *A. oryzae* are high pure.

The inductively coupled plasma optical emission spectrometry analysis showed that the concentration of the AgNPs solution was 134.5 µg/ml.

Antimicrobial activity

AgNPs solution and NanoMAC impregnated with AgNPs

The antimicrobial activity with the AgNPs solution

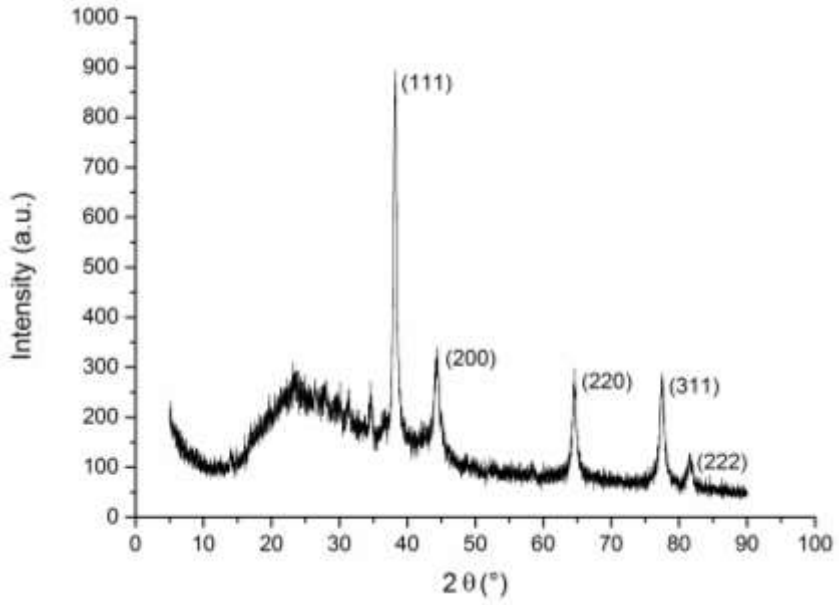

Figure 4. XRD patterns of lyophilized AgNPs biosynthetized by *A. oryzae* mycelial extract.

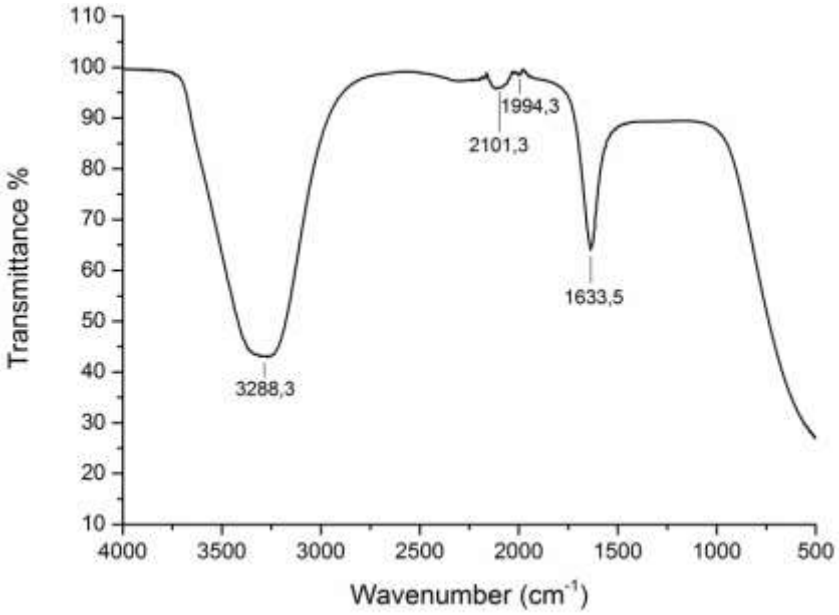

Figure 5. FTIR spectra of AgNPs biosynthetized by *A. oryzae* mycelial extract.

showed that *E. coli* CBAM 0001 and *S. aureus* ATCC 25923 were resistant to the AgNPs (134 µg/ml), while *C. albicans* DPUA 1706 was sensible. The yeast growing was completely inhibited by the AgNPs showing an inhibition zone of 23.6 cm (Figure 7).

Xue et al. (2016) reported similar results with AgNPs mediated by *Arthroderma fulvum*. The particles presented antifungal activity against many fungi including *C. albicans*.

AgNPs present efficient antimicrobial properties compared to other molecules due to their large superficial area that promotes a better contact with the micro-organims (Rai et al., 2009; Kon and Rai, 2013). However, according to Segala et al. (2015), the antimicrobial activity of AgNPs depends on some factors such as crystallinity, geometry, size, superficial oxidation,

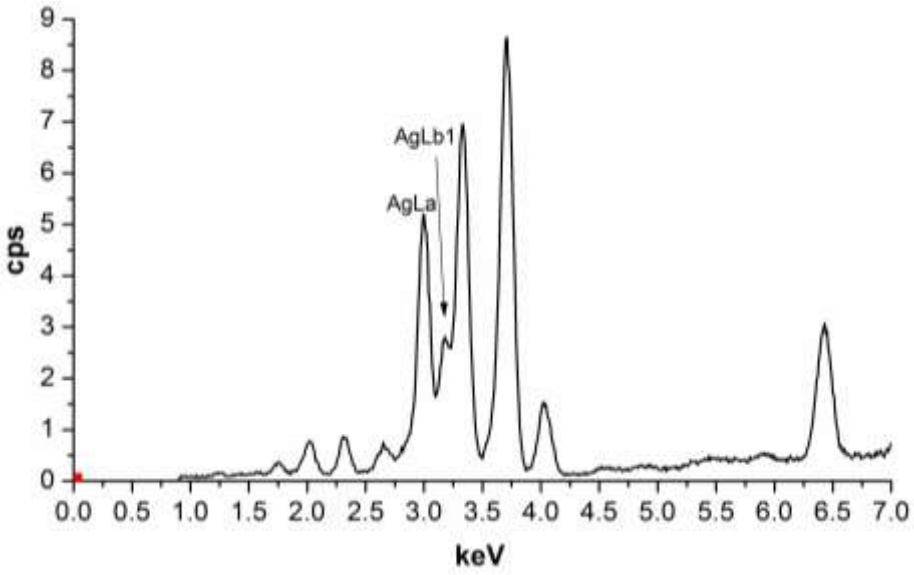

Figura 6. Energy dispersive X-ray spectra (EDX) of AgNPs solution biosynthetized by *A. oryzae* mycelial extract.

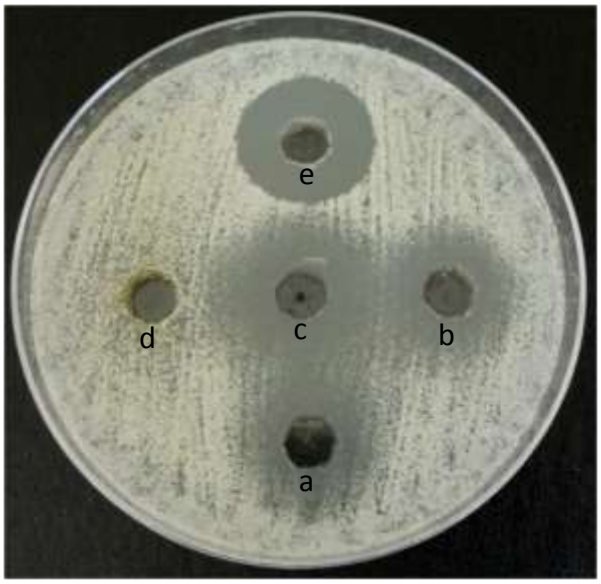

Figure 7. Antimicrobial activity of *A. oryzae* AgNPs against *C. albicans*: (a), (b) and (c) inhibitions zones formed by the AgNPs [134 µg/ml], (d) 1 mmol L^{-1} of $AgNO_3$, (e) itraconazole (50 µg/ml).

agglomeration in the biological environment, superficial charge, groups present in the nanoparticles surface, and others. For that reason, we can deduce that the properties of *A. oryzae* AgNPs enabled a better interaction with the yeast cells promoting the penetration of the nanoparticles through the plasmatic membrane of the micro-organism causing cell death.

The AgNPs antimicrobial effects are widely known, although their action mechanism in yeast cells not been completely described. Kim et al. (2009) observed that the AgNPs interact with *C. albicans* cells causing significant changes in their membranes resulting in the formation of “pits” and finally, the formation of pores and death. The authors also believe that the AgNPs break the membrane permeability barrier of *C. albicans* by the disarray of the lipid bilayer. It causes the scape of ions and other materials and the formation of pores that dissipate the membrane electrical potential.

Although many studies report the cytotoxic and genotoxic action of AgNPs in human and animal cells, there is still a lack of consistent and trustable data about them. Some of them are contradictory and, for that reason, there is no general consense about the action of these molecules in living organisms and in the environment (Rai et al., 2017). The cytotoxicity of AgNPs depends on many factors as size, shape, stability, given dose and time of exposition (Tran et al., 2013; Rai et al., 2017).

The AgNPs biosynthetized by the mycelial extract of *A. oryzae* DPUA 1624 can be evaluated as efficient agent for use in clinical application, especially in treatments of infections caused by *C. albicans.*

Yeasts highlight as main etiologic agent of fungal diseases primarily dermatomycosis, a skin infection that have been considerably increasing in the last decades. One of the causes that favor the incidence of this disease is due to the resistance of these fungi to the medications. For that reason, a considerable motivation occurs which is to find new anti-yeasts agents (Sidrim and Rocha, 2010; Xue et al., 2016).

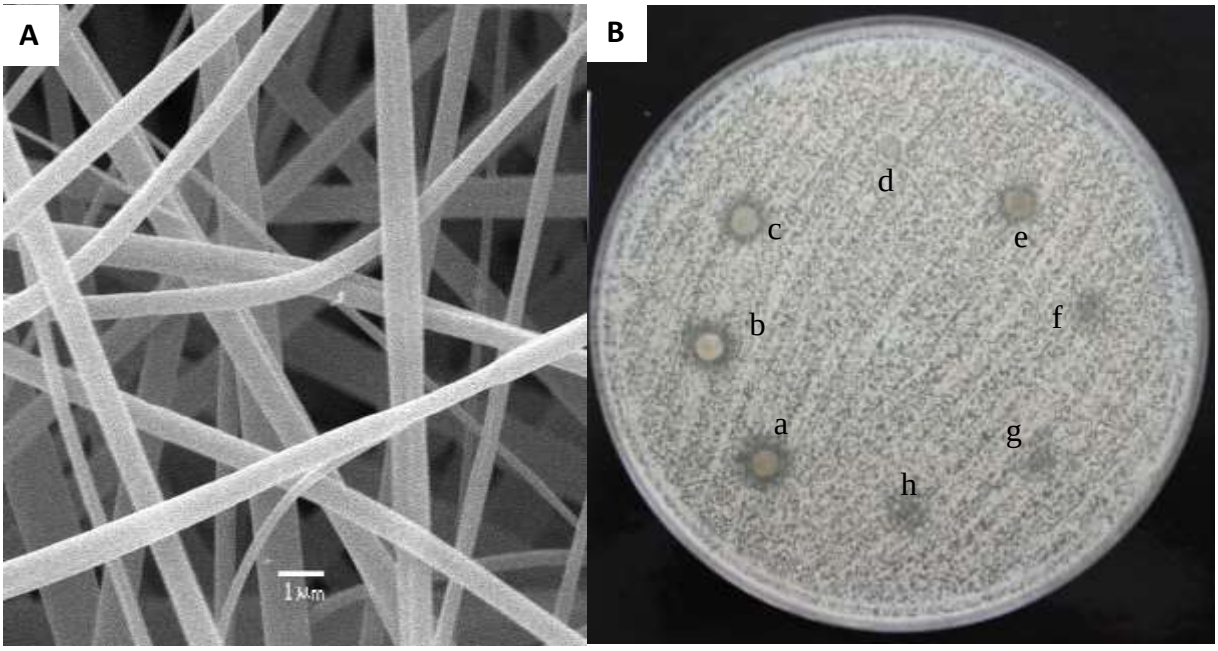

Figure 8. (A) Image in scanning electron microscope of cellulose acetate membrane (nanoMAC). **(B)** Antimicrobial activity of AgNPs impregnated in nanoMAC against *C. albicans*: (a), (b) and (c) nanofibers with AgNPs (6.73 µg); (d) nanofiber without AgNPs; (e) nanofiber with itraconazole + AgNPs (6.73 µg each); (f), (g) and (h) AgNPs 6.73 µg.

Table 1. Antimicrobial activity of AgNPs from *A. oryzae*.

Micro-organisms		Antimicrobial activity [Inhibition zone (mm)]		
		AgNPs [134 µg/ml][a]	AgNPs (6.73 µg)[a]	NanoMAC impregnated with AgNPs (6.73 µg)
Bacteria	*Escherichia coli* CBAM 0001	Resistance	na	na
	Staphylococcus aureus ATCC 25923	Resistance	na	na
Yeast	*Candida albicans* DPUA 1706	23.6±3.82	9.66±0.44	12±0.67

[a]AgNPs in colloidal solution-agar diffusion method. Na, not avaiable.

According to the necessity to develop new strategies against skin infections caused by yeasts, this study carried out the antimicrobial activity of AgNPs impregnated in cellulose acetate membrane (15%) and DMAc: acetone (1:2) (NanoMAC). The nanoMAC contains nanofibers with average diameter of 410.76 nm. They are random deposited forming a thinny and homogeneous tissue without beads formation (Figure 8A).

The nanoMAC impregnated with AgNPs (Table 1), allowed the increase of 24.22% in antifungal action against *C. albicans* when compared with the antimicrobial activity using the colloidal solution of AgNPs (Figure 8B). This result is relevant because it demonstrates that the AgNPs impregnation in cellulose acetate membrane can potentiate the action against yeasts of *Candida* genus.

NanoMAC impregnated with biological AgNPs present a large potential to be used in medical and pharmaceutical industries. They can be considered as sustainable nanocomposites with anti-yeast action.

Conclusion

The fungi *A. oryzae* DPUA 1624 produces molecules capable to reduce silver (Ag) ions and mediate stable silver nanoparticles synthesis. They are monodisperse with antimicrobial activity. This result provides a viable and eco-friendly alternative to antifungal production based on AgNPs without causing any polluting residues. The impregnation of AgNPs in cellulose acetate membrane increases the anti-yeast action of these particles resulting in the development of a nanocomposite with antifungal properties that can be used in medical and pharmaceutical industries.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Generation of *gua1* deletion using polymerase chain reaction (PCR)-mediated gene disruption method in fission yeast, *Schizosaccharomyces pombe*

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***Gua1* gene in *Schizosaccharomyces pombe* encodes inosine 5′-monophosphate dehydrogenase (IMPDH), which catalyzes the first step in *de novo* biosynthesis of guanosine monophosphate (GMP). Knock-out cassette was constructed with polymerase chain reaction (PCR)-based gene targeting technique for deletion of *gua1* gene in *S. pombe* and this knock-out cassette was transformed to *S.s pombe* wild type (*972h*$^-$). Knock-out cassette contains 653 and 285 bp sequences which are upstream and downstream of *gua1* gene, respectively, in *S. pombe* genome and kanamycin resistance gene obtained from *pFA6* plasmid. After transformation using lithium acetate method, knock-out cassette is aimed at replacing with the sequences of *gua1* gene via homologous recombination. The transformant *972h*$^-$ colonies which integrated knock-out cassette to the genome via homologous recombination are selected in YEA medium with antibiotic G418 after transformation and in this step, possible mutant colonies in *gua1* gene were determined. Finally, colony PCR techniques were performed to control whether the deletion is made in the right place or not. The results show that the *gua1* gene deleted strain was obtained.**

Key words: Fission yeast, deletion, *gua1* gene, knock-out cassette, inosine 5′-monophosphate dehydrogenase (IMPDH).

INTRODUCTION

Inosine 5′-monophosphate dehydrogenase (IMPDH) is the key enzyme in *de novo* biosynthesis pathway of purine nucleotides. The role of IMPDH at metabolic branch point in *de novo* biosynthesis pathway of purine nucleotides makes IMPDH a target for drug discovery for immunosuppression (Ratcliffe, 2006), cancer (Chen and Pankiewicz, 2007; Oláh et al., 2006), antiviral (Nair and Shu, 2007) and antimicrobial chemotherapy (Shu and Nair, 2008). *Schizosaccharomyces pombe* is a free living unicellular fungus which has common features with

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Table 1. Primer sequences and size of products.

Primer	Outside Primer	Inside Primer	Product size (bp)
Upstream primers	tgcagcaaggaaaccatatcactgg	***ttaacccggggatccgtcgac***tttaggccgcttggggtgtg	653
Downstream primers	ccgaagactcgacaaagcctcag	***atgaatcggccaacgcgcgg***tgcattacgaacgaattgtgcaag	285

*Sequences are homologous to kanamycin resistance gene (bold type).

higher eukaryotic organisms and characterized with properties of Ascomycetes (Wood et al., 2002). IMPDH is a product of *gua1* gene in *S. pombe* and this gene is located near the centromeric region of chromosome II (Karaer et al., 2006).

Knock-out technology is carried out successfully in different organisms from single-celled eukaryotic organisms to mammalian including human cells (Kim et al., 2010; Shen et al., 2015; Rong and Golic, 2000). This technique is based on gene targeting and it modifies the genome of the living organism via homologous recombination. In gene targeting with homologous recombination technique, exogen DNA fragments (plasmid, cassette) produced *in vitro* are transformed into the cell in order to generate homologous recombination between target sequence and exogen DNA fragment which causes desired genetic change (Sawitzke et al., 2013; Gardner and Jaspersen, 2014).

Knock-out technology has made feasible research initiatives such as *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.edu/group/ yeast_deletion_ project/deletions3.html) which aimed to bring about a complete set of yeast deletion strains. Assigning specific functions to all open reading frames (ORFs) in one organism through phenotypic analysis of the mutants is the most common purpose of knock-out studies.

Korean BIONEER Corporation attempted to create a knock-out library for all *S. pombe* genes (http://pombe.bioneer.co.kr). The study aimed to obtain haploid mutant *S. pombe* with *gua1* deletion, which does not take part in mutant collection of BIONEER. In this study, this cassette was similar to the modular kanMX6 cassette system described by Hentges et al. (2005) and Palabiyik et al. (2016). The lengths of homologous flanking sequences (60 to 80 bp) to the target gene were shorter in these studies, but our cassette had long sequences (653 to 285 bp) flanking to the *gua1* gene. The *gua1* gene deletion strain was obtained, although the strain is not viable for long time, as seen in other studies (Jiang et al., 2010).

MATERIALS AND METHODS

Strains, media, growth conditions

S. pombe Linder str. *Liquefaciens* haploid wild type (*972h-*) and *Escherichia coli DH5α* strain containing *pFA6-KanMX4* plasmid were used and these organisms were obtained from Istanbul University Department of Molecular Biology and Genetics Laboratory collection.

In this study, media which were described by Gutz et al. (1974) were used for production of *S. pombe* cells. *S. pombe* cells were produced in yeast extract with supplements (YES) media (supplemented with adenine, histidine, leucine, uracil, lysine and guanine at the concentration of 50 mg/L for each), yeast extract agar (YEA), minimal media agar (MMA). Luria Bertani (LB) media with ampicillin (50 mg/L) was used for *E. coli* containing *pFA6-KanMX4* plasmid.

Isolation of *pFA6-KanMX4* plasmid from *E. coli DH5α* strain and genomic DNA from *S. pombe*

E. coli DH5α strain containing *pFA6-KanMX4* plasmid was grown overnight using LB media broth with ampicillin (50 mg/L) at 37°C and 150 rpm. Isolation of *pFA6-KanMX4* plasmid was performed according to the manufacturer's instructions (DSBIO Eco Friendly Plasmid Miniprep Kit).

972h- was grown in yeast extract agar plate 2 days at 30°C. Then, 1x 10^6 cells/mL were incubated overnight in yeast extract media at 30°C and 180 rpm. The isolation of genomic DNA from these cells was performed with DSBIO Quick Yeast Genomic DNA Extraction Kit according to the manufacturer's instructions. Mechanical disruption was carried out with 0.3 g 0.45-0.55 mm glass beads in dismembrator (Sartorius Mikro-Dismembrator S) at 3000 rpm for 1 min and this step is repeated 5 times for each sample.

Production of knock-out cassette

Primers which include both homologous sequences for downstream and upstream sequences of *gua1* gene and short sequences that are homologous to kanamycin resistance gene are designed as shown at Table 1.

Knock-out cassette was obtained in two steps. In the first PCR, genomic DNA of *S. pombe* was used as a template and DNA fragments sized 652 and 285 bp were obtained. After purification, DNA fragments were used together for second PCR in which *pFA6-KanMX4* plasmid was used as a template and outside primers. Knock-out cassette was observed by using agarose gel electrophoresis and gel extraction of cassette was carried out by Roche High Pure PCR Product Purification Kit.

Control of knock-out cassette with DNA sequence analysis

Upstream-outside primer of knock-out cassette and a reverse primer (inside primer) which complements kanamycin resistance gene (Table 2) were used in DNA sequence analysis. Sequence analysis was carried out with Sanger method by using 3500XL Genetic Analyzers (Applied Biosystems).

Transformation of knock-out cassette to *S. pombe*

Transformation of knock-out cassette to *S. pombe 972h-* wild type

Table 2. Primers which was used in nucleotide sequence analysis of knock-out cassette.

Primer	Sequences of primer
Upstream-outside primer	tgcagcaaggaaaccatatcactgg
İnside primer	ttgcccgacattatcgcgag

Table 3. Primers which was used in colony PCR and size of products.

	Forward primer	Reverse primer	Product size
gua1	gggctgcagatgtctgcctttaagcc	gaagatctagtaaagacgcttttc	~ 1.5 kb
kan^r	atgggtaaggaaaagactcacg	ttagaaaaactcatcgagca	~1 kb

strain was carried out using lithium acetate method according to the method of Gregan et al. (2006). Solutions in transformation procedure (LiAc solution (0.1 M LiAc, 1X TE (pH 7.5)), LiAc-PEG solution (0,1 M LiAc, 40% PEG 3350, 1X TE (pH 7)) were sterilized by autoclave for 20 min at 121°C and 1.2 atm pressure.

Selection of transformant colonies

After the transformation of knock-out cassette to wild type *S. pombe,* for the detection of the transformant cells, these cells were inoculated into yeast extract agar plate with antibiotic G418 (200 mg/ml) (Sigma G418 disulfate salt) and incubated at 32°C for 5 days. Then resistant *972h*$^-$ cells to antibiotic G418 were inoculated separately into selective media (YEA, YEA with G418, MMA and MMA with guanine) at the same time and incubated at 32°C for 5 days.

Control of transformant colonies

Colonies which grown with G418 were used as templates in PCR in order to determine the homologous recombination and colony PCR was separately performed with primers which are suitable for kanamycin resistance and *gua1* genes (Table 3).

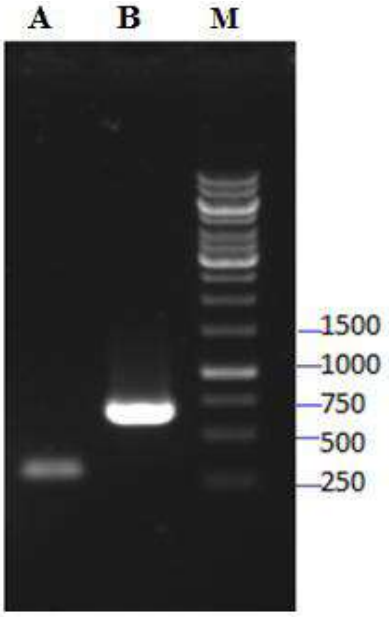

Figure 1. A. Downstream flanking region of *gua1* (285 bp). **B.** Upstream flanking region of *gua1* (653bp). M. Thermo Scientific™ O'GeneRuler 1 kb DNA Ladder.

RESULTS

Production of knock-out cassette

In the genome of *S. pombe* 653 and 285 bp homologous sequences which is located upstream and downstream flanking regions of gua1 gene respectively were amplified by two separate PCR (Figure 1). Knock-out cassette (2.5 kb) which includes kanamycin resistance gene was obtained at the second PCR (Figure 2).

Control of knock-out cassette with DNA sequence analysis

Results of DNA sequencing was confirmed with BLAST analysis in National Center for Biotechnology Information (NCBI). Sequencing results of knock-out cassette were compared with *S. pombe 972h*$^-$ genome and *pFA6-KanMX4* plasmid. Outcomes of sequencing which carried out with upstream-outside forward primer were compared with genome of *S. pombe 972h*- and it was observed that this regions showed at the rate of 95% identification with downstream and upstream regions of *gua1* gene.

Sequencing with inside reverse primer of kanamycin resistance gene was compared with *pFA6-KanMX4* plasmid. According to the result of BLAST analysis, it was observed at the rate of 99% identification to kanamycin resistance gene and expectation value was found to be “0.0”.

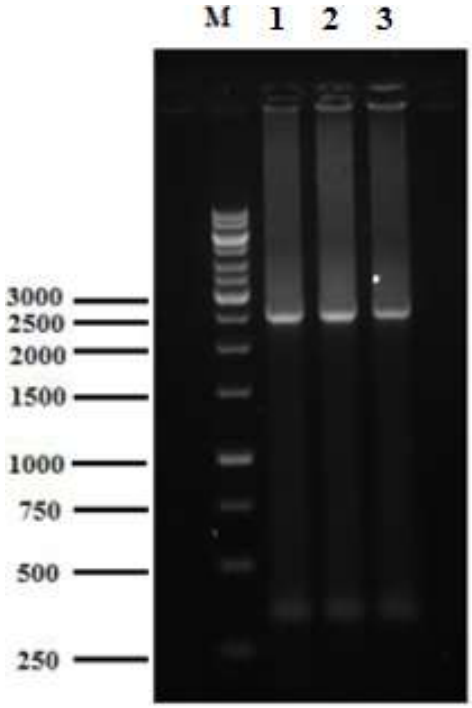

Figure 2. Agarose gel electrophoresis of knock-out cassette. 1, 2, 3, Knock-out cassette. M, Thermo Scientific™ O'GeneRuler 1 kb DNA ladder.

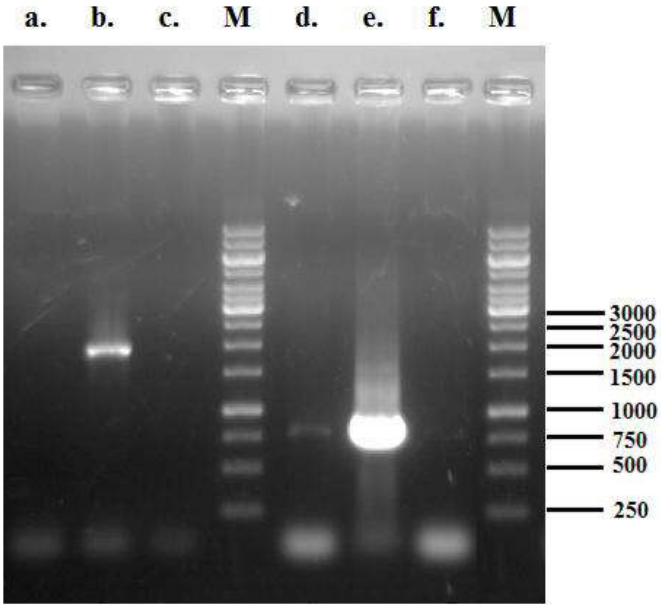

Figure 3. Colony PCR images of *gua1* mutant. a. PCR product with primers of *gua1* gene in *gua1* mutant colony; b. PCR product with primers of *gua1* gene by using *972h*$^{-}$ genomic DNA; c. No template with primers of *gua1* gene; d. PCR product with primers of *kan*^{r} gene in *gua1* mutant colony; e. PCR product with primers of *kan*^{r} gene by using *pFA6-KanMX4* plasmid DNA; f. No template with primers of *kan*^{r} gene; M. Thermo Scientific™ O'GeneRuler 1 kb DNA Ladder.

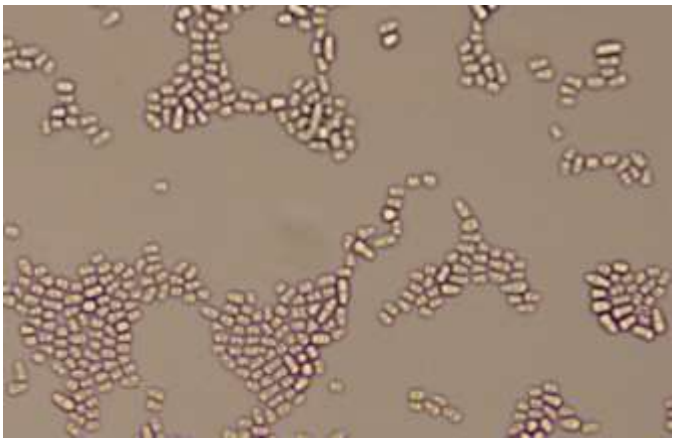

Figure 4. Images of putative mutant cells under fluorecent microscope.

Observation of null *gua1* mutant of *S. pombe*

24 independent transformation experiments with the knock-out cassette yield, 3604 positive colonies which were obtained according to kanamycin resistance, was carried out. Subsequently, colonies were inoculated into YEA, MMA, YEA with G418 and MMA with guanine in order to confirm whether cassette was inserted to *gua1* gene region or not. 56 colonies which could not live in media without guanine were detected after incubation for 5 days at 32°C.

After colony PCR with these 56 transformant colonies and primers which are appropriate to *gua1* and *kan*^{r} gene, samples were analyzed on agarose gel electrophoresis. In 55 colonies, it was observed that insertion of knock-out cassette into the location of *gua1* gene was unsuccessful. *Gua1* gene fragment does not exist in only one colony but a band with primers of *kan*^{r} gene in the PCR of this colony (Figure 3).

Observation of *gua1* deletion mutant cells using the phase-contrast microscope

When *gua1* deletion mutant cells (Figure 4) and wild type *S. pombe* cells (Figure 5) were investigated under the fluorescent microscope, it was seen that mutant cells were smaller than wild type and mutant cells tend to be aggregate.

DISCUSSION

Over the past 60 years, *S. pombe* has become an important model organism along with *S. cerevisiae* to study the molecular biology of eukaryotes (Hoffman et al., 2015). Yeasts help to improve comprehension of basic cellular processes and metabolic pathway in human. This

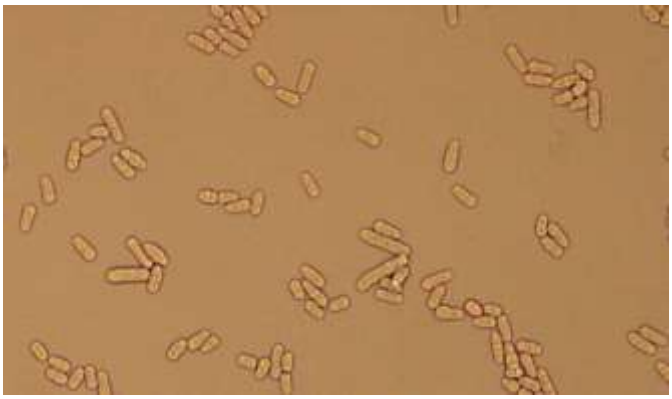

Figure 5. Images of wild type *S. pombe* cells under fluorescent microscope.

organism makes easier molecular analysis of several genes related to diseases (Suter et al., 2006). *S. pombe* is a very convenient model organism for investigating specific cancer pathway, owing to its cell cycle and cell control point mechanisms (Wood et al., 2002).

Deletions of *S. pombe'*s ORFs were carried out at the rate of 98.4% (Kim et al., 2010; Spirek et al., 2010). *Gua1* gene in which deletion was performed in this study was not yet deleted within the scope of the "*S. pombe* Genome Deletion Project". Obtaining a viable *gua1* gene deleted strain will make a contribution regarding the determination of the effect of IMPDH on the various cellular pathway such as cancer and immunosuppression.

In this study, the deletion of *gua1* gene was carried out by creating knock-out cassette via homologous recombination according to Kim et al. (2010). Before transformation knock-out cassette, it was analyzed with sequencing.

Colonies which were phenotypically selected were confirmed by colony PCR. In the results of colony PCR, one single candidate transformant colony from *972h-* showed the expected band profile on the agarose gel electrophoresis. PCR was performed in the candidate *972h-* colony using primer set of *kan*^{r} gene, the band of interest at the correct size was observed. However, no band was shown when PCR was carried out with same colony but using primer sets of *gua1* gene. After the transformation, 56 colonies were reproduced at selective media and it was observed that only one colony was inserted knock-out cassette to the target region in genomes.

When these mutant cells were analyzed microscopically, it was observed that the cells were small and displayed aggregation similar to the results of Fernández-Álvarez et al. (2009). In this study, these mutant colonies were phenotypically more flattened than wild type colonies. Jiang et al. (2010) studied on human fungal pathogen *Candida albicans* and aimed at the deletion of *gua1* gene in *Candida albicans*. They reported that *gua1/gua1* diploid mutant colonies could not live longer than a week at 4°C. Similar to the results of Jiang et al. (2010), it is also observed that these mutant cells with *gua1* deletion are not viable at 4°C for a long time. The authors passaged this mutant cells six times in every other day and inoculated at 30°C, then it was observed that the mutant cells can survive for six or seven generations. This study could be maintained with glycerol stocks of the single colony which was obtained after the transformation. Although, mutant cells with deletion of g*ua1* gene have no long life span, the deletions in their genome are permanent and after some optimization, this mutant strain could be used in other studies.

Decottignies et al. (2003) carried out a pilot gene deletion project in *S. pombe* to determine the number of essential genes. They reported that, obtaining systematic deletion of all *S. pombe* ORFs is more difficult than *S. cerevisiae* when similar studies are compared. The reason is that the homologous recombination efficiency of *S. pombe* is lower than *S. cerevisiae* (Kaur et al., 1997).

In addition, they reported that the genome of *S. pombe* includes some regions which contain inadequately transcribed genes and these genes have closed chromatin structure. The other possible reason for low efficiency may be the deletion cassette integrated into genome but kanamycin resistance gene was deficiently expressed by the reason of heterochromatin structure (Decottignies et al., 2003). The location of a gene in the centromeric region reduces the homologous recombination frequency. In this context, location of the *gua1* gene makes the deletion to be more difficult than the other genes which are in euchromatic region. Finally, data presented here support the fact that the obtained colony is the first mutant strain of *S. pombe* with deletion of *gua1* gene.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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Full Length Research Paper

Fourier transform infrared spectroscopy (FTIR) profiling of red pigment produced by *Bacillus subtilis* PD5

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Pigments play an important role in physiological and molecular processes such as photosynthesis, survival to oxidative damage and resistance to ultra violet radiation in microorganisms. This study isolated and characterized pigment produced by *Bacillus subtilis* PD5 from soil sample of rice field. The pigment was extracted using three solvent system, namely, methanol, n-hexane and ethyl acetate. Fourier transform infrared spectroscopy (FTIR) technique was used for pigment characterization, while beta carotene was taken as standard. The molecular identification of bacterial strain was done by polymerase chain reaction (PCR) amplification of 16S *rRNA* gene sequence analysis. Sequence was submitted to National Center for Biotechnology Information (NCBI) database with accession number KY306687. Sequence analysis showed that the PD5 bacterial strain which produce red pigment is a novel strain of *B. subtilis* with 98 to 99% similarity. FTIR analysis of extracted red pigment showed similarity with beta carotene, indicating that the pigment is a derivative of carotenoid.

Key words: *Bacillus subtilis* PD5, 16S *rRNA,* Red pigment, Fourier transform infrared spectroscopy (FTIR).

INTRODUCTION

Microbial cells accumulate pigments under certain culture conditions, with important industrial applications. Micro-organisms can serve as sources of carotenoids, the most widespread group of naturally occurring pigments. Colour is an important attribute for the consumer's preference to buy foods. As a result various synthetic food colours have been manufactured but many of them comprise various hazardous effects (Fabre et al., 1993). The toxicity of synthetic colourants used as natural additives increases interest, therefore, emphasis on the production of bio-colour or natural colour extracted from fruits, vegetables, plant roots and microorganisms (Pattnaik et al., 1997). The industrial production of natural food colourants is already well-established and expanding. However, the use of natural colour is gaining attention. Production of microbial pigments can cut down the production cost of natural colours and it may be a cheaper source of natural food colourants. Hence, microbial pigment production is now one of the emerging fields of research which needs to be explored for commercial pigment production

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(Dufossé, 2009). Red biopigments produced as a secondary metabolite by some *Serratia* species (*Serratia marcescens*), actinomycetes and fungus *Monascus* species show antimicrobial activity and have a strong potential to develop antitumor drugs (Mekhael and Yousif 2009). The present study aimed at the screening of bacterial isolate capable of producing pigment in different organic solvents and of possible commercial importance. Promising pigment producing bacterial isolate was classified and identified through molecular characteristics. FTIR based characterisation of extracted pigment was done to find out chemical nature of pigment.

MATERIALS AND METHODS

Isolation of bacteria

Pigment producing bacteria was isolated from the soil sample collected from the rice field where herbicide pendimethalin was supplemented for controlling weed. Soil sample (~1.0 kg) from 0 to 15 cm depth were taken and stored at 4°C. About 10 g soil was treated with triple recommended dose of crude pendimethalin at 30% Emulcified Concentration (EC) as it was used in the rice field and incubated for one week at room temperature for soil enrichment. After incubation, serial dilution, 10^{-1} to 10^{-8} was performed in sterile water and 10 µl from each dilution, was spread on nutrient agar plate. Plates were incubated at 28°C for 24 h. After incubation, five bacterial isolates PD1, PD2, PD3, PD4 and PD5 have been selected randomly, one bacterial isolate PD5 was found to produce pigment. The pigment producing bacterial isolate was used for pigment extraction.

Growth of bacterial isolate in mineral salt medium (MSM) having pendimethalin as carbon source

The bacterial isolate was allowed to grow in minimal salt media. Minimal medium as mineral salt liquid (MSL) and Nutrient Broth (NB), a complete medium were used throughout this study as described by Sambrook et al. (1989). Minimal salt media was supplemented with pendimethalin at a concentration of 50 mg/L. Single colony of bacteria was inoculated in 100 ml Nutrient broth and incubated at 28±2°C in incubator shaker at 120 rpm. Overnight grown primary culture with optical density 0.6 was taken for further inoculation in Nutrient broth and minimal salt liquid media having pendimethalin (sole carbon and energy source). Growth of bacteria was measured by taking absorbance after 2 h interval till 60 h at 660 nm wavelength.

Extraction of pigment from bacterial isolate

The pigment producing bacterial isolate was further grown in nutrient broth in rotary shaker incubated at 28±2°C for three days. After that, the bacterial cells were harvested by centrifugation at 10000 rpm for 15 min. The pellet was washed with sterile water and spin for 15 min at 8000 rpm. The pellet was suspended with 5 ml of methanol. It was incubated in water bath at 55°C for 15 min until all visible pigments were extracted and centrifuged (8000 rpm) for 15 min. The coloured supernatant was separated and filtered through Whatman filter paper (No.1). The coloured extract was analysed by scanning the absorbance in the wavelength ranging from 300 to 800 nm using the spectrophotometer (Schimadzu UV-Vis 1800).

Extraction of pigment in different solvents

The pigment was extracted by different solvents. Primary extraction was done in methanol followed by secondary extraction in hexane and final extraction was done in ethyl acetate in which complete extraction of pigment takes place. Red pigment was extracted in ethyl acetate and yellow pigment in methanol and hexane. Extracted pigments in different solvents were scanned spectrophotometrically to analyse maximum absorbance (λmax). Ethyl acetate was shown to be better than methanol and hexane as the latter two chemicals are not very efficient in the extraction (Henriques et al., 2007).

Effect of change of pH of growth medium on pigment production

Pigment producing bacterial isolate was allowed to grow in nutrient broth medium at pH range 5, 7, 9, and 11 for 3 days. After incubation, pigment was extracted by the same method as described earlier and extracted pigment was analysed spectrophotometrically.

Fourier transform infrared spectroscopy (FTIR) analysis of extracted pigment

The methanolic extract of pigment with optical density (1.5) was taken for FTIR analysis. Beta carotene with a concentration of 1 mg/ml (methanol as solvent) was taken as standard. A quantity of 10 µl of pigment and beta carotene was placed on a diamond window of the spectrophotometer under standard room temperature. A 32 scans with a resolution of 4 cm^{-1} was adapted. The available spectrum ranges were 400 to 4000 cm^{-1}.

A 16S *rRNA* based identification and phylogenetic analysis

Total bacterial genomic DNA was isolated using DNA extraction kit (Himedia, India). The amplicon size of 1500 nt fragment of 16S *rRNA* was amplified using specific primer, forward Gm 3f (5'-AGAGTTTGATCMTGG-3') and reverse Gm 4r (5'-TACCTTGTTACGACTT-3') targeting 16S *rDNA*. PCR amplified product was purified using DNA purification kit, Himedia, India; HiPura™. The purified product was sent for sequencing. The consensus sequences were aligned and analysed for determination of specific bacterium using the NCBI web-based BLAST program (http:// www.ncbi.nlm.nih.gov/BLAST/). The closest known species were compared with nucleotide identity matrix. The nucleotide sequences were aligned using the clustal W program. The phylogenetic analysis of the 16S *rDNA* sequences of the isolate was conducted with MEGA 5.1 using neighbour- joining method with 1,000 bootstrap replicates (Tamura et al., 2011).

RESULTS AND DISCUSSION

In the present study, a bacterial strain from soil sample was isolated by serial dilution and studied for pigment production. Yassin and Almouqatea (2010) suggested “open plate technique” is suitable for microbial isolation particularly for pigment producing bacteria. The isolation of carotenoid producing microbes from some abnormal environmental condition was also reported by Ramasamy and Udayasuriyan (2006).

Based on coloured colonies production on Nutrient

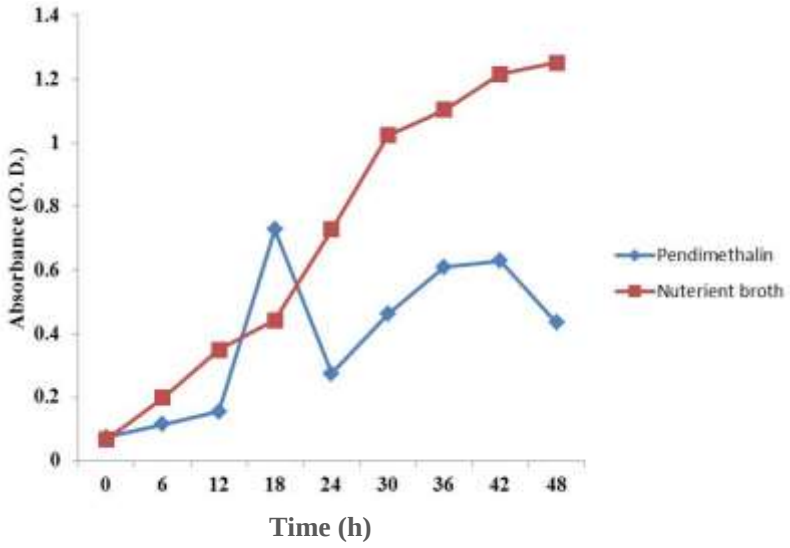

Figure 1. Growth of bacteria in Nutrient Broth and MSM media (having pendimethalin as sole carbon and energy source).

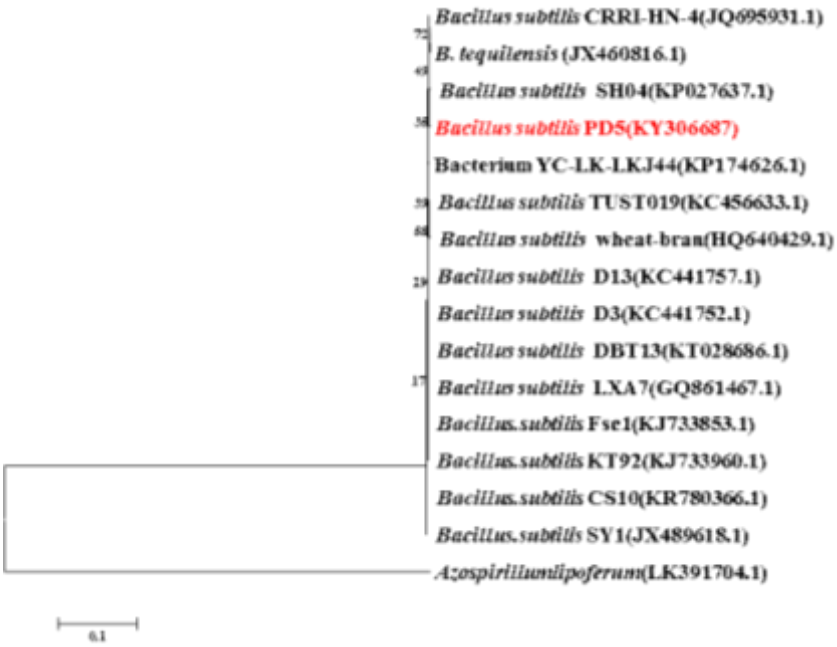

Figure 2. Pigment producing bacterial isolate PD5 showing similarity with *Bacillus subtilis* in phylogenetic tree.

Agar plates, isolate PD5 was selected. The nutrient agar plate shows red colonies with no fluorescence emission under ultra violet (UV) light. It grows at 28 to 40°C, but the optimum temperature is 30°C. It can grow at pH 6 to 10, but optimum growth was found at pH 7. Growth study of bacterial isolate showed that it is able to grow in MSM having pendimethalin as carbon source at 50 mg/L concentration (Figure 1). Identification of bacterial isolate was done using 16S *Rrna* based polymerase chain reaction (PCR) amplification followed by sequencing.

The sequence data of the 16S *rRNA* was subjected to BLAST analysis. As 16S *rRNA* gene sequence provide accurate grouping of organism even at subspecies level, it is considered as a powerful tool for the rapid identification of bacterial species (Jill and Clarridge, 2004). The sequence analysis of 16S *rRNA* sequences of isolate PD5 showed its maximum identity of 99% to *Bacillus subtilis* (Figure 2). The 16S *rRNA* sequences of

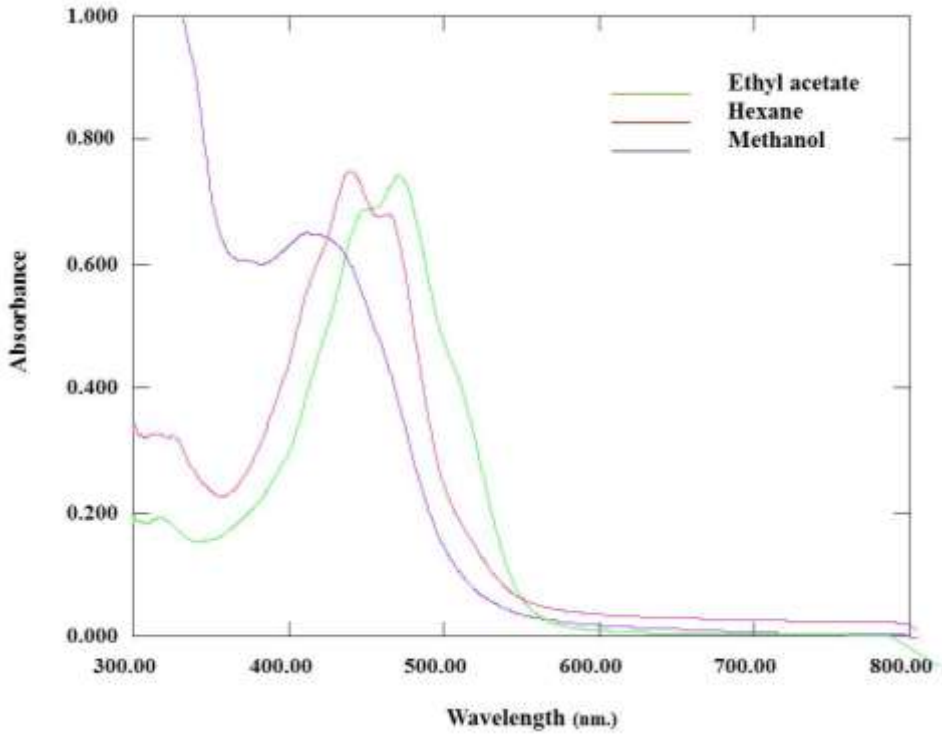

Figure 3. Spectrophotometric scan of extract of pigment in methanol, hexane and ethyl acetate as solvent.

the bacterial isolate were submitted to NCBI under the accession number (KY306687).

Extract of pigment in different solvents showing characteristic spectra (Figure 3). Methanol extract of the pigment shows λmax at 490 nm, nearer to beta carotene spectra. Change in pH of culture medium has effect on colour. Spectrophotometric scan showed that at pH 7, pigment extract were green in colour at λmax 290 nm, while at pH 9, orange pigment at λmax 310 nm was observed (Figure 4).

FTIR technique is used to obtain an infrared spectrum, emission, photoconductivity of a solid, liquid or gas. FTIR that operates in the mid infrared region (4000 to 400 cm^{-1}) is a powerful tool for quantitative analysis of fats, oils and palm carotene (Moh et al., 1999). The bands of the characteristic functional groups (CH3, CH2, C=C, C=O, OH, etc.) can be assigned when possible. Some special functional groups such as C=C=C, 'cross epoxides', etc., which cannot be easily identified by give full form (1H-NMR) methods, can be detected in the FTIR spectra (Lorand et al., 2002).

FTIR spectrum of extracted pigment from bacterial isolate, showed similarity with beta carotene in fingerprinting patterns of peaks (Figure 5). It indicates that extracted pigment is a beta carotene or carotenoid derivative. Analysis of pigment highlighted the stretching of different functional groups with peaks at 1661, 1653, 1654 and 1655 cm^{-1}, respectively. FTIR peaks in the range of 1600 to 1670 correspond to protein (Suresh et al., 2016). The next bands were approximate at 1425, 1426 and 1424 cm^{-1}, usually caused by the bending and vibration of methylene -CH_2 (scissoring) seen in beta carotene standard at 1450.68 cm^{-1} peak height. This could be attributed to lycopene pigments (Parlog, 2011).

Bacillus cereus group members are found to be versatile producers of secondary metabolites, such as antimicrobial substances (Abriouel et al., 2011), extra cellular enzymes (Liang et al., 2013) or fluorescent pigments (Banerjee et al., 2013). Melanin producing *Bacillus weihenstephanensis* isolated from the soil can offer considerable benefit for commercial production of biopigments like carotenoids, anthraquinone, chlorophyll, melanin, flavins, quinones, prodigiosins, monascins, violacein, etc (Drewnowska et al., 2015).

The red pigment producing bacterium isolated from rice field was characterized based on morphology, coloured colony production on Nutrient Agar plate and identified by 16S *rRNA* gene sequencing. The bacterial strain was identified as a novel strain of *Bacillus* species. The isolated strain produced intracellular red pigment in nutrient broth medium. The optimum conditions for pigment production were determined which revealed the maximum production of pigment at 30 to 37°C and at pH 7 after 3 days. The red color was extracted with methanol and the extract was analyzed by scanning the absorbance with a UV-VIS spectrophotometer. FTIR analysis of

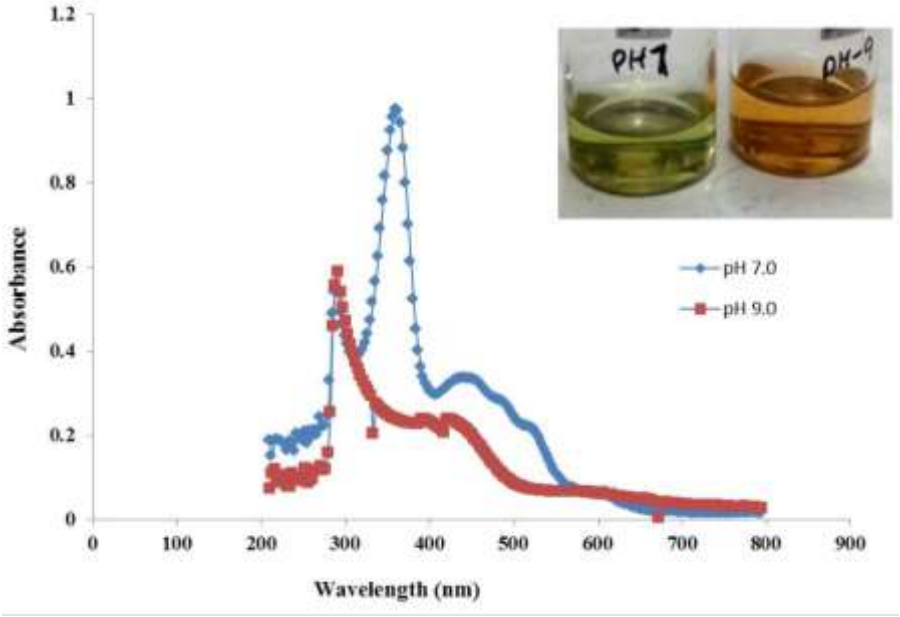

Figure 4. Effect of change of pH of culture medium on the colour of pigment.

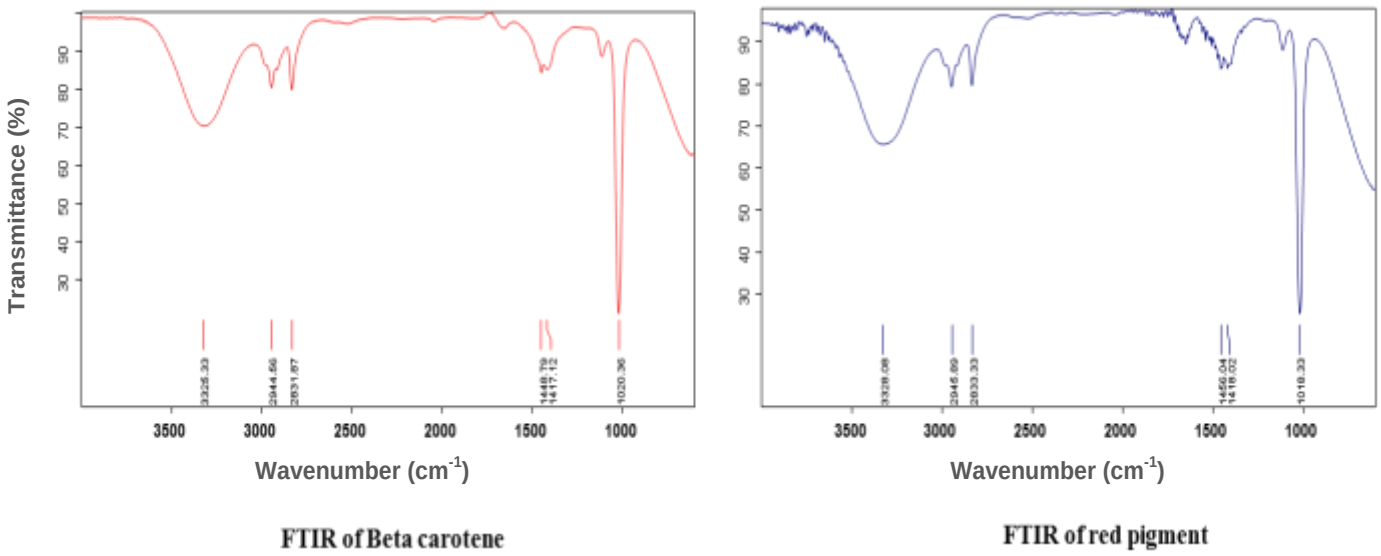

Figure 5. FTIR spectra of beta carotene and extracted pigment showing similarity in band stretching pattern of methanolic extract of pigment with beta carotene.

pigment showed similarity with chromatogram given by beta carotene. These results suggest that the pigment producing *B. subtilis* PD5 can be used for the commercial production of pigment. This strain is able to grow in minimal salt media, so pigment production by fermentation can be commercialized at low cost.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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African Journal of Biotechnology

Full Length Research Paper

Evaluation of antihyperglycemic, antiradical and acute oral toxicity activities of aqueous leaves extract of *Moringa oleifera* Lam (*Moringaceae)* from Benin in normal rats

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***Moringa oleifera* is a commonly cultivated plant in the world. It has a high economic impact owing to its medicinal and nutritional values. Leaves of *M. oleifera* also contain various constituents that are useful for therapeutic purposes. The aim of this study is (i) to perform phytochemical screening, and to check antiradical and antihyperglycaemic activities of *M. oleifera* aqueous leaves extract (ii) to control the impact of this aqueous extract on the liver parameters of wistar rats. The analysis was carried out on a few large families of bioactive molecules such as triterpenoids, alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, anthocyanes, leucoanthocyanes, reducing sugar, mucilage and cyanogenic. With a concentration of 2.47 ± 0.26 mmol EAA/mg, the plant has a good antioxidant activity. Antihyperglycemic capacity was evaluated by using oral glucose tolerance test. The α,α-diphenyl-β-picrylhydrazyl (DPPH) method was used to show antiradical activity. The impact of aqueous leaves extract of *M. oleifera* on some biochemical parameters of Wistar rats (150 to 200 g) was shown. The contents of total phenolic, flavonoids and condensed tannins were respectively 46.242±2.44 (mmolEAA/mg of extract); 10.80±0.64 (mg Eq of rutine/mg of extract); 2.85±1.018 (mg Eq of catechine/mg of extract). The results of the acute oral toxicity test showed that the dose of 2000 mg/kg was non-toxic to the rats. The haematological and blood parameters did not change after administration of the plant aqueous extract. Glycemia reduction was observed with the dose of 500 mg/kg. The important results obtained in this study explain the abundant use of this plant by the populations in the treatment of diabetes. However, it would be necessary to confirm the non-toxicity of the plant by histological studies of the liver and kidneys.**

Key words: *Moringa oleifera,* aqueous leaves extract, antihyperglycemic activity, acute oral toxicity, *in vivo*.

INTRODUCTION

Diabetes mellitus is a major degenerative disease in the world today (Ogbonnia et al., 2008). The fundamental defect in diabetes mellitus is an absolute or relative lack of biologically active insulin, which results in the impairment of uptake and storage of glucose, and reduced glucose utilization for energy purpose (Tiwari and Rao, 2002). According to World Health Organization projection, the prevalence of diabetes is likely to increase by 35% (Sangeeta et al., 2010). Currently, there are over 150 million diabetics world-wide and this is likely to increase to 300 million or more by the year 2025. The prevalence of diabetes mellitus in Benin is 2.9% (Djrolo et al., 2011).

Although, anti-diabetic drugs are accessible, in many cases, the treatment is not suitable because these drugs are not able to heal this infection and present side effects. In the same way, the high cost of the anti-diabetic drugs directs the populations of low and middle-income countries to resort to the medicinal plants. Medicinal plants constitute an important source of potential therapeutic agents for diabetes and hyperlipidemia (Adisakwattana et al., 2011). Most of the plants prescribed for diabetes mellitus are not edible and therefore, the studies on edible plants which have an antihypoglycemic effect would be of great value in the dietary management of the disease. *M. oleifera* is used in traditional medicine all over the world to cure many diseases (india, Pakistans, ancient Rome, Greece and Egypt).

Different parts of this plant such as the leaves, roots, seed, bark and flowers were used in indigenous medicine for the treatment of some pathologies. Leaves were known to act as cardiac and circulatory stimulants, possess antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, cholesterol lowering, antioxidant, antidiabetic, hepatoprotective, antibacterial and antifungal activities, and are being used for the treatment of different ailments in the indigenous system of medicine, particularly in South Asia (Farooq et al., 2007). Fresh root is acrid and vesicant (has the taste of horse-radish). Internally, it is used as stimulant, diuretic and antilithic. Seeds are acrid and stimulant. Bark is emmenogogue and even abortifacient, antifungal and antibacterial. Flowers are cholagogue, stimulant, tonic and diuretic, and useful to increase the flow of bile. The plant is also a cardiac circulatory tonic and antiseptic.

Ethnobotanical studies along different phytodistricts in Benin showed the use of *M. oleifera as* one of the plants involved in the traditional medicine in the treatment of diabetes pathology (Laleye et al., 2015). As in most cases, a lot of plants are used in indigenous medicine to treat diseases, it is very difficult to say either if the results are from action of a single plant or a synergy of all plants. To elucidate this aspect, there is a need to evaluate the action of this plant extract in order to provide scientific proof and its role in the treatment of pathologies. In the same way, the phytochemical screening of aqueous extract of *M. oleifera* was done to evaluate its action on diabetes.

MATERIALS AND METHODS

Plant material

The leaves of *M. oleifera* were collected from Abomey-Calavi (Benin) city during the month of January to February, and were certified at the National Herbarium of Benin. A voucher specimen (AA6533/HNB) was reserved in this herbarium. The plant material was air dried at room temperature in the laboratory and further dried in the oven for 1 week at a temperature of 24°C. The leaves were then powdered using mortar and pestle.

Preparation of the water extract

The powder was hot macerated with distilled water and then the extract obtained was filtered, concentrated by rotary vacuum pump to get the solid mass because the main methods of *M. oleifera* remedy preparations in Benin is the maceration.

Animal treatments

In this study, albino Wistar rats (male and female) weighing 150 to 200 g and 4 months old were used. Animals were maintained under standard conditions and guarded in iron cages with easy access to water and food. The temperature of the room was maintained at 25°C and the relative humidity is between 35 and 60%. Animals were submitted to an alternated cycle of light (12 h) and dark (12h).

Drugs and chemicals

Different drugs and chemicals were obtained from Sigma (USA), Aldrich (Milwaukee, USA): sodium carbonate (Na_2CO_3), gallic acid, methanol, hydrochloric acid (HCl), Folin-Ciocalteau reagent, sodium nitrite ($NaNO_2$), vanillin, aluminum chloride ($AlCl_3$), sodium hydroxide (NaOH), 2,2-diphenyl-1-picrylhydrazyl, ferric chloride ($FeCl_3$), ammoniac, sulfuric acid, acetic anhydride, chloroform, toluene, dinitrobenzoic acid, phosphomolybdenum, catechin and potassium ferricyanide

Phytochemical screening

The freshly prepared crude leaves extract of *M. oleifera* was subjected to qualitative phytochemical analysis for the presence of

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various classes of active chemical constituents such as tannins, saponins, glycosides, flavonoids, alkaloids using the methods described by Houghton and Raman (1998).

Quantification of the bioactive molecules

Determination of total phenolic content

Singleton et al. (1999) was used to determine total phenolic content. 125 µl of aqueous extract at the concentration of 1 to 5 mg/ml was diluted into 250 µl of distilled water. Then, 625 µl of Folin -Ciocalteu reagent was added and gently mixed. After 2 min of homogenization, 500 µl of 20% sodium carbonate was added. The contents were mixed and allowed to stand for 2 h. The optical density of the blue colored samples was measured at 760 nm. Gallic acid was used as standard. The total phenolic content was expressed in mg/g of gallic acid equivalent of mixture.

Determination of total flavonoid content

Total flavonoid content of the extract was measured based on method described by Kim et al. (2003) with slight modifications. According to this method, 400 µl of extract was mixed with 500 µl of distilled water and then 120 µl of 5% sodium nitrite was added to the mixture and allowed to stand for 5 min, followed by the addition of 120 µl of 10% of $AlCl_3$ to the mixture which was mixed using a vortex. 800 µl of 1 M NaOH was added after 6 min and the mixture was incubated for 15 min in darkness. Rutin solution was used as reference and the content in flavanoids was expressed as mg of rutin equivalent/1 mg of sample. The calibration curve established with the reference solution was used to read directly the concentration values.

Determination of condensed tannins content

Broadhurst and Jones (1978) method modified by Heimler et al. (2006) was used to appreciate condensed tannins content. 1.5 ml of vanillin solution initially dissolved in methanol for a final concentration at 4% was mixed with 500 µl of extract, 2 ml of methanol and 1.5 ml of concentrated hydrochloric acid. The mixture was incubated for 15 min and the absorbance was taken at 500 nm. Catechin solution was used as reference and the content of condensed tannins was expressed as mg of catechin equivalent/mg of extract.

Total antioxidant capacity assay (TAO)

The ability of the plant extract to trap free radicals explains their antioxidant capacity. The method described by Lamien-Meda et al. (2008) was used. 0.75 ml of different concentrations (from 0.5 to 3.5 mg/ml) of leaves extract were mixed with 1.5 ml of DPPH solution (0.4 mg/ml in methanol). Then, the mixture was incubated at room temperature for 15 min in a dry bath room. Ascorbic acid solution is used as standard and the total antioxidant activity was expressed as millimol of ascorbic acid equivalent per gram of aqueous extract.

Acute toxicity studies

This assay was done in accordance with Organization for Economic Cooperation and Development (OECD, 2001) guideline. Three female and non-gravid Wistar rats weighing 150 to 200 g received by gavage, 2000 mg/kg aqueous extract of *M. oleifera* after being kept on fasting the previous night. The animals are observed closely during the first four hours and daily for 14 days (for weight change, tremors, convulsion, salivation, diarrhea, lethargy, sleep, coma, and death, changes in the skin, fur, eyes and behavioral pattern). At the end of 14 days, a histological study was performed on some organs (liver and kidney) and biochemical parameters were measured.

Collection of blood and serum samples

Anesthetized rats underwent cervical decapitation. The blood was collected in heparinized bottles for hematological studies. Blood samples taken from non-heparinized bottles were allowed to coagulate. The serum was separated from the clot and centrifuged in clean bottles for biochemical analysis.

Hematological measurements

The hematological examinations were done using blood samples collected from retro-orbital of the experimental rats and conserved in capillary tubes (EDTA). These examinations using Toro and Ackermann (1975) and Duncan et al. (1994) included red blood cell counts, white blood cells and platelets hemoglobin, hematocrit, mean globular volume (VGM), average corpuscular content in hemoglobin (TCMH), the mean corpuscular concentration in hemoglobin (CCMH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Creatinine levels and serum urea were measured using the method described by Coles (1986).

Anti-hyperglycemic activity of *M. oleifera*

Pareek et al. (2009) method was used to evaluate anti-hyperglycemic activity by oral glucose tolerance test (OGTT). 30 rats divided into 6 lots of five rats were constituted before the experiment (OGTT), all the rats were beforehand submitted to a non-hydric fast for 16 h. 500 and 1000 mg/kg of aqueous extract were administered to the lots 3 and 4. Lots 1 and 2 received each, a dose of 5 ml/kg of Glibenclamide. The control received distilled water with a dose of 10 ml/kg. D-glucose was administrated to the rats with a dose of 3 g/kg after half an hour. SD CHECK glucometer was used to take the glycemia in a sequential way every 30 min from start point corresponding to T0 up to 120 min corresponding to T120. The final measure was taken at T 180 min.

Statistical analysis

Statistical analysis of the data was done and expressed as a mean using Minitab Version 1.0 software. The differences obtained from the univariate variance analysis (ANOVA) were reported using the Kruskal-Wallis test. Correlations between different values were expressed graphically using Graph Pad Prism version 5. For $p \leq 0.05$, the results are statistically significant.

RESULTS AND DISCUSION

Phytochemical screening and bioactive molecules quantification

Only three families of compounds were absent from the ten important families of compounds sought in the extract (Table 1). They are leucoanthocyan, coumarin and cyanogenic glycoside. The content of phenolic compounds,

Table 1. Phytochemical analysis of *M. oleifera* leaves extract.

Chemical compounds class	Test	Test result
Alkaloids	General test: Dragendorff reagent	+
Catechin tannins	Stiasny reagent	+
Gallic tannins	Saturation of Na acetate + a few drops of $FeCl_3$, 1%	+
Flavonoids	Shinoda reagent (cyanidine reaction)	+
Anthocyanes	Adding some drops of HCl 5% to 1 ml of decocted + alalinisation (with drops of ammoniac 50%)	+
Leucoanthocyanes	Shinoda reagent (chlorhydric alcohol)	-
Quinonic derivatives	Born- Trager reaction: concentrated HCl, diluted HCl	-
Saponosides	Foam index (FI) of diluted aqueous decoction (positive if IF≥ 100, measuring foam height ≥1 cm)	+
Reducing sugars	Fehling's test	+
Triterpenoids	Liebermann-Buchard reaction (acetic anhydride-sulfuric acid 50: 1) Kedde reaction (dinitrobenzoic acid 2% in ethanol + NaOH (1 N) 1:1)	+
Cyanogenic derivatives	Ammoniac at 25%: high fluorescence	-
Mucilages	Chloroform + ammoniac: red ± highy coloration	+
Coumarins	Extraction, elution with toluen + AcEt (97/3), revelation with sulfuric anysaldehyde or vanilin	-

+ Present, - absent.

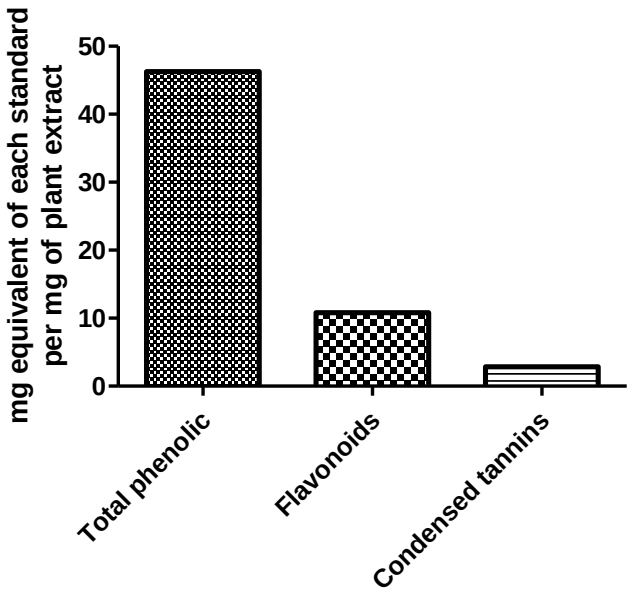

Figure 1. Total phenolic, flavonoids and condensed tannins content of *M. oleifera* leaf extract.

flavonoids and condensed tannins respectively in the aqueous leaves extract of *M. oleifera* are 46.242±2.44, 10.80±0.64 and 2.85±1.018 (Figure 1).

Total antioxidant capacity assay (TAO)

The antioxidant capacity of the plant leaves extract was 2.47 ± 0.26 mmol equivalents of ascorbic acid per mg of leaves extract. Total antioxidant capacity represents both oil soluble and water soluble antioxidants that are capable of scavenging reactive oxygen species and protects from chronic diseases such as cancer, diabetics and arthritics (Mrudula et al., 2014).

Acute toxicity studies

It was observed that no rat that received the aqueous extract of the plant at a dose of 2000 mg/kg died. This result shows that the concentration administered to the rats is below the toxicity threshold, 2000 mg/kg which is therefore lower than the LD50. It was observed that the rats used in this test gained weight as those of the control group. This is contrary to the effects of toxicity. Figure 2 presents the evolution of animal's weight during the 14 days.

Determination of hematological and biochemical parameters

Table 2 presents the results obtained from different measurements of hematological parameters. No differences were observed between control animals and experimental animals. These results showed that the aqueous extract of *M. oleifera* leaves has no impact on the metabolic parameters of rats.

Table 3 presents evolution of some biochemical parameters in comparison with control rats. Maintaining of metabolic equilibrium requires that these parameters

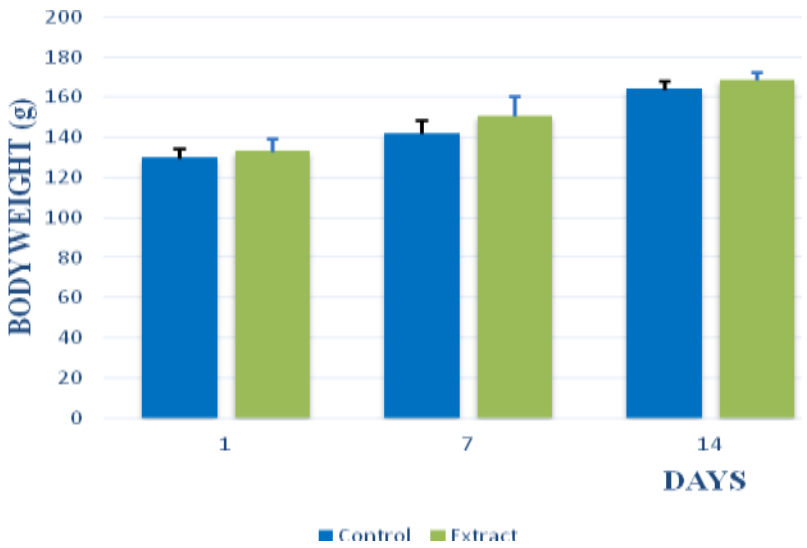

Figure 2. Effects of *M. oleifera* on the body weights of rats.

Table 2. Hematological parameters in rats after administering *M. oleifera* extract.

Parameter	RBCs (T/L)	PLT (G/L)	WBCs (T/L)	Hb (g/dl)	HTE (%)	MCV (fL)	MCHC (%)	MCH (pg)
Control rat	5.22±0.10	821.30±29.6	3.17±0.57	11.4±0.2	32±1.0	62.33±2.52	27.93±0.40	21.80 ±0.40
Experimental rats	5.26±0.22	831.30±4.60	3.50±0.78	11.60±0.65	32.67±3.51	62.33±2.52	28.10±1.45	22.03±0.80
P value	0.805	0.753	0.592	0.664	0.782	0.773	0.866	0 .696

Hb: Hemoglobin; MCV: mean cell volume; MCHC: mean cell hemoglobin concentration; RBCs: red blood cells count; WBCs: white blood cells count; PLT: platelets; HTE: hematocrit; MCH: mean corpuscular hemoglobin; Hb: heamoglobin.

Table 3. Determination of biochemical parameters of rats after treatment with *M. oleifera* extract.

Parameter	Creatinine (mg/L)	Urea (g/L)	ALT (UI/L)	AST (UI/L)
Control	7.47±1.10	0.30±0.01	88.67±4.16	144.67±7.57
Aqueous extract	7.67±0.42	0.31±0.04	79±10.5	152.0±30
P -value	0.796	0.826	0.277	0.738

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

are constant. As compared to the 2000 mg/kg dose, no significant differences were observed in these parameters, as the p>0.005. For both hematological and biochemical parameters, no significant difference (p>0.005) was observed.

Anti-hyperglycemic activity of *M. oleifera*

From the analysis of the graphs presented in Figure 3, distilled water showed a high level of hyperglycemic activity from T0 to T90, after which a low decrease up to T180 was noticed. For plant leaves extract, the two concentrations (500 and 1000 mg/kg) presented the same effect on the hyperglycemic activity. An increase of the hyperglycemic level was observed only for the first half of time. Then, from T30, hyperglycemic activity decreased progressively until T180 for both concentrations.

DISCUSSION

Traditional medicine is the oldest medicine used for the treatment of many diseases and by rural as well as urban people. This medicine was based mostly on the use of

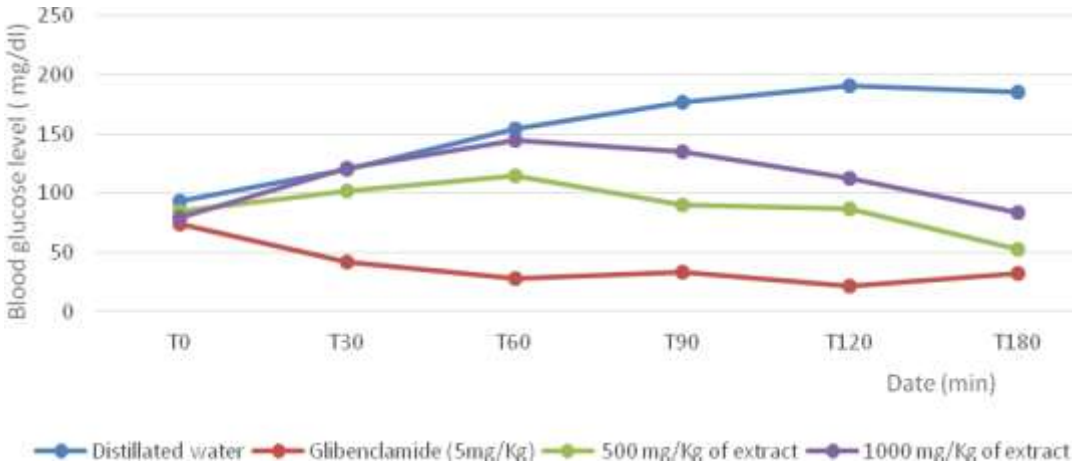

Figure 3. Effect of *M. oleifera* leaves extract on blood glucose level in rats. T0, T30, T60, T90, T120 and T180 stands for time interval of hyperglycemic activity in minutes.

diverse plants which revealed different pharmacological properties. These properties were attributed to the presence of some secondary plants metabolites. Indeed, the phytochemical screening carried out on the aqueous extract of *M. oleifera leaves* revealed the presence of phenolic compounds, sugar and other substances. Their presence confirms in certain proportions, their efficiency in the treatment of many diseases. Most medicinal plants are the important source of bioactive molecules that contribute to the reduction of risk and progression of certain acute and chronic diseases such as heart diseases, cancer, diabetes, etc. (Ali et al., 2008). Farooq et al. (2007) also revealed the presence of alkaloids, tannins and flavonoids in *M. oleifera* leaves extract but in different proportions (Teteh et al., 2013). The age of the plant, the harvest period of the plant, the climate, the method of extraction, the type of soil could explain the differences noticed. In this case, phenolic compounds content was higher than the remainder and indicated that it was the most important compound in *M. oleifera* leaves extract. This result could probably justify the antioxidant activity of the leaves. Phenolic compounds were reported to have multiple biological effects, including antioxidant activity (Jaehak et al., 2014). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Singleton et al., 1965).

Regarding antihyperglycemic activity, it was shown that leaves extract presented this activity. A maximum action on blood glucose level decrease was obtained with a dose of 500 mg/kg. The findings are in agreement with the result of Edoga et al. (2013) who showed that the leaves extract of *M. oleifera* reduced blood sugar levels in the normoglycemic rats, this effect was dose dependent. The antihyperglycemic effect of the plant may be due to the presence of alkaloids, flavonoids, steroids and other constituents present in the leaves extract which could act synergistically or independently in lowering the blood sugar level. Some alkaloids are known hypoglycemic agents. For instance berberine, a quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids has been used successfully in experimental models of diabetes mellitus, berberine was shown to possess insulin sensitizing effect (Wang et al., 2010). Also, flavonoids, being polyphenolics, are known to be hypoglycemic. The antidiabetic activity of four flavonoids-boswellic acid, ellagic acid, quercetin and rutin were demonstrated in rats and the proposed mechanism of action was by increasing the peripheral utilization of glucose and inhibiting the glucose transporter activity from intestine (Jadhav and Puchchakayala, 2012). Thus, *M. oleifera* might decrease the velocity of gastric emptying, a major determinant of postprandial glycemia (Horowitz et al., 2002). Some herbal medicines were reported to inhibit gastric emptying and also glucose uptake in the small intestine (Matsuda et al., 1998). High fiber diets decrease postprandial blood glucose by slowing the rate of food passage from the stomach to the small intestine (Tsai and Peng, 1981).

Administration of *M. oleifera* leaves extract did not affect blood biochemical parameters. In the same way, the toxicity test on Wistar rats revealed a gain of body weight with all groups of rats even when the dose was 2000 mg/kg. The increase in the body weight of rats might be due to the fact that *M.* oleifera is rich in amino acids, vitamins and minerals, particularly iron (Booth and Wickens, 1988; Mori et al., 2009). The results obtained show that the LD50 is greater than 2000 mg/kg. This result is in concordance with the work of Ojo et al. (2013) who used the dose of 5000 mg/kg. At doses above this level; however, the animals may exhibit some toxic changes. The plant is safe for consumption and for

medicinal uses.

Conclusion

The result obtained from this research study revealed that aqueous leaves extract of *M. oleifera* exhibited diverse bioactive compounds with different properties. The plant leaves extract presented no acute toxicity and could be safe for consumption as traditional vegetable and for medicinal uses. With its contribution in the decrease of blood glucose level, this result confirmed its use by traditional therapist for treatment of diabetes. However, it is important that future study should contribute to identifying and characterizing the crude extract specific molecules involved in the treatment of each type of the disease.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Molecular discrimination of *Trypanosoma vivax* and *Trypanosoma congolense* isolates of Nigerian origin

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The present study was conducted to assess the molecular discrimination of field isolates of *Trypanosoma congolense* and *Trypanosoma vivax* of Nigeria origin *in vitro* (PCR) whether there will be genetic alteration of the parasites as the infections of the isolates in Yankasa sheep progresses from acute through relapses to chronic stages. A total of thirty Yankasa sheep were acquired from Kastina State in the northern part of Nigeria, screened for haemo-, ecto- and endo-parasites, treated against anaplasmosis, coccidiosis and conditioned in arthropod-free pens for two weeks. They were randomly divided into five groups of six animals each. Two groups each were infected intravenously with approximately 2.0×10^6 of *T. vivax* and *T. congolense*. One group served as uninfected control. A group each from *T. vivax*-infected and *T. congolense*-infected groups were treated with trypanocide 6 days post parasitaemia. The other infected groups were left untreated. The animals were monitored for 8 weeks post infection (pi). Incubation period of 6 days was recorded for both parasite species. Multiplex primers polymerase chain reaction (PCR) and *T. vivax* species-specific PCR confirmed that the isolates used were *T. congolense* and *T. vivax* at molecular band sizes of 750 and 175 bp, respectively. Randomly amplified polymorphic DNA (RAPD)-PCR showed that the band sizes associated with the chronic form of the infection of *T. congolense* differed from those of the acute and relapse forms, whereas the band weights of the relapse form of the *T. vivax* differed from those of acute and chronic forms. Molecular characterization of *T. vivax* and *T. congolense* revealed differences through the variations in band weights of the parasites derivatives in the acute, chronic and relapse infections and this may translate into differences in antigenicity.

Key words: Trypanosoma, infection, parasitaemia, parasite, relapse, band, amplicon.

INTRODUCTION

African trypanosomosis is currently resurgent across a great part of tropical Africa, reaching epidemic levels in many places (Stich et al., 2002; Delespaux et al., 2008; Ogbaje et al., 2015). *Trypanosoma vivax* and

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Trypanosoma congolense are the major species responsible for African Animal Trypanosomosis (AAT) or Nagana in Nigeria and sub-Saharan African (Takeet et al., 2013; Isaac et al., 2016; Morrison, 2016). *T. vivax* and *T. congolense* infect large variety of domestic and wild animals (D'avila et al., 1997; Auty et al., 2012).

Polymerase chain reaction (PCR) was introduced for detection of trypanosome DNA in biological samples. PCR have been proved to be highly sensitive and specific and has been widely used in the detection of trypanosomes (Masiga et al., 1992; Ventura et al., 2001). The techniques have been found to be specific and sensitive for large scale analysis of trypanosome samples (Hide and Tait, 1991). It is also capable of detecting minute quantities of DNA of specific pathogens through amplification of a defined DNA segment and the discrimination in one reaction between different organisms even if they are closely related (Hatta and Smits, 2007).

Previous studies showed that the sensitivity of PCR in detecting trypanosomes is about two times higher than that of Ag-ELISA and four times higher than with the parasitological techniques, especially the Wet blood film and thin blood smear (Narendra et al., 2004; Clausen et al., 1998; Solano et al., 1999).

The accurate identification of a parasite to the species level has major implications for various aspects in veterinary parasitology, including diagnosis as well as treatment and control. The resurgence and difficulty in the control of the disease due to antigenic variation and development of drug-resistant strains have necessitated the need for more research in the study of the gene and nature of DNA elements within the genome of the parasites for possible vaccine and new drug development (Greif et al., 2013; Jackson et al., 2015; Morrison, 2016).

The aim of this research was to carry out molecular characterization of *T. congolense* and *T. vivax* isolates of Nigeria origin and to check whether there will be genetic alteration of the parasites as the infections of the isolates in Yankasa Sheep progresses from acute through relapses to chronic stages.

MATERIALS AND METHODS

Parasite isolates used

The isolates of *T. vivax* and *T. congolense* used in this study were isolated from sedentary white Fulani cattle in Makurdi, Benue State and Idon, Southern part of Kaduna State, Nigeria, respectively. About sixteen cattle were selected from the herd in Makurdi and twenty one at Idon based on their history of dullness, pica appetites by the herd men and high pyrexia on physical examinations.

Experimental animals

Thirty (30) Yankasa sheep, aged between 2 and 3 years were used for the study. The sheep were purchased from an open market at Karfur in Kastina State considered to be free of tsetse flies and consequently pathogenic *Trypanosoma* species. On arrival, the animals were screened using microscopy for endoparasites, haemoparasites and also through physical examination for presence of ecto-parasites. Two milliliters (2 ml) of blood was obtained from the jugular vein of each of the sheep, and examined for haemoparasites using wet mount, thin blood smear and microhaematocrit methods as described by Woo (1971). Three grams of faeces was scooped from the rectum of each sheep using a clean polythene bag and taken to Helminthology Laboratory, processed and examined for helminthes eggs using floatation and sedimentation methods as described by Cole (1986).

All the experimental sheep were dewormed orally using Albendazole® at the dose rate of 7.5 mg/kg. Ecto-parasites infestations were treated and controlled with deltamethrin® pour-on preparation and asuntol® spray. Those found with *Anaplasma* infections were treated with oxytetracycline long acting at the dose rate of 20 mg/kg body weight. Amprolium was administered to the ones that were infected with Coccidia for 5 days according to the manufacturer's recommendation. The sheep were also vaccinated each with 1 ml subcutaneous injection of monoclonal *pestes de petit ruminantes* (PPR) vaccine, against PPR. The sheep were kept in arthropod-free pens of the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria and pre-conditioned for two weeks before the commencement of the experiment.

The experimental animals were tagged and randomly divided into five groups (A, B, C, D and E) of six animals each per group. Base line data were obtained from each of the animal in all the groups for a period of one week prior to infections. Two different sheep were used as donor animals, one sheep each for *T. vivax* and *T. congolense* to multiple the parasites. Each of the sheep in groups A and B was inoculated through the jugular vein with 2 ml of blood containing approximately 2.0×10^6 *T. vivax* as quantified using the improved Naubauer haemocytometer (Petana, 1963). Each animal in groups D and E were also inoculated through the jugular vein with 2 ml of blood containing approximately 2.0×10^6 *T. congolense* as quantified using the improved Naubauer haemocytometer. Group C served as uninfected control. Groups A and E animals were treated with diminazene aceturate at the height of parasitaemia (++++).

The DNA profiles of the parasites were examined at different stages of the infections (acute, relapse and chronic) using randomly amplified polymorphic DNA analysis (RAPD-PCR). The following samples/forms of the parasites were collected from only trypanocidal treated animals and preserved at -20°C and later subjected to *Trypanosoma* Genus specific PCR, *Trypanosoma* species-specific PCR and RAPD-PCR analysis:

1. Acute form of the *T. vivax* and *T. congolense* (a week post infection)
2. Relapse forms of the *T. vivax* and the *T. congolense* (first phase of parasitaemia after treatment)
3. Chronic form of the infections with *T. vivax* and *T. congolense* (8 week post infection)

The *Trypanosoma* Genus specific PCR primers (Table 1) for *Trypanosoma* and *T. vivax* species-specific primers were supplied by Inqaba Biotechnical Industries LTD, Hatfield- Pretoria, S/Africa and RAPD-PCR primers were obtained from Bioneer-Biotech Company, Washington DC, USA. The experiment was divided into three different phases:

1. Each of the two isolates was subjected to *Trypanosoma* Genus specific PCR using primers for identification of different species and to rule out possibility of mixed infections in any of the samples.
2. *T. vivax* species-specific PCR was conducted on the two field isolates for *T. vivax* species identification of the fields samples (confirmatory test for *T. vivax* isolate).

Table 1. PCR methods and primers used.

S/N	PCR Type	Primers
1	*Trypanosoma*-Genus specific primers PCR	F: 5'GCGTTCAAAGATTGGGCAATG-3' R: 5'-CGCCCGAAAGTTCACC-3'
2	*Trypanosoma vivax* species-specific	TVW A- dGTG CTC CAT GTG CCA CGT TG TVW B- dCAT ATG GTC TGG GAG CGG GT
3	RAPD-PCR	Pbs-5'GGAAACAGCTATGACCATGA-3' Tpnr-5' CCAAGTCGACATGGCACAAC-3'

3. RAPD-PCR was conducted on the three different forms (the acute, relapse and chronic forms) of the two isolates.

Identification of different Trypanosoma species using Trypanosoma genus specific primers PCR

DNA extraction from whole blood: DNA was extracted using QIAamp® DNA Mini Kit (Qiagen, Germany). The method was conducted as described by Desquesnes et al. (2001). Briefly, a pair of primers designed for ribosomal DNA was used. The amplification was performed in a final volume of 50 µl containing 5 µl of isolated DNA (template), (20 µM = 1 µL) of each primer (Desquesnes et al., 2001), 2 mM of each dNTP, 32.5 µL of Nuclease-free water and 0.5 unit of Taq DNA polymerase. Initial denaturing step at 94°C for 5 min was followed by 30 amplification cycles. Each cycle consisted of a denaturation step at 94°C for 30 s, an annealing step at 58°C for 30 s, 54°C for 30 s and an extension step at 72°C for 1 min, and a final extension at 72°C for 10 min. The total number of cycles was 42 (4 cycles for 58°C, 8 cycles for 56°C and 30 cycles for 54°C).

The amplified products of the PCR were resolved on 2% agarose gel at 100 V for 60 min containing 1.0 mg/µl of ethidium bromide. The gels were observed on ultraviolet light and photographed.

Trypanosoma vivax species-specific PCR

In order to confirm the presence or absence of *T. vivax* in any of the samples, because of the low sensitivity of the *Trypanosoma* Genus specific primers PCR, they were subjected to *T. vivax*- specific primers PCR.

DNA extraction from whole blood: The DNA was extracted using QIAamp® DNA Mini Kit (Qiagen, Germany).

Primers: A pair of specific primers for *T. vivax* was used in this study as described by Desquesnes et al. (2001)

DNA amplification

The amplification was performed in a final volume of 25 µl containing 2 µl of isolated DNA (template), (1 µM = 1 µL) of the primer (TVW A- dGTG CTC CAT GTG CCA CGT TG TVW B- dCAT ATG GTC TGG GAG CGG GT), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of dNTPs, 10 µL of Nuclease free water, chelex 10 µl. Initial denaturing step was carried out at 94°C for 5 min and then 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 60 s and extension at 72°C for another 5 min.

RAPD-PCR of *T. vivax* and *T. congolense*

Stabilates of the two isolates (*T. vivax* and *T. congolense*) were retrieved from the freezer (-20°C).

DNA extraction from the *T. vivax* and *T. congolense* isolates

Two hundred and fifty microlitres (250 µl) of blood was mixed with 250 µl of lysis buffer (0.31 M sucrose, 0.01 M Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100). The mixture was centrifuged at 15,000 ×g for 20 s, the supernatant was removed and 500 µl of lysis buffer was added and mixed by vortexing. Centrifugation and addition of lysis buffer was repeated twice. After the last centrifugation and removal of the supernatant, the pellet was re-suspended in 250 µl of 1× PCR buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 1% Triton X-100) and 1.5 µl of proteinase-K (10 mg/ml) was added, mixed and incubated at 56°C for 1 h. Finally, the mixture was incubated at 95°C for 10 min to inactivate the proteinase-K. The resulting eluate was stored frozen at -20°C until used (Clausen et al., 1998).

Characterization of the extracted DNA using randomly amplified polymorphic DNA PCR (RAPD-PCR)

The extracted DNAs from the two isolates were subjected to RAPD-PCR using short random primers as described by Welsh and McClelland (1990) and modified by Tibayrenc et al. (1993). Genomic DNA samples (20 ng) were amplified in 60 µl of specific buffer (10 mM Tris- HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), in the presence of 0.2 µM of primers, 4x 100 mM, dNTP and 0.9 µl of Taq DNA polymerase. The RAPD-PCR was based on the use of twenty base pair primers (Tibayrenc et al., 1993). The total number of amplification was 45 cycles at 94°C for 1 min and 60°C for 2 min and a final elongation at 72°C for 7 min. The amplification was repeated three times for the pair of the primers. The amplified product was separated by electrophoresis on 1.6% agarose gels with TAE buffer (40 mM Tris- acetate pH 7.5, 1 mM EDTA) at 5 V/cm and DNA fragments were visualized after staining with ethidium bromide. The patterns of amplified fragments of each isolate were used to compare the isolates.

RESULTS

Identification of different species of trypanosomes (mixed infections) using multiplex *Trypanosoma* primers-PCR

The result shows that the isolate from the Northern Guinea savannah (Idon in Kaduna State) is purely Savannah type of *T. congolense* with a band of 750 bp (Plate 1). Sample from Southern Guinea Savannah (Makurdi-Benue state) did not contain *T. congolense*.

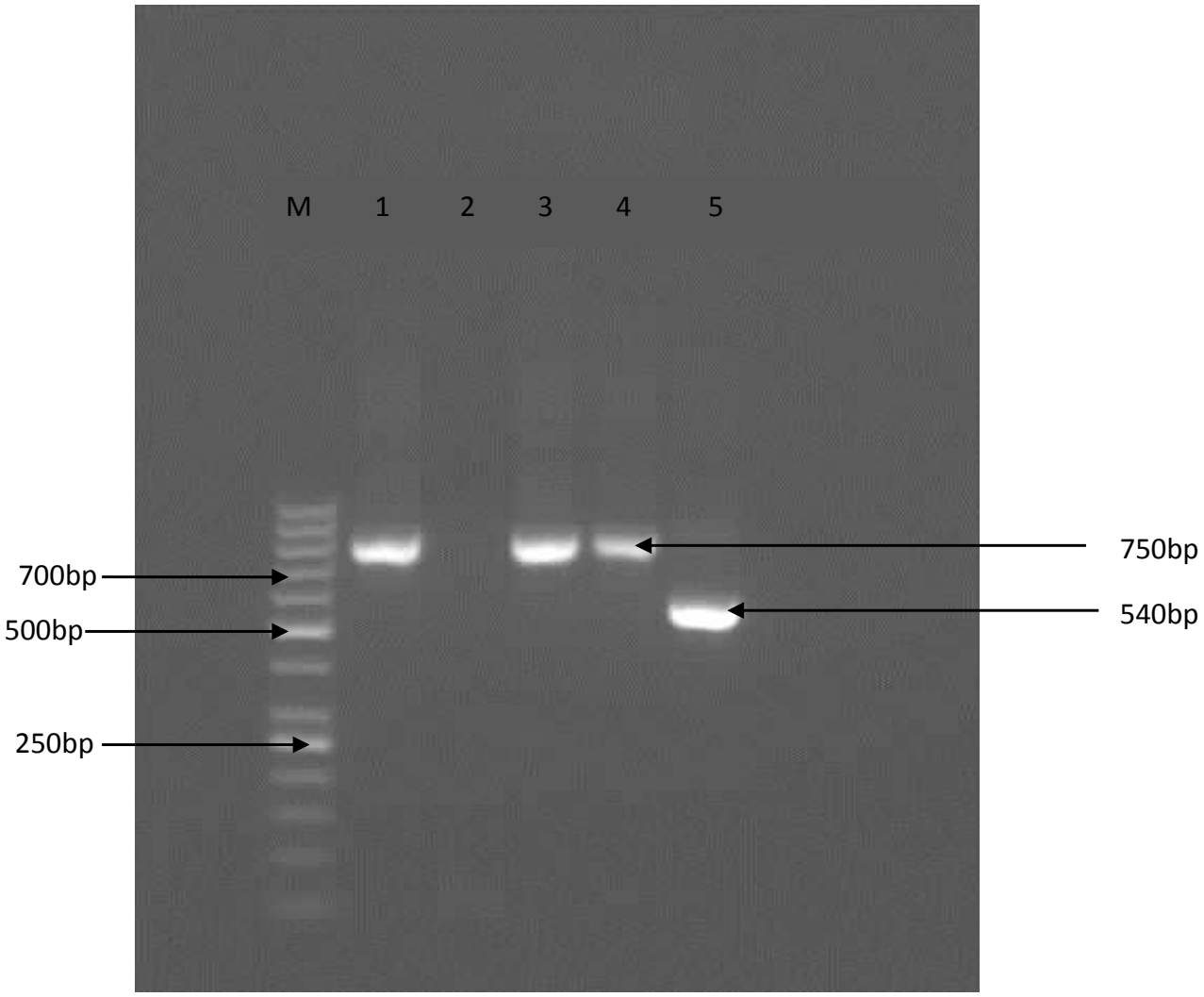

Plate 1. Identification of different species of trypanosomes using multiplex *Trypanosoma* primers PCR. Lane M - 50 bp DNA Molecular Wight Marker (Thermo-Scientific), Lane 1- Positive control of *T. congolense* (750 bp) [Savannah], Lane 2- Acute isolate of *T. vivax*, Lane 3- Acute form of *T. congolense* (750 bp) [Savannah]), Lane 4– Chronic stage of *T. congolense* (750 bp) [Savannah]), Lane 5- positive control (540 bp- *T. brucei*).

T. vivax species-specific PCR

In order to confirm the presence or absence of *T. vivax* in any of the samples (Kaduna and Makurdi), the samples were subjected to *T. vivax*-specific primers PCR. The acute and relapse samples of *T. vivax* (Makurdi sample) yielded the species specific amplicon of 175 bp (Plate 2), indicating pure *T. vivax*.

The results revealed that sample from Southern Guinea Savannah (Makurdi) was pure *T. vivax* isolates since it was negative to multiplex primers PCR for other trypanosomes, but positive to *T. vivax* which yielded an expected amplicon of 175 bp. However, the Northern Guinea Savannah sample (Kaduna sample) was negative to *T. vivax* species-specific PCR. These two tests confirmed that the isolates used in the study were pure *T. vivax* and *T. congolense*.

Observation on the RAPD-PCR of *T. vivax* and *T. congolense*

The experiment aimed at checking whether there was alteration of the DNA through change in bands of the *T. vivax* and the *T. congolense* at different stages (acute, relapse and chronic) of the disease or not. The results of the experiment revealed that the amplicons of the acute (N) and chronic forms (C) of the *T. vivax* were the same, whereas that of the relapse form (K) was different.

However, in *T. congolense*, the acute (A) and the relapse forms (D) yielded bands of the same molecular sizes but the chronic form (R) had a different pattern

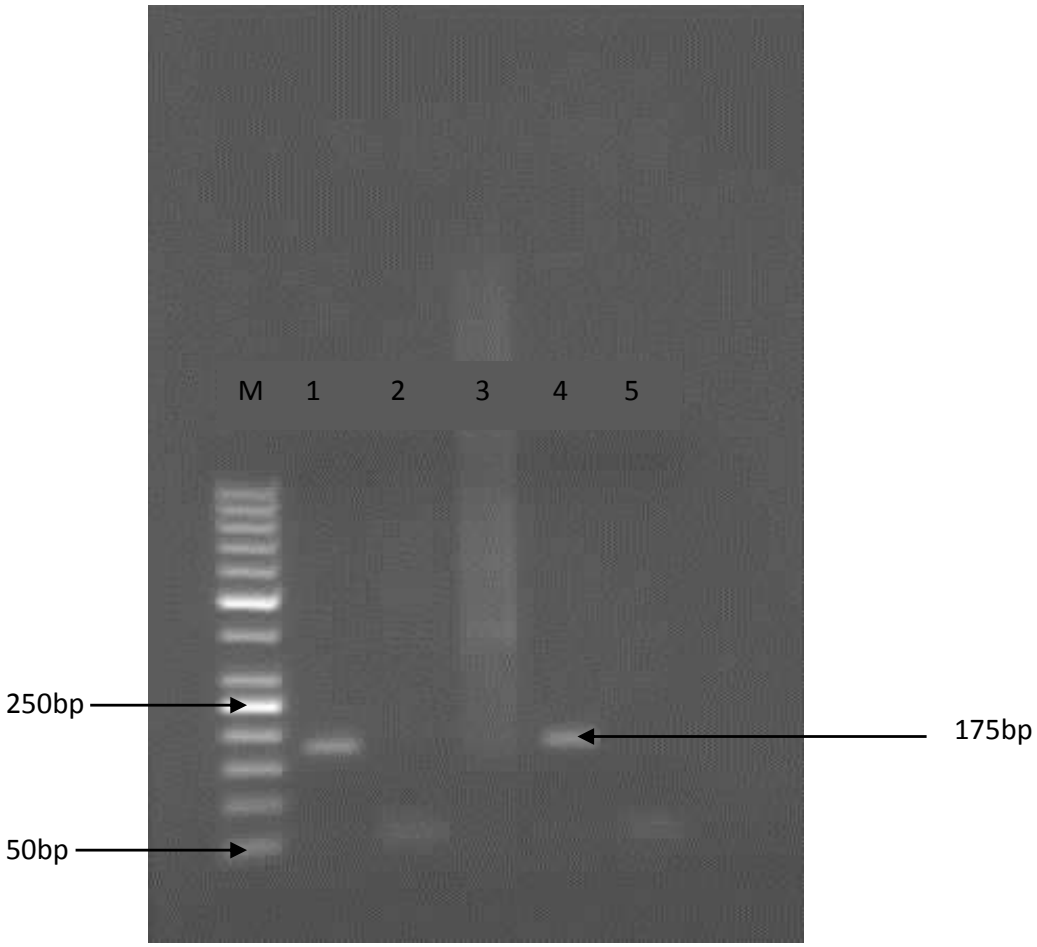

Plate 2. *T. vivax* PCR with TVWA/TVWB Primer. Lane M- 50 bp DNA Marker (Thermo Scientific), Lane 1- Acute isolate of *T. vivax* (175 bp), Lane 2- Acute isolate of *T. congolense* (Savannah type), Lane 3- Relapse form of *T. congolense* (Savannah type), Lane 4- Relapse form of *T. vivax* (175 bp), Lane 5- chronic form of *T. congolense* (Savannah type).

from the other two forms (Plate 3).

DISCUSSION

The *Trypanosoma* Genus specific and *T. vivax* species-specific PCR that the samples were subjected to confirm the species of the isolates to be pure *T. vivax* and *T. congolense* by having the same band sizes with other *T. vivax* and *T. congolense* from different parts of the world. It is known that Genus-specific *Trypanosoma* PCR can simultaneously detect multiple infections with *T. vivax*, *T. congolense* and *T. brucei brucei* in ruminants. The method was reported to have almost zero percent error in detecting and differentiating the different species and even subspecies and strains of trypanosomes (Hutchinson et al., 2007; Garcia et al., 2014).

The isolates were confirmed through the detection of bands of 750 and 175 bp for the primers used in amplifying the fragments from *T. congolense* and *T. vivax*, respectively. The results obtained from the RAPD-PCR shows that there may be genetic alteration in the field isolate as seen in the acute, relapse and chronic forms of the experimental disease, since the fragments seen with the field isolate (acute form) and chronic form (about 350 and 175 bp) were the same but differed from that of the relapse form (about 350 and 195 bp) for *T. vivax*. In *T. congolense*, the bands observed with the acute and relapse forms were the same and differed from the bands of the chronic form. The different bands may be as a result of new mutant formation that resulted from the use of drugs in this study. The difference in band weights of the three forms of the two isolates may be responsible for the differences in the pathogenicity of the parasites. The genetic alterations (change in bands) may also contribute to the widely reported antigenic variation in trypanosomosis (Zambrano et al., 2002; Pays, 2005; Deitsch et al., 2009). It is reported that species differ in the organization of their silent VSG archive (Greif et al., 2013); genomic analysis of the different stages of these

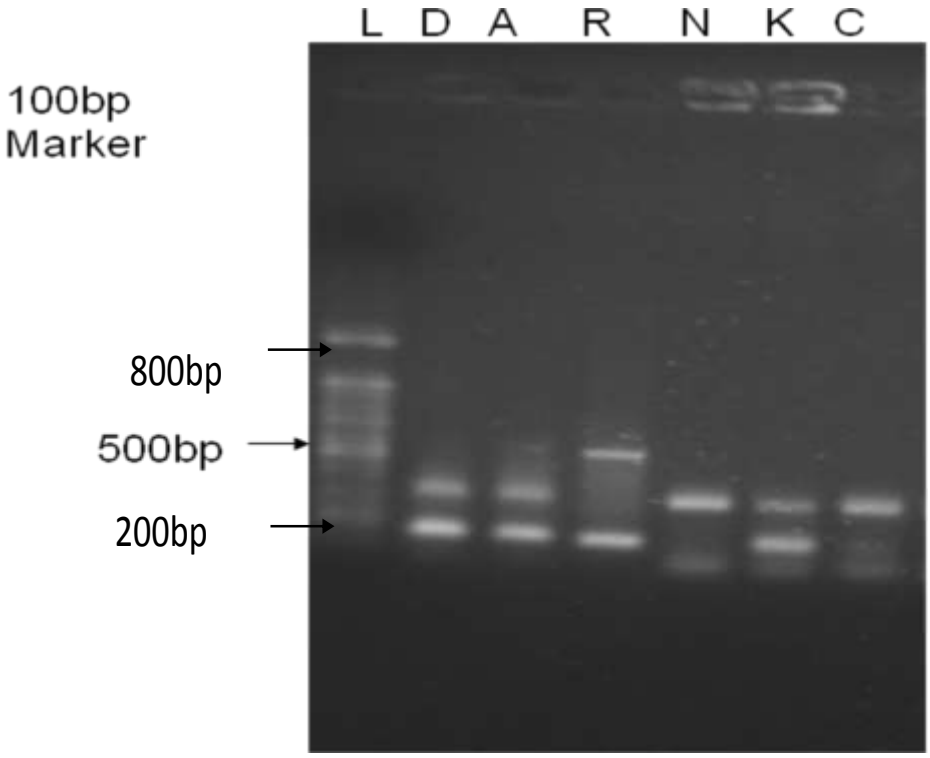

Plate 3. RAPD-PCR of *T. vivax* and *T. congolense* different forms. L= Marker (100bp), D= relapse form of *T. congolense* (approximately 420 and 290 bp), A = acute isolate of *T. congolense* (approximately 420 and 290 bp), R= chronic form of *T. congolense* (Approximately 510 and 290 bp), N = acute isolate of *T. vivax* (approximately 390 and 220 bp), K= Relapse form of *T. vivax* (Approximately 390 and 290 bp), C = chronic form of *T. vivax* (Approximately 390 and 220 bp).

infections assists in knowing the genes responsible for the changes in these band weights observed. It would have been very helpful if the amplicons obtained from the RADP-PCR were also sequenced to know the nucleotides that were altered, as this may assist in the possible production of DNA vaccine and drugs development that will knock off that nucleotide region for the control or prevention of this disease. The findings of this work may serve as a stepping stone to the discovery of a DNA vaccine against the disease.

CONFLICT OF INTEREST

The authors have not declared any conflict of interest

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Full Length Research Paper

Evaluation of hypolipidemic activity of different extracts of *Chomelia asiatica* (Linn) in rat fed with atherogenic diet

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In the present investigation, the hypolipidemic activity of different extracts of aerial parts of *Chomelia asiatica* (Linn) is shown. The evaluation dose of different extracts (petroleum ether, ethyl acetate and methanol) of *C. asiatica* was fixed at 200 mg/kg/day. Hypolipidemic activity was screened by inducing hyperlipidemia with the help of atherogenic diet (AD) in Wistar albino rats and plasma levels of different biochemical parameters such as total cholesterol, triglycerides, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) cholesterol, were determined. Thirty six male Wistar rats were divided into six groups, comprising six rats each. The rats in group 1 were treated with Standard chow diet, group 2 rats were treated atherogenic diet, group 3 rats were treated with AD + Pet.ether extracts of *C. asiatica* (200 mg/kg B.wt), group 4 rats were given AD + ethyl acetate extracts of *C. asiatica* (200 mg/kg B.wt), group 5 rats were given AD + methanol extracts of *C. asiatica* (200 mg/kg B.wt), group 6 AD + standard drug atorvastatin (1.2 mg/kg B.wt). At the end of 9 weeks, all rats were sacrificed by cervical dislocation after overnight fasting. The ethyl acetate extracts administrated rats were significantly ($p<0.001$) reduced in body weight, plasma and tissue total cholesterol, TG, phospholipids, plasma Low density lipoprotein and very low density lipoprotein; along with an increase in plasma high density lipoprotein when compared to that of AD treated groups of rats. Taking into account the outcomes, it can be concluded that the ethyl acetate extract of *C. asiatica* is a significant hypolipidemic agent, having preventive and curative activity against hyperlipidemia.

Key words: *Chomelia asiatica, atherogenic diet*, rats, hypolipidemia.

INTRODUCTION

Coronary heart disease (CHD) is the main cause of death in Western countries and Asia. Among CHDs, ischemic heart disease (IHD) leads to increase in mortality rate. The number of heart patients suffering from IHD worldwide is gradually increasing. About 41% of the deaths in the United States are due to heart diseases

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(Dallas, 2001). Extensive epidemiological studies reported that increased blood cholesterol level is a major cause of coronary heart diseases (Gambhir et al., 2001). Studies have also demonstrated that the relationship between plasma cholesterol levels and the development of IHD hypercholesterolemia is generally associated with an increase in plasma concentration of low density lipoprotein and very low density lipoprotein. The lowering of elevated levels of LDL cholesterol can slow the progression of atherosclerotic lesions.

The current hostility to hyperlipidemic drugs like statins and manufactured cancer prevention agents like probucol are broadly used to treat hyperlipidemic patients. Shockingly, these medications are not free of reactions. To give novel medications to hyperlipidemia, it has been centered on the common items that do not have very many reactions. The world ethnobotanical data reported different home grown drugs from the herbs that are utilized for normalling atherosclerosis and the complexities in patients. Around 80% of the third world populaces are totally reliant on conventional medications. Medicinal plants contain large number of pharmacological active phytoconstituents that might serve as lead for the development of activity, and they are safe and cheap novel drugs. A number of medicinal plants have shown their beneficial activity on the cardiovascular disease (CVD) by virtue of their hypolipidemic, antianginal, antioxidant, and cardioprotective activities (Wang and Ng, 1999; Dwivedi, 2004). Hence, the objective of the present investigation is to ascertain the hypolipidemic activities of different extracts of aerial parts of *C. asiatica* in experimental animals.

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals and reagents were purchased from Sigma, SD fine chemicals, and Fisher. The Fisher chemicals were of analytical grade.

Plant materials (study species)

Chomelia asiatica (Linn) belongs to the Rubiaceae family, commonly known as *Tharani in tami*: *Kuppipoovu, Tharana* in Makayalam. *Chomelia Gaertn* has about 370 species distributed in tropical and subtropical Africa, Asia, Madagascar, and the Pacific Islands. *C. asiatica* (Linn) is a common species found in India, Srilanka, and China. The leaves or powder extracts of *C. asiatica* are used as antimicrobial activities (Jayasinghe et al., 2002). It accounts for pain relieving and mitigating activities (Amutha et al., 2012). The parts of *Tarenna asiatica* (Rubiaceae) plants are customarily used to advance suppuration (Anonymous, 1976), as anthelmintic (Ramarao and Henry, 1996) and antiulcer operator (Rao et al., 2006). Its phytochemical constituents are accounted to be antiseptic (Vinothkumar et al., 2011), injury healing (Anjanadevi and Menaga, 2013) and antioxidant (Ramabharathi et al., 2014). Moreover, the extract of shoots, leaves and fruits are purportedly dynamic against *Mycobacter phlei* (Rajakaruna et al., 2002).

Collection and identification of plant materials

The aerial parts of *C. asiatica* (Linn) were gathered from Shencottai, Tirunelveli District, Tamil Nadu, and India. Taxonomic identification (Plant deposition no is Au/CA/810) was made from Botanical Survey of Medical Plants Unit Siddha, Government of India. Palayamkottai, Tirunelveli. The aerial parts of *C.asiatica* (Linn), were dried under shade, separated, and pulverized by a mechanical processor, through a 40 mesh sieve.

Preparation of different extracts

The above powered materials were successively extracted with Petroleum ether (40 to 60°C), using hot continuous percolation method in Soxhlet apparatus (Harborne, 1984) for 24 h. The marc was subjected to ethyl acetate (76 to 78°C) for 24 h and then marc was subjected to methanol for 24 h. The extracts were concentrated, using a rotary evaporator and subjected to freeze drying in a lyophilizer, until a dry powder was obtained. The percentage yields of pet.ether extract was 2.7%, ethyl acetate extract was 7.4 and 8.2% methanol extract was obtained.

Animals and treatment

Male Wistar rats of 16 to 19 weeks age, weighing 150 to 175 g were procured from the Central Animal House, Nizam Institute of Pharmacy and Research Centre, near Ramoji Film City, Deshmukhi, Hyderabad, Telugana, India. The rats were kept in cages, 2 per cage, with 12:12 h light and dark cycle at 25±2°C. The rats were maintained on their respective diets and water *ad libitum.* Animal Ethical Committee's clearance (Approval number is NIPRC/IAEC/Ph.D/2015/01) was obtained for the study.

Experimental design

The rats were divided into the following 6 groups, 6 rats each:

Group I: Standard chow pellet; Group II: Atherogenic diet (AD); Group III: AD plus treated with pet.ether extract of *C. asiatica* (200 mg/kg B.wt); Group IV: AD plus treated with Ethyl acetate (EA) extract of *C. asiatica* (200 mg/kg B.wt); Group V: AD plus treated with methanolic extract of *C.asiatica* (200 mg/kg B.wt); and Group VI: AD plus treated with Standard drug atorvastatin (1.2 mg/kg B.wt)

Animal diet

The compositions of the two diets were as follows (Kottai Muthu et al., 2005).

Normal diet

Wheat flour, 22.5%; simmered bengal gram powder, 60%; skimmed milk powder, 5%; casein, 4%; refined oil, 4%; salt blend with starch, 4%; and vitamin and choline blend, 0.5%.

High fat diet

Normal diet with coconut oil, 9%; cholesterol, 0.4%.

Assessment of hypolipidemic activities

Rats of III, IV and V groups were orally fed with the different

Table 1. Average body weight gain in normal and treated rats**.**

Treatment groups	Before treatment weight (g)	After treatment weight (g)	Average Body weight gain (g)
I	147.63±0.87^{bNS}	193.83±0.88^{b}*	46.20±0.38 ^{b}*
II	148.21±1.13^{aNS}	285.24±1.34^{a}**	137.03±1.19^{a}**
III	152.96 ± 0.33$^{aNS, bNS}$	265.35± 0.71$^{aNS, b*}$	112.39 ± 0.39$^{aNS, b}$*
IV	152.30±0.92$^{aNS, bNS}$	223.37±0.66$^{aNS, b**}$	70.65±0.22$^{aNS, b}$*
V	150.65±0.46^{aNS,bNS}	255.26±0.09$^{aNS, b*}$	104.61 ± 0.36 $^{aNS, b}$*
VI	162.68±0.88$^{aNS, bNS}$	222.01±0.43$^{aNS, b**}$	59.33±0.52$^{aNS, b}$**

Values are mean ± SE of 6 rats; *P* values: *<0.001, **<0.05; NS: Non-significant; a → group I compared with groups II, III, IV, V, VI. b → group II compared with groups III, IV, V, VI. Group I: standard chow pellet. (Normal); Group II: atherogenic diet. III Group: AD + Pet.ether extracts of *C. asiatica* (200 mg/kg B.wt); Groups IV: AD + Ethyl acetate extracts of *C. asiatica* (200 mg/kg B.wt); Group V: AD + Methanol extracts of *C. asiatica* (200 mg/kg B.wt); Group VI: AD + standard drug atorvastatin (1.2 mg/kg B.wt).

extracts of *C. asiatica* (200 mg/kg/day) and rats of VI group were fed with standard drug atorvastatin. Both the extracts and atorvastatin were suspended by 2% tween 80 (Waynforth, 1980) separately and nourished to the respective rats, using oral intubation. Toward the end of sixty three days, every one of the creatures was sacrificed by cervical dislocation after overnight fasting. The liver, heart and aorta were cleared of sticking fat, weighed precisely and utilized for the preparation of homogenate. Animals were given enough care as per the Animal Ethical Committee's recommendations.

Biochemical estimation

Plasma samples were analyzed for TC, HDL-cholesterol and TG were estimated using Boehringer Mannheim kits by Erba Smart Lab analyzer USA. Low density lipoprotein and very low density lipoprotein were calculated, using Friedewald et al. (1972) method. Ester cholesterol and free cholesterol (Sperry and Webber, 1950) were analyzed, using digitonin. Parts of the tissues from liver, heart and aorta were marked, weighed, and homogenized with methanol (3 volumes). The lipid extracts were obtained using Folch et al. (1957) method. Extracts were used for the estimation of ester cholesterol and free cholesterol, triglycerides (Foster and Dunn, 1973) and phospholipids (Zilversmit and Davis, 1950). Plasma total cholesterol: High density lipoprotein cholesterol ratio. Low Density Lipoprotein cholesterol: High density lipoprotein ratio was also utilized to access the atherogenic risk.

Statistical analysis

The results were expressed as mean ± SE of 6 rats in each group. The statistical significance between the groups was analyzed, using one way analysis of variance (ANOVA); followed by Dunnet's multiple comparison tests. The significance level was fixed at $p < 0.05$.

RESULTS

The body weight changes in normal and treated rats were appeared in Table 1. The body weight of AD treated rats (Group II) were increased significantly ($p<0.001$) in comparison with normal rats (group I). The average body weight gain was reduced significantly by the administration of EA extracts of *C. asiatica* at the dose of 200 mg/kg body weight, as well as atorvastatin 1.2 mg/kg in comparison with AD rats (Group II).

The activities of different extracts of *C. asiatica* on plasma lipid parameters were summarized in Table 2. The lipid parameters levels were significantly ($p<0.001$) increased in the rats fed with AD in comparison with the normal rats (Group I). The treatment of ethyl acetate extracts of *C. asiatica* of rat fed with AD significant ($p<0.001$) reduction in the level of plasma total cholesterol, free cholesterol, ester cholesterol, TG, phospholipids and free fatty acids as compared to AD rats (Group II). The treatment of ethyl acetate extracts of *C. asiatica treated* rats with AD showed that the plasma lipid parameters were restored to normalcy as well as standard drug treated rats. Promising results in reduced atherogenic index by ethyl acetate extracts of *C. asiatica* was found in Table 2. The AI is utilized as a marker to assess the susceptility of atherogenesis. The ethyl acetate extracts of *C. asiatica* showed an improvement of the cardio vascular risk level by decrease of AI in the treated group by more than 61% ($p < 0.01$), as comparison to AD rats (Group II).

As shown in Table 3, the plasma HDL-cholesterol levels were reduced in AD rats (Group II) as compared to normal rats. After treatment with ethyl acetate (EA), extracts of *C. asiatica* significantly increase the high density lipoprotein cholesterol concentration in rats fed with AD as compared to AD rats. Rats fed with AD (Group II) had elevated levels of plasma low density lipoprotein and very low density lipoprotein cholesterol, when compared with the normal rats (Group I). The treatment of EA extracts of *C. asiatica* significantly reduces the levels of low density lipoprotein and very low density lipoprotein cholesterol in plasma, when compared to AD rats (Group II). The ratios of TC: high density lipoprotein cholesterol and low density lipoprotein: high density lipoprotein-cholesterol are presented in Table 3. AD rats caused significant ($P<0.001$) increase in the ratios of TC: high density lipoprotein cholesterol and low density lipoprotein: high density lipoprotein cholesterol. Treatment of EA extracts of *C. asiatica* along with AD was found to significantly reduce the ratios of high density lipoprotein cholesterol. TC and low density

Table 2. Activity of different extracts of *C. asiatica on* plasma lipid parameters in normal and treated rats.

Group	Total cholesterol (mg/dl)	Free cholesterol (mg/dl)	Ester cholesterol (mg/dl)	Free fatty acid mg/dl)	Phospholipid (mg/dl)	Triglyceride (mg/dl)	AI
I	114.79± 0.35^{b}*	26.05±0.07^{b}*	88.73±0.39^{b}*	42.01±0.11^{b}*	104.87±0.17^{b}**	85.79±0.15^{b}*	1.91.05±0.01^{b}*
II	179.68±0.49^{a}*	45.31±0.23^{a}*	134.36±0.63^{a}*	59.96±0.36^{a}*	149.99±0.24^{a}**	170.39±0.44^{a}*	4.48±0.03^{a}*
III	163.83±0.44^{a}**,^{b}**	41.71±0.30^{a}*,^{b}*	122.18±0.21^{a}**,^{b}*	54.68±0.27^{a}*,^{b}**	143.01±0.17^{a}*,^{b}**	153.89±0.16^{a}*,^{b}**	3.81±0.01^{a}**,^{b}**
IV	99.85±0.15 ^{a}**,^{b}*	23.24±0.34^{a}*,^{b}*	76.61±0.32^{a}*,^{b}*	39.76±0.19^{a}*,^{b}**	108.35±0.29^{a}*,^{b}*	78.74±0.21^{a}*,^{b}*	1.75±0.01^{a}*,^{b}*
V	123.29±0.45^{a}*,^{b}*	36.78±.0.23^{a}*,^{b}*	86.52±0.50^{a}*,^{b}*	48.34±0.34^{a}*,^{b}**	133.61±0.32^{a}*,^{b}**	119.93±0.41^{a}*,^{b}**	2.78±0.01^{a}*,^{b}*
VI	97.57±0.29 ^{a}*,^{b}*	21.34±0.33^{a}*,^{b}*	76.23±0.15^{a}*,^{b}*	38.49±0.16^{a}*,^{b}*	100.42±0.20^{a}*,^{b}*	70.46±0.30^{a}*,^{b}*	1.72±0.01^{a}*,^{b}*

Values are expressed as mean ± SE (n=6 rats), *P* values: * < 0.001, ** < 0.05.

Table 3. Activity of different extracts of *C. asiatica* on plasma lipoprotein in normal and treated rats.

Group	High density lipoprotein (mg/dl)	Low density lipoprotein (mg/dl)	Very low density lipoprotein (mg/dl)	LDL- c/HDL-c ratio	HDL-c/ TC ratio
I	59.94±0.24^{b}*	36.35±0.22^{b}*	17.15±0.04^{b}*	0.60±0.01^{b}*	0.52±0.01^{b}*
II	40.02±0.24^{a}*	101.40±0.16^{a}*	34.07±0.08^{a}*	2.53±0.01^{a}*	0.22±0.01^{a}*
III	42.89±0.18^{a}**,^{b}*	80.55±0.20^{a}*,^{b}*	30.77±0.03^{a}*,^{b}*	1.87±0.01^{a}**,^{b}*	0.26±0.01^{a}*,^{b}*
IV	56.93±0.18^{a}*,^{b}*	30.21±0.24^{a}*,^{b}*	15.75±0.04^{a}*,^{b}*	0.53±0.01^{a}*,^{b}**	0.56±0.01^{a}*,^{b}*
V	44.23±0.15^{a}*,^{b}*	44.73±0.20^{a}*,^{b}*	23.98±0.08^{a}*,^{b}**	1.03±0.01^{a}*,^{b}**	0.35±0.02^{a}*,^{b}*
VI	56.35±0.22^{a}*,^{b}*	30.15±0.21^{a}*,^{b}*	14.06±0.04^{a}*,^{b}*	0.53±0.01^{a}*,^{b}*	0.57±0.05^{a}*,^{b}*

Values are expressed as mean ± SE (n=6 rats), *P* values: * < 0.001, ** < 0.05.

Table 4. Activity of different extracts of *C. asiatica* on tissues ester cholesterol profile in normal and experimental rats.

Group	Ester cholesterol (mg/g tissue)		
	Liver	Heart	Aorta
I	2.00±0.01^{b}*	2.83±0.02^{b}*	2.30±0.06^{b}*
II	3.45±0.02^{a}*	7.19±0.04^{a}*	7.21±0.03^{a}*
III	2.97±0.07^{a}*,^{b}**	5.36±0.03^{a}*,^{b}**	6.67±0.04^{a}*,^{b}*
IV	1.86±0.04^{a}*,^{b}*	2.80±0.04^{a}**,^{b}*	2.79±0.02^{a}*,^{b}**
V	2.70±0.04^{a}*,^{b}*	4.72±0.08^{a}*,^{b}*	5.22±0.04^{a}*,^{b}*
VI	1.84±0.01^{a}*,^{b}*	2.58±0.07^{a}*,^{b}*	2.65±0.04^{a}*,^{b}*

Values are expressed as mean ± SE (n=6 rats), *P* values: * < 0.001, ** < 0.05.

lipoprotein cholesterol: high density lipoprotein cholesterol compared to AD rats.

Activity of free and ester cholesterol in tissue were shown in Tables 4 and 5. The significant (P<0.001) elevated levels of both free and ester cholesterol were observed in tissues of rats fed

Table 5. Activity of different extractss of *C asiatica* on tissues free cholesterol in normal and treated rats.

Group	Free cholesterol (mg/g tissue)		
	Liver	Heart	Aorta
I	0.89±0.01^{b}*	0.79± 0.01 ^{b}*	0.55±0.17^{b}*
II	1.44±0.01^{a}**	1.12±0.02 ^{a}*	2.51±0.02^{a}*
III	1.24±0.02^{a}**,^{b}**	1.01±0.01 ^{a}*,^{b}**	1.87±0.01^{a}*,^{b}**
IV	0.90 ±0.01^{a}*,^{b}*	0.64±0.01 ^{a}*,^{b}*	0.79±0.01^{a}*,^{b}*
V	1.03±0.01^{a}*,^{b}*	0.88±0.01 ^{a}*,^{b}**	1.39±0.02^{a}*,^{b}**
VI	0.84±0.01^{a}*,^{b}*	0.61±0.01 ^{a}*,^{b}*	0.72±0.01^{a}*,^{b}*

Values are expressed as mean ± SE (n=6 rats), *P* values: * < 0.001, ** < 0.05.

Table 6. Activity of different extracts of *C. asiatica* on tissues TG level in normal and treated rats.

Group	Triglyceride (mg/g tissue)		
	Liver	Heart	Aorta
I	9.54±0.04^{b}*	11.43±0.08^{b}*	11.56±0.12^{b}*
II	30.50±0.02^{a}*	49.36±0.08^{a}*	23.19±0.10^{a}*
III	28.29 ± 0.09 ^{a}*,^{b}**	46.78±0.26^{a}**, ^{b}**	22.41±0.14^{a}*, ^{b}**
IV	10.42±0.08^{a}*, ^{b}*	19.24± 0.10 ^{a}*, ^{b}*	14.25±0.05^{a}*, ^{b}*
V	26.41±0.08^{a}*, ^{b}*	40.17± 0.10 ^{a}**, ^{b}*	19..37±0.05^{a}*, ^{b}*
VI	11.56±0.08^{a}*,^{b}*	20.26±0.04^{a}*, ^{b}*	13.31 ± 0.05 ^{a}*, ^{b}*

Values are expressed as mean ± SE (n=6 rats), *P* values: * < 0.001, ** < 0.05.

Table 7. Activity of different extracts of *C. asiatica on* tissues phospholipids level in normal and treated rats.

Group	Phospholipids (mg/g tissue)		
	Liver	Heart	Aorta
I	18.46±0.07^{b}*	25.49 ±0.08^{b}*	9.37±0.09^{b}*
II	27.46±0.14^{a}*	37.39±0.13^{a}*	18.26± 0.07^{a}*
III	26.34± 0.10^{a}**,^{b}*	35.29±0.10^{a}**,^{b}**	12.37 ± 0.06^{a}*, ^{b}**
IV	19.36 ± 0.09^{a}*,^{b}*	26.24±0.04 ^{a}*,^{b}*	10.30± 0.04^{a}*, ^{b}*
V	24.67 ± 0.24^{a}*,^{b}**	34.527±0.07 ^{a}*,^{b}**	16.35± 0.04^{a}*, ^{b}*
VI	18.66±0.14^{a}*,^{b}*	27.33±0.12^{a}*,^{b}*	11.48± 0.08^{a}*, ^{b}*

Values are expressed as mean ± SE (n=6 rats), *P* values: * < 0.001, ** < 0.05.

with AD (Group II), when compared to normal rats (Group I). Both tissues were free and ester cholesterol reduction remarkably treats the AD rats with EA extracts of *C. asiatica* (Group IV) compared with other two extracts treatment group.

The activity of different extracts of *C. asiatica* on tissue TG is shown in Table 6. The levels of TG tissue were increased in rats fed with AD (Group II), as compared to normal rats (Group I). Both plasma and tissue TG levels were significantly reduced in rats treated with EA extracts of *C. asiatica,* at the dose of 200 mg/kg and as well as standard drug along with AD, when compared to AD rats (Group II).

Activities of different extracts of *C. asiatica* on tissue phospholipids and free fatty acid are presented in Tables 7 and 8. The levels of tissue phospholipids and free fatty acids were significantly increased in rats fed with AD (Group II), as compared to normal rats (Group I). After the treatment of EA extracts of *C. asiatica* along with AD were shown, significantly (p<0.001) reduced the level of phospholipids and free fatty acids in comparison with AD fed rats (Group II).

DISCUSSION

The male albino rats utilized in the current investigation were reported as ideal hypercholesterolemic models in

Table 8. Activity of different extracts of *C. asiatica* on tissues free fatty acids level in normal and treated rats.

Group	Free fatty acids (mg/g tissue)		
	Liver	Heart	Aorta
I	10.66±0.15^{b}*	14.28±0.04^{b}*	10.30±0.06^{b}*
II	31.38±0.10^{a}*	49.38±0.06^{a}*	27.53±0.12^{a}*
III	29.21 ± 0.08 ^{a}*,^{b}**	46.24±0.50^{a}**, ^{b}**	25.31±0.07^{a}*, ^{b}**
IV	11.39±0.09^{a}*, ^{b}*	20.87± 0.10 ^{a}*, ^{b}*	14.34±0.08^{a}*, ^{b}*
V	25.29±0.06^{a}*, ^{b}*	37.39± 0.08 ^{a}**, ^{b}*	22.40±0.05^{a}*, ^{b}*
VI	10.34±0.05^{a}*,^{b}*	19.36±0.07^{a}*, ^{b}*	13.51 ± 0.10 ^{a}*, ^{b}*

Values are expressed as mean ± SE (n=6 rats), *P* values: * < 0.001, ** < 0.05.

previous studies (Mary et al., 2003). The plasma lipid parameters in plasma and tissue were increased in rats fed with AD (Group II) (Chandar et al., 1996; Guido and Joseph, 1992). The decrease in the lipid parameters levels in rats fed with ethyl acetate extracts of *C. asiatica* may be attributed to the elevated level of serum HDL, increase in the activity of lipoprotein lipase and plasma LCAT; which are known to be involved in transport of tissue cholesterol to liver for its excretion. Hence the hypolipidemic activity of the extracts seems to be mediated through elevated hepatic clearance of cholesterol, reduced the regulation of lipogenic enzymes like glucose-6-phosphate dehydrogenase and malate dehydrogenase as well as cholesterol biosynthetic enzyme HMG-CoA reductase.

Earlier investigations demonstrated constructive association in LDL cholesterol level in serum and risk of atherosclerosis (Kannel et al., 1971). The elevated levels of Low density lipoprotein and very low density lipoprotein cholesterol are the main risk factor for atherosclerosis (Temme et al., 2002; Parthasarathy et al., 1989). The low level of HDL-C was found in AD rats. It has been demonstrated that an elevated concentration of High density lipoprotein correlates inversely with atherosclerosis (Mayes et al., 1996). The present report demonstrates a significant reduction in plasma low density lipoprotein and very low density lipoprotein level, as a function of treatment of ethyl acetate extracts of *C. asiatica* in experimental animals. The decrease in the serum total cholesterol concentration may be due to increase in HDL cholesterol, which normally facilitates catabolism of excess cholesterol.

The high level of plasma and tissue triglyceride in rats fed with AD. AD rats significant elevated level of plasma TG due to reduction in the activity of lipoprotein lipase (Kavitha and Nalini, 2001). Similarly, there is a high level of plasma and tissue phospholipids also in rats fed with AD; this may be due to the reduced phospolipase effect (Mirhadi et al., 1991; Whereat and Robinowitz, 1975). Treatment of ethyl acetate extracts of *C. asiatica* significantly decreases the level of TG and phospholipids when compared to AD rats. The plant extracts may have prompt lipoprotein lipase activities resulting in reduced plasma TG and might enhance the uptake of TG from plasma by skeletal muscle and adipose tissues (El-Hazmi and Warsy, 2001).

Conclusion

The current investigation demonstrated that AD-induced hyperlipidemic was associated with ethyl acetate extracts of aerial parts of *C. asiatica,* reducing plasma free cholesterol (FC), esterified cholesterol (EC), total cholesterol (TC) triglyceride, low density lipoprotein and increase plasma high density lipoprotein-C. Also, the treatment with the ethyl acetate extracts of aerial parts of *C. asiatica* gives the protection against AD induced damage to the cardiac tissues probably through constructive modulation of the cardiac antioxidant system. The findings hence support the therapeutic use of the ethyl acetate extracts of aerial parts of *C. asiatica* in the management of cardiovascular complications like atherosclerosis.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Microbial diversity in leaves, trunk and rhizosphere of coconut palms (*Cocos nucifera* L.) associated with the coconut lethal yellowing phytoplasma in Grand-Lahou, Côte d'Ivoire

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The diversity of culturable bacterial and fungal communities was assessed from leaves, trunk and rhizosphere of coconut palms infected and non-infected by the Côte d'Ivoire lethal yellowing (CILY) phytoplasma. Bacterial and fungal microbes were isolated from leaves, trunk and rhizosphere samples collected from two villages of Grand-Lahou, Braffedon and Yaokro, by using a high-throughput 'dilution-to-extinction' cultivation method coupled with PCR and sequencing with primers that amplified both the 16S ribosomal RNA and intergenic transcribed spacer (ITS) genes. The relative abundance was higher for *Bacillus* and *Candida* in Braffedon, and *Burkholderia* and *Neodeightonia* in Yaokro. Commonly genera identified from rhizosphere included *Bacillus, Burkholderia, Pseudomonas, Streptomyces, Cryptococcus, Penicillium, Purpureocillium* and *Trichoderma.* The most abundant endophytes identified were *Pantoea*, *Candida*, *Cryptococcus, Bacillus, Pseudomonas, Penicillium, Aspergillus* and *Rhodotorula.* Genera limited to symptomless palms included *Arthrininum*, *Chaetomium*, *Phialemonium*, *Fusarium*, *Klebsiella* and *Candida*. Results indicate that the CILY phytoplasma may be a factor determining the level of diversity of a microbial community in a given location. Our research provides the basis to investigate the possible effect of endophytic and rhizosphere microbes against the CILY phytoplasma to further effectively improve the management of CILY in Grand-Lahou.

Key words: Côte d'Ivoire lethal yellowing phytoplasma, endophytes, biocontrol, coconut.

INTRODUCTION

Grown in tropical and subtropical regions, the versatile coconut (*Cocos nucifera* L.) is an important source of

food, shelter, and household income for smallholder farmers around the globe (Batugal et al., 2005). Côte d'Ivoire is among the top 20 coconut producers in the world, with an annual production of 182,170 tonnes (FAOSTAT, 2015), and it is the top African exporter of coconut oil from copra to Europe and West Africa. However, the Ivoirian coconut industry is currently impacted by the Côte d'Ivoire lethal yellowing disease (CILY) (Konan Konan et al., 2013a). Lethal yellowing-like diseases are considered a serious world threat for coconut-producing countries in Central America and the Caribbean, West and East Africa, and Mozambique (Danyo, 2011).

The disease CILY has been associated with a phytoplasma of the group 16SrXXII, subgroup B, '*Candidatus* Phytoplasma palmicola'-related strains (Harrison et al., 2014). It is widespread throughout the south coast littoral of Grand-Lahou, where it has caused the destruction of more than 400 ha within the last ten years, and losses of 12,000 tonnes of copra per year (Kra et al., 2017).

Phytoplasmas are bacteria-like phloem-inhabiting pathogens of the class *Mollicutes* transmitted by Hemiptera insect vectors that cause diseases in over a thousand plant species, including crops, fruit trees, and ornamental plants (Maejima et al., 2014). Management and control of phytoplasma diseases have been based on the removal of infected plants that can act as an inoculum source (Romanazzi et al., 2009), the use of resistant varieties, and the control of insect vectors; however, no effective disease control has been achieved so far. No genotypes are available that show reliable resistance to phytoplasma diseases, and particularly for coconut lethal yellowing, the sort of unexplained resistance breakdown seen in some widely used hybrids is a global concern for replanting programs (Baudoin et al., 2008). Phytoplasma vector control has been focused on insecticidal treatments against natural vectors such as *Macrosteles quadripunctulatus,* the vector of chrysanthemum yellows phytoplasma (Saracco et al., 2008), and *Cacopsylla pyri*, the vector of pome fruit phytoplasmas (Bangels et al., 2010).

Some other strategies tested to control phyoplasma diseases include transgenic plants as in the case of tobacco plants expressing antibodies against the stolbur phytoplasma (Malembic-Maher et al., 2005); the use of elicitors such as indole-3-acetic acid/butyric acid (Perica, 2008; Lherminier et al., 2003); organic fertilizers or algal extracts; natural and synthetic peptides and essential oils; or recovery promoted by abiotic stress in grapevines affected by 'bois noir' (Romanazzi et al., 2009). Previous studies have shown that applying the arbuscular mycorrhizal fungus *Glomus intraradices* can improve tolerance to pear decline phytoplasma (Garcia-Chapa et al., 2004). Similarly, it has been shown that plant-growth promoting bacteria like *Pseudomonas*, isolated from the rhizosphere of daisy plants, when applied to phytoplasma-infected daisies, are able to extend the daisy's life span (D'Amelio et al., 2007).

The plant rhizosphere harbors numerous bacteria capable of stimulating and aiding plant growth and are termed plant growth promoting rhizobacteria (Lugtenberg and Kamilova, 2009). They exert their beneficial effects through biofertilization, stimulation of root growth, rhizo-remediation, plant stress control, or biological control including antibiosis, induction of systemic resistance and competition for nutrition and niches, besides they have become the new inoculants for biofertilizers. Studies on the bacterial population isolated from the rizhosphere of coconut palms resistant and tolerant to Kerala wilt disease phytoplasma in India (Gopal et al., 2005) have suggested that rhizosphere microflora could play a role in evading phytoplasma infection.

Over the last few years, there has been an increasing interest in the use of endophytes to control plant pathogens (Romanazzi et al., 2009). Indeed, one of the latest trends is the identification of fungal or bacterial endophytes with biocontrol potential against phytoplasmas (Compant et al., 2013; Martini et al., 2009; Romanazzi et al., 2009). Endophytes refer to endosymbionts that colonise the inside of the plants without causing any disease to the plant host (Schulz and Boyle, 2006; Wilson, 1995). They can inhabit different plant parts such as tubers, stems, leaves and roots, and can enter the plant via insect sucking or by passive diffusion or active selection from the adjacent rhizosphere (Romanazzi et al., 2009).

Endophytes establish mutualistic relationships with plants and produce important secondary metabolites and compounds that can act as plant growth promoters or enhancers of plant resilience to certain pests and plant pathogens, as well as to abiotic factors such as environmental stress and drought (Golinska et al., 2015; Rodriguez et al., 2009).

The interaction of endophytic bacteria and fungi in phytoplasma-affected plants has been poorly studied, and limited to a few crops like grapevine infected with the "flavescence dorée" and "bois noir" phytoplasmas (Bulgari et al., 2011; Grisan et al., 2011), and apple infected with the apple proliferation phytoplasma (Bulgari et al., 2012; Musetti et al., 2011). Recently, endophytic communities associated with healthy and phytoplasma-infected plants have been characterised (Bulgari et al., 2009; Martini et al., 2009) to find possible biocontrol agents. Martini et al. (2009) identified *Aureobasidium pullulans* and *Epicoccum nigrum* as fungal endophytes

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associated with grapevine varieties that spontaneously recovered from phytoplasma disease. Musetti et al. (2011) showed that periwinkle plants, *Catharanthus roseus* L. G. Don, grafted with 'Ca. Phytoplasma mali'-infected apple scions, and treated with *E. nigrum* exhibited reduced symptoms, and lower concentration of the phytoplasma when compared with untreated control plants. Grisan et al. (2011) identified *A. pullulans* and other fungal species when characterising the fungal endophytic bio-diversity in both healthy and phytoplasma-infected grapevines. Other species included *Alternaria* sp., *Phoma* sp., *Cladosporium* sp., *Pestalotiopsis* sp., and *Pestalotia* sp.

A number of endophytes have been studied for their potential as biocontrol in coconut palms. A member of the genus *Cordyceps* has been reported as a biocontrol against *Lecopholis coneophora*, the causal agent of coconut root grub (Kumar and Aparna, 2014). Endophytic bacteria such as *Pseudomonas, Bacillus*, and *Enterobacter* have been commonly isolated from a wide range of monocots and dicots including coconut (Rajendran et al., 2008). Rajendran et al. (2015) used endophytic bacteria *Bacillus subtilis* and *Pseudomonas fluorescens* in a bioconsortium with *Trichoderma viride* as a soil application against *Ganoderma lucidum* (Leys) Karst, the causal agent of basal stem rot on coconut palm, and showed a higher induction of defense related enzymes.

High-throughput cultivation – 'dilution-to-extinction' cultivation – has proven to be an effective method to assess endophytic diversity (Unterseher and Schnittler, 2009). The method increases the diversity of cultivable species as inoculum density decreases (Collado et al., 2007). It has been primarily used to lower species richness so functional ability can be correlated with biodiversity.

A multi-well plate is used to provide spatial separation and reduce interspecific interactions. This increases the possibility of detecting slow-growing and weak competitors, while, at the same time, allows the isolation of ubiquitous and dominant taxa. The 'dilution-to-extinction' cultivation method has been widely used to study the modified microbial diversity in different materials such as mineral soils (Wertz et al., 2006, 2007; Griffiths et al., 2001), peat (Dimitriu et al., 2010) and sewage (Franklin and Mills, 2006). It has been applied to assess the fungal endophytic community of leaf-litter fungi in beech, *Fagus sylvatica* L. (Unterseher and Schnittler, 2009), and to isolate ammonia-oxidizing bacteria from arable and lead-contaminated soil (Aakra et al., 1999).

The present work aimed at characterizing the culturable bacterial and fungal communities residing in leaves, trunk, and the rhizosphere of CILY phytoplasma-infected and CILY phytoplasma-free coconut palms by using the 'dilution-to-extinction' cultivation method coupled with PCR and sequencing approaches.

MATERIALS AND METHODS

Sampling from leaves, trunks and rhizosphere from coconut palms

Samples were collected in 2016 from Braffedon during April, and from Yaokro in June. Braffedon and Yaokro are two coconut-growing villages of the Grand-Lahou village area, located at 18 and 43 Km from Grand-Lahou downtown, respectively (Arocha Rosete et al., 2017). Samples included leaves, trunk borings and rhizosphere from PB121 coconut palm ecotypes. Two symptomless coconut palms were sampled per village. Coconut palms exhibiting CILY-like symptoms were also surveyed. Samples were collected from two palms with symptoms resembling those of each disease stage 1, 2 and 3 for a total of six symptom-bearing coconut palms per village. Symptoms of disease stage 1 (S1) corresponded to palms with initial yellowing in the older leaves and initial blackening of the inflorescences. Symptoms of disease stage 2 (S2) were related to the progress of yellowing from the older to the younger leaves, and increase of the inflorescence blackening. In palms showing symptoms similar to those of disease stage 3 (S3), the older yellowed leaves turned brown and desiccate, and in some cases hanged down forming a skirt around the trunk. This latter sign along with full blackening of the inflorescences were only observed in coconut palms from Yaokro.

Rhizosphere samples were collected from clay-rich sandy tertiary soils, characteristic of the Grand-Lahou coastal littoral zone at 10 cm from the trunk, and 26 cm deep in the soil with a stainless steel soil-probe sampler (Konan Konan et al., 2013b). Three young leaves emerging from the coconut heart were collected per palm, then individually surface sterilised with both 0.5% sodium hypochlorite and 70% ethanol, and rinsed in Sigma-graded sterile deionized water (SDW). Small plastic bags were used to collect around 2 g of trunk borings by boring into the trunk at one meter from the trunk base using an eight cm long drill bit sterilised with 70% ethanol before collecting each sample (Harrison et al., 2013). For the trunk borings, 1 g of each sample was weighed and placed in a 15 mL Falcon™ tube, and sterilized with 10 mL of 0.5% sodium hypochlorite, followed by 10 mL of 70% ethanol, and rinsed in 10 mL of Sigma-graded SDW. UV-sterilized Whatman paper was used to dry up the wet trunk borings after the final rinse. The trunk borings were transferred to a clean sterile 1.5 mL microtube. All samples were kept at 4°C until further analysis.

Culture isolation of bacterial and fungal organisms

Bacterial and fungal organisms were isolated by using a modified 'dilution-to-extinction' cultivation method (Collado et al., 2007) with some modifications explained below and illustrated in Figure 1.

Rhizosphere culture sample preparation

One gram of rhizosphere was weighed from each palm, and the weighed amounts from each two replicates representing each disease stage (1, 2 and 3) were pooled in a 15 mL Falcon™ tube, and mixed by a 10 sec vortexing. A total of 0.1 g of each mixture was taken with a small spatula and added to 900 µL of SDW in individual 2 mL microtubes resulting in a 10^{-1} dilution.

Trunk culture sample preparation

A total of 0.5 g of trunk borings collected was weighed from each tree, and the weighed amounts from each two replicates representing each disease stage (1, 2, and 3) were pooled in a 15 mL Falcon™ tube, and mixed by a 10 sec vortexing. A total of 0.1 g

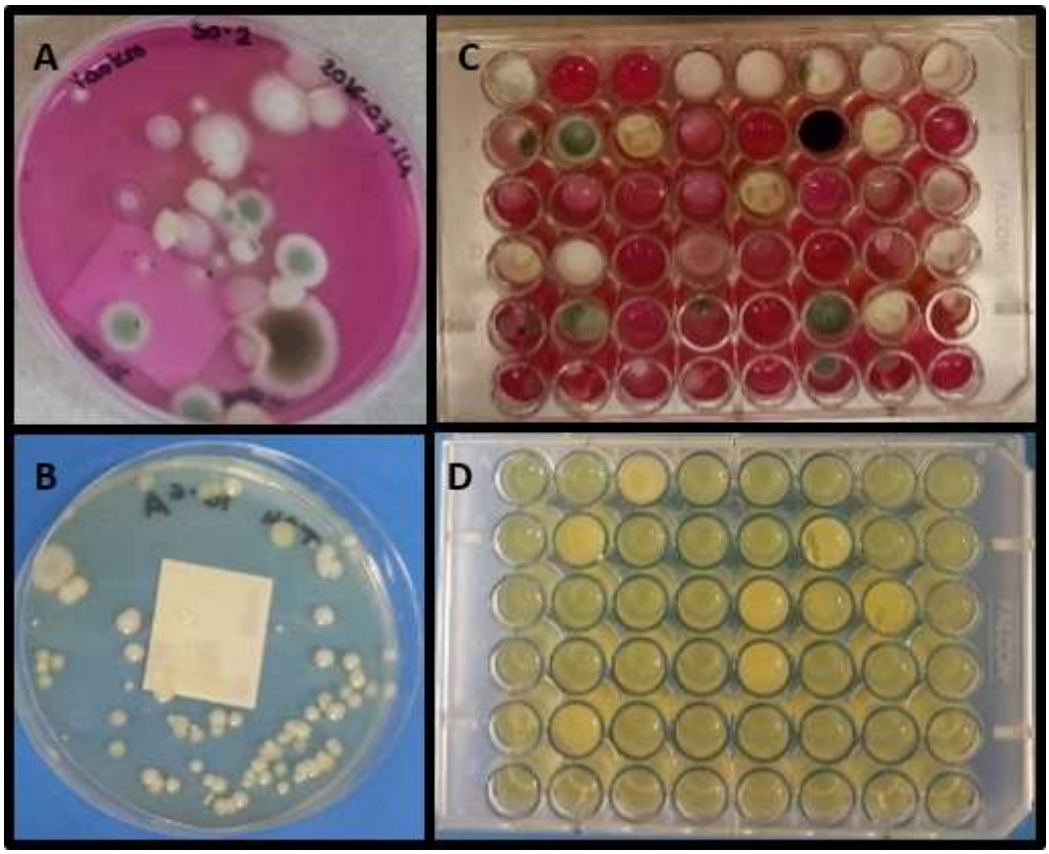

Figure 1. Conventional isolation method in 35 mm Petri dishes with 100 µL of fungal (A) or bacterial (B) suspensions. Fungal (C) and bacterial (D) isolations obtained by 'dilution-to-extinction' cultivation method in 48-well plates. Fungal (A and C) isolates were grown on Rose Bengal Agar with chloramphenicol. Bacterial (B and D) isolates were grown on Tryptic Soy Agar with amphotericin B.

of each pool was taken with a small spatula and added to 900 µL of SDW in individual 2 mL microtubes resulting in a 10^{-1} dilution.

Leaf culture sample preparation

Since three leaves were collected per palm, 0.5 g of leaf midrib from each leaf sample were finely cut into 0.5 cm square pieces with a sterile scalpel, and pooled together in a 15 mL Falcon™ tube, and briefly vortexed. A total of 1 g from each pool representing each disease stage was weighed, placed in a sterile porcelain mortar and macerated with a sterile pestle in 2 mL of SDW. The homogenate was collected per sample in 2 mL microtubes and diluted six-fold (10^{-6}) in SDW.

Sample plating

An aliquot of 100 µL of each dilution series was plated in duplicate in 35 mm Petri dishes with Tryptic Soy Agar (TSA) medium (BD Difco™) supplemented with amphotericin B (4 µg/mL; TSAA) for bacterial isolation. Plates were incubated for 2 days at 35°C. For fungal isolation, fresh 100 µL aliquots were plated in 35 mm Petri dishes with Rose Bengal agar (RBA medium; Oxoid) supplemented with chloramphenicol (50 mL; RBAC). Plates were incubated at 25°C for 4 to 6 days.

Endophytic sterility check

For the verification of the effectiveness of the disinfection procedure and the confirmation that microbial growth was endophytic and not from leaf surface or trunk contamination, sterility checks were carried out for each leaf and trunk sample. For these checks, 0.1 mL from the final SDW rinse were plated out on TSAA and RBAC plates. Plates were incubated as described above. The effectiveness of the sterility checks was measured based on the absence of bacterial or fungal growth in both TSAA and RBAC plates after the corresponding incubation period.

Colony counts

Colony forming units (CFU) were counted for each duplicate sample and averaged to approximate the dilution factor in SDW to 100 CFU/mL. Duplicates of 48-wells of RBAC and TSAA Falcon™ Polystyrene Microplates (Fisher) were used per sample and inoculated with 10 µL of the final dilution resulting in approximately 1 colony per well. TSAA and RBAC microplates were incubated for 2 days at 35°C, and for 4 to 6 days at 25°C, respectively.

Colony morphology identification

Bacterial and fungal colonies were morphologically identified based

on the colony shape and appearance, texture and pigmentation. Colonies of similar morphologies were assigned the same number. A colony representative of each number was subcultured into corresponding TSAA or RBAC Petri dishes. TSAA plates were incubated for 2 days at 35°C, while RBAC plates were incubated for 4 to 6 days at 25°C.

Total DNA extraction

After incubation, plates were used for total DNA extraction using the FastDNA® SPIN Kit (MP Biomedicals, USA) following manufacturer's recommendations. Total DNA was also extracted directly from leaf and trunk boring samples (Harrison et al., 2013).

PCR amplification

All PCR reactions (25 µL) were performed with illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, UK) with a starting concentration of 50 ng of genomic DNA and 0.4 µM final primer concentrations. The CILY phytoplasma was detected by nested PCR with universal 16S rRNA PCR primers P1 and P7 (Schneider et al., 1995; Deng and Hiruki, 1991) with an initial denaturation of the template at 94°C for 3 min, followed by 35 cycles: 94°C for 40 s, 54°C annealing for 40 s, 72°C extension for 1 min 40 s, and a final 10 min extension at 72°C. One microliter of the first PCR reaction was diluted 1:30 in SDW and used as a template for the nested PCR with primers G813f and AwkaSR (Tymon et al., 1998) specific for West African phytoplasma strains. The nested PCR protocol followed 35 cycles of initial denaturation of the template at 94°C for 3 min, 94°C for 40 s; 53°C annealing for 40 s; 72°C extension for 1 min, and a final 10 min extension at 72°C. P1 and P7 primers were nested with phytoplasma generic primers U5 and U3 (Lorenz et al., 1995) to confirm the detection of any other phytoplasma group in samples yielding no amplification with primers G813/AwkaSR.

For bacterial identification, the 16S rRNA gene was amplified with universal primers 27f and 907r (Lane, 1991) with an initial denaturation of the template at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 50°C annealing for 30 s, 72°C extension for 1 min and 20 s, and a final 7 min extension at 72°C. For fungal identification, the ITS region of the rRNA gene was amplified using the universal primers ITS5 and ITS4 (White et al., 1990) with the following amplification protocol: initial template denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 55°C annealing for 30 sec, 72°C extension for 1 min and 15 s, and a final 5 min extension at 72°C.

Sequencing and sequence analyses

All PCR amplicons were purified using the E.Z.N.A.® Cycle-Pure Kit (Omega Bio-tek, USA), and sequencing reactions were prepared using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following the manufacturer's recommendations. Samples were directly sequenced at the Centre for the Analysis of Genome Evolution and Function (CAGEF; University of Toronto). The sequences obtained were assembled into contigs using the Sequencher 4.9 software (Gene Codes Corporation, USA). Bacterial and phytoplasma contigs were compared to NCBI (Genbank https://www.ncbi.nlm.nih.gov), and in the case of bacteria, the sequences were also analyzed at the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/). Fungal contigs were compared to the CBS Fungal Biodiversity Centre (http://www.cbs.knaw.nl/) by pairwise sequence similarity search. A sequence similarity greater than 97% was used to assign a genus to the bacterial and fungal isolates. Multiple sequence alignments of the obtained contigs were made with Clustal W and phylogenetic analyses were performed with MEGA version 7 (Kumar et al., 2016), using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) with a bootstrap analysis of 1000 replicates.

Diversity analyses

Shannon (H') and Simpson (D) diversity indices were calculated for the microbial communities using PAST version 3.14 (Hammer et al., 2001). A modified t-test for the above-mentioned diversity indices (Brower et al., 1998; Hutcheson, 1970) was used in order to assess the differences in diversity between the microbial communities identified. Relative abundance was calculated by dividing the number of species from one given group (bacterial or fungal) by the total number of species from leaves, trunk and rhizosphere.

RESULTS

Screening for the presence of CILY phytoplasma

Total DNA from leaf and trunk boring samples was subjected to nested PCR for the detection of the CILY phytoplasma. No amplification was obtained for any of the two symptomless palms collected in Braffedon or Yaokro with any of the primer combinations. The six coconut palms exhibiting S1, S2 and S3 CILY symptoms collected from Yaokro tested positive for the presence of the CILY phytoplasma with the specific primers G813/AwkaSR. Sequences were 99% identical to those of the CILY phytoplasma isolates from Grand-Lahou from previous studies (Harrison et al., 2014). Interestingly, no CILY phytoplasma was detected with primers G813/AwkaSR in any of the leaf or trunk samples collected in Braffedon regardless the presence of CILY-like symptoms. Only four out of the six palms exhibiting CILY-like symptoms collected in Braffedon: two palms with S3-like symptoms (from trunk and leaf, respectively), one palm with S2-like symptoms (from trunk), and one palm with S1-like symptoms (from trunk) yielded P1/P7 amplicons. After PCR purification and sequencing, the partial P1/P7 sequence was determined to belong to the Gram positive bacterium *Bacillus megaterium*. Besides, nested PCR with phytoplasma generic primers U5/U3 yielded no amplification for all the six coconut palms surveyed in Braffedon that exhibited CILY-like symptoms.

Characterization of isolated microbial communities

Six palms per location and a total of 24 leaf, 40 rhizosphere (24 from Braffedon and 16 from Yaokro), and 32 trunk (24 from Braffedon and 8 from Yaokro) samples were processed. Eighty-seven bacterial and 97 fungal isolates were taxonomically identified and their respective 16S rDNA and ITS sequences compared. The resulting 95 operational taxonomic units (OTUs) represented 3 phyla (Actinobacteria, Firmicutes, and Proteobacteria), 5 classes, and 26 genera for the bacterial isolates (Table 1

and Figure 2), and 3 phyla (Ascomycota, Basidiomycota, and Zygomycota), 7 classes, and 27 genera for the fungal isolates (Table 1 and Figure 3). One representative sequence of each OTU was deposited in GenBank (Accession numbers shown in Figures 2 and 3).

Diversity of bacterial and fungal communities isolated per geolocation

There was no significant difference with regards to the whole microbial community diversity (combined analysis of bacterial and fungal genera/species) identified from Yaokro and Braffedon (Shannon and Simpson's indexes 3.0975 and 0.068594, respectively, for Braffedon and 3.1489 and 0.066162, respectively for Yaokro, Table 2). However, statistical differences were observed among diversity levels when the bacterial and fungal microbial communities were analyzed independently by village.

The bacterial community in Braffedon was more diverse than that of Yaokro (Shannon and Simpson's indexes 2.7085 and 0.091545, respectively, for Braffedon; Shannon and Simpson's indexes 2.4027 and 0.147, respectively, for Yaokro, Table 2). Six bacterial genera were commonly identified in both locations, which included *Bacillus, Burkholderia, Enterobacter, Pseudomonas*, *Ralstonia* and *Streptomyces*. Eight bacterial genera were only found in Braffedon (Table 1 and Figure 4A, 5A and 5C): *Agrobacterium*, *Alcaligenes*, *Chryseobacterium*, *Curtobacterium*, *Leifsonia*, *Paenibacillus*, *Pantoea* and *Streptococcus*, while thirteen genera were only found in Yaokro: *Blastococcus*, *Dyella*, *Herbaspirillum*, *Kitasatospora*, *Herbaspirillium, Klebsiella*, *Lysinibacillus*, *Ochrobactrum*, *Rhodococcus*, *Serratia*, *Sinomonas*, *Stenotrophomonas*, and *Variovorax*. Althoug Braffedon showed a higher bacterial diversity level than Yaokro, the latter scored a higher number of genera limited to that particular location.

Conversely, the fungal community was more significantly diverse in Yaokro when compared to that observed in Braffedon (Shannon and Simpson's indexes 2.0339 and 0.20588, respectively, for Braffedon and Shannon and Simpson's indexes 2.5142 and 0.11985, respectively, for Yaokro). Seven fungal genera were isolated from both Braffedon and Yaokro: *Aspergillus, Candida*, *Cryptococcus*, *Fusarium*, *Penicillium*, *Purpureocillium*, and *Trichoderma*. Five genera were only found in Braffedon (Table 1 and Figure 4B, 5B and 5D): *Exophiala*, *Meira*, *Pestalotiopsis*, *Pseudozyma*, and *Rhodosporidium*, while fifteen genera were only found in Yaokro: *Arthrininum*, *Chaetomium, Cladophialophora*, *Clonostachys*, *Cunninghamella*, *Eupenicillium*, *Gibberella*, *Gliocladium*, *Neodeightonia*, *Nirograna*, *Phialemonium*, *Phialocephala*, *Pseudallescheria*, *Rhodotorula*, and *Triplosphaeria*.

In Braffedon, the largest number of bacteria belonged to the genus *Bacillus*, representing 15% of all the bacterial isolates identified, while *Candida* was the most abundant fungal genus, representing 24% (Table 1). In Yaokro, *Burkholderia* was the most abundant bacterial genus, while *Neodeightonia* was the most abundant fungal genus, corresponding to 19 and 12% of all the bacterial and fungal isolates identified, respectively (Table 1).

When comparing Braffedon and Yaokro, no difference was found on the microbial community diversity levels among the different CILY stages S1, S2 or S3. The commonly identified bacterial and fungal genera: *Bacillus, Burkholderia, Enterobacter, Pseudomonas*, *Aspergillus, Penicillium*, *Purpureocillium*, and *Trichoderma* previously found indistinctly across the symptomatic palms in Braffedon and Yaokro were also found in the symptomless palms surveyed in both locations (Figure 5A to 5D). The genus *Fusarium* although found in both locations was limited to symptomless palms in Braffedon, and palms showing S2-like symptoms in Yaokro. However, there were isolates identified solely in each specific location. In Braffedon, *Paenibacillus* was only isolated from palms showing CILY S3-like symptoms. In Yaokro, four bacterial genera were only associated with symptomatic palms such as *Pseudomonas* and *Herbaspirillum* in those with S1-like symptoms, *Blastococcus* in those with S2-like symptoms and *Ralstonia* in those with S3-like symptoms. The fungal genera *Cunninghamella* was limited to palms with S1-like symptoms, and *Cladophialophora* and *Gibberella* to those with S3-like symptoms, while six other fungal genera *Eupenicillium*, *Hypocrea*/*Trichoderma*, *Nirograna*, *Phialocephala, Pseudallescheria*, and *Triplosphaeria* were only found in palms with S2-like symptoms (Figure 5A to 5D).

Microbial community in the rhizosphere

The most abundant genera found in the rhizosphere in Braffedon (Table 1) were *Bacillus* (9.9%) followed by *Penicillium* (3.3%) then *Candida* (2.3%), while in Yaokro, these were *Bukholderia* (19%) followed by *Neodeightonia* (12.5%), *Penicillium* (6.6%) then *Trichoderma* (4.4%). Commonly genera identified from rhizosphere samples in both locations included *Bacillus, Burkholderia, Pseudomonas, Streptomyces, Cryptococcus, Penicillium, Purpureocillium* and *Trichoderma.* Four fungal genera *Arthrininum*, *Candida*, *Chaetomium*, and *Phialemonium* were solely isolated from symptomless palms in Yaokro.

Endophytic community in leaves and trunks

The most abundant genera found in leaves in Braffedon (Table 1) were *Pantoea* (9.9%) followed by *Cryptococcus* (6.8%) then *Bacillus* (4.7%). For trunk samples, the most

Table 1. Relative abundance of bacterial and fungal isolates identified from leaves, trunk and rhizosphere in Braffedon and Yaokro.

Bacteria	Braffedon			Yaokro	
	Leaves	Trunk	Rhizosphere	Trunk	Rhizosphere
***Agrobacterium* sp.**			**0.5070**		
Alcaligenes faecalis			**0.4225**		
Bacillus	**4.6895**		**9.8859**	**6.0558**	**3.2669**
B. aquimaris			1.2674		
B. cereus					1.5139
B. indicus			0.5492		
B. megaterium	4.6895		0.5915		
B. mycoides			0.6337		1.1952
B. niacini					0.3187
B. pseudomycoides			0.5915		
B. pumilus				0.0797	
B. subtilis				0.2390	0.0797
B. vietnamensis			1.2674		
Bacillus sp.			4.9852	5.7371	0.1594
***Blastococcus* sp.**					**0.0797**
Burkholderia		**0.3802**	**0.2535**		**18.7251**
B. anthina			0.2535		
B. cepacia		0.3802			0.3984
B. glumae					0.1594
B. nodosa					4.8606
Burkholderia sp.					13.3068
Chryseobacterium sp.			**0.5915**		
Curtobacterium	**1.8166**	**0.8450**			
C. flaccumfaciens		0.8450			
Curtobacterium sp.	1.8166				
Dyella					**0.7171**
D. japonica					0.6375
Dyella sp.					0.0797
Enterobacter	**2.6193**		**0.6337**	**3.2669**	
E. hormaechei			0.6337		
Enterobacter sp.	2.6193			3.2669	
***Herbaspirillum* sp.**					**0.0797**
***Kitasatospora* sp.**					**1.1952**
Klebsiella pneumoniae				**0.1594**	
***Leifsonia* sp.**	**3.1263**		**1.8589**		
Lysinibacillus sphaericus					**0.2390**
***Ochrobactrum* sp.**				**0.9562**	
Paenibacillus assamensis			**0.0422**		
Pantoea	**9.8859**	**1.1407**			
P. agglomerans	1.2252				
P. dispersa	8.6608				
Pantoea sp.		1.1407			
Pseudomonas	**1.8166**	**8.7452**	**1.0984**		**0.0797**
P. denitrificans					0.0797
P. plecoglossicida		8.7452	1.0984		
P. psychrotolerans	1.8166				
***Ralstonia* sp.**		**0.5492**			**0.0797**
***Rhodococcus* sp.**					**0.2390**

Table 1. Contd.

***Serratia* sp.**					**2.4701**
Sinomonas atrocyanea					**2.3904**
Stenotrophomonas				**0.0797**	**0.1594**
S. acidaminiphila					0.1594
Stenotrophomonas sp.				0.0797	
***Streptococcus* sp.**	**4.1403**				
***Streptomyces* sp.**			**1.3519**		**0.7171**
Variovorax paradoxus					**1.5936**
Fungi					
***Arthrininum* sp.**					**0.3984**
Aspergillus	**0.1267**	**0.0422**		**1.5936**	**1.0359**
A. aculeatus		0.0422		1.5936	0.7968
A. flavipes					0.0797
A. nomius					0.1594
Aspergillus sp.	0.1267				
Candida	**1.5632**	**19.9831**	**2.2814**	**8.1275**	
C. carpophila	0.2535	6.8441		8.1275	
C. tropicalis	1.3097	13.1390	2.2814		
***Chaetomium* sp.**					**0.1594**
***Cladophialophora* sp.**					**0.1594**
Clonostachys candelabrum					**0.3187**
Cryptococcus	**6.8019**		**1.6054**		**2.6295**
Cryptococcus aff taibaiensis	6.8019				
Cryptococcus podzolicus			1.6054		2.6295
Cunninghamella bainieri				**0.9562**	
Eupenicillium javanicum					**0.0797**
Exophiala alcalophila			**0.4647**		
Fusarium		**0.0845**			**0.3984**
F. solani					0.3984
Fusarium sp.		0.0845			
Gibberella fujikuroi				**4.0637**	
Gliocladium cibotii					**0.1594**
***Meira* sp.**	**0.2957**				
Neodeightonia subglobosa					**12.5100**
***Nirograna* sp.**					**0.7171**
Penicillium		**1.9434**	**3.3376**		**6.6135**
P. citreonigrum		0.3802			0.0797
P. citrinum	0.8450	1.4787			0.7171
P. coffeae					0.0797
P. daleae					0.3984
P. oxalicum		0.0845	0.0845		
P. radicum					0.3187
P. rapidoviride					0.3187
P. simplicissimum			2.7038	4.8606	3.7450
P. soppii					0.1594
P. verruculosum			0.5492		0.7171
Penicillium sp.					0.0797
***Pestalotiopsis* sp.**		**0.2112**			
Phialemonium curvatum					**0.0797**
***Phialocephala* sp.**					**0.1594**
***Pseudallescheria* sp.**					**0.0797**

Table 1. Contd.

***Pseudozyma* sp.**	**0.4225**		
Purpureocillium lilacinum	**0.1690**		**0.3984**
Rhodosporidium sphaerocarpum	**1.8166**		
Rhodotorula mucilaginosa		**7.4900**	
Trichoderma/Hypocrea	**1.6054**		**4.3825**
T. erinaceum			3.2669
T. helicum			0.6375
T. koningiopsis			0.3187
T. lixii	0.2535		0.0797
T. virens	1.3519		0.0797
***Triplosphaeria* sp.**			**0.0797**

Relative abundances are presented as proportions. Braffedon isolates are shown as light gray shaded rows. Yaokro isolates are shown as dark gray shaded rows. Commonly found bacterial and fungal isolates in Yaokro and Braffedon are shown as not shaded rows.

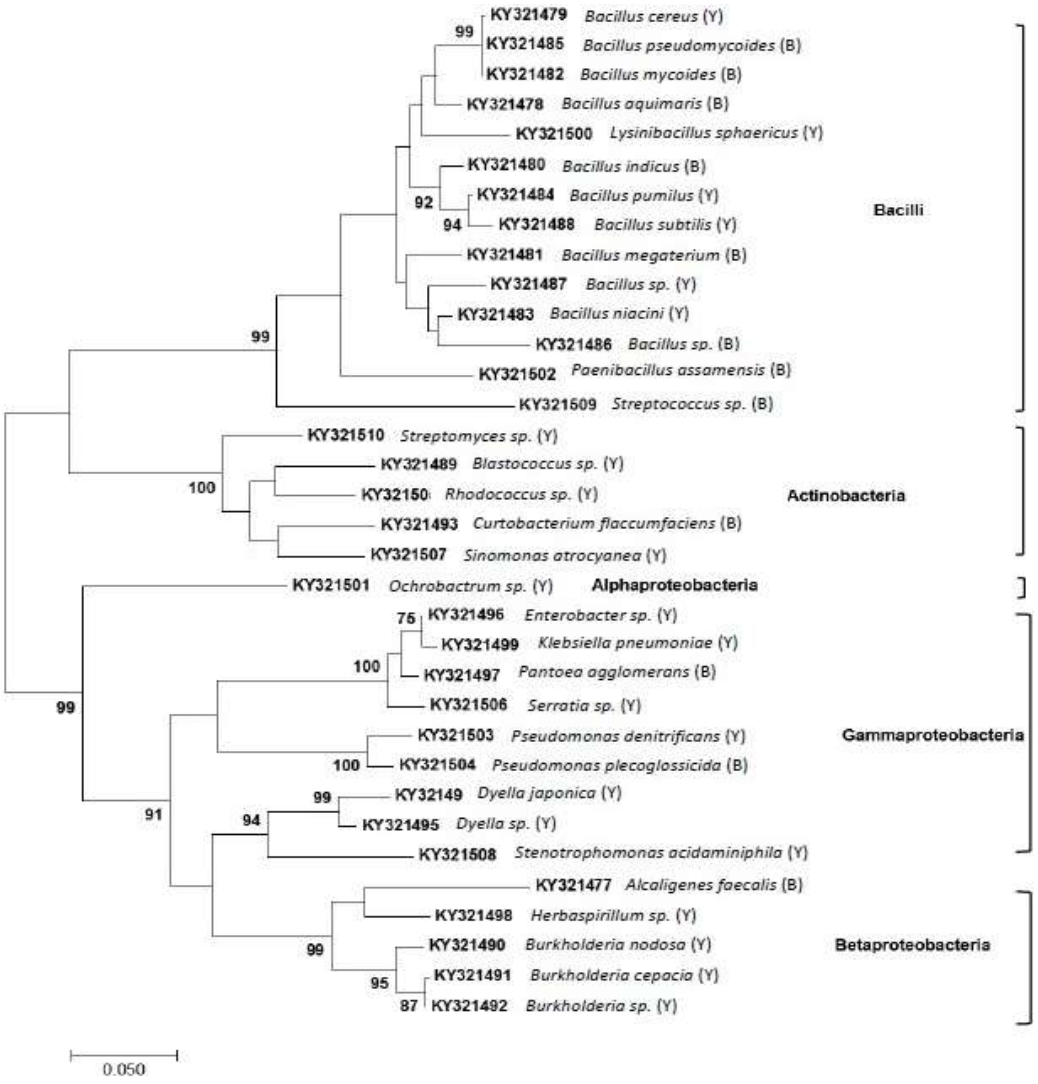

Figure 2. Phylogenetic analysis based on the16S rRNA gene sequences of the bacterial isolates. Branches are grouped by classes. Bootstrap percentages from 1000 replicates > 75 are shown above branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. (B): Braffedon. (Y): Yaokro.

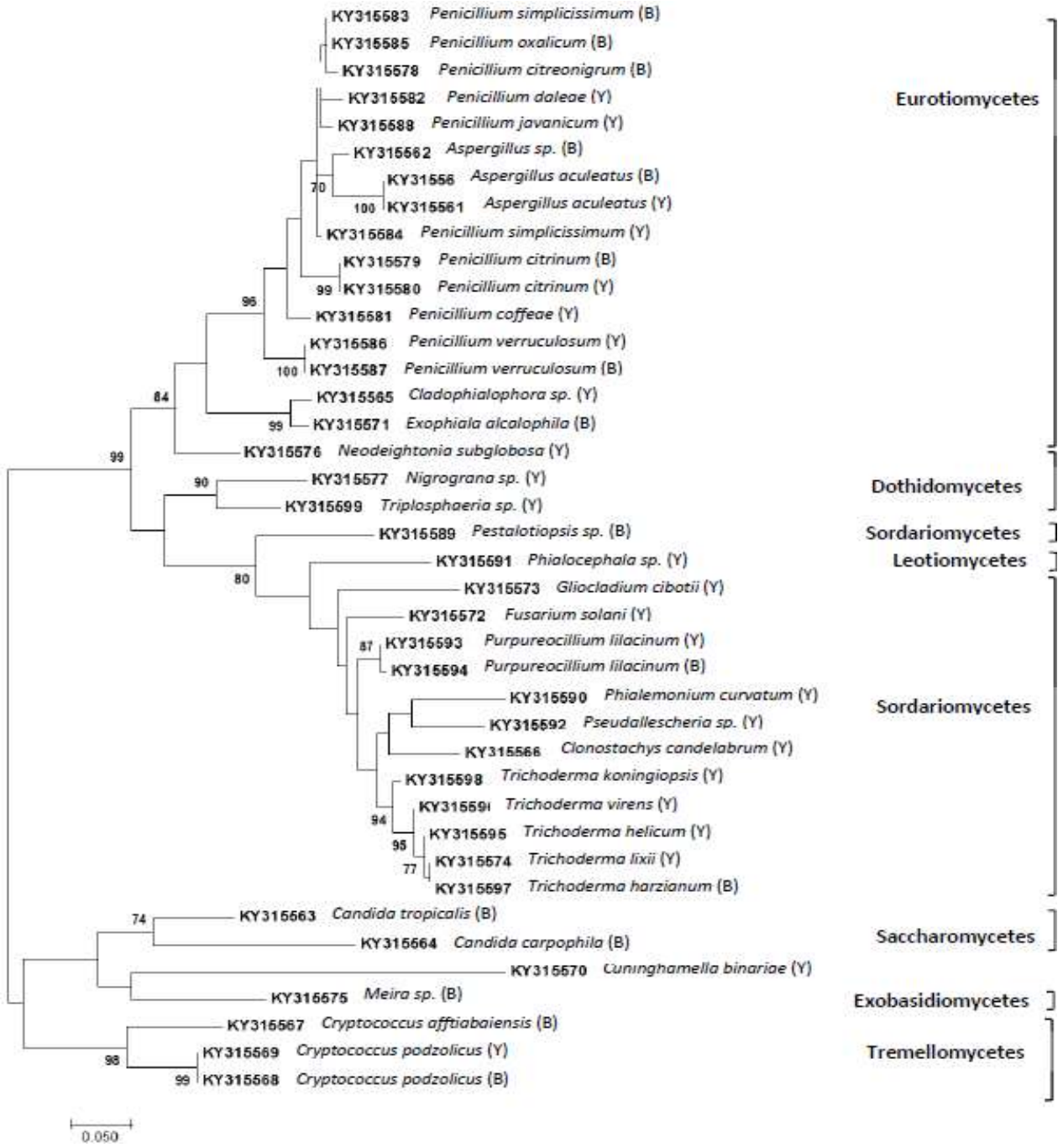

Figure 3. Phylogenetic analysis based on the ITS region of the rRNA gene sequences of the fungal isolates. Branches are grouped by classes. The percentage of trees (based on 1000 replicates) in which the associated taxa clustered together is shown above branches > 75. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. B: Braffedon. Y: Yaokro.

abundant genera in Braffedon were *Candida* (20%) followed by *Pseudomonas* (8.7%) then *Penicillium* (1.9%); and in Yaokro, *Candida* (8.1%) followed by *Rhodotorula* (7.5%) then *Penicillium* (4.8%). No bacteria were commonly isolated from trunk samples in Yaokro and Braffedon. However the opposite occurred for fungal isolates, since commonly fungal genera identified from trunk samples in both locations included *Aspergillus* and *Candida.* The genus *Fusarium* was limited to trunk samples from Braffedon symptomless palms, while *Klebsiella* and *Candida* were only found in trunk samples from symptomless palms from Yaokro.

Table 2. Diversity of microbial communities associated with geolocation of coconut plantations in Yaokro and Braffedon.

Isolate	Shannon H		Simpson D	
	Braffedon	Yaokro	Braffedon	Yaokro
Bacteria	2.7085[a]	2.4027[b]	0.091545[a]	0.147[b]
Fungi	2.0339[a]	2.5142[b]	0.20588[a]	0.11985[b]
Bacteria + fungi	3.0975[a]	3.1489[a]	0.068594[a]	0.066162[a]

[a,b]Different letters indicate significant differences between geolocations at $p < 0.05$ level with t test.

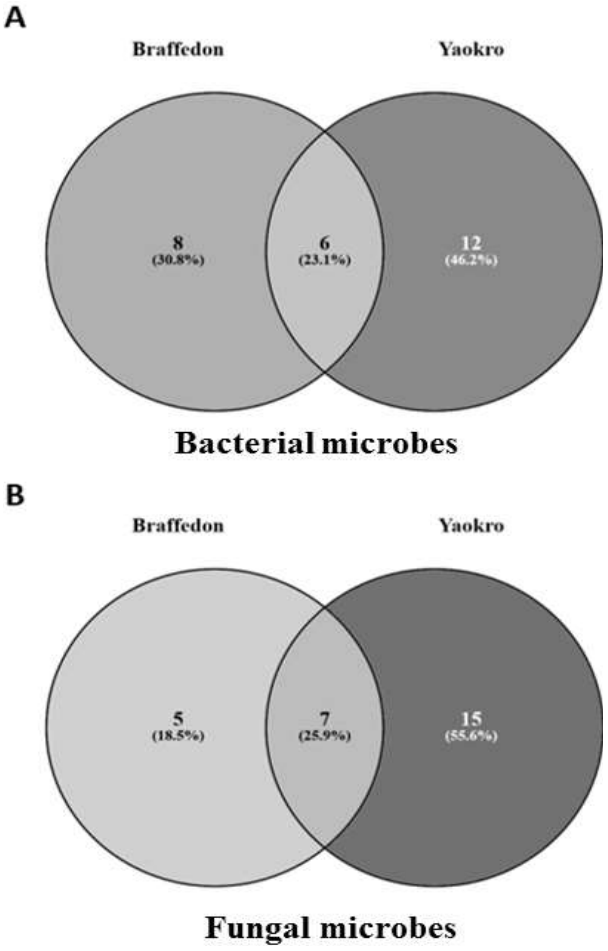

Figure 4. Venn diagram of the bacterial and fungal genera from Braffedon and Yaokro. **A.** Comparison of bacterial microbes either commonly for both geolocations or only in Braffedon or Yaokro. **B.** Comparison of fungal microbes either commonly for both geolocations or only in Braffedon or Yaokro. Venn diagram was done using VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

DISCUSSION

The diversity of bacterial and fungal communities in coconut palms infected and not infected by the CILY phytoplasma was investigated by coupling a high-throughput culturing method ('dilution-to-extinction') and sequence analysis, in order to increase the range of diversity explored in each sample. The 'dilution-to-extinction' cultivation methodology allows for a better recovery of species since it eliminates interaction between colonies, which supports the findings of bacterial and fungal species that seem to be limited to either Braffedon or Yaokro village.

It is noteworthy that no CILY phytoplasma was detected after PCR testing with the specific primers G813/AwkaSR in any of the six coconut palms collected in Braffedon exhibiting symptoms resembling those of CILY. No phytoplasma DNA was neither detected with the phytoplasma generic primers U5/U3. It is known that phytoplasmas are present at very low concentrations in the phloem of plants they infect (Maejima et al., 2014), so this may have prevented phytoplasma detection in the coconut palms. However, the partial 16S rDNA sequence recovered from P1/P7 amplifications from four out of six symptomatic palms confirmed the presence of *Bacillus megaterium*. Previous studies have confirm the amplification of bacterial DNA particularly *Bacillus* species with primers P1/P7 due to their lack of specificity (Harrison et al., 2002; Yankey et al., 2014). Harrison et al. (2002) reported the detection of *B megaterium* from Canary Island date palms infected with the lethal decline phytoplasma. Moreover, PCR and sequencing results from our study were supported by the isolation of *B. megaterium* (4.7%, Table 1) from the endophytic bacterial community of coconut palm leaves in the Braffedon village, not found in Yaokro.

The fact that no phytoplasma DNA was detected from the symptomatic palms in Braffedon with the second phytoplasma generic primer pair U5/U3 ruled out the possibility of any detectable phytoplasma in those palms. Instead, *B. megaterium* was detected in the coconut palm phloem and amplified with universal primers P1/P7, hence, the probably higher titre of *B. megaterium* may have prevented any phytoplasma to be detected, if any. CILY-like signs seen in the coconut palms surveyed may be related to other factors, for instance, environmental. Sampling in Braffedon was conducted during April 2016 within the dry season, which fell within a period of a very severe drought during that season in early 2016. This was registered by SODEXAM (*Société d'Exploitation*

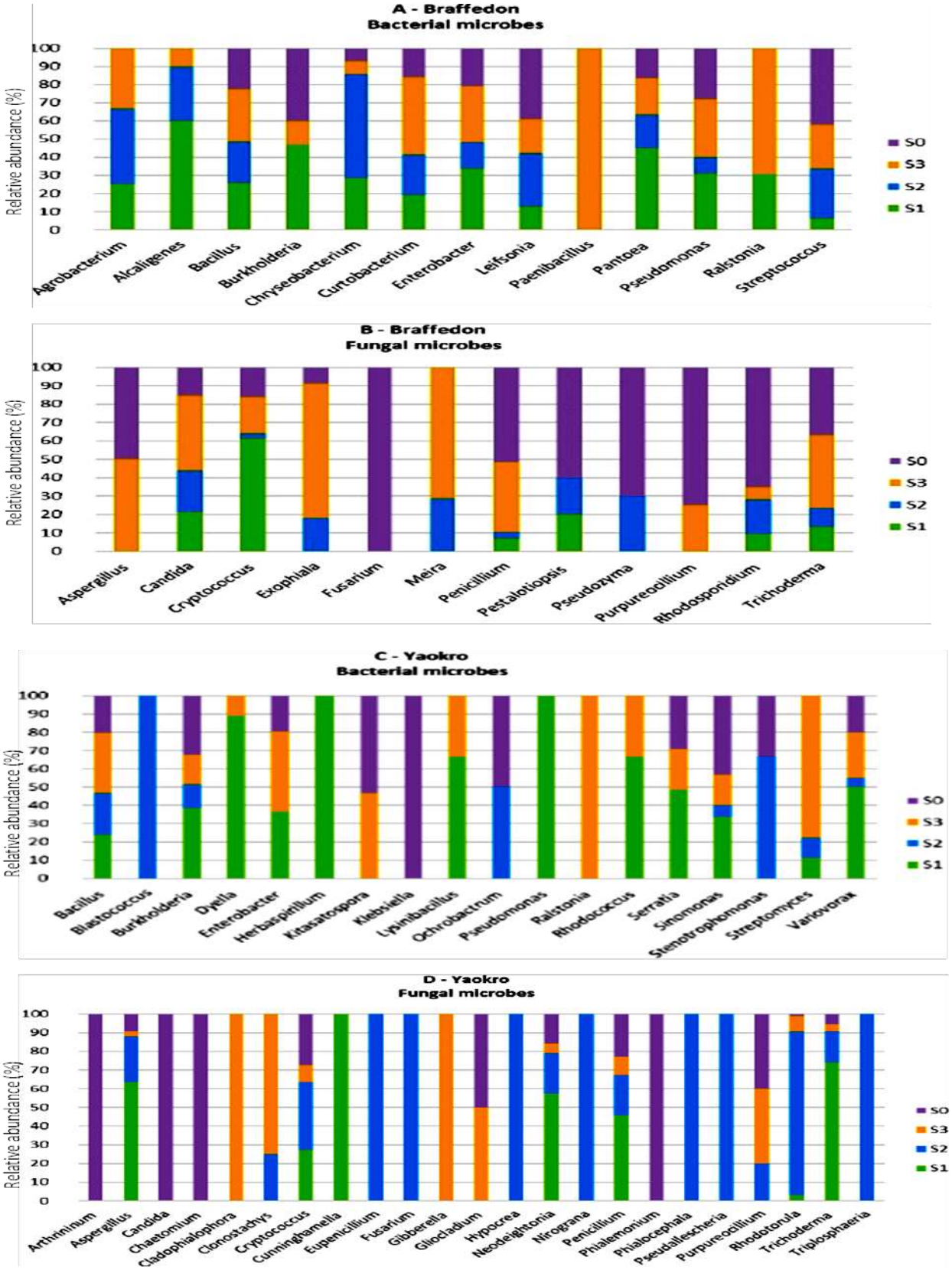

Figure 5. Relative abundance of bacterial and fungal isolates per disease stage S1, S2, S3 and in symptomless palms (S0). Braffedon bacterial (A) and fungal (B), and Yaokro bacterial (C) and fungal (D) genera are grouped according to the disease stages (S1, S2, S3) from where they were isolated.

et de Devéloppement aéroportuaire, Aéronautique et Météorologique) in Côte d'Ivoire (Diallo, personal communication), so the drought may have had an impact on the agronomic performance of those six coconut palms in Braffedon.

No significant differences were observed in the overall diversity levels for fungal and bacterial organisms regardless the origin (leaves, trunk or rhizosphere) between Braffedon and Yaokro (Table 2). However, when bacterial and fungal communities were screened separately per location, Yaokro scored a higher number of both bacterial (13) and fungal (15) species limited to the location when compared to Braffedon. Since the CILY phytoplasma was detected in all symptomatic coconut palms from Yaokro, this suggests that the presence of the CILY phytoplasma in the symptomatic coconut palms may be a factor determining the level of diversity of a microbial community in that given geolocation. Bulgari et al. (2014) confirmed that indeed the endophytic bacterial community composition in grapevine is correlated to phytoplasma infection from studies of the endophytic bacterial community in grapevines healthy and infected with the "flavescence dorée" phytoplasma.

Burkholderia (18.7%) and *Neodeightonia* (12.5%) were the most abundant bacteria identified from the rhizosphere in Yaokro. Species of *Burkholderia* included *B. cepacia* and *B. nodosa,* known as plant growth promoters, nitrogen-fixing enhancers, and siderophore producers have been used for soil bioremediation for better water management, and biocontrol of soil-borne plant pathogenic fungi (de los Santos-Villalobos et al., 2012; Parke, 2000) and bacteria (Nion and Toyota, 2008). Although it is not clear its role, the fact that *Burkholderia* was mostly isolated from symptomless and S1-like symptom bearing palms suggests it as a candidate to further assess as a possible biocontrol against the CILY phytoplasma.

The plant-growth promoter *Enterobacter* was isolated from rhizosphere only from Braffedon, while *Penicillium* and *Trichoderma* were identified from Yaokro. In all cases these genera were present in both symptomless and symptomatic palms. *Enterobacter* has been isolated from the rhizosphere of coconut palms in India and proposed as a candidate for further biofertilizer development (Gupta et al., 2014); while *Penicillium* and *Trichoderma* have been combined and used as biocontrol against Ganoderma wilt of coconut (Srinivasulu et al., 2001). Since these genera have been tested in coconut palms, further investigation may help clarifying their possible role as potential bioinoculant or biofertilizer to mitigate CILY.

There are no records of *Neodeightonia* as biocontrol. This genus was isolated from symptomless coconut palms, as well as palms with S1-, S2-, and S3-like symptoms; however, it may be more directly associated to the early stages of the CILY phytoplasma colonization since it was mostly isolated from palms exhibiting S1-like symptoms. *Bacillus* (9.9%) was the most abundant genus isolated from the rhizosphere in Braffedon. In India, *Bacillus* sp. have been isolated from coconut rhizosphere and proved to have antagonistic activity against coconut fungal pathogens (George et al., 2011). *Bacillus* sp. have been also proven as plant growth promoting rhizobacteria to be used as effective bioinoculants to support coconut organic farming (Geetanjali et al., 2015; George et al., 2012). Coconut palms in Braffedon showed signs that although not associated with the CILY phytoplasma, may be related to drought exposure, so more investigation could support a possible role of *Bacillus* sp. in enhancing coconut palms resilience to this environmental constraint.

Arthrinium, *Chaetomium* and *Phialemonium* were solely isolated from the rhizosphere of symptomless coconut palms in Yaokro. *Chaetomium* and *Phialemonium* have been long used as biocontrols of several fungal diseases (Hung et al., 2015; Shanthiyaa et al., 2013; Hiratsuka and Chakravarty, 1999), while *Arthrinium* has been recommended as a potential biocontrol of the bayoud disease in palm trees (Calvo et al., 2005). The fact that specific fungal organisms were limited to the rizosphere of symptomless palms in Yaokro raises many expectations for the possibility of assessing these for their biocontrol potential against the CILY phytoplasma.

Previous studies have shown that the endophytic species composition and frequency vary with different tissues of host plants. In fact, Petrini et al. (1993) suggested that plant organs resemble distinct microhabitats for endophyte colonization. Therefore, endophytic species richness and composition within a tree species will always differ among individual trees, even within homogenous investigation sites (Collado et al., 2007; Petrini et al., 1993). The results of the present study are in consonance with these observations; hence, every palm at a given location is at different risk of CILY, compared to its neighbouring palms.

The occurrence of bacterial and fungal endophytes identified in either Braffedon or Yaokro varies upon the microhabitat and location. For instance, *B. megaterium* was restricted to leaves in Braffedon, while *B. pumilus* and *B. subtilis* were only isolated from trunk samples from Yaokro; *Enterobacter* was found in trunks from Yaokro, and in leaves in Braffedon, while *Curtobacterium, Pantoea* and *Streptococcus* were limited to leaf samples from Braffedon. For fungal species, *Aspergillus* and *Candida* were found in trunks and leaves in Braffedon, and in trunk in Yaokro; *Pestalotiopsis, Pseudozyma* and *Fusarium* in trunks from Braffedon, and *Meira* sp. and *Cryptococcus* sp. from leaves in Braffedon. *Pantoea agglomerans, Curtobacterium, Bacillus, Burkholderia*, *Pestalotiopsis* have been recorded as biocontrol agents against a broad spectrum of plant pathogens, or part of endophytic communities in a number of crops (Bulgari et al., 2011; Grisan et al., 2011). The above mentioned observations support the fact that apart from the nature of

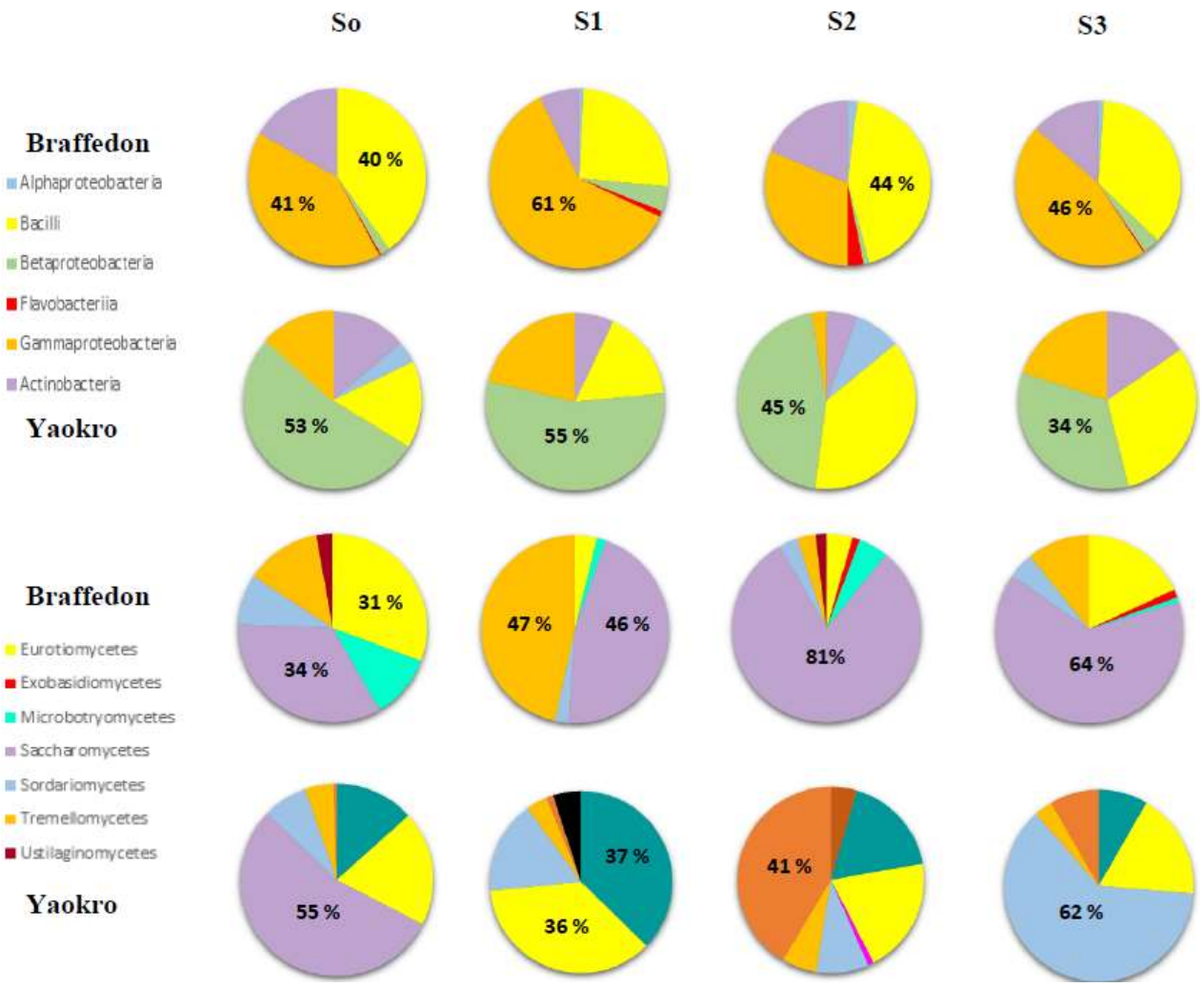

Figure 6. Relative abundance of isolated bacterial and fungal classes from Braffedon and Yaokro.. S0, Symptomless palms. Coconut palms with CILY-like symptoms; S1, Disease stage 1; S2, Disease stage 2; S3, Disease stage 3.

the host tissue, the geographic location may influence the host composition of the microbial community, including the endophytic population (Suryanarayanan and Vijaykrishna, 2001; Petrini et al., 1993), aspects that would worth be further investigated.

Klebsiella pneumoniae and *Candida* were solely found in trunk samples corresponding to symptomless coconut palms in Yaokro. There are records of *K. pneumoniae* as part of endophytic communities such as that from soybean (Kuklinsky- Sobral et al., 2005), but not yet from coconut palms. Interestingly, Tagliavia et al. (2014) recently identified *K. pneumoniae* as the major bacterial microbe within the gut microbiota of *Rhyncophorus ferrugineus*, the red palm weevil, one of the major pests of palms, including coconut. *Candida* is a known antagonistic agent used as biological control of postharvest diseases, available as commercial products such as Aspire and Yield-Plus (Sui et al., 2015). The fact that *K. pneumoniae* and *Candida* are only present in symptomless palms in Yaokro opens up expectations of their possible potential as biocontrol against the CILY phytoplasma, which should be further studied. On the other hand, *Fusarium* was limited to trunk samples from symptomless palms in Braffedon, which may make it a good candidate as bioinoculant to boost plant resilience against drought.

As previously reported, *Proteobacteria* is the most abundant phylum identified in plants (Bulgari et al., 2012). From our study, the dominant bacterial classes identified in Yaokro from all the disease stages, including the symptomless palms were *Betaproteobacteria* followed by *Bacilli*, while in Braffedon the dominant bacterial classes were *Gammaproteobacteria* followed by *Bacilli* (Figure 6). These results coincide with those from Bulgari et al. (2012), where *Betaproteobacteria* was the dominant bacterial class isolated from apple tree roots, healthy and infected with '*Ca*. P. mali'.

The dominant fungal classes in Yaokro corresponded

to *Saccharomycetes* for the symptomless palms and *Dothiomycetes, Urediniomycetes* and *Sordariomycetes*; whereas in Braffedon, the fungal dominant class was *Saccharomycetes.* Results show that there is more diversity for the fungal endophytic communities in CILY phytoplasma-infected coconut palms, which suggests that the presence of the CILY phytoplasma may influence the composition of the fungal endophytic community. Furthermore, the 'dilution-to-extinction' cultivation method proved to be a reliable high-throughput method for isolation of both endophytes and rhizosphere-limited species from either Braffedon or Yaokro villages.

Since the endophytic control is one of the desirable sustainable approaches for the possible control of phytoplasma diseases, bacterial and fungal endophytes identified in the present study from coconut palms from either Braffedon or Yaokro should be further investigated to assess their biocontrol potential against the CILY phytoplasma. Most of the bacterial and fungal endophytes isolated from coconut palms from Braffedon and Yaokro have been already reported as potential biocontrols for a number of bacterial, fungal and phytoplasma diseases around the world. These are so far the first results that refer to the rhizosphere, as well as the bacterial and fungal endophytic populations isolated through a high-throughput cultivation method, 'dilution-to-extinction' from coconut palms symptomless and with CILY-like symptoms, as well as, infected and non-infected with the CILY phytoplasma.

The study offers a baseline for future research on culturable endophytic or rhizosphere microbial communities that may be used as future biocontrols, or bioinoculants to enhance plant resilience against CILY or environmental constraints like drought in Côte d'Ivoire. At this stage it may be too early to recommend which bacterial or fungal species could be effectively used to tackle CILY in Côte d'Ivoire without having further evidences on the assessment of their role as potential biocontrols. Further testing should be done to explore the richness of the endophytic microbial population profiles, and to further clarify the possible environmental and physiological factors that may govern their occurrence in both Braffedon and Yaokro. Once more data and research evidence become available, the present research outcomes can be used as groundwork to help designing new strategies for the effective management of CILY in Grand-Lahou.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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Generating virus-free cassava plants by *in vitro* propagation with chemical and heat treatment

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Cassava production in eastern and Central Africa is severely threatened by the current epidemic of cassava brown streak disease (CBSD). The disease is caused by cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV) of the genus *Ipomovirus*, family Potyviridae. Using virus-free planting material is one effective way to reduce CBSD yield losses in farmer fields. The effects of chemical and heat treatment on tissue cultured plants was investigated to eliminate CBSV and UCBSV infections in three cassava varieties with differing levels of resistance to CBSD. Cassava node buds taken from young shoots of the varieties Kaleso (CBSD resistant), Kiroba (tolerant) and Albert (susceptible) infected with CBSV and UCBSV were grown *in vitro* in standard Murashige and Skoog tissue culture media, which alone eliminated viruses from up to 30% of the plants. To increase the number of virus-free plants, two-week old virus-infected *in vitro* plants were exposed to constant temperature regimes of 30, 35, 40 or 45°C for three weeks. Heat treatment at 40°C had the optimum effect as up to 50% virus-free plants were obtained for Kiroba; however, incubation at 45°C was lethal to all three varieties while the lower temperatures produced fewer virus-free plants. Chemical treatment of *in vitro* plants was performed using the antiviral chemical ribavirin at various concentrations. Ribavirin at 0.10 mM generated up to 40% virus-free plants in all three varieties but concentrations of 0.21 mM were lethal. These findings indicate important methods to produce virus-free planting material; their use by breeders and in the field, will help develop effective control strategies for CBSD management.

Key words: Africa, cassava, chemical and heat treatment, viruses, tissue culture, disease resistance.

INTRODUCTION

Cassava productivity in sub-Saharan Africa (SSA) was estimated at around 9.2 tons per hectare compared to the world average of 12.3 tons for 2014 (FAOSTAT, 2015). The low African cassava yields are attributed to pests and diseases, among which cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) are the most prominent (Hillocks et al., 2015; Legg et al., 2011, 2015; Mohammed et al., 2016; Patil et al., 2015). Epidemics of one or both these diseases have occurred over the last two decades and have severely affected

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cassava production and threatened livelihoods and food security in eastern and central African countries (Legg et al., 2015). Susceptible plants infected as cuttings are severely stunted and produce poor yields.

Since the 1930s, CBSD has been endemic in areas along the Indian Ocean coast of eastern Africa, from the north-eastern border of Kenya across the Tanzanian border down as far as the Zambezi River in Mozambique; it was also widespread along the shores of Lake Malawi. In 2004, however, an outbreak of CBSD was reported in Uganda which has now spread into neighbouring countries in the Great Lakes region of East and central Africa, including Burundi, Rwanda and the eastern part of the Democratic Republic of Congo (Hillocks and Jennings, 2003; Alicai et al., 2007; Legg et al., 2011; Mulimbi et al., 2012). This is of great concern because incidence of CBSD up to 100% in cropping areas has been recorded, and in sensitive varieties the disease causes rotting of the tuberous roots, reducing both the quality and quantity. Recent estimates indicate that CBSD causes economic losses of up to US$726 million annually for African farmers (Maruthi et al., unpublished data). The disease is the most important cause of food insecurity in the coastal and lake zone areas of eastern Africa, and has been a serious threat to the entire cassava-growing belt of SSA (Legg et al., 2014). CBSD is caused by two RNA viral species; cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV) belonging to the family Potyviridae of the genus *Ipomovirus* (Mbanzibwa et al., 2009; Monger et al., 2010; Winter et al., 2010), and are collectively referred to as cassava brown streak ipomoviruses (CBSIs).

CMD has been managed through the development and dissemination of virus-resistant varieties. Progress in managing CBSD, however, has been slower. A small number of varieties expressing a range of resistance levels are currently available in CBSD affected countries but they can still be infected with CBSIs. To-date, this represents the best solution available for CBSD control. Recent findings about CBSD epidemiology, however, have provided new opportunities for control. These include the finding that CBSIs are not retained for periods of more than 48 h in whitefly vectors and that an isolation distance of about 100 m is sufficient to notably minimize the spread of CBSIs between infected and disease-free plots (Maruthi et al., unpublished data). Furthermore, it has been found that tolerant cassava varieties support low viral titres and cuttings from them have a higher recovery from CBSD compared to susceptible cultivars (Maruthi et al., 2014; Mohammed et al., 2016). These results provide a strong indication that phytosanitary measures, involving the use of virus-free planting material coupled with isolation from surrounding potential sources of infection, offer excellent potential for CBSD control. Meristem culture and heat treatment have been used with considerable success to eliminate CBSD in the past (Kaiser and Teemba, 1979; Wasswa et al., 2010).

The study therefore aimed to use tissue culture and chemical and thermal treatments to eliminate CBSIs infections from cassava varieties with different levels of field resistance, with the aim of identifying optimum conditions for effective treatment. The three therapies were compared for their efficiency on plant regeneration as well as for the elimination of viruses.

MATERIALS AND METHODS

Cassava varieties and virus isolates

Healthy cassava plants of the varieties Kaleso (CBSD resistant), Kiroba (tolerant) and Albert (susceptible) were graft-inoculated with either CBSV or UCBSV (Mohammed et al., 2012) and infection was confirmed by reverse transcription polymerase chain reaction (RT-PCR; Abarshi et al., 2010, 2012; Otti et al., 2016). The two viral isolates were collected as stem cuttings from farm fields in Uganda and Mozambique, respectively (Mohammed et al., 2012; Maruthi et al., 2014).

Media preparation for cassava tissue culturing

The tissue culture method of Frison (1994) was optimised and used in this study. Basal medium, 2.2 g Murashige and Skoog (1962) (MS; Sigma, UK), 20 g of sucrose and 2 ml of plant preservative mixture (PPM) were dissolved in about 800 ml of single distilled water. PPM is a broad-based effective pesticide against bacteria and fungi. Fifty micro litres of the growth regulator 1-Naptherlenacetic acid (NAA) was added to enhance rooting. 2 g of phytagel (Sigma, UK) was then added to the solution and the volume adjusted to 1 litre and pH 5.8. The medium was boiled, and 10 ml was dispensed into 25 ml glass tubes (Sterlin, UK). Tubes were closed with plastic caps and sterilized by autoclaving.

Surface sterilization and inoculation of nodal buds into the media

Single node cuttings from young stems were excised from each of the three cassava varieties. The nodes were washed with tap water and pre-sterilized with 70% ethanol for 3 to 5 s. The nodes were then transferred into 10% (v/v) bleach solution of sodium hypochlorite containing 2-drops of Tween-20, and sterilized by vigorous shaking for 30 min in glass jars. The nodes were subsequently washed in sterile distilled water (SDW) 3 to 4 times until no foam was left in the jar. Using sterile conditions, nodes were transferred into sterile tubes containing MS basal medium. The tubes were covered with sterile plastic lids, labeled and incubated in a tissue culture control growth room for 4 to 6 weeks at 25 ± 3°C, RH 60% and 14:10 h of light and darkness (L14:D10).

Transplanting and hardening of *in vitro* cassava plants

Following the growth period above, *in vitro* plants were gently removed from glass tubes and residual medium was washed away with running tap water. Older and middle leaves were then removed from the plants to prevent excessive moisture loss through transpiration but, the upper 3 to 4 leaves were retained. Plantlets were rinsed in a fungicide solution of dithane (6 g/l) (Mancozeb 80% a.i.) for 10 min to prevent fungal infection. They were then planted in small pots (10 cm diameter) with 1:1 compost and soil, and grown at 28 ± 5°C, 50 to 60% relative humidity (RH), 14:10 light:

dark hours under propagator lids. Pots were soaked with a *Bacillus thuringiensis* (Bt)-based biological insecticide Gnat-Off (Hydro gardens, UK) at a dilution rate of 1 ml/l of water following manufacturer's instructions for the control of fungus gnat insect (black flies). High humidity was created by spraying water inside the lids and the vents remained closed for two weeks. The vents were then opened for one week to acclimatize the plants to ambient humidity, and the lids were gradually lifted to further harden the plants. Lids were taken off completely by the end of four weeks and 1 g/L of NPK fertilizer with Mg 30 + 10 + 10 + 2 was applied (Sinclair Ltd., UK). Plants were hardened-off by gradually reducing the amount of water provided over the next four weeks.

Effect of stem position and varieties on virus elimination

Nodes from each plant were numbered 1 to 10 from top to bottom and classified into two categories; top (node numbers 1 to 5) and bottom (node numbers 6 to 10), and grown *in vitro* as described previously. Fifteen nodes generated from each stem position from the three different cassava varieties, Albert, Kiroba and Kaleso were planted in MS media in three replications. Regenerated plants were grown and scored for CBSD symptoms. Plants that remained symptomless for six months were tested for the presence or absence of CBSV and UCBSV by RT-PCR using virus-specific primers (Abarshi et al., 2010, 2012).

Effect of thermal treatment on virus elimination

Ten node cuttings each from UCBSV-or CBSV-infected plants of the three varieties were excised (~0.4 mm size), and grown in tubes containing MS media. The plantlets were then kept in incubators (Leec, UK) under different temperature regimes of 30, 35, 40 and 45°C for three weeks at L14:D10 h. Plantlet survival was recorded from each temperature regime and for each variety-virus combination. The plantlets were then transferred into a tissue culture growth room for stabilization for 1 week at 25 ± 3°C, RH 60% and L14:D10 h. They were then grown in soil and compost as described above. Presence or absence of CBSD symptoms was recorded monthly by visual observation and tested for viruses using RT-PCR. The experiment was repeated three times using 10 nodal cuttings for each variety-virus combination and the four temperature regimes. Twenty healthy nodal cuttings from each variety for each treatment served as the control.

Effect of chemical treatment on virus elimination

The antiviral chemical ribavirin (1,ß-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide; Sigma R9644), supplied in powder form, was tested for its efficiency in the elimination of UCBSV and CBSV from the three cassava varieties. Extreme care was taken when handling ribavirin due to its toxicity and broad spectrum of anti-viral activities (Dawson, 1984; Fletcher et al., 1998). Ribavirin treatment was carried out at three concentrations: 15 mg/l (0.06 mM), 25 mg/l (0.1 mM) and 50 mg/l (0.21 mM) in MS medium on nodes from five plants of the three cassava varieties infected with UCBSV or CBSV. The experiment was repeated three times using 50 nodes for each variety-virus combination and for three ribavirin concentrations. Twenty healthy nodal cuttings from each variety for each treatment served as the control.

Combined application of the therapies

Combinations of chemical and heat treatments were carried out on tissue-cultured plants of the three cassava varieties for UCBSV and CBSV elimination. Thirty nodes per variety were transferred into glass tubes containing the medium supplemented with ribavirin at 0.1 mM concentration. The plantlets were kept in the incubator (Leec, UK) at 40°C for three weeks with L14:D10 h. Plantlet survival was recorded from each variety-virus combination. After three weeks, the plantlets were removed from the incubator and transferred into a tissue culture growth room for one week and then planted in pots in a quarantine glasshouse. Plants were grown under propagator lids for four weeks. Presence or absence of CBSD symptoms was recorded monthly by visual observation of treated plants. After six months, leaf samples were tested for UCBSV and CBSV by RT-PCR. The experiment was repeated three times. Twenty healthy nodal cuttings from each variety exposed to identical treatments served as the control.

Assessment of treatment efficacy and statistical analysis

The tissue culture plantlets resulting from the trials outlined above were tested for the presence or absence of UCBSV and CBSV six months after growing in soil. The efficiency of the treatment (ET) was determined using the formula described previously (Hormozi-Nejad et al., 2010; Meybodi et al., 2011):

$$\% ET = \frac{\% \text{ plant regenerated} \times \% \text{ virus-free plants}}{100}$$

The ET of tissue culture, heat, chemical- and co-treatments were compared for their efficacy in eliminating UCBSV and CBSV from *in vitro* cassava plants. Experimental data were analysed using the statistical platform R (R Development Core Team, 2011). The proportions of virus-free plants after the treatments were analysed using a generalised linear model with binomial errors with logit link function. Statistical inference was based on resulting analysis of deviance and estimated standard errors.

RESULTS

Cassava nodal bud culture for virus elimination

Elongation of auxiliary buds and the emergence of new leaves were observed two weeks after seeding of the explants into the MS medium, while root formation took three weeks. The node position, cassava variety and virus significantly affected the number of virus-free plants generated (Table 1). Nodes from lower stem positions produced a lower number of virus-free plants for both CBSV and UCBSV (5.1 and 25.3%, respectively) than from upper stem positions (34.3 and 51.4%, respectively). A greater number of virus-free plants were recorded from UCBSV-infected plants than CBSV (Figure 1). Kaleso (18.3 and 51.7%) and Kiroba (26.3 and 44.3%) produced a significantly higher number of virus free plants than Albert (14.6 and 19.0%) from CBSV- and UCBSV-infected plants, respectively (Figure 1). All plants that remained symptom-free after six months were also shown to be virus-free by RT-PCR.

More node buds developed roots and leaves from the lower part (75.4%) compared to the upper part (62.2%) of the plant (Table 2). About 62.6% of the *in vitro* plants grown from nodes taken from the lower plant section

Table 1. Analysis of deviance to investigate the effect of cassava nodes, variety and virus on eliminating cassava brown streak virus and Ugandan cassava brown streak virus from infected cassava plants grown under tissue culture.

Parameter	df	Deviance	Residual df	Residual deviance	P value
Null			58	181.43	
Node	1	44.123	57	137.3	3.084e-11***
Variety	2	22.706	55	114.6	1.173e-05***
Virus	1	26.164	54	88.4	3.136e-07***
Node:Variety	2	0.076	52	88.36	0.96258
Node:Virus	1	4.913	51	83.45	0.02665*
Variety:Virus	2	7.977	49	75.47	0.01853*
Node:Variety:Virus	2	4.330	47	71.14	0.11472

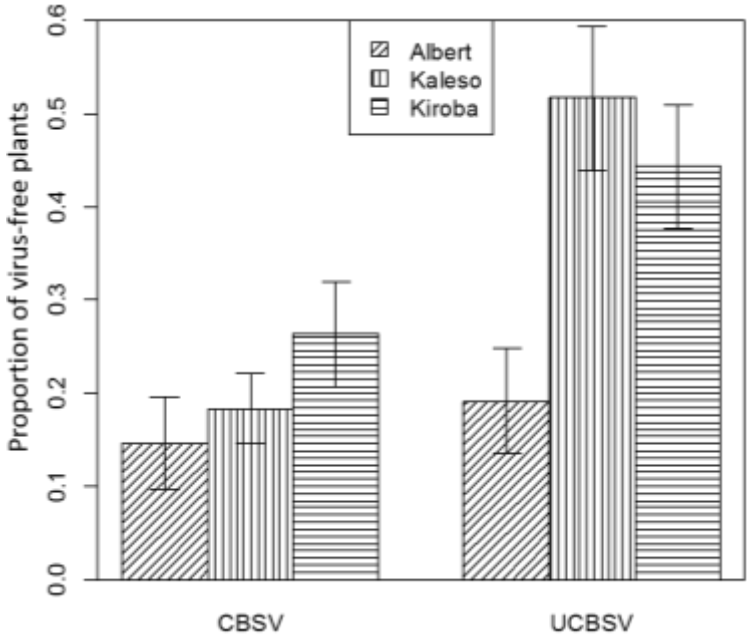

Figure 1. Mean proportion of virus-free plants generated after cassava nodal bud culture from cassava brown streak virus and Ugandan cassava brown streak virus-infected cassava plants.

Table 2. Comparison of the survival of cassava nodes four weeks after growth in the tissue culture MS media and transfer to soil.

Tissue culture plants that survived in both media and after transfer to soil					Successfully grown[c]
Plant part	Contaminated (%)	Dead (%)	Alive but not grown[a] (%)	Grown[b] (%)	(%)
Upper (nodes 1-5)	5.4	24.3	71.3	62.2	54.3
Lower (nodes 6-10)	19.2	5.1	76.8	75.4	71.4

[a]Nodes that were still green or exhibited callus formation were all considered as 'green state'. [b]Growth includes roots, stems and/or leaves. [c]Nodes that developed into fully grown plants after transfer into soil, including those of the control treatment.

(positions 6 to 10) survived after being transferred into soil. This number was reduced to 57.2% for nodes (positions 1 to 5) from the upper plant section. Contamination from bacteria and fungi was also higher for nodes from the lower (19.2%) than upper (5.4%) sections (Table 2).

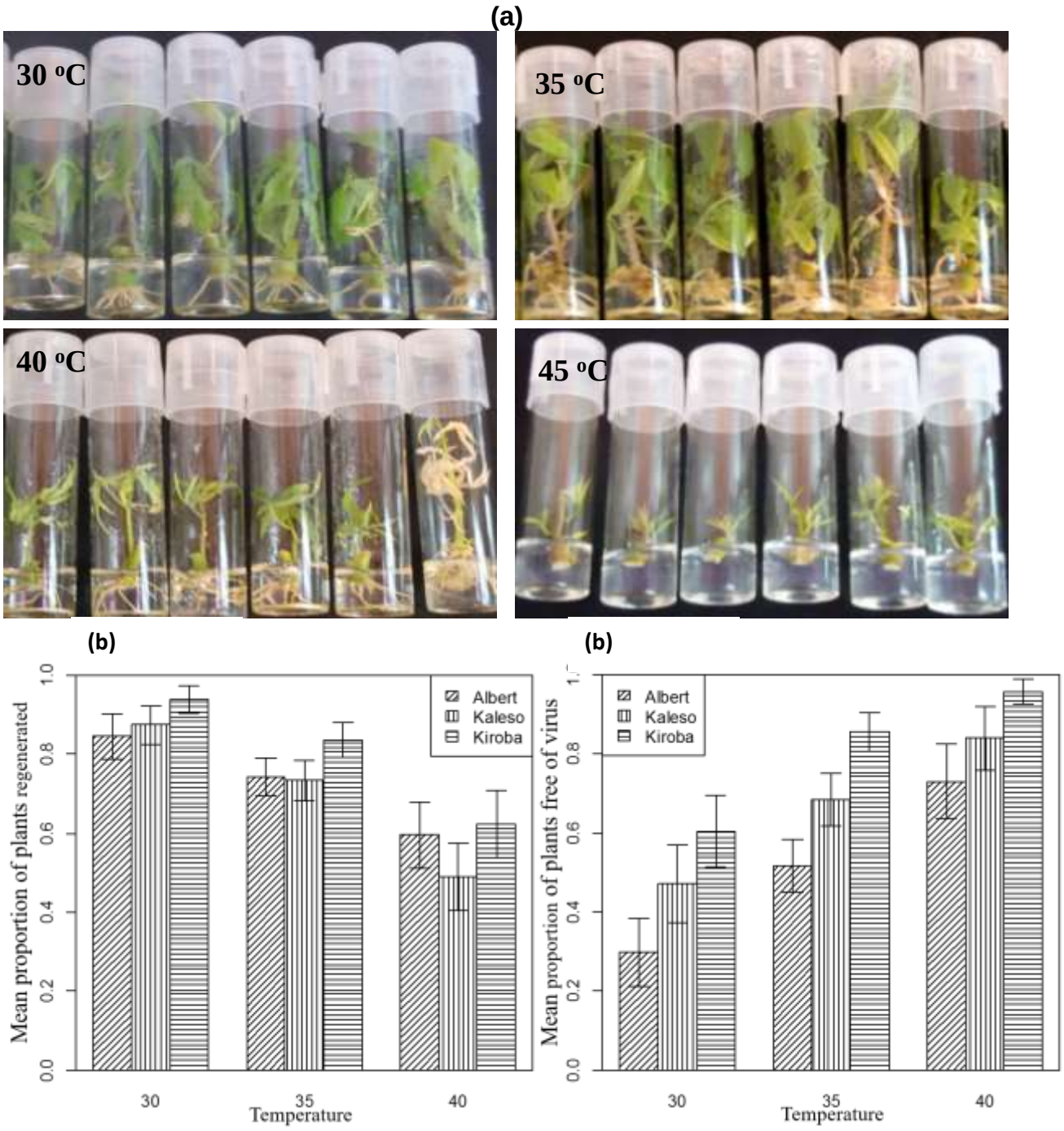

Figure 2. Heat treatment and growth of cassava nodal buds at different temperatures. Data for 45°C not included as all plants in this treatment died.

Heat treatment

Heat treatment significantly affected the number of plants regenerated ($P < 0.001$). Heat stress caused a reduction in the size of leaves and shoots tips at 40°C, and caused plantlet mortality at the highest temperature of 45°C (Figure 2 a and b). Maximum plant growth was achieved at 30°C (88.6%) and 35°C (77.1%) as compared to 40°C (56.9%; Figure 2c).Temperature and cassava varieties had a significant effect on virus elimination (Table 3). The maximum number of virus-free plants was obtained at 35 (68.3%) and 40°C (84.2%) as opposed to 30°C (46.5%). A greater number of virus-free plants were obtained from tolerant Kiroba and Kaleso varieties than the susceptible

Table 3. Analysis of deviance to investigate the effect of temperature, variety and virus on cassava brown streak virus and Ugandan cassava brown streak virus elimination from infected plants following heat treatment.

Parameter	df	Deviance	Residual df	Residual deviance	P value
Null			17	80.93	
Temp	1	39.53	16	41.40	3.226e-10 ***
Variety	2	30.66	14	10.74	2.193e-07 ***
Virus	1	1.36	13	9.37	0.2432
Temp:Variety	2	1.35	11	8.02	0.5083
Temp:Virus	1	0.47	10	7.55	0.4936
Variety:Virus	2	1.65	8	5.89	0.4373
Temp:Variety:Virus	2	1.13	6	4.77	0.5692

Temp = temperature.

Albert (Figure 2c).

Chemical treatment

The phytotoxic effects of ribavirin were observed at 0.21 mM which resulted in severe stunting of plantlets, thin stems, stunted leaflets, sluggish root development (Figure 3a) and finally death of all plantlets of all three cassava varieties. The number of plants generated at 0.1mMribavirin (65.6%) was like that of 0.06 mM (71.0%). However, the development of roots was sluggish (at 0.1 mM) while plantlets regenerated at 0.06 mM were morphologically identical to those regenerated fromthe non-treated control. Ribavirin concentrations and the virus type had a significant effect on the number of virus-free plants generated (Table 4). Ribavirin at 0.1 mM generated a significantly higher (59.6%, P≤ 0.01) number of virus-free plants than 0.06mM (41.1%; Figure 3b). Number of virus-free plants obtained from UCBSV-affected plants were also significantly higher (53.6%; P ≤ 0.05) compared to CBSV (47.2%; Figure 3c).

Combined application of the therapies

Of the 50 nodes inoculated in the tissue culture media supplemented with ribavirin at 0.10 mM and simultaneously exposed to heat treatment at 40°C, a high percentage of plantlets were regenerated from node cuttings and this varied between therapies and varieties (Table 5). For instance, 55.4% Kaleso, 65.6% Kiroba and 58.6% Albert plantlets were regenerated from nodal buds infected with UCBSV/CBSV after tissue culture. Similarly, heat treatment of *in vitro* plants at 35°C resulted in good regeneration of plants (77.1%) and a high generation of virus-free plants. Simultaneous application of the three treatments resulted in decreased regeneration of plantlets in Kiroba (46.6%), Kaleso (41.6%) and Albert (51.6%) for both viruses.

Similarly, dual effects of heat and chemical treatments, applied to *in vitro* cassava plants, were more efficient in eliminating UCBSV and CBSV from the three cassava varieties than either of the single methods. The highest number of virus-free plants was found from UCBSV-infected plants compared to CBSV (Table 5). Tissue culture alone eliminated UCBSV and CBSV from Kaleso (25.2 and 10.3%, respectively), Kiroba (30.0 and 17.1%), and Albert (11.3 and 9.2%). Simultaneous application of the three treatments resulted in an increased elimination of both UCBSV and CBSV. Combining the three treatments resulted in the lowest ET (27.2%) from CBSV and highest (50.0%) from UCBSV on Kaleso (Table 5).

DISCUSSION

Experiments to produce virus-free cassava plants were carried out on node cuttings of CBSD-infected cassava plants of three different cassava varieties by tissue culture, chemical and heat treatments. Regeneration of plants from the lower nodes, 6 to 10, was high compared to the upper node positions of 1 to 5 in all treatments applied including the controls. This was expected because the upper nodes were tender and fragile, and did not survive the rigors of sterilization (treatment with 10% bleach and alcohol) and subsequent treatments. However, despite the rigorous sterilization procedures, a higher proportion of plants from nodes 6 to 10 became contaminated with fungi and bacterial growth. This was likely due to the nature of the mature cassava stem that has a cracked surface, so complete removal of microbes from the cracks was not always possible. Where possible, a balance can be achieved in cassava node bud culture experiments by taking nodes from close to positions 3 to 7 to prevent high plant losses from the tender uppernodes and minimizing contamination from the lower nodes. The size of the node cuttings was also an important factor for initial plant growth, particularly in chemical and heat treatment experiments. The effect of

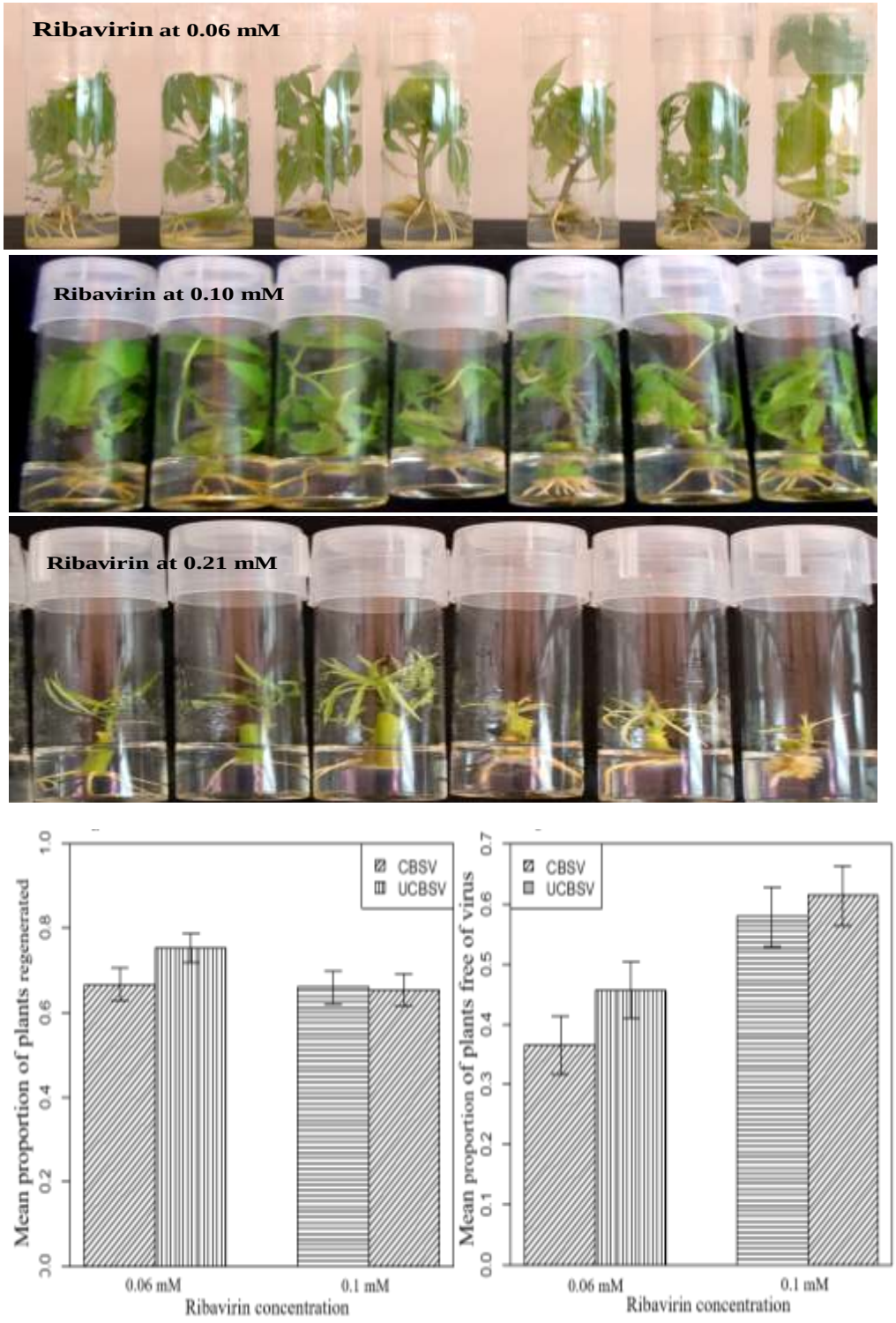

Figure 3. Chemical treatment and growth of node buds from the three cassava varieties in different concentrations of ribavirin and mean proportion of plants regenerated from cassava brown streak virus and Ugandan cassava brown streak virus-infected plants.

the size of node bud on plant regeneration or virus elimination was not investigated; however, this work has been done previously in cassava by Kartha and Gamborg (1975). They observed a 60% virus elimination rate when explants were excised at a length of 0.4 mm; an increase in node size to 0.5 to 0.8 mm resulted in complete plant

Table 4. Analysis of deviance to investigate the effect of ribavirin concentration, variety and virus on the elimination of cassava brown streak virus and Ugandan cassava brown streak virus from infected plants after chemical treatment.

Parameter	df	Deviance	Residual df	Residual deviance	P value
Null			11	49.85	
Concentration	1	40.41	10	9.44	2.058e-10 ***
Variety	2	1.48	8	7.96	0.47725
Virus	1	5.18	7	2.78	0.02289 *
Concentration:Variety	2	0.11	5	2.67	0.94726
Concentration:Virus	1	0.98	4	1.69	0.32116
Variety:Virus	2	0.25	2	1.44	0.88446
Concentration:Variety:Virus	2	1.44	0	0	0.48645

Concentration = Concentrations of ribavirin treatments.

Table 5. Combined effect of the therapies on the regeneration of cassava plants for cassava brown streak virus and Ugandan cassava brown streak virus elimination.

Tissue culture	**% Plantlets regenerated**		**% Virus elimination**		**ET**	
(TC)	**UCBSV**	**CBSV**	**UCBSV**	**CBSV**	**UCBSV**	**CBSV**
Variety	%	%	%	%	%	%
Kaleso	50.7	55.1	49.4	18.2	25.2	10.3
Kiroba	69.3	61.8	43.2	27.0	30.0	17.1
Albert	56.5	60.0	19.1	14.3	11.3	9.2
Heat treatment	**% Plantlets regenerated**		**% Virus elimination**		**ET**	
(TT)	**UCBSV**	**CBSV**	**UCBSV**	**CBSV**	**UCBSV**	**CBSV**
Variety	%	%	%	%	%	%
Kaleso	52.5	53.2	65.2	62.2	33.6	33.3
Kiroba	58.1	62.0	84.0	73.3	49.0	45.0
Albert	53.3	57.1	51.1	47.0	27.4	27.2
Chemical treatment (CT)	**% Plantlets regenerated**		**% Virus elimination**		**ET**	
	UCBSV	CBSV	UCBSV	CBSV	UCBSV	CBSV
Variety	%	%	%	%	%	%
Kaleso	73.1	66.3	53.3	46.0	38.3	30.4
Kiroba	66.0	67.2	56.2	50.1	37.2	34.2
Albert	72.2	66.3	51.0	46.2	37.0	30.0
TC + TT + CT	**% Plantlets regenerated**		**% Virus elimination**		**ET**	
	UCBSV	CBSV	UCBSV	CBSV	UCBSV	CBSV
Variety	%	%	%	%	%	%
Kaleso	53.2	30.2	94.1	89.4	50.0	27.2
Kiroba	50.1	43.1	87.0	92.2	43.6	43.3
Albert	66.3	47.4	53.2	71.4	35.0	33.0

regeneration but 100% viral infection. Thus, anode size of ~0.4 mm or smaller would be ideal for virus elimination and regeneration of cassava plants.

The three cassava varieties subjected to various treatments responded differently to tissue culture. A comparatively high number of nodes were developed from Kiroba and Albert than from Kaleso. Plantlets from tissue culture and the ones that underwent chemical treatment developed at a slower rate than those exposed to temperature regimes of 30 and 35°C. High temperature treatments are known to stimulate plant growth as mentioned previously by Kartha and Gamborg

(1975). Similar results were obtained in yams (Mantell et al., 1980; Chandler and Haque, 1984) and potatoes (Salazar and Fernandez, 1988), although the reasons behind such a growth spurt are not well understood. Differences in the rate of virus elimination through heat treatment were greater than other treatments in the three varieties and it was also more efficient in eliminating mild UCBSV than the severe CBSV (Mohammed et al., 2012). This is consistent with the elimination of potato viruses, in which heat treatment was more efficient in eliminating mild potato virus X (PVX) than the severe potato virus S (PVS) (Stace-Smith and Mellor, 1968). Thus, the elimination of virus by heat depended on the temperature regime used and the virus isolate as well as the host plant species. The highest number of plantlets was regenerated at 35°C compared to other treatments. Similarly, the largest number of plantlets was virus-free at 40°C when tested by RT-PCR, suggesting that the viruses were inhibited by higher temperature, and new shoots produced during the heat treatment could be virus-free (Kassanis, 1957). It has been suggested that the bonds between protein sub-units that protect the virus nucleic acid become weaker at high temperatures, resulting in temporal fissures and allowing attack by nucleases (Allam, 2000). The high rate of UCBSV and CBSV elimination at 40°C could therefore be attributed to the fact that increased temperatures destroy essential chemical processes in the virus life cycle.

The high percentage of virus-free plants obtained from heat treatment in this study (47 to 84%) was generally higher than the 49% achieved by Wasswa et al. (2010) at 40°C. Walkey (1976) further demonstrated that cucumber mosaic virus (CMV) did not multiply at 30°C in the experimental host *Nicotiana rustica*; the virus was inactivated at 32°C and was eliminated after 30 days. The highest temperature used in this study (45°C) resulted in the death of plants of all three cassava varieties, thus indicating the temperature threshold at which cassava nodes cannot survive. The use of ribavirin at different concentrations did not positively influence plant development. The maximum tolerance was reached at 0.21 mM ribavirin concentration, at which all node cuttings from the three cassava varieties died. Highest plant regeneration was obtained at 0.10 mM ribavirin although plant development was slow. Ribavirin has also been shown to slow the regeneration of potatoes (Klein and Livingston, 1982; Slack et al., 1987). These observations confirmed the toxic nature of ribavirin on the *in vitro* development of cassava and other plants above 0.10 mM. Developing virus-free plants by chemical treatment (Klein and Livingston, 1982) will take longer than heat treatment (Stace-Smith and Mellor, 1968) or tissue culture alone. Together with the toxic nature of ribavirin, these factors make it the least favourable choice of treatment for generating virus-free cassava plants.

Regeneration of plants was also slow in the co-treatments, although they were more efficient in eliminating the two viruses. Treatments that included the addition of ribavirin at 0.10 mM into the tissue culture media and exposure to 40°C resulted in increased virus-elimination compared to single treatments as was also achieved with PVY elimination in potato plants (Nascimento et al., 2003). The ET for UCBSV and CBSV elimination varied between the therapies and cassava varieties used. Heat treatment was most efficient for eliminating both viruses from Kaleso and Kiroba when compared to Albert, in which chemical treatment alone was more efficient than simultaneous application of the three therapies. Considering the toxic nature of ribavirin, maximum gains can be obtained without its inclusion with *in vitro* cultures and heat treatments (Klein and Livingston, 1982; Ng et al., 1992; James et al., 1997). Phytosanitary measures have been used to eliminate viruses from cassava by heat (Nyland and Goheen, 1969) and chemical treatments (Quak, 1961), while tissue culture alone has been found to be sufficient for eliminating DNA viruses (cassava mosaic begomoviruses) from cassava (Kartha, 1981; Roca et al., 1984). Wasswa et al. (2010) demonstrated CBSV elimination in cassava through a combination of tissue culture and heat treatment. The result of the study confirmed these findings in the three cassava varieties with different levels of resistance to CBSD, indicating that these effects were not variety specific. Tissue culture together with heat treatment is therefore the preferred methods for virus elimination in cassava due to high levels of plant regeneration, rapid plant growth and high success rates.

There has been an increased demand for healthy and certified cassava planting material in recent years because of the recent CBSD outbreak in eastern and central Africa. Developing methods for efficient elimination of UCBSV and CBSV from infected cassava plants is critical in providing healthy planting materials as required by farmers. The methods will also aid in effective germplasm exchange between countries and thus facilitate CBSD control regionally. The availability of virus-free planting material will facilitate the implementation of new disease management strategies and greater effective control over CBSD in East Africa.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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