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Table of Content

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Full Length Research Paper

Sales environment, microbiological and biochemical quality of beef skins intended for human consumption

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The quality and composition of meat and its derivatives are influenced by many factors namely physicochemical, organoleptic factors and microbial contamination. The aim of this study was to evaluate *kpakouma* consumption risk through its chemical and microbiological contaminants. The methodological approach was composed of (i) observation of selling environment, (ii) pesticides and antibiotics residues quantification by HPLC, and (iii) microbiological analysis using selective media and biochemical tests. The data shows that aminoglycosides, penicillin and nitrofuran were not determined both in the black and the white *kpakouma*. Macrolides (0.094±0.004) and beta lactams (0.016±0.0036) are noted only with white *kpakouma*. Lindane (0.215±0.003) and HCH (1.0003±0.003) were only detected among some samples whereas chlorpyrifos, malathion and parathion were not detected in all the tested samples. Concerning the microbial contaminants, according to the European Regulation, all *kpakouma* samples were highly contaminated with *Staphylococcus* species, *Escherichia coli* and *Salmonella*. The isolated *Staphylococcus* spp. were mostly (90%) resistant to vancomycin, no *Staphylococcus* spp. resistance was recorded for ciprofloxacin. *E. coli* and *Salmonella* were all resistant to oxytetracycline, no resistant isolate of *E. coli* was recorded for ciprofloxacin but *Salmonella* strains were at 25% resistant to ciprofloxacin. These results show the non-compliance with the hygiene rules during the sale of *kpakouma* and reaffirm the potentially critical role that can be played by commensals in public health.

Key words: Environment, bacteria, pesticide and antibiotics residues, *kpakouma*, Cotonou.

INTRODUCTION

Given the importance of livestock, the valuation of the by-products is still modest in Africa. The fifth quarter of carcasses is a large group representing 20 to 55% of the living animal weight and is divided into two parts, namely

an edible part that groups the offal and an inedible part that includes the exits (Fiems, 2012). Thus, they include edible parts such as tongue, offal, fat and skins; which can be used for human consumption (Jayathilakan et al., 2012).

Except few countries, export marketing is often in the form of pre-tanned or rawhide (Strasser, 2015). Thus, the global trade in hides and leather goods has undergone a profound change over the past twenty years. Raw hides and skins from developed countries are imported to prepare, process and re-export them as products with added value (Bereda et al., 2016). However, industry professionals and industrialists with good development potential in Africa may, benefit from this historic reversal.

Meat products have been traditionally reported to be the vehicle for many foodborne diseases in humans (Heredia and García, 2018). Its hygienic quality depends on the contamination occurring during transformation process and during cooling, storage and distribution (El Okki et al., 2005; Rani et al., 2017). The bad hygienic quality can be the cause of food poisoning. In addition, in many tropical countries, foods are sold at public places and roadside shops. The main critical points of meat products hygiene are in slaughterhouses where microbial contamination may be occurred (Soepranianondo and Wardhana, 2019). It is reported that 80 to 90% of the microflora of meat products reaching the consumers resulted from contamination occurring at the slaughterhouse (Odeyemi et al., 2020). The presence of microorganisms that can cause food poisoning are viruses and bacteria (Hernández-Cortez et al., 2017). Organisms mostly reported to be involved in food poisoning are *Salmonella*, *Escherichia coli* and *Staphylococcus aureus* (Sina et al., 2011; Attien et al., 2017). The presence of microorganism causing food poisoning is related to several factors such as pathogenicity and resistance to antibiotics (Fisher et al., 2018). Thus, the damage caused by bacteria can be due to their capacity to not only colonize their host but also production of toxins (Kim et al., 2017).

The transformed beef skin ready to be eaten is commonly called *kpakouma* in Benin local language *barriba*. The *kpakouma* is a very important part of cooking habits. This importance is explained by its availability, its accessibility to any type of portfolio and the satisfaction it provides to the consumers. Once considered food for the poor, beef skin is now a "star" in Beninese cuisine. Indeed, it is found more and more in dishes cooked in important events like vegetable sauce. However, it can be cook it in different ways. Due to its importance, it will be interesting to investigate the major critical points of *kpakouma* hygiene. Despite its appreciation by the population, information on the microbiological quality and importance of this commodity is lacking in Benin. Thus, the aim of this study was to appreciate the *kpakouma* selling environment and to evaluate its chemical and microbiological contaminants qualities.

MATERIALS AND METHODS

Experimental design and *kpakouma* sales environment investigation

For this study, the sample size is determined using the work published by Kadam and Bhalerao (2010). Thus, 300 samples of vendors' goods on show were selected using the "convenience" sampling technique (Etikan et al., 2016) for the investigation. The survey was carried out in 10 markets (Dantokpa, St Michel, Aïdjèdo, Midonbo, Wologuèdè, Gbégamey, Vèdoko, Zogbo, Minnontin and Degakon) of Cotonou to collect information on the *kpakouma* selling environment. For the microbial analysis in the laboratory, nine samples were collected from each market.

Sample's collection for chemical and microbial analysis

Three samples of *kpakouma* were randomly collected from three different sellers per market in ten markets. Thus, 90 samples were collected. Once collected, samples were collected in sterile Stomacher papers then carried to laboratory in icebox (4-8°C) for their microbial analysis.

Chemical contaminant detection

Preparation of standards solutions

Stock standards solutions (1000 µg.ml^{-1}) were prepared by dissolving 10 mg of each analyte in 10 ml HPLC grade water. Further dilution was obtained using acetonitrile to obtain working solutions (40, 20, 10, 5, 2.5 and 1.25 µg/ml). All standards were protected from light with aluminium foil; diluted solutions were stored at -20°C and used after a week.

Determination of antibiotics and pesticides residues

The antibiotics and pesticide residues were extracted from *kpakouma* samples using the method previously described by Lehotay et al. (2005). Briefly, 15 g of *kpakouma* samples were mixed with 15 ml of acetonitrile + 1% acetic acid. Extraction salts (6 g of magnesium sulphate + 1.5 g of sodium acetate) were added and mixed before centrifuged (5000 rpm for 1 min). The organic phase (1 ml) was mixed to purified salt (150 mg PSA+ 50 mg $MgSO_4$ + 50 mg C_{18}) and then centrifuged (5000 rpm for 1 min). One hundred microliters of the supernatant were filtered and then transferred to the HPLC (Agilent Technologies 1260 infinity, GmbH & Co. KG, Waldbronn, Germany) vials for antibiotics (sulfamide, tetracycline, macrolides, b-lactams, aminoside, penicillin and nitrofuran) and pesticides residues (lindanes, HCH, DDT,

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Figure 1. Picture showing *kpakoumas*' sales environment.

chlorpyrifos malathion and parathion) quantification. Each solution was analysed on three replications according to HPLC quantification protocol. About 10 µl was injected in the HPLC analytical column at room temperature and the chromatographic peaks for each sample were identified and compared to the determined retention time of the standard solution of each antibiotic and pesticide residues. The limits of detection (LOD) and of quantification (LOQ) of each antibiotic and pesticides residues were determined using the linear regression method as previously described (Shrivastava and Gupta, 2011).

Microbial analysis

Once in the laboratory, 10 g of each *kpakouma* sample was added to 90 ml of sterile bacteriological peptone (Oxoid, Hampshire, England) and then incubated at 37°C for 1 to 3 h for the enrichment (Akoachere et al., 2009). In this study, microbiological analyses focus on staphylococci, *E. coli*, *Salmonella*, and Mesophilic Aerobic Flora (MAF) were enumerated. From the incubated suspension, a decimal dilution was made with peptone water (BioRad, Paris, France) and used for bacterial identification and enumerations. Each of the dilution (0.1 ml) was spread on Baird Parker agar (Biokar Diagnostics, France) with egg yolk (Baird-Parker, 1990; Dennaï et al., 2001) before its incubation at 37°C for 48 h for Gram positive cocci's. EMB agar (Biokar Diagnostics, France) incubated at 37°C for 24 h was used to isolate *E. coli*. The research of *E. coli* was completed by indole production test (Riegel et al., 2006).

For the identification of *Salmonella* spp. 10 g of each sample was cut into small pieces in sterile blender jar containing 90 ml of peptone water as a pre-enrichment broth and incubated at 37°C for 24 h. After incubation, 0.1 ml of pre-enrichment culture was transferred into sterile tubes containing 10 ml of Rappaport Vassiliadis broth, and incubated at 42°C for 24 h. After incubation, a loopful of each tube was cultured on *Salmonella Shigella* agar and incubated for 24 h at 37°C. Typical colony of *Salmonella* appears as transparent colonies with or without black centers (depending on the species isolated).

The contamination frequency was calculated from the ratio of contaminated products on all the products whereas the prevalence was obtained by the ratio of the strains isolated on all the biological products tested. Once isolated, the microorganisms were identified using classical morphological (gram staining, shape of bacteria, and Ziehl-Neelsen staining) and biochemical characters (sugar fermentation, Oxidase, Methyl red-Voges-Proskauer, indole and Catalase test) related to the genus identification techniques (Janda and Abbott, 2002).

Susceptibility to antibiotics

Antimicrobial susceptibility of isolated bacteria was determined by the disc diffusion method of Kirby-Bauer on agar Mueller-Hinton (bioMérieux, Marcy l'Etoile, France) using EUCAST recommendations and interpretation (CA-SFM/EUCAST, 2019). After 24 h of incubation, inhibition zone was measured.

The tested antibiotics (Bio Mérieux, France) against *Staphylococcus* spp. were ampicillin (A 10 µg), chloramphenicol (C 30 µg), ciprofloxacin (CIP 5 µg), gentamicin (GM 10 µg), oxacillin (OX 5 µg), oxytetracycline (OT 30 µg), penicillin G (P 10 µg), and vancomycin (VA 30 µg). For *Salmonella* and *E. coli* stains, the tested antibiotics were ampicillin (A 10 µg), cephalothin (KF 30 µg), chloramphenicol (C 30 µg), ciprofloxacin (CIP 5 µg), nalidixic acid (NA 30 µg), oxacillin (OX 5 µg) and oxytetracycline (OT 30 µg).

Data proceeding and statistical analysis

Field investigations have identified the manufacturing methods and sources of microbiological contamination of *kpakouma* samples. The data obtained from the survey and the laboratory analyses were coded and entered with the Excel 2016 spreadsheet. Descriptive statistics such as prevalence, mean and standard deviation were calculated for the quantitative variables. A significant difference between the mean was determined. The Graph Pad Prism 7.00 software was used for statistical analysis and graphs. The test is considered statistically significant if $p<0.05$.

RESULTS

kpakouma sales environment in the markets of Cotonou

In the markets covered by our investigation, it is found that the *kpakouma* is exposed unprotected (Figure 1). Thus, exposition was observed either on a plate (Figure 1a), in the uncovered basin (Figure 1b) or directly on tables (Figure 1c). In addition, considering the color of the products, two kinds were observed: white (Figure 1c) and

Table 1. Average values of antibiotics residues detected in *kpakouma* samples (µg/kg).

Antibiotics	Black (µg/kg)	White (µg/kg)
Sulfamides	0.0615±0.0026	0.0893±0.0006
Tetracyclines	0.0652±0.0024	0.4823±0.0025
Macrolides	ND	0.094±0.004
B-lactams	ND	0.016±0.0036
Aminoglycosides	ND	ND
Penicillin's	ND	ND
Nitrofuran	ND	ND

ND: Not detected.

Table 2. Average values of pesticides residues detected in *kpakouma* samples (µg/kg).

Pesticide family	Pesticides residues	Black	White
Organochlorines	Lindane	ND	0.215±0.003
	HCH	ND	1.0003±0.003
	DDT	ND	ND
Organophosphorus	Chlorpyrifos	ND	ND
	Malathion	ND	ND
	Parathion	ND	ND

brown (Figure 1a and b).

Chemical residues contaminant detected

Two residues of antibiotics (sulfamides and tetracyclines) were founded in both white and black *kpakouma* at variable concentration (Table 1). Macrolides (0.094±0.004) and beta lactams (0.016±0.0036) are noted only with white *kpakouma*. Three antibiotics residues (aminoglycosides, penicillin and nitrofuran) were not determined both in the black and the white *kpakouma*.

Organophosphorus residues (chlorpyrifos, malathion and parathion) were not detected in all the tested samples (Table 2). Among the organochlorin, DDT was not detected in both white and black samples whereas lindane (0.215±0.003) and HCH (1.0003±0.003) were only detected among white samples.

Microbial load of collected *kpakouma* samples

The *kpakouma* samples have variable microbial loads depending on the place of collection and the bacterial strain (Table 3). Thus, it is found that all bacterial loads are high, and therefore dangerous for consumption. Globally, the highest loads were recorded with the Mesophilic Aerobic Flora (MAF).

Resistance profile of isolated bacteria from *kpakouma*

Susceptibility of E. coli strains

Figure 2 shows that the susceptibility of *E. coli* strains highly varies with antibiotics ($p<0.0001$). However, it was noted that all strains have been resistant to ampicillin, oxacillin, and oxy-tetracycline while they are sensitive to ciprofloxacin.

Susceptibility of Staphylococcus strains

Figure 3 shows that susceptibility of *Staphylococcus* strains considerably varies with antibiotics ($p<0.0001$). Our data shows that all strains were resistance to ampicillin, oxacillin, oxy-tetracycline and penicillin G while they are sensitive to ciprofloxacin.

Susceptibility of Salmonella subsp. strains

Figure 4 shows that susceptibility of *Salmonella* strains highly varies with antibiotics ($p<0.0001$). The analysis of this figure shows that all *Salmonella* strains were resistant to ampicillin, cephalothin, oxacillin, and oxy-tetracycline while 75% of the isolated strains were sensitive to ciprofloxacin and chloramphenicol.

Table 3. Average number of microbial loads per germ of *kpakouma* samples collected in Cotonou (CFU/g).

Market	*Staphylococcus* spp.	*MAF*	*E. coli*	*Salmonella*
Dantokpa	1.88×10^5	2.50×10^9	6.30×10^4	++
St Michel	2.73×10^5	4.00×10^9	1.33×10^3	++
Gbegamey	4.00×10^5	4.55×10^9	1.70×10^5	++
Wologuèdè	4.00×10^5	5.25×10^9	5.50×10^4	++
Vèdoko	2.15×10^5	3.06×10^9	1.59×10^5	++
Minnontin	2.45×10^5	5.00×10^9	3.00×10^4	++
Midonbo	1.80×10^5	5.00×10^9	3.00×10^5	++
Degakon	2.12×10^5	4.07×10^9	4.10×10^4	++
Aidjèdo	4.50×10^5	5.06×10^9	7.40×10^4	++
Zogbo	3.09×10^5	6.17×10^9	1.70×10^5	++

++: High presence.

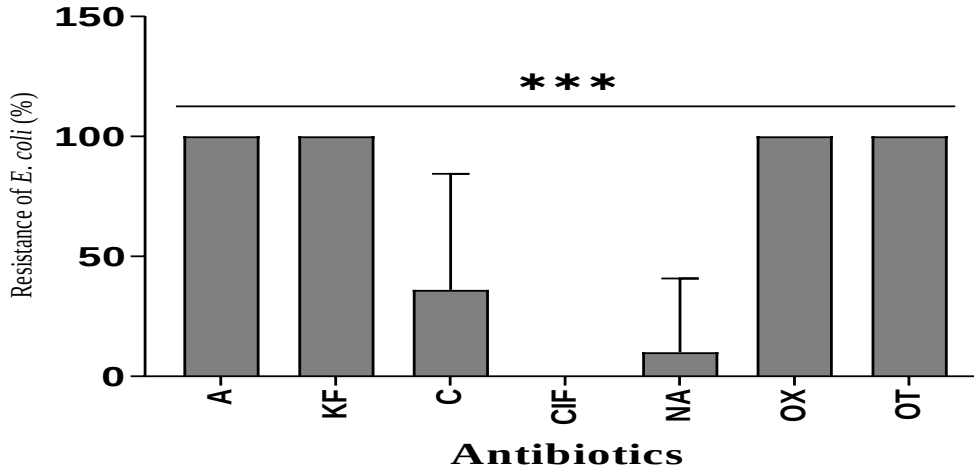

Figure 2. Resistance profile of *E. coli* strains isolated from *kpakouma* to the seven antibiotics. Ampicillin (A), cephalothin (KF), chloramphenicol (C), ciprofloxacin (CIP), nalidixic acid (NA), oxacillin (OX), and oxytetracycline (OT). ***p<0.0001.

DISCUSSION

In this study, the presumptive isolation and identification of bacterial isolates indicate the presence of *Staphylococcus* spp., *E. coli* and *Salmonella* spp. The presence of these three genera of bacteria isolated from this food ingredient could be a matter of serious concern. Indeed, those microorganisms were reported to be pathogenic and can be involved in food poisoning (Sina et al., 2011; Musa and Hamza, 2013). This result can also be explained by the existence of multiple sources of contamination, such as the contact of *kpakouma* with the hands of contaminated buyers and sellers. Overall, the present study revealed that 80% of sampled *kpakouma* samples were contaminated with *E. coli* strains. This rate recorded in our study is much higher than that of 10 and 57.92% observed in beef and chicken carcass, respectively in Australia (Phillips et al., 2006) and Benin (Ahouandjinou et al., 2015). It was noticed that the absence of *E. coli* is an indicator of good hygiene practices whereas its presence is an indication of fresh fecal contamination and the potential presence of ecologically similar pathogens. This indicator is a measure of the implementation of good selling process practices, and the prevention of the occurrence and spread of fecal contamination during sales.

Overall, most of the collected samples were contaminated by *Staphylococcus* spp. strains. However, there was a slight variability in the contamination rate depending on the collection site (market). *Staphylococcus* spp. particularly *S. aureus* causes diseases such as folliculitis, furuncles, erysipelas, cellulites, scalded skin syndrome, impetigo, pneumonia, osteoporosis, toxic shock syndrome (Toxemia), and staphylococcal food

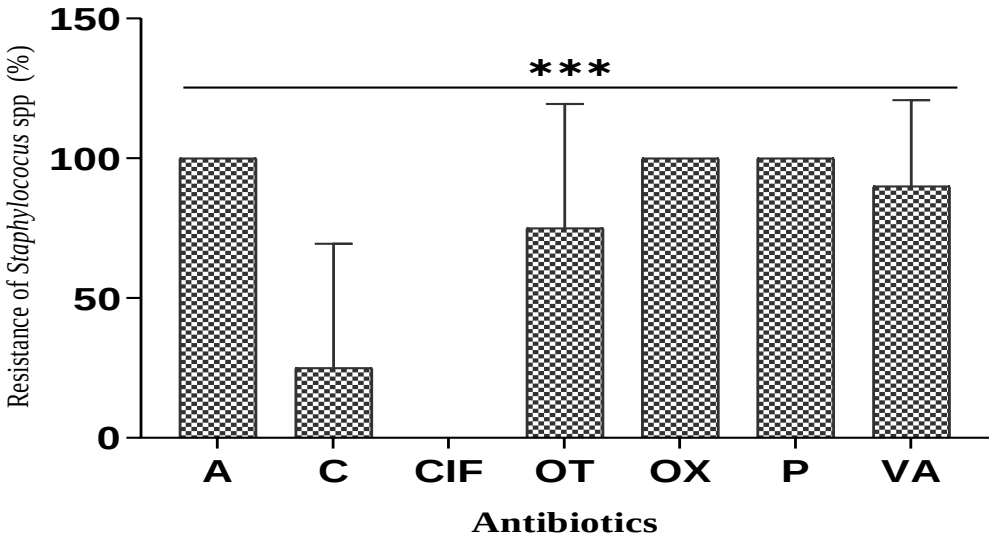

Figure 3. Resistance profile of *Staphylococcus* strains isolated from *kpakouma*. Ampicillin (A), chloramphenicol (C), ciprofloxacin (CIP), oxacillin (OX), oxytetracycline (OT), penicillin G (P) and vancomycin (VA). ***p<0.0001.

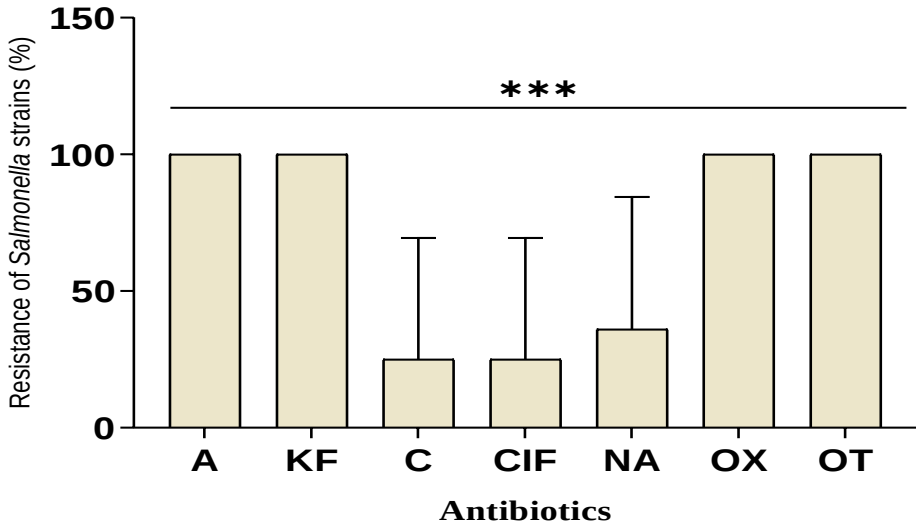

Figure 4. Resistance profile of *Salmonella ssp.* strains isolated from *kpakouma*. Ampicillin (A), cephalothin (KF), chloramphenicol (C), ciprofloxacin (CIP), nalidixic acid (NA), oxacillin (OX), and oxytetracycline (OT). ***p<0.0001

poisoning (Sina et al., 2011; Abdulkadir and Mugadi, 2012). Several authors have found a lower level of contamination than that obtained in this study. This may be due to the sales environment. This result can also be explained by the fact that the mixture water and *kpakouma* would be a broth for the development of aerobic mesophilic germs.

Salmonella prevalence rates recorded in this study are 10 out of 20 samples. *Salmonella* spp. is a strict pathogen and has no habitat other than humans and animals' body; the bacteria is excreted in the faeces or urine and transmitted by food or water which is ingested by another subject (Mølbak et al., 2006). *Salmonella* spp. can cause any of these three types of infections: bacterial food poisoning, enteric fever and systemic fever (Septicemia) (Mølbak et al., 2006).

For most bacteria, there is evidence that increased usage of a particular antimicrobial correlates with

increased levels of bacterial resistance (Granizo et al., 2000); perhaps this explains the high resistance to amoxicillin by the isolates because of its common and prevalent use. Indeed, all isolated strains expressed variable susceptibility to the antibiotics tested. For staphylococci, the highest resistance was observed for vancomycin (90%). The results of the present study have confirmed the frequent resistance to tetracyclines observed previously by some authors (Werckenthin et al., 2001; Aarestrup et al., 2000). It has long been considered the antibiotic of last resort for multi-resistant staphylococcal infections (Schoenfelder et al., 2017). There was no resistance in *Staphylococcus* spp. for ciprofloxacin (quinolone family), which is also the most effective in *Staphylococcus* spp.

All strains of *Salmonella* are resistant to oxacillin, ampicillin, and oxy-tetracycline. This resistance to these molecules, which are old molecules widely used in first intention, is commonly observed in *Salmonella* isolates. This resistance has been reported in several studies on poultry and poultry products (Nayak et al., 2004; Elgroud et al., 2009). *Salmonella* resistance could be transfer from animals to humans, through meat consumption. *Salmonella* strains are 25% resistant to ciprofloxacin. These strains came from the markets of Verdoko, Dégakon, Wologèdè and Dantokpa. Although *Salmonella* strains, highly resistant to quinolones, are still rarely isolated (Schwarz and Chaslus-Dancla, 2001), the decrease in sensitivity should be considered as an alert, since quinolones are antibiotics of last resort against *Salmonella* strains, multi resistant. The evolutionary nature of resistance mechanisms therefore calls for caution.

The observed resistance of *E. coli* to oxy-tetracycline can be attributed to a mutation affecting the structure of porins or decreasing their synthesis. One or more modifications of the porins are at the origin of acquired resistance to beta-lactams, quinolones, chloramphenicol, sulfonamides, trimethoprim and tetracyclines (Liwa and Jaka, 2015). In our study, 10% are resistant to nalidixic acid. These strains came from the markets of Dantokpa and Wologèdè. These results are inferior to those found by Bodering et al. (2017) where they found that *E. coli* strains were resistant to nalidixic acid of 23.08%. Quinolones are currently the largest group of antibiotics. Their interest is related to their low toxicity and especially to the absence of plasmid resistance (Pham et al., 2019). Studies in Nigeria have shown that environmental strains are more resistant to quinolones/fluoroquinolones than clinical strains, and this is explained by the intensive use of these antibiotics in veterinary medicine as growth factors and in the treatment infections (Chigor et al., 2010). Sometimes, the resistances can be of chromosomal origin and this by means of a mutation at the level of the gene, causing a modification of the site of attachment of the antibiotic, or by active efflux; but most often, they are of plasmid origin, therefore transferable horizontally between bacteria of the same species, or even bacteria of distant species (Gassama-Sow et al., 2006).

In this study, strains of *E. coli*, *Salmonella* and *Staphylococcus* spp. showed high resistance prevalence. It is likely that these isolated strains have been subjected to selection pressure by previous use of antibiotic therapy. The consequence is the selection of many resistant strains from the outset to several families of antibiotics that can contaminate consumers of *kpakouma* and make it difficult, if not impossible, all treatments with antibiotics (Amarasiri et al., 2020).

Sulfamides and tetracyclines were founded in all the *kpakoumas'* samples whereas macrolides and beta lactams are noted only with white *kpakouma*. As therapeutic agents, antibiotics have been used in animal production to prevent or control infectious diseases (Dixon, 2001). Meanwhile, growth promoting agents are used to improve the feed conversion efficiency, while antimicrobial agents are added to make more nutrients available to the animal. Those practices have increasingly contributed to the development of bacterial resistance to certain antibiotics (Butaye et al., 2001). Most veterinary drugs used are recommended to be only administered for therapeutic purposes under strict control (van Peteghem and Daeselaire, 2004) with the aim to reduce the development of antimicrobial resistance (Reig and Toldrá, 2009). Comparing the two kinds of *kpakouma*s, the white one shows the high number of residues. This observation can be explained by the fact that the black *kpakouma* is exposed to heat source. Indeed, the black one obtained after grilling is the fresh skin. The heat probably contributes to destroy original molecules.

Concerning the pesticides residues contaminants, some well-known are dioxins, organophosphorus and organochlorine pesticides. In our study, organophosphorus residues are not detected whereas among the organochlorine, DDT was not detected. Pesticide contaminants are quite extensive worldwide, making their control very difficult and can be accumulated in foods and cause damage to consumers. Thought most of the organochlorine pesticides were banned, they are persistent and stable. They can be present in feeds used for farm animals because of the remaining environment for many years, constituting a risk of long-term exposure (Moats, 1994). In our study, the reasons for such contamination can include the use of contaminated ingredients to feeding beefs, lack of control and inadequate processing when using antibiotics (Croubels et al., 2004). However, environmental contaminants are rather difficult to control particularly in our conditions where cows are fed in open air with wild herbs. This habit is favorable for potential toxicity in the meet product (Heggum, 2004). The use of pesticides in farms has no doubt helped developing countries' green revolution, but these are involved in polluting air, water and land. It also

posing serious public health problem by entering in to food chain, thus, it will be interesting to investigate the toxins production capability of the isolated pathogens after their molecular characterization.

Conclusion

The manufacturing techniques of the *kpakouma* and the various apprehensions of the populations consuming this commodity were established. Some collected samples contain antibiotics and pesticides residues. In addition, the microbiological quality of the *kpakouma* samples reveals that all the samples were dissatisfaction for the three criteria, namely, FAM, *E. coli* and *Salmonella* independently to the collection place. Considering the resistance profiles against the antibiotics tested, the results are alarming because of the high resistance rates recorded. Thus, the dangers associated with *Staphylococcus*, *E. coli* and *Salmonella* can be aggravated by the noticeable increases in this antibiotic resistance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Review

Vertical-horizontal subsurface flow hybrid constructed wetlands for municipal wastewater treatment in developing countries: A review

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The use of low-cost on-site wastewater treatment technologies, including constructed wetlands (CWs), is wide spread. Despite the purported high performance of vertical-horizontal subsurface flow (V-H SSF) hybrid CWs, data on implementation and performance in developing countries is scarce. Here, the design, operation and performance of V-H SSF hybrid CWs for treatment of municipal sewage in an effort to encourage and direct future research and assist technology choice were reviewed. Literature reveals that successful performance of V-H SSF hybrid CWs depends mainly on system design and is independent of mode of feeding. Moreover, performance and final effluent quality is high for biological oxygen demand (BOD), chemical oxygen demand (COD) and total suspended solids (TSS) which are all reduced by over 80%. Despite high removal of ammonium-nitrogen (NH_4^+-N), concentration in the final effluent remains above desired levels, which is attributed to the design of V-H SSF hybrid CWs based on BOD as a parameter of choice, rather than nitrogen. It was argued that further research on performance of V-H SSF hybrid CWs based on designs that consider both nitrogen in the form of NH_4^+-N and BOD and assessment under different climatic conditions, is essential prior to mass implementation of this technology in developing countries.

Key words: Hybrid constructed wetlands, municipal wastewater, pollutant removal.

INTRODUCTION

Pollution of surface water resources due to municipal discharge is a major environmental challenge due to enrichment of these systems with pollutants thus, posing a threat to aquatic ecosystems and public health (Edokpayi et al., 2017; Balthazard-Accou et al., 2019). In Africa for instance, most sewages are not subjected to treatment mainly due to the current state of disrepair of deployed wastewater treatment plants (WWTPs) and poor appreciation of the associated technologies. In many developing countries, centralized conventional WWTPs are the currently preferred choice by engineers, planners, and decision makers and the reason; these are the most tried and tested technologies (Tsagarakis et al., 2003). The major drawbacks of this implementation strategy are high construction costs, chemical and energy demands, in addition to the requirement for high skilled

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and non-skilled personnel for operation and maintenance. This has, in part, resulted in failure of WWTPs and discharge of effluent that does not always conform to national and international guidelines, and in most instances is blamed on insufficient funding for municipalities to meet day-to-day running costs (Bakir, 2001).

To overcome the high costs associated with establishment of wastewater treatment infrastructure in developing countries, there is a shift away from construction of centralized WWTPs to decentralized natural, low-cost wastewater treatment technologies including CWs, waste stabilization ponds (WSPs), bio-filtration (BF) and integrated algae pond systems (IAPS) to mention a few. These systems are typically passive and utilize natural processes that depend mainly on the interaction of bacteria and algae and/or macrophytes powered by sunlight as a major source of energy (Mahmood et al., 2013). When these biochemical and physical processes occur in a more natural environment rather than tank reactors, the resulting system consumes less energy, is more reliable, and requires less operation and maintenance and hence the overall cost is lower (Makopondo et al., 2020). Among the decentralized wastewater treatment technologies, WSPs are the most widely adopted technology for the treatment of both domestic and municipal wastewater in tropical and subtropical regions. Unfortunately, many of these systems perform below the required standard especially concerning nitrogen (Mburu et al., 2013), which is attributed mainly to improper operation and maintenance (Magayane and Mwanuzi, 2006). Moreover, the performance of WSPs is dependent upon the prevailing climatic conditions, which is disadvantageous in high rainfall regions, and open systems pose a risk to public health, as they can be breeding grounds for mosquitoes in malaria prone countries. Furthermore, passive treatment processes with exposed anaerobic ponds such as WSP systems are also considered major contributors to greenhouse gas emissions (Coggins et al., 2019). For this reason, there is a growing interest in evaluating alternative wastewater treatment technologies with less environmental impact that consistently produce a quality effluent for discharge particularly regarding nitrogen.

This review focuses on CWs as an alternative passive wastewater treatment technology for application in developing countries. Most other studies address the general performance of different types of hybrid CWs (Vymazal, 2013) and their potential for adoption in developing countries (Mthembu et al., 2013). However, one of the major aims of wastewater treatment is protection of the integrity of aquatic and public health through production of a quality effluent that meets discharge standards and, at the lowest possible cost. Despite the different types of hybrid CWs reported in the literature, the most popular hybrid system used for treatment of both domestic and industrial wastewater is the vertical-horizontal subsurface flow (V-H SSF) hybrid CW (Lavrnic et al., 2020). The major objective of V-H SSF hybrid CWs is to maximize nitrogen removal through nitrification (in the VSSF) and denitrification (in the HSSF) processes (Vymazal, 2013, 2017). While application of CWs is regarded as a potential and novel biotechnology for wastewater treatment, information about their performance and factors influencing the quality of treated water from these systems especially in the tropics is scarce (Mburu et al., 2012). Furthermore, Avellán et al. (2019) emphasize that policy and decision makers who might have an influence on choice of an appropriate wastewater treatment technology and/or process system often lack the necessary information. In an effort to reduce this knowledge gap, this review therefore seeks to contribute to the body of information about effluent quality and performance with attention to single stage V-H SSF hybrid CWs in sewage treatment. Additionally, factors affecting performance and effluent quality of SSF CWs in general are also surveyed with particular attention to design consideration, influence of mode of operation and type of macrophyte used.

CONSTRUCTED WETLANDS FOR WASTEWATER TREATMENT

Constructed wetlands for wastewater treatment are engineered systems planned, designed and constructed to imitate natural wetland systems utilizing natural wetland processes including wetland plants, soil, and associated microorganisms to remove contaminants from wastewater in a controlled environment (Vymazal and Kröpfelová, 2008). This remedial technology is a low cost and environmentally friendly sanitation alternative to conventional methods and recommended for on-site wastewater treatment in small communities to meet required effluent discharge standards (Rousseau et al., 2004; Massoud et al., 2009). Furthermore, it is recognized as dependable for the treatment of different types of wastewaters including municipal and domestic Chang et al., 2012), mine drainage (Sheridan et al., 2013), agricultural runoff (Tyler et al., 2012), landfill leachate (Białowiec et al., 2012) and abattoir wastewater (Odong et al., 2013) to mention a few. Invented in the middle of the 20th century (Vymazal, 2008), CW technology has potential in developing countries in particular those located in warm tropical and sub-tropical regions which favour high biological activity and productivity, and thus better treatment performance (Zhang et al., 2015).

Constructed wetlands are preferred to conventional WWTPs due to simplicity of operation and maintenance costs (Puigagut et al., 2007; Dhir, 2013), and low energy demand (Álvarez and Bécares, 2008). Unlike WSPs and mechanized WWTPs, CWs offer other ecosystem services when operated on a commercial scale. These include: the provision of habitat to wildlife such as fish,

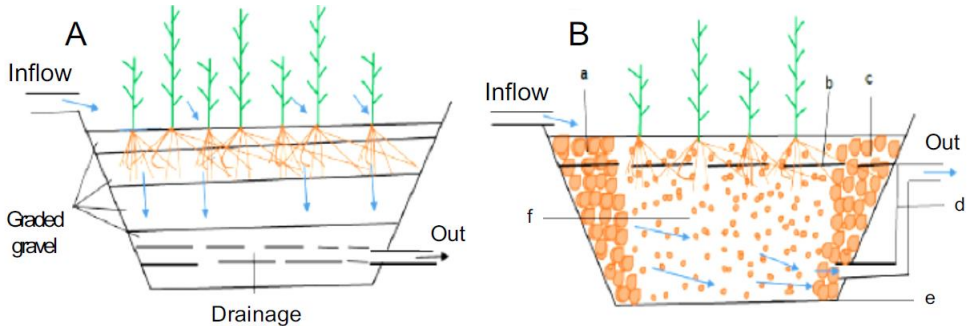

Figure 1. Diagrammatic representation of a typical subsurface flow (SSF) constructed wetland (CW) system. A) Vertical subsurface flow (VSSF) constructed wetland (CW); and, B) Horizontal subsurface flow (HSSF) constructed wetland (CW). Distribution (a) and collection zones (c) filled with large gravel; water level in the filtration bed (b); outlet structure (d) for maintaining the water level in the wetland; impermeable liner (e) such as Polyvinyl chloride and filtration zone (f), mainly gravel.
Source: Vymazal (2007).

birds, amphibians and reptiles (Lee et al., 2009), flood control, provision of educational and recreational opportunities when designed and built at schools, hospitals or even in municipal parks, provision of water for reuse and a visually attractive and functional landscape (Shutes, 2001; Lee et al., 2009; Stefanakis, 2020). Even so, like any other natural system, CWs are a biologically complex ecosystem with various components that interact non-linearly (Banzhaf and Boyd, 2012), and a better understanding of their effective performance is therefore required.

Categories of constructed wetlands

According to Kadlec and Knight (1996), CWs are categorized as either subsurface flow (SSF) or free water surface (FWS) systems depending on the type of flow. The former is further differentiated into vertical subsurface flow (VSSF) and horizontal subsurface flow (HSSF) CWs (Figure 1). A single stage CW either as vertical or horizontal subsurface flow system is preferred to FWS due to many environmental benefits. Thus, SSF conditions prevent odours and the breeding of mosquitoes especially in tropical regions. The bottleneck for SSF systems is however, the requirement of large land area (Zapater-Pereyra et al., 2014) and capital investment, which usually contributes significantly to the cost of the treatment media needed (Kadlec and Knight, 1996).

Horizontal subsurface flow CWs are designed so that wastewater continuously flows horizontally below ground through the substrate and effluent is then collected on the opposite side (Brix, 1994). The transfer of oxygen in this system is limited due to the fact that opportunities for contact between air and water are limited. Despite the fact that plants transport some oxygen from the atmosphere to the proximate roots, thereby creating some aerobic zones, the main part of the bed remains anaerobic (Vymazal, 2005). Nonetheless, there has been a growing interest in achieving completely nitrified conditions, which are seldom attained in the HSSF due mainly to insufficient oxygen supply (Vymazal, 2005).

In contrast to a HSSF system, wastewater is dosed onto the entire surface of the VSSF wetland system from above using a mechanical dosing system, allowing it to flow vertically through the treatment medium (sand or gravel bed) and discharge at the base (Brix and Arias, 2005). The bed is usually allowed to dry until the next dose of wastewater is applied, which allows diffusion of oxygen into the adjacent environment. The next dose traps air and this together with the aeration caused by the rapid dosing into the beds, leads to good oxygen transfer and hence nitrification (Brix and Arias, 2005). Aerobic conditions and direction of flow path are therefore two important features that differentiate VSSF from HSSF CW. The higher availability of oxygen in the VSSF CW increases nitrification thereby facilitating conversion of ammonium to oxidized nitrogen (Vymazal, 2007, 2013).

Phosphorus removal in VSSF CW is very similar to that of HSSF CW since the mechanisms are mainly physical and include adsorption to the substratum and plant root surface and/or precipitation with ions such as calcium, alluminium, and iron present in the rooting medium, and neither is influenced by oxygen concentration (Brix et al., 2001; Arias et al., 2001). It has therefore been proposed that, to improve phosphorous retention in CWs, treatment media with higher phosphorus adsorption capacities, higher calcium, iron or alluminium contents, larger particle surface areas, and appropriate hydraulic conductivities should be used (Vymazal et al., 1998).

Despite the higher removal of nitrogen by VSSF than HSSF CWs, the choice of application of CW type depends on the treatment objective. For example, where

guidelines exist for receiving waters sensitive to eutrophication, nutrients, especially nitrogen, must be reduced to the required discharge limit and hence, a VSSF is recommended. In contrast, where wastewater is to be reused for agriculture, aquaculture, or even recreation (such as swimming), pathogenic microorganisms, helminths, BOD_5 and TSS are the target components for remediation. In this case, a HSSF CW is more suitable (Vymazal, 2005).

In the past two decades, intensive research has been carried out on the performance of SSF CW for treatment of domestic wastewater, especially in sub-Saharan Africa (Mashauri et al., 2000; Keffala and Ghrabi, 2005; Abidi et al., 2009) and accordingly, SSF CWs have proved efficient in the removal of BOD_5, COD, TSS, and pathogenic microorganisms. Despite the high performance of SSF CWs, it has been reported that independent systems in operation with either HSSF or VSSF show difficulty in reducing nitrogen to the levels required for discharge into surface water courses (Molle et al., 2008). Although some authors have reported a reduction in NH_4^+-N in the final effluent from HSSF CW (Vymazal, 2005), others have observed an increase (Mburu et al., 2013), which was attributed to prevailing anaerobic conditions. Likewise, the removal of oxidized forms of nitrogen is considered a bottleneck for many VSSF systems since the prevailing aerobic conditions in the system lead to production of nitrates (Molle et al., 2008).

To improve total nitrogen removal, studies have thus focused on using hybrid constructed wetlands. A hybrid constructed wetland system is defined as a combination of different types of CWs aimed at achieving higher treatment efficiency than a single CW, and particularly for nitrogen (Vymazal, 2013). This is due to the fact that single-stage CWs, which are a popular method adopted for removal of nitrogen and other pollutants from domestic wastewater, hardly achieve high removal of TN due to the inability to simultaneously provide both aerobic and anaerobic conditions (Tuncsiper, 2009). Among the hybrid CWs, a combination of VSSF and HSSF is a popularly adopted system for wastewater treatment (Vymazal, 2013); that exploits the uniqueness of each system (Tuncsiper, 2009). First, wastewater is treated in a VSSF CW, in which the aerobic environment makes nitrification possible, to convert the main part of nitrogen into nitrate. The effluent is then passed into and treated by a HSSF CW where the anoxic environment facilitates denitrification, converting nitrate to nitrogen gas (Tuncsiper, 2009). Despite insufficient data on the performance of hybrid CWs, Vymazal (2013) established that V-H SSF hybrid CWs are slightly better at ammonia removal than H-V SSF or multi-stage V-H SSF hybrid CWs.

Nitrogen removal from wastewater is important for health and protection of aquatic ecosystems especially in areas where discharge limits for NH_4^+-N and NO_3^--N into surface waters exist. In South Africa for example, NH_4^+-N and NO_3^--N limits are ≤ 6 and ≤ 15 mg/L, respectively for disposal of treated wastewater into a water resource that is not a listed water resource and to irrigation of any land up to 2 ML on any given day (DWS, 2013). In water resource areas where effluent cannot be reused in agriculture and must be discharged into a fragile watercourse, nitrogen removal is therefore paramount, and adoption of a V-H SSF hybrid CW could be essential.

Earlier, authors particularly Vymazal (2008, 2013) presented a detailed history of V-H SSF CW. Our analysis reveals that, whereas a few pilot scale V-H SSF hybrid CWs are reported in the literature for sub-Saharan Africa, there appears to be a deficiency in information on the full-scale application of the technology on the continent of Africa. Stagnation in the implementation of the V-H SSF CW technology in developing countries could be attributed to the fact that aid programs from developed countries tend to favour more overt technologies that have commercial spin-off to donors (Denny, 1997). Added to this is the fact that engineers and decision makers tend to prefer tried and tested technologies rather than the risk that may be associated with newer technologies (Verburg et al., 2006).

PERFORMANCE OF V-H SSF HYBRID CW FOR DOMESTIC WASTEWATER TREATMENT

Hybrid CWs involving the use of combined VSSF and HSSF CWs to maximize removal of contaminants from wastewater have been used widely to attain high removal efficiency, particularly for nitrogen (Vymazal, 2005, 2013). These CWs have been successfully used for domestic wastewater treatment particularly in small communities and in remote areas. Domestic wastewater originating from toilets, bathing, sinks and laundry is the major source of organic matter, TSS, the soluble nutrients NO_3^-, PO_4^{3-}, NO_2^-, NH_4^+, the particulate nutrients TP and TN, indicator organisms (e.g. *Escherichia coli*), pathogenic organisms like *Salmonella* and *Shigella* species and other organic contaminants (Sayadi et al., 2012). Many authors have documented the effluent quality and performance of V-HSSF hybrid CWs and results from these studies are summarized in Table 1 and, an overall evaluation of the surveyed hybrid CWs is as shown in Figure 2.

The survey shows that there is a paucity of data regarding the performance of hybrid systems on the African continent. Out of the sixteen surveyed hybrids, only three were reported from Africa and these were from one country (Tunisia). The effluent quality and performance of the surveyed V-HSSF hybrid CWs appears to be very high with regards to BOD_5: 19 ± 7.5 mg/L (92 ± 2% removal); COD: 74.0 ± 1.5.7 mg/L (86 ± 2% removal); TSS: 8.0 ± 1.8 mg/L (94 ± 2% removal); and TP: 3.0 ± 0.8 mg/L (64 ± 4% removal). Despite the

Table 1. Effluent quality and performance of single stage V-H hybrid constructed wetlands for sewage treatment from 2000-2014.

Example by country	Water quality parameter						Macrophyte species	Area (m^2)	Reference
	BOD_5	COD	TSS	TP	TN	NH_4^+-N			
Belgium									
Quality ($mg.L^{-1}$)	5.8	43	5	2.9	27	*i*	*P. australis*	2250	Lesage et al. (2007)
Removal (%)	92	81	90	45	43	*i*			
Quality ($mg.L^{-1}$)	4	47	4.8	3.4	*i*	*i*	*P. australis*	2250	Lesage (2006)
Removal (%)	92	81	95	32	*i*	*i*			
Quality ($mg.L^{-1}$)	9	49	4.3	3.4	26	*i*	*P. australis*	1080	Lesage (2006)
Removal (%)	96	90	98	47	53	*i*			
Quality ($mg.L^{-1}$)	10.3	57	15	4.3	23	*i*	*P. australis*	660	Lesage (2006)
Removal (%)	93	84	87	38	60	*i*			
Estonia									
Quality ($mg.L^{-1}$)	5.5	*i*	5.8	0.4	19	9.1	*P. australis*	432	Öövel et al. (2007)
Removal (%)	94	*i*	87	91	70	84			
Tunisia									
Quality ($mg.L^{-1}$)	*i*	*i*	*i*	*i*	*i*	30	*Typha* spp., *P. australis*	1.8	Abidi et al. (2009)
Removal (%)	*i*	*i*	*i*	*i*	*i*	19			
Quality ($mg.L^{-1}$)	30	134	18	7.2	*i*	47	*Typha* spp., *P. australis*	1.8	Keffala and Ghrabi (2005)
Removal (%)	93	90	98	77	*i*	19			
Quality ($mg.L^{-1}$)	*i*	*i*	*i*	*i*	*i*	*i*	*P. australis, Typha* spp.	327	Kouki et al. (2009)
Removal (%)	93	89	98	72	*i*	*i*			
Spain									
Quality ($mg.L^{-1}$)	24	71	3.6	*i*	*i*	11	*P. australis, Scirpus* spp.	0.88	Herrera Melián et al. (2010)
Removal (%)	85	74	95	*i*	*i*	91			
Quality ($mg.L^{-1}$)	66	172	16.2	8.8	26	40	*Typha latifolia*	450	Vera et al. (2010)
Removal (%)	84	77	95	35	43	51			
China									
Quality ($mg.L^{-1}$)	*i*	21	3.2	0.4	*i*	2.2	*P. australis*	3716	Zhai et al. (2011)
Removal (%)	*i*	84	97	85	*i*	80			
Quality ($mg.L^{-1}$)	*i*	26	7.2	0.9	*i*	5.3	*Cyperus alternifolius*	1400	Zhai et al. (2011)
Removal (%)	*i*	90	85	77	*i*	84			
Quality ($mg.L^{-1}$)	*i*	28	1.6	0.6	14	6.2	*Cyperus alternifolius*	4459	Zhai et al. (2011)
Removal (%)	*i*	84	99	68	65	72			
Italy									
Quality ($mg.L^{-1}$)	*i*	36	*i*	0.2	17	11.4	*P. australis*	6.75	Foladori et al. (2012)
Removal (%)	*i*	94	*i*	98	78	80			
Brazil									
Quality ($mg.L^{-1}$)	*i*	29	*i*	4	*i*	5.6	*Typha* spp., *Zizaniopsis bonariensis*	110	Phillipi et al. (2010)
Removal (%)	*i*	95	*i*	69	*i*	89			

Table 1. Contd.

Mexico									
Quality (mg.L^{-1})	*i*	*i*	*i*	12	102	19	*Z. aethiopica*	3.66	Zurita and White (2014)
Removal (%)	*i*	*i*	*i*	0	26	85			

i= not indicated.
Source: Vymazal (2013).

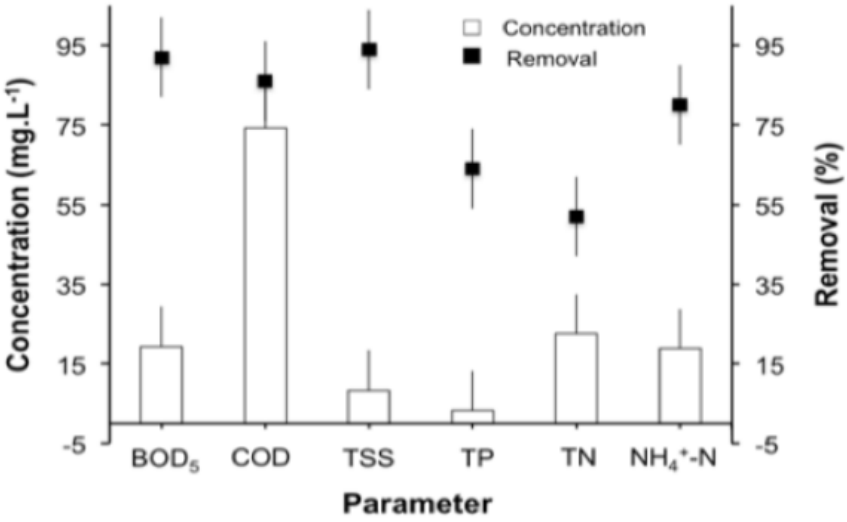

Figure 2. Surveyed data on effluent quality and pollutant removal efficiency of hybrid CW treating municipal sewage.

high removal of nitrogen species (NH_4^+-N and TN), the effluent quality from the surveyed systems is low at 19.0 ± 6.2 mg/L and 23 ± 2.1 mg/L for NH_4^+-N and TN, respectively. Some factors that could be contributing to poor effluent nitrogen concentration in the surveyed CWs are discussed subsequently.

FACTORS AFFECTING PERFORMANCE OF CONSTRUCTED WETLANDS AND EFFLUENT QUALITY

Several factors influence performance of a CW including design and construction, hydraulic and organic loading rate, operation, maintenance, media type and size, macrophyte, hydrology and environmental variables, particularly temperature (Varma et al., 2021). Literature on the influence of some of these factors on performance of CWs has been discussed in detail by others and includes construction (UN Habitant, 2008), hydraulic loading rate (HLR) and hydraulic retention time (HRT) (Chazarenc et al., 2007) and environmental variables (Varma et al., 2021) among others. Here, the influence of design, mode of operation and vegetation on performance of CWs are considered in more detail.

Design and size determination

Proper design and construction are a major consideration for successful deployment and performance of a CW. The components of a well-designed CW include a primary treatment unit and the various wetland compartments; the wetland itself, comprising substrate/ treatment media, vegetation, and micro-organisms (Steiner et al., 1989). The primary treatment unit is important for reducing heavy solids and organic load, and may require installation of an Imhoff tank or septic tank for individual households or a primary sedimentation tank or stabilization pond(s) for small communities (Korkusuz, 2004). Primary treatment units have the associated problem of sludge accumulation and may require desludging from time to time. However, this may be overcome by incorporating a fermentation pit in the primary treatment unit for accelerated anaerobic digestion with the added benefit of methane generation for use in energy derivation (Rose et al., 2002). A properly designed and constructed primary treatment unit for a CW should be able to remove up to 60% of influent BOD at 20°C (Magayane and Mwanuzi, 2006) and it is estimated, that inclusion of a fermentation pit will yield methane equivalent to 30% of the influent organic carbon

(Green et al., 1995).

Primary treatment is followed by secondary treatment, in this case the CW, and performance is dependent not only on efficient removal of organic matter and suspended solids from the primary treatment unit, but also optimum design. The latter, is to attain better nutrient removal while mitigating operational problems. Hence, optimum performance of a CW depends on using an appropriate area for a given hydraulic and organic load (or population) as smaller areas for large flows result in lower treatment efficiency. As a consequence of this requirement, different methods have been proposed for sizing the effective area of a CW including, the population equivalent (PE) method, pollutant loading method, and non-mechanistic models.

Non-mechanistic models have been widely adopted for sizing the effective area required for a SSF CW. Literature shows that by employing the equation described by Kickuth (1977) and Reeds et al. (1995), in which BOD is the design parameter, the required area for SSF systems for domestic wastewater treatment can be estimated. The bottleneck of using non-mechanistic models for CW area estimation is that BOD, which is widely used as a target pollutant in the design, results in under estimation of the area. Hence, this method is suitable for organic matter and TSS removal but not appropriate for nutrient removal (Vymazal, 2005). Thus, and as pointed out by Huang et al. (2000), nitrogen removal from a SSF CW is an important design criterion despite the fact that it has not been fully explored. Until now, no information on performance of SSF flow CWs designed for nitrogen removal has been available, and it is not clear if designs based on nitrogen will allow CW to meet organic matter effluent standards. Thus, the need to evaluate performance of CWs designed based on nitrogen removal.

Mode of operation versus macrophyte selection

The mode of operation of a CW greatly influences the redox potential and consequently the performance of the CW (Faulwetter et al., 2009). Mode of operation is thus categorized as either batch, intermittent or continuous feeding.

During batch feeding, the CW is fed wastewater in doses for a specific time period and then allowed to drain completely until the next dose is applied (Caselles-Osorio and Garcia, 2007). This mode of operation results in variation of redox potential with time in the wetland. Typically, redox potential declines when wastewater is dosed and this is then followed by a gradual increase in redox condition as pollutants are removed (Allen et al., 2002). This variation in redox condition may select for a microbial community that is adapted to changes in redox and nutrient conditions (Stein et al., 2003). Stein and Kakizawa (2005) reported that operating a CW under batch feeding promotes better oxidizing conditions and hence better nitrogen and organic matter removal.

Intermittent feeding is closely related to batch operation but differs slightly in a way that the CW does not completely drain before the next dose is applied. This allows the wetland to accumulate more dissolved oxygen (DO) which enhances mineralization of organic compounds particularly in the VSSF systems (Knowles et al., 2011). According to Knowles et al. (2011), problems associated with clogging of HSSF CW may also be overcome by intermittent operation where re-aeration of the subsurface may occurs. Studies have shown that intermittently fed CW perform better than continuously fed systems in terms of nitrogen and organic matter removal (Caselles-Osorio and Garcia, 2007) since, like batch feeding, intermittent feeding creates temporal and spatial variation in redox potential throughout the whole length of the wetland (Headley et al., 2005). Additionally, it may also increase oxygenation, which will reduce or eliminate the development of anaerobic zones within biofilms and minimize release of volatile fatty acids and ammonia.

The simplest and most common mode of operating a CW is by continuous flow (Faulwetter et al., 2009). However, there is debate by some authors over its use in CWs. Stein et al. (2003) for instance claim that, the major limitation of this mode of operating CWs is that it lowers DO concentration and consequently, reduces removal efficiency of some pollutants that require aerobic conditions for their elimination such as NH_4^+-N. In contrast, Toet et al. (2005) suggested that pollutant removal in CWs depends largely upon hydraulic retention time (HRT) and hydraulic loading rate (HLR) regardless of the mode of feeding. The HRT and HLR affect the time of contact between pollutants and the microbial population within the CW system. It has been revealed that operating a CW for longer HRT results in higher redox potentials and thus greater pollutant removal. Headley et al. (2005) for instance, reported redox potentials in the range of -92 to +103 mV when the HRT was 10.1 days and -109 to +186 mV when the HRT was 16.1 days, under intermittent operation of the wetland.

However, in our view, effluent quality from CWs especially concerning NH_4^+-N may be influenced by several factors; especially macrophyte species other than the mode of wastewater feed and flow. For instance, under intermittent operation, Zurita and White (2014) operated a hybrid CW planted with *Zantedeschia aethiopica* at a hydraulic loading rate (HLR) of 0.28 m/d with influent NH_4^+-N concentration of 128.2 mg/L. They reported effluent NH_4^+-N concentration of 19 mg/L (85% removal) from their system. In contrast, at a much lower HLR (0.08 m/d) and influent NH_4^+-N concentration (37 mg/L), Keffala and Ghrabi (2005) reported effluent NH_4^+-N concentration of 30 mg/L (19% removal) from a V-H hybrid system, planted with *Typha* species and *Phragmites australis* in the vertical and horizontal

systems, respectively. Surprisingly, Herrera-Melián et al. (2010) were able to achieve an effluent NH_4^+-N concentration of 11 mg/L (91%) from a continuously operated hybrid system planted with *P. australis* and *Scirpus* species in the vertical and horizontal systems, respectively at 0.4 m/d with an influent NH_4^+-N of 122 mg/L.

In as much as the review by Brisson and Chazarenc (2009) on the effect of macrophyte species selection on pollutant removal in SSF CWs revealed that macrophyte species selection does not influence the effluent quality from CWs, based on results obtained by Zurita and White (2014), Keffala and Ghrabi (2005) and Herrera-Melián et al. (2010), we argue that if nitrogen is the target pollutant for removal especially in hybrid systems, it could be important to pay more attention to the macrophyte species used particularly in the HSSF CW than the mode of feeding. Hence, the difference in the NH_4^+-N effluent quality reported by Zurita and White (2014) and Keffala and Ghrabi (2005) under intermittent feeding based on the type of macrophyte used can be explained in two ways: (1) wetland plants have been reported to dispatch oxygen to the vicinity of the root system that is responsible for oxidation of ammonium (Brix, 1994); and status and health of the root system which may impact ammonia removal directly. While different plants do appear to show differences in oxygen released into the rhizosphere, to date, there is little information regarding oxygen release rates among CW plants. This is surprising as oxygen release rates could be important in selecting plant species for use in CWs in particular to target specific pollutants and especially nitrogen. Among the macrophytes, *P. australis* is the most studied. Using different methods, several studies have reported oxygen release rates from *Phragmites*. Brix (1990) and Gries et al. (1990) for instance reported oxygen release of up to 5-12, 0.02 and 1-2 g/m^2.d, respectively. Unfortunately, there is little or no information regarding oxygen release rates of *Z. aethiopica.* An earlier study on the use of this ornamental species in HSSF CWs by Belmont and Metcalfe (2003) however, showed considerable reduction in the influent ammonium concentration indicating that *Z. aethiopica* has a positive effect on ammonium removal. While SSF CWs are known to be anaerobic/anoxic, throughout the course of their study, however, Belmont and Metcalfe (2003) observed an increase in the effluent oxygen concentration that could be linked to the high oxygen release rates from the test species, *Z. aethiopica.* Although it may require further investigation, *Z. aethiopica* could have higher oxygen release rates than *P. australis*, which may account for the significant difference in the ammonium removal reported by Zurita and White (2014) and Keffala and Ghrabi (2005). (2) Bezbaruah and Zhang (2004) reported lower oxygen release rates in the range 0.00021-0.00155 and 0.00083-0.00288 g/m^2.d from brown and white roots of *Scirpus*, respectively than reported for *Phragmites*. Whereas firm, fleshy white roots are a sign of plant health, root rot typically manifests as the presence of soft, brown roots and may be the outcome of anoxic conditions within the rhizosphere caused by water logging or the result of fungal infection. In response, a pathogen-induced response occurs in which plants mount a defense that includes the production of secondary products (e.g. alkaloids) but if weak, leads to organ senescence and death. The lower NH_4^+-N concentration reported by Herrera-Melián et al. (2010) in a continuously operated hybrid CW than in intermittently operated hybrids reported by either Keffala and Ghrabi (2005) or Zurita and White (2014) could thus, be attributed to the difference in root function and/or alkaloid concentration of the macrophyte. It has been reported that some wetland macrophytes for example *P. australis* contain high concentrations of alkaloids especially N,N-dimethyltryptamines (DMT) in their rhizomes (Khan et al., 2012). During the treatment process, these alkaloids may be released as extracellular polymeric substances (EPS) containing nitrogenous compounds. It can therefore be hypothesized that the alkaloid concentration in the three different macrophytes follows the order *P. australis* > *Z. aethiopica*> *Scirpus* species.

Therefore, future study should aim at investigating the physiology and mechanisms of the different macrophyte species that go beyond a comparison in pollutant removal efficiency, such that unique species are selected for use in CWs depending on the treatment objective. Despite the simplicity of operation associated with continuous in comparison to intermittent fed CWs, there is insufficient data regarding performance of V-H SSF hybrid CW operated continuously and the resulting effluent quality. Feeding the wetland intermittently requires energy input, which may not be available or supplied reliably in developing countries but may be achieved using innovative and appropriate engineering. Nevertheless, research into performance of these systems under continuous feeding is essential if they are to be implemented in rural communities or developing countries where energy supply is unreliable.

CONCLUSION

This review shows that a combined VSSF with HSSF is a widely recognized and adopted hybrid system for the treatment of domestic wastewater. Despite the high removal efficiency of the V-HSSF hybrid CWs in reducing BOD_5, COD, TSS, TP and NH_4^+-N, water quality concerning nitrogen is low. The latter is attributed to design limitations as most systems are designed based solely on BOD as a target pollutant and not nitrogen. This overview of the current state of the technology also reveals that there is insufficient published information regarding the performance of V-HSSF hybrid CW systems in developing countries particularly in sub-

Saharan Africa. Out of the sixteen surveyed hybrids, only three were reported from Africa (Northern Africa). Further study is therefore required to shed light on final water quality and performance of systems designed on the basis of nitrogen removal and, operating under different climatic conditions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Efficient conditions for in vitro establishment and regeneration of disease-free Ugandan farmer-preferred cassava genotypes

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Cassava (*Manihot esculenta* Crantz) is majorly devastated by two viral diseases, cassava brown streak disease (CBSD) and cassava mosaic disease (CMD), resulting in 100% yield loss. Being a clonal plant, nodal cuttings (NC) and shoot apical meristems (SAMs) are the best explants for production of disease free planting materials. In this study, NCs and SAMs were used to determine reliable indicators for successful *in vitro* establishment of cassava. Eight cassava genotypes were used for the study. Leaf samples were collected from 30 stakes of each of the eight genotypes planted in the screen house. The leaf samples were pooled and screened for presence and/or absence of CBSD and CMD by PCR using virus specific primers. Nodal cuttings were excised from screen house grown plants, surface sterilized to rid-off contaminants and established on Murashige and Skoog (MS) Medium. Using the sprouted stakes, 5-mm sized SAMs were excised, surface sterilized and reduced to 0.5-1 and 2-3 mm sizes. The SAMs were established on MS medium with varying concentrations of plant growth regulators (0.5, 1, 2) ml/L Benzylaminopurine (BAP) and (2, 4) ml/L Naphthalene acetic acid (NAA), Kinetin (K) and BAP respectively. PCR results revealed the pooled leaf samples were free of both CBSD and CMD for all genotypes. Establishment and regeneration of NCs was possible with MS medium for all genotypes. For the SAMs, the concentrations of (2, 4) ml/LBAP followed by 2 ml/LNAA facilitated their establishment and regeneration in comparison to KIN.SAMs of 2-3 mm sizes regenerated better than 0.5 - 1 mm size. Both NCs and SAMs of the different genotypes produced leaves, nodes, roots and there was an increase in plant length. These parameters are critical indicators for *in vitro* establishment and regeneration of cassava.

Key words: Cassava genotypes, cassava diseases, shoot apical meristems, nodal cuttings, growth regulators.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) of the family Euphorbiaceae, is the most important staple food crop grown by low-income African farmers. It is a vegetatively propagated crop and is recycled over seasons. The use

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of planting material from previous generation to establish the next generation provides an easy way for disease-causing pathogens, particularly viruses to pass from one generation to the next resulting in yield reduction, poor seed quality, and lack of available disease-free planting materials (Legg et al., 2011). There has been a decline in production of cassava from 5.5 million metric tonnes (2005) to 2.5 million metric tonnes (FAOSTAT, 2018), as a reult of cassava brown streak disease (CBSD) and cassava mosaic disease (CMD) epidemics (Alicai et al., 2007; Beyene et al., 2016).

The devastating CBSD is caused by at least two viral species: Cassava brown streak virus (CBSV) and Ugandan cassava brown streak virus (UCBSV), both belonging to genus Potyviridae. The CBSD infected tuberous roots are necrotic, rendering the tuber unfit for human consumption (Mbanzibwa et al., 2009). The CMD is caused by cassava mosaic geminiviruses (family Geminiviridae, genus Begomovirus) (Legg and Thresh, 2000). In Uganda, the dominant strains are; African cassava mosaic virus (ACMV) and the East African cassava mosaic virus Uganda variant (EACMV-UG2) (Harrison et al., 1997). Both diseases are transmitted by the whitefly vector (*Bemisia tabaci*) (Maruthi et al., 2005) and through the use of infected cuttings (Thresh et al., 1994) during vegetative propagation. A number of local and improved cultivars are grown by farmers, for their inherent preferred traits including high yield, taste (bitter/sweet), early maturity or long storage in soil, softness, high dry matter content, good root tuber qualities (for cooking and flour), and ready market for fresh roots (Otim-Nape et al., 2001). Unfortunately, many of the landraces were lost to CMD, and improved cultivars bred for resistance to CMD are all susceptible to CBSD (Abaca et al., 2012).

In Uganda, CBSD continues to spread and devastate cassava production and productivity (Alicai et al., 2019). With the help of the eye, foliar and stem symptoms are used by trained field assistants to determine the presence or absence of CBSD disease, of the plants using a known scale of 1-5 (Alicai et al., 2007, 2019). However, research has shown that some genotypes show severe foliar symptoms and others show mild symptoms, even though the plant is carrying the disease (Maruthi et al., 2005; Maruthi et al., 2019). The differences in the foliar symptoms is attributed to factors such as; variety, age of the plant, the viral species present and the environment in which the crop is growing (Maruthi et al., 2019). The levels of expression of the disease in the leaves vary from genotype to genotype. This necessitates validation of the health status of the plant material picked, from different agroecological zones, making it possible to establish virus free materials in tissue culture (Maruthi et al., 2019),

Plant tissue culture (PTC) methods have played an integral part of plant science research because it enables detailed study of plant development and facilitates bulking up of the plant materials (Thorpe, 2007). PTC relies on a phenomenon known as totipotency, which allows a single plant cell to grow, divide and differentiate into a complete new plant. PTC involves the cultivation of cells, tissues, organs in an appropriate nutrient medium, in a sterile environment to facilitate growth and maintain it, in vitro (Thorpe, 2007; George et al., 2008; Lone et al., 2020). PTC techniques have made it possible to eliminate plant pathogens like bacteria and fungus. However, it has not been the same for virus infections in plants (Baker and Jeyaprakash, 2015).

According to Nakabonge et al. (2020), the use of meristem tip culture in combination with hot water treated removed the cassava mosaic viruses (CMV) and reduced the UCBSV in the system of the plant. To further certify the absence of viruses in the explants to be used, molecular biology techniques like the polymerase chain reaction (PCR) are being used. PCR is a simple technique used to amplify and detect DNA and RNA sequences. It allows for enzymatic replication of DNA, amplification of specific regions of a DNA strand, making it possible to detect the viral sequences that are associated with a specific disease (Mullis and Faloona, 1987; Sambrook and Russell, 2001). The demand by farmers to access disease-free planting materials is on the increase, as there is need to certify these materials and accelerate their production in large quantities (Maruthi et al., 2019). In order to understand the response of each genotype and what can be used to measure their ability, with regard to bulking up of materials, nodal cuttings (NC) and the shoot apical meristems (SAMs) were used as explants and morphological features like roots, leaves and so on were documented.

Shoot apical meristems (SAMs) are a population of small, isodiametric cells with embryogenic characteristics found at the extreme end of the shoot axis. SAMs are considered a virus free region, given that most plant viruses do not infect it (Mochizuki and Ohki, 2015). In sweet potatoes (Wondimu et al., 2012) and sugar cane (Cha-um et al., 2006), sizes ranging from (0.5 - 0.7) mm and (0.5 - 1.5) mm respectively have been used for producing disease-free planting materials. Establishment and maintenance of SAMs is facilitated by plant growth regulators (PGR) like auxins and cytokinins that regulate plant growth and development (Donald, 1994; Ha et al., 2010; Murray et al., 2012; Fouracre and Poethig, 2019). Cells arising from the peripheral zones of the apical meristem contribute to formation of organs. The SAMs continuously regenerate itself and also produces leaves, lateral buds as well as stem tissues (Donald, 1994; Ha et al., 2010; Murray et al., 2012; Fouracre and Poethig, 2019), which further facilitates micropropagation or clonal propagation of plants. Thus, plants can be propagated either through the sexual or asexual developmental life cycle.

The sexual cycle of new plants arise from the fusion of

the parental gametes forming zygotic embryos within seeds, each embryo having a new combination of genes due to meiosis, resulting into a new individual plant (George et al., 2008). In the asexual or vegetative cycle, selection of the mother plant or stock plant is crucial. The genes of the mother plant are copied exactly at mitosis, resulting into a new plant that is considered a clone of the mother plant. The asexual approach is considered a more reliable method for true-to-type *in vitro* propagation (George et al., 2008). Micro propagation is a method used for rapid proliferation of tiny stem nodal cuttings, facilitating conservation of germplasm and massive production of disease-free planting materials (Thorpe, 2007; George et al., 2008).

The response of the explant used *in vitro* is dependent on its physiological state, the genotype, the culture media and the health status of the explant (Isah, 2019). The medium is critical because it provides the appropriate nutrients that facilitate growth of the plant. Plant growth involves cell volume increase, cell division, and developmental programmes that specify tissue and organ identity (Lastdrager et al., 2014). Growth of the explants can be measured by using parameters such as number of leaves and roots because they act as reliable indicators for photosynthetic activity and the support system of the plant respectively. The number of nodes can be used to ascertain the number of plants that can be obtained, therefore facilitating the multiplication of plants all year round (Isah, 2019).

Earlier reports on cassava sprouting and regeneration were focused on the use of the stem cuttings (EL-Sharkawy, 2004). Majority of the reports to date have been demonstrated through somatic embryogenesis in the presence of plant growth regulators for Kenyan cultivars (Nyaboga et al., 2013), Ugandan cassava genotypes (Apio et al., 2015), Ghanaian cultivars (Elegba et al., 2021) and so on, in an effort to develop genetic transformation systems for the crop. However, there is no clear documented information on the multiplication rate and regenerative ability of plants generated from in vitro nodal cuttings (NC) and shoot apical meristems (SAMs), as an incentive for farmers. This study was conducted to identify indicators suitable for growth and multiplication of disease-free Ugandan cassava genotypes using nodal cuttings (NC) and shoot apical meristems (SAMs).

MATERIALS AND METHODS

Cassava genotypes

The experiments were conducted at the screen house setup at the National Crops Resources Research Institute (NaCRRI). A total of eight (8) cassava genotypes, seven (7) improved varieties: NASE 3, NAROCASS 1, NAROCASS 2, NASE 13, TME 204, NASE 19, NASE 12 and one (1) land race: Alado, were collected from different agroecological zones of the country. The Northern (Nase 13, Nase 12, Alado, TME 204), Western (NAROCASS 1, NAROCASS 2), Eastern (Nase 3) and Central (Nase 19). These genotypes were selected for their resistance to cassava mosaic disease (CMD) (Nase 3, Nase 13, Nase 12 and TME 204), tolerance to cassava brown streak disease (CBSD) (Nase 19, NAROCASS 1 and NAROCASS 2) and susceptibility to both diseases (Alado) and to CBSD (TME 204). A total of 10 L of the insecticide Cypermethrin at 2 ml/L concentration was prepared. With the help of a 16-ml capacity knapsack sprayer, the prepared cypermethrin was sprayed in and out of the screen house to get rid of whiteflies, mites and other insects a day earlier. Using black forest soil, a total of thirty-one (31) four-nodes stakes were planted for each genotype. The total number of stakes planted was two hundred forty-eight (248). Each pot contained a stake that was watered every morning and evening. Their ability to sprout fully was monitored. The experiment was laid out in completely randomized design (CRD) and was repeated twice. Data on the number of stakes that fully sprouted was taken. Data was analysed using ANOVA at P≤0.05 with Genstat 12th Edition. These stakes were established as a source of explant material for the in vitro experiments in tissue culture and the disease diagnostics in the molecular laboratory.

Leaf samples collection

Leaf samples were collected from a total of 30 sprouted stakes for each of the eight (8) genotypes growing in the screen house. Leaf samples were picked from the top three leaves and placed in 1.5 ml of screw capped vials, which were placed in a styrofoam box containing ice packs and carried to the molecular laboratory for extraction. Leaf samples from 10 plants for each genotype were pooled to achieve one sample. Each genotype therefore had a total of three pooled samples for CMD and CBSD detection. Samples 1-3 represented NASE 19, 4-6 (NAROCASS 2), 7-9 (Alado), 10-12 (TME 204), 13-15 (NASE 12), 16-18 (NAROCASS 1), 19-21 (NASE 3) and 22-24 (NASE 13). Samples for CMD and CBSD detection were handled separately. The total number of samples used was 24 for CMD and 26 for CBSD. For CBSD two samples, one from tissue-cultured and field grown TME 204 plant was added as internal and external checks.

Extraction of total nucleic acids for CMD and CBSD detection

Total nucleic acids were extracted from leaf samples following the cetyl trimethyl ammonium bromide (CTAB) method as described by Lodhi et al. (1994) with some modifications. Firstly, One hundred (100) ml of the CTAB extraction buffer (100 mM TrisHCl, 20 mM EDTA, 1.4 mM NaCl, 5%β-Mercaptoethanol) was prepared. To the CTAB extraction buffer, 0.1 mg/ml of proteinase K enzyme was added before adding to the samples. Secondly, 0.2 g of asymptomatic mature cassava leaves were collected and placed in 1.5 ml screw capped vials, 700 µl of CTAB extraction buffer were added to each sample and homogenized using a Tissue lyser (Model Percellys 24, bertin technologies, France, 2013). The samples were incubated at 65°C for 30 min, and mixed by inversion every 10 min. The samples were kept for 10 min at room temperature, then 700 µl of 24:1 mix of chloroform: Isoamyl alcohol was added to each sample, mixed by inversion for 10 min and centrifuged at 12000 rpm for 10 min. The 550 µl of the upper aqueous phase was transferred to a clean 1.5 ml Eppendorf tube. This step was repeated and 0.7 volume of the upper aqueous phase was added to ice cold (-20ºC) isopropanol, mixed gently then incubated at -20ºC for 1 h, spun for 10 min at 13000 rpm, after which the isopropanol was decanted. To purify the pellet, 500 µl of 70% ethanol was added to the samples, washed by tapping, then centrifuged at 13000 rpm for 10 min. The ethanol was decanted and the samples left to air dry for 40 min. The same approach was used for CBSD samples. The air-dried pellets were resuspended in

300 µl of nuclease free water and stored at -20ºC for CMD analysis and in 50 µl of nuclease free water for CBSD analysis. The purity and quality of each nucleic acid sample was assessed using a NanoDrop spectrophotometer (Model 2000C, Thermo Scientific, Waltham, MA).

Polymerase chain reaction (PCR) for detection of CMGs

Total nucleic acids was used as a template for the standard PCR reaction to detect cassava mosaic Geminiviruses (CMGs) using ACMV and EACMV-UG2 specific primers (ACMV-AL1/F and ACMV-ARO/R) and (ACMV-CP/R3 and uv-AL1/F1) respectively. Fragments produced for ACMV and EACMV-UG2 are about 1000 and 1500 bp, respectively (Harrison et al., 1997). The PCR reaction was performed in a volume of 20 µL containing 12.4 µl of nuclease free water, 2.5 µl of 5X PCR reaction buffer, 1.5 µl of 25 mM $MgCl_2$, 1.0 µl of 10 mM dNTPs (2.5 mM), 10 pmoles of forward primer, 10 pmoles of reverse primer, 0.2 µl of 5U/µl of Taq polymerase and 2 µl of DNA template was added. Two master mixes were prepared for ACMV and EACMV with two different primer pairs for 26 samples each respectively. The PCR conditions were as follows: a one cycle of the denaturation step at 94°C for 2 min, 55°C for 1 min, 72°C for 2 min followed by the annealing step with 30-35 cycles at 94°C for 1 min, 55°C for 1.30 min, 72°C for 2 min and lastly one extension cycle 94°C for 1 min, 55°C for 1 min, 72°C for 10 min and held at 4°C for ACMV and EACMV. The reaction was run for 3 h. The PCR products were electrophoresed in a 1.2% agarose gels stained with ethidium bromide and run at 80 V for 1 h in X1 Tris-Acetate-EDTA (TAE) buffer of pH 8. The gels were visualized under UV light and photographed using a GelDoc system (Model: uGenius3, SYNGENE, UK). The absence of the expected band sizes, indicated absence of the Gemini viruses in the sample.

RT-PCR for CBSD detection of CBSVs

To prepare the cDNA that was used in the PCR reaction, 50 µl of total nucleic acid for each sample plus 7.5 µl of the DNase mix (DNase 1 and DNase 1 buffer) was added to each sample and mixed well. The twenty six (26) samples together with the controls were left to incubate at 37ºC for 30 min. The samples were left at room temperature for 2 min. 1 µl of 0.5M EDTA was added and heated at a temperature of 75ºC for 10 min. The RNA was quantified using a NanoDrop spectrophotometer (Model 2000C, Thermo Scientific, Waltham, MA). To a sterile RNase-free microfuge tube, 1 µg of the RNA sample, 2 µl of the primer d(T)23 VN and a calculated amount of nuclease-free water was added to achieve 8 µl and were mixed together. The sample RNA/d(T)23VN was denatured for 5 min at 65ºC, spun and placed on ice, to generate the first strand cDNA, 10 µl of the ProtoScript II Reaction Mix (2X) and 2 µl of the ProtoScript II Enzyme Mix (10X) were added to achieve a volume of 20 µl. The 20 µl cDNA synthesis reaction was incubated at 42ºC for 1 h and the enzyme deactivated at 80ºC for 5 min. This was done for 28 samples. The reverse transcription reaction product was used for PCR reaction to detect cassava brown streak viruses (CBSVs) using the CBSVDF2 and CBSVDR primers, the fragment sizes obtained for UCBSV and CBSV are 437 and 343 b p respectively (Mbanzibwa et al., 2010). The PCR reaction was performed in a volume of 20 µL containing 6 µl of nuclease free water, 10 µl of One Taq Quick-Load 2X Master Mix with Standard buffer, 1 µl of 10 µM forward primer, 1 µl of 10 µM Reverse primer and 2 µl of the template. The samples were gently mixed. The PCR conditions used were as follows: 94°C denaturation step of 30 s followed by 30 cycles of 30 s at 94°C, 30 s at 51ºC and 30 s at 72ºC; then the final elongation step of 10 min at 72°C and held at 4ºC. The reaction ran for 1 h 46 min. The PCR products were electrophoresed in a 1.2% agarose gels stained with ethidium bromide and run at 80 V for 1 h in X1 Tris-Acetate-EDTA (TAE) buffer of pH 8. The gels were visualized under UV light and photographed using a GelDoc system (Model: uGenius3, SYNGENE, UK). The absence of the expected band sizes, indicated absence of the viruses in the sample.

Establishment of nodal cuttings (NC) in vitro

Nodal cuttings were excised from the fully grown tested disease free cassava plants in the screen house. NC was surface sterilized using 2% of 3.85 m/v Sodium hypochlorite (NaOCl), 2-3 drops of Tween-20, and swirled for 10 min to ensure microbes are removed. The NC was rinsed using tap water and transferred to sterile conditions under the laminar flow hood for further sterilization. To the 500 ml of 2% NaOCl, 4-5 drops of tween-20 was added, stirred and dispensed into a jar carrying NC of each genotype. These were further swirled for 10 min and rinsed five times with sterile distilled water under a laminar flow hood. The edges of the nodal cuttings which were affected by bleach were cut off. The remaining explants was placed on Murashige and Skoog (MS) basal medium with vitamins (Duchefa Biochemie), with 2% of sucrose (Merck) and 3 g of gelrite (Duchefa Biochemie) and monitored for their response *in vitro*. This was done for all eight genotypes. A total of 31 NC were used for each genotype. The cultures were placed in a growth room at temperature of 28 ± 2°C and photoperiod of 16 h of light and 8 h of darkness. Completely randomized design was used. The experiment was repeated twice. Data on sprouting, number of leaves, nodes, length of plants were documented. Data was analysed using ANOVA at P≤0.05 with Genstat 12th Edition.

Establishment of shoot apical meristems (SAMs) *in vitro*

The shoot apical meristems (SAMs), 5 mm in size, were excised from the 2 months old mother plant confirmed to be disease-free. The SAMs were surface sterilized using 1% of 3.85 m/v Sodium hypochorite (NaOCl), 2-3 drops of Tween-20, and swirled for 5 min to remove microbes. The SAMs were rinsed using tap water and transferred to sterile conditions under the laminar flow hood for further sterilization. To the 200 ml of 1% NaOCl, 2-3 drops of tween-20 were added, stirred and dispensed into the jar carrying the SAMs of each genotype. These were further swirled for 5 min and rinsed three times with sterile distilled water under the laminar flow hood. The protective layers of the SAMs were excised under a dissecting microcospoe using a hypodermal needle to achieve, 2-3 mm size explants and placed on MS basal medium supplemented with Benzylaminopurine (BAP) at varying levels (0.5, 1 and 2 ml/L). This was done for eight genotypes. Using 90 x 15 cm sized petri dishes, 5 explants of 2-3 mm sized SAMs were plated. Four plates were setup for each genotype. A total of 20 SAMs 2-3 mm were established for each genotype. The subsequent experiments were conducted using 0.5 – 1 mm size SAMs, which were placed on MS basal medium with vitamins (Duchefa Biochemie) containing three plant growth regulators: Benzylaminopurine (BAP), Naphthalene acetic acid (NAA) and Kinetin (KIN) at two concentration levels (2 and 4 ml/L), 2% sucrose (Merck) and 3 g of gelrite (Duchefa Biochemie) and monitored for their response in vitro. This was done for all the six genotypes. Each petri dish 90 x 15 cm size contained 4 explants. Three petri dishes were set up for each genotype. A total of 12 SAMs of 0.5-1 mm were established for each genotype. The cultures were placed in a growth room at temperature of 28 ± 2°C and photoperiod of 16 h of light and 8 h of darkness. Completely randomized design was used. Each experiment was repeated twice. Data on sprouting, number of leaves, nodes, roots and plant length were taken. Data was analysed using ANOVA at P≤0.05 with Genstat 12th Edition.

Figure 1. Establishment of cassava stakes in the screen house. a) Uniform size stakes for planting, b) Stakes of cassava planted in soil, c) Sprouted stakes of Nase 12, with the yellow arrow indicating a plant that dried out after sprouting.

RESULTS

Establishment of nodal cuttings (NC) in the screen house

The cassava stem cuttings (stakes) were established insterilized soil and watered morning and evening to ensure their growth. Stakes from all the genotypes sprouted (Figure 1). Significant differences were observed among the genotypes (P = 0.017) for sprouting at 5% CI. According to the results obtained, Alado and TME 204 had the highest number of stakes that sprouted while Nase 13 had the least (Table 1).

CBSD and CMD detection

Leaf samples collected from all screen house-grown cassava genotypes tested negative for the presence of the African Cassava Mosaic Virus (ACMV) with a band size of 1000bp and the East African Cassava Mosaic Virus (EACMV) with a band size of 1500bp (Figure 2a). Sample 24 was lost during processing to obtain DNA (Figure 2a). Similarly, all leaf samples analyzed for CBSD were negative for the presence of the cassava brown streak viruses (CBSVs) (Figure 2b). A field Sample 26 from CBSD symptomatic plant used as a check, tested positive for CBSD (Figure 2b). A disease-free tissue culture lab Sample 25, was used as an internal check (Figure 2b). The band size of 437bp represented CBSVs, while the Uganda cassava brown streak virus (UCBSV) were represented by the band size of 343bp (Figure 2b).

Responses of nodal cuttings (NC) to *in vitro* conditions

All the eight genotypes were subjected to the steps as illustrated in Figure 3. Most of the nodal cuttings sprouted to produce leaves, nodes and roots (Figure 3g) and for others, bacterial contamination was observed (Figure 3e). Significant differences were observed among the genotypes for production of leaves (P = <.001). The genotype NASE 19 had the highest average number of leaves (3.9±0.35) while NAROCASS 1 had the least (2 ± 0.14) (Figure 4a). Significant differences were observed among the genotypes for the number of nodes observed (P =<.001). The highest average number of nodes was observed in NASE 19 (4.0 ± 0.44) and least in NAROCASS 1(1.4±0.13) (Figure 4b). Similarly, significant differences were observed among the genotypes for the ability to produce roots (P = <.001). NASE 12 had the highest average number of roots (7.2 ±0.56), while NASE 3 had the least (0.9 ±0.43) (Figure 4d). No roots were produced by NAROCASS 1 (Figure 3h; Figure 4d). Also, significant differences were observed among the genotypes for plant length (P = 0.005). NASE 13 had the highest average of plant length (2.24±0.25) and NAROCASS 1 had the least (0.55 ± 0.18) (Figure 4c).

Table 1. Percent sprout of the selected farmer-preferred cassava genotypes.

SN	Genotype	Sprouting (%)*
1.	Alado	100
2.	Nase 19	94
3.	Nase 12	97
4.	Nase 13	81
5.	Nase 3	97
6.	NAROCASS 1	94
7.	NAROCASS 2	97
8.	TME 204	100

*Mean values of sprouted stakes of the genotypes from two experiments.

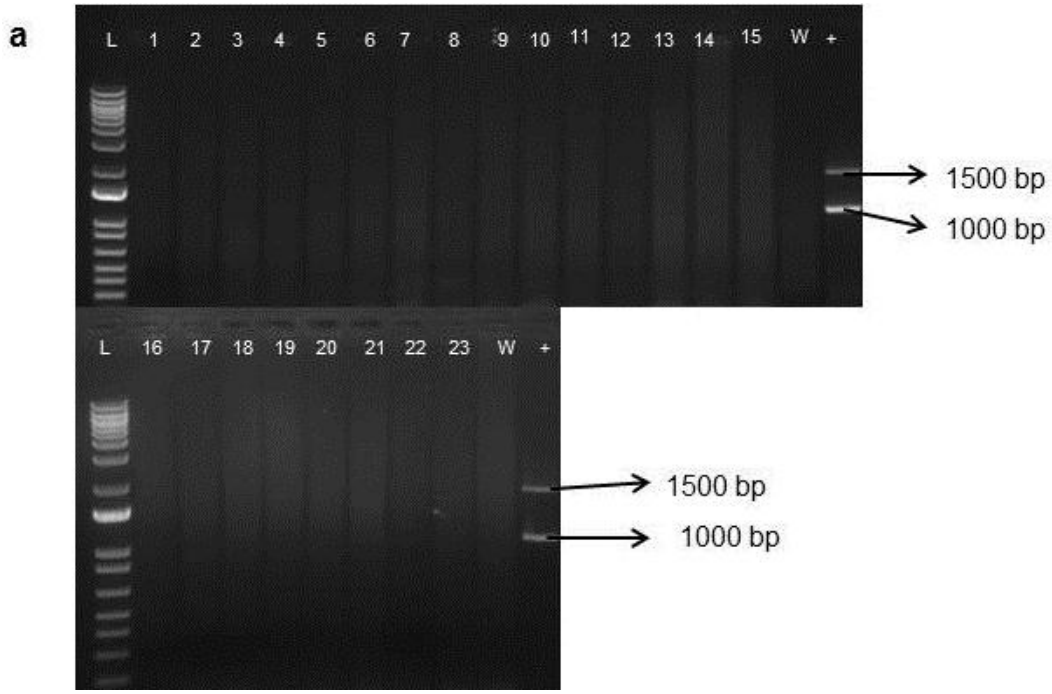

Figure 2a. PCR results for plants tested for the cassava mosaic disease (CMD). The plants were all negative for the disease. L – 1 Kb plus ladder, W – Negative check and + - Positive check, 1-23 leaf samples from test plants.

Responses of shoot apical meristems (SAMs) to *in vitro* conditions

In general, most SAMs obtained from all genotypes, sprouted and regenerated into plantlets (Figure 5) in the presence of plant growth regulators *in vitro* for 2-3 mm size (Figure 6a). In the case of 0.5 - 1 mm size, not all SAMs from genotypes regenerated (Table 2); this was dependent on the plant growth regulator (PGR) used, the concentration of the PGR and the genotype (Table 2). Preliminary experiments on SAMs conducted with 2-3 mm sized explant on varying concentrations of Benzylaminopurine indicated that the SAMs were able to regenerate (Figure 6a). Significant differences were observed in relation to effect of genotype ($P \leq 0.001$) while no significant differences were noted for the auxin levels ($P = 0.741$) and the interaction ($P = 0.610$) at 5% CI (Figure 6a). Highest regenerative ability was observed in TME 204, whereas NASE 12 had the least regenerative ability (Figure 6a). In the case of 0.5 – 1 mm sized SAMs, not all explants sprouted for the genotypes given the PGR concentrations used (Table 2). Overall, Nase 19 had plants sprout with all the PGR concentrations. The highest percentage of sprouting was in Nase 19 (100%)

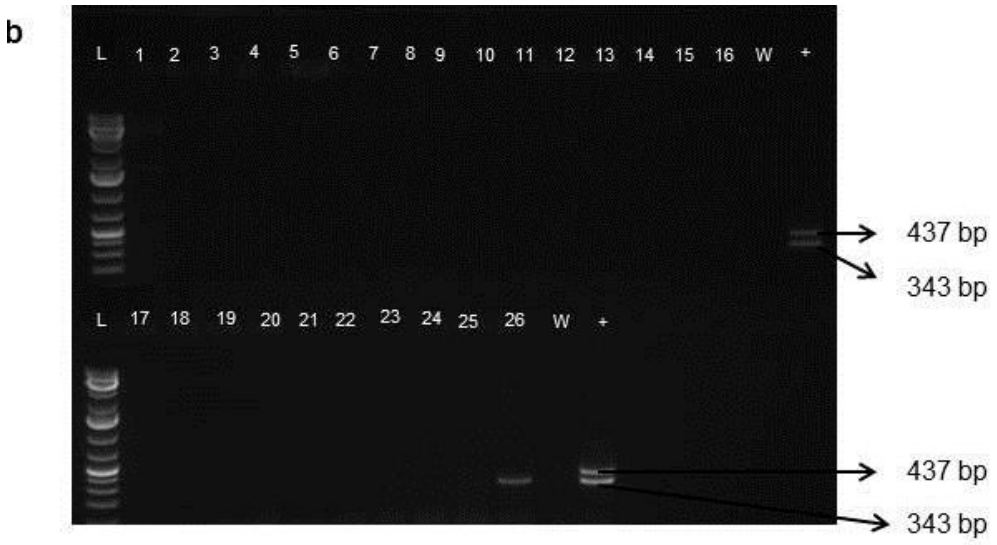

Figure 2b. PCR results for plants tested for cassava brown streak disease (CBSD). The plants were all negative for the disease, except for field sample 26 which showed symptoms of the disease. L- 1Kb plus ladder, W – Negative check and + - Positive check, 1 – 26, leaf samples from test plants.

in media with (2, 4) ml/L BAP (Table 2). Significant differences were observed due to the effect of the plant growth regulators ($P=1.19X10^{-20}$), genotypes ($P=2.98x10^{-43}$) and the interaction ($P=3.76x10^{-31}$) at 5% CI (Table 2).

Responses of SAMs (2-3) mm sized in vitro

Given the demand and response of these genotypes *in vitro*, five (5) genotypes out of the eight were selected as a sample representation, to ascertain the number of roots, nodes and leaves, as indicators for their establishment of 2-3 mm SAMs (Figure 6b, c, d). Explant sizes of 2-3 mm produced leaves with an average number of 4-6 leaves. Significant differences were observed in the number of leaves produced in presence of auxins (P≤0.001), genotype (P≤0.001) and the interaction between auxin and genotype (P≤0.001). The highest average number of leaves were produced by Alado (7.25±0.58) in media with 2 ml/L BAP, while the least average number leaves were produced by NASE 14 (4.29±0.45) at 1 ml/L BAP (Figure 5c and Figure 6b). Significant differences was also observed in the number of nodes produced in the genotypes (P≤0.001), and interaction of the auxin and the genotype (P=0.005). No significant differences were observed in number of nodes produced due to presence of the auxins (P = 0.435). The highest average number of nodes were observed in Alado (3.74±0.29) in media with 1 ml/L BAP and the least average number of nodes in NAROCASS 1 (0.33±0.16) at 1 ml/L BAP (Figure 5c and Figure 6c). Significant differences were observed in number of roots produced due to the auxins (P≤0.001) and the interaction between auxin and the genotype (P = 0.007). No significant differences were observed for the number of roots produced in relation to the genotype (P = 0.140). The highest average number of roots were observed in Alado (3.38±0.42) at 0.5 ml/L BAP, whereas NAROCASS 1 had the least average number of roots (0.33±0.22) at 2 ml/L BAP (Figure 5c and Figure 6d).

Responses of SAMs (0.5-1) mm sized in vitro

Given the responses of the 2-3 mm sized SAMs, subsequent experiments on SAMs were done on 0.5 -1 µM explant sizes for the six genotypes (Alado, NAROCASS 1, NAROCASS 2, TME 204, NASE 19 and NASE 12) on three different plant growth regulators (PGR) (Benzylamionpurine (BAP), Naphthaleneacetic acid (NAA) and Kinetin (KIN)). The responses of the genotypes varied with the PGRs used and their concentrations (Table 2). All genotypes produced leaves, nodes, roots and showed an increase in plant length (Figures 5d and 7). In some instances, depending on the plant growth regulator (PGR) used; no roots were produced by some genotypes (Figure 7). Overall, NASE 19 performed better than all other genotypes, while Benzylaminopurine (BAP) facilitated regeneration across all genotypes in comparison to the plant growth regulators (Figure 7). At 2 ml/L, BAP facilitated production of leaves generally in all the genotypes followed 4 ml/L BAP. Significant differences were noted in the ability of genotypes to produce leaves from the 0.5 -1 mm sized SAMs, in relation to the PGRs levels (P≤0.001), genotype (P≤0.001) and the interaction

Figure 3. Pictorial representation of collection of plant materials from screen house and the establishment of nodal cuttings in vitro. a) Collected plant material of Alado, b) Single plantlet of Alado, c) Nodal cuttings with no branches and leaves, d) Excising of the nodal cutting to achieve a single node e) Sprouted nodal cuttings of NAROCASS 1, seven days after in vitro establishment on MS medium, with the white arrow showing the bacterial contamination and f) Fully developed plants from nodal cuttings of NASE 13 after 28 days, g) A sample plant to show data collected on leaves, nodes, roots and plant length, h) A plant of NAROCASS 1 which developed roots, leaves, plant height and no roots.

(P≤0.001) (Figures 5d and 7a). Alado did not perform well compared to the rest of other genotypes. The interaction between the PGR levels and genotypes indicated that 2 ml/L BAP, 4 ml/L BAP and 2 ml/L NAA promoted the production of leaves across genotypes (Figures 5d and 7a).

Significant differences were noted in the ability of genotypes to produce number of nodes on the stem from the 0.5 -1 mm sized SAMs, in relation to the auxin levels (P≤.001), genotype (P≤0.001) and the interaction (P≤0.001) (Figure 7b). The auxin concentrations that facilitated the production of nodes were 2 ml/L NAA and the 2 ml/L BAP across the genotypes. At interaction of the auxin and the genotypes, 2 ml/L BAP, 4 ml/L BAP and 2 ml/L NAA facilitated production of nodes (Figure 7b). Significant differences were also noted in the ability of genotypes to produce roots from the 0.5 -1 mm sized SAMs, in relation to the auxin levels (P = 0.021) and the interaction (P≤0.001). No significant differences were noted in relation to the genotypes (P = 0.294) (Figure 7d). In most instances, production of roots was observed in most of the genotypes, in the presence of 2 ml/L BAP, 2 ml/LNAA and in some cases 2 ml/L KIN. The different levels PGRs impacted on the production of roots negatively in Alado compared to the rest of the genotypes (Figure 7d). The interaction of the auxin and genotypes indicated that 2 ml/L NAA, 2 ml/L BAP and 4 ml/L BAP facilitated growth of roots in the genotypes (Figure 7d). Significant differences were noted in the ability of genotypes to have their plant length increase from the 0.5 - 1 mm sized SAMs, in relation to the auxin levels (P = 0.009), genotypes (P≤0.001) and the interaction (P≤0.001) (Figure 7c). The PGRs at 2 ml/L NAA and 2 ml/L BAP promoted increase of the plant length. Alado had the highest plant length among the genotypes. The interaction of PGRs and genotypes facilitated increase in plant length among the genotypes at 2 ml/L BAP, 4 ml/L BAP and 2 ml/L NAA (Figure 5i; Figure 7c).

DISCUSSION

The growth of plants *in vitro* is influenced by factors such as physiological state of the explant, the genotype, the health status of the plant and the culture media. The health status is a very crucial indicator as to how the plant responds *in vitro* (Isah, 2015, 2019). The results indicate that all the genotypes tested for the presence and absence of the cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) were negative indicating that the pre-basic planting materials were free from viruses, therefore certified as healthy. However, it is

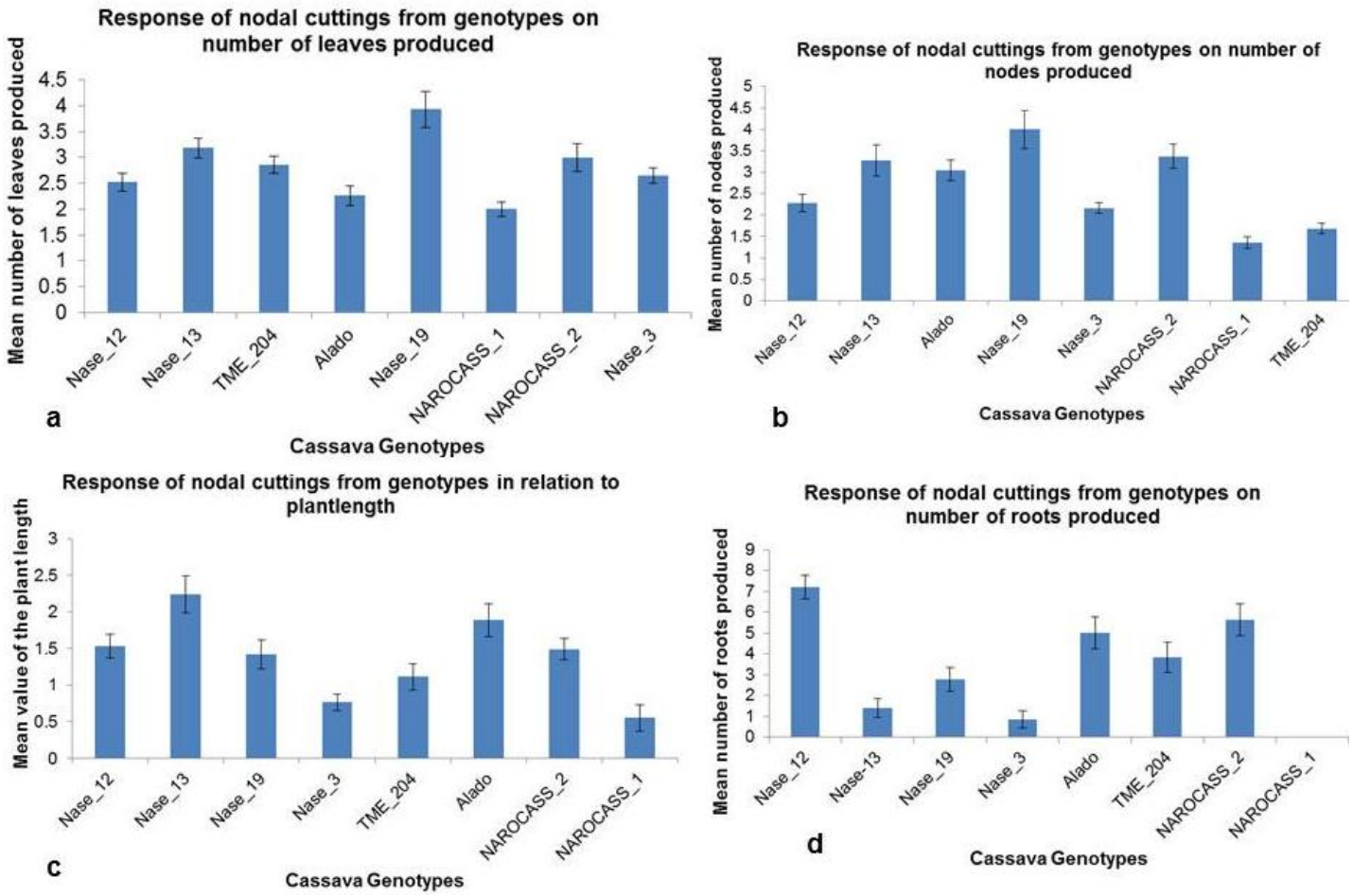

Figure 4. The average number of leaves, nodes, roots and plant height from selected cassava genotypes established in vitro using nodal cuttings. Values are mean ± SD of two independent experiments.

important to note that in both situations (nodal cuttings and shoot apical meristems), some explants were lost due to bacterial or fungal contamination as noted in Figure 3e for nodal cuttings and Table 2 for the 0.5-1 mm SAMs. The death of the explants indicated that the plant innate immune response was triggered on detection of microbe-associated molecular patterns (MAMPs) (He et al., 2007; Bolton, 2009), and the plants ability to recognize pathogen effectors through the resistance proteins was low (Bent and Mackey, 2007; Bolton, 2009); therefore, the available defense mechanisms were not as operative against the pathogens that attacked the explants (Katagiri, 2004; Bolton, 2009).

In some instances, it was noted that the potential of the explants to grow fully into complete plants, was curtailed or reduced, which was observed in plants with reduced plant height. Research in Arabidopsis has indicated that when allocation of resources towards defense responses associated with bacteria and fungus occur in plants *in vitro* (Heil et al., 2000), the plant is forced to adjust growth to reduced levels of carbon available (Bolton, 2009), resulting in poor growth and yields.

Growth in plants is linked to an increase in size associated with cell division and cell elongation (Lastdrager et al., 2014). Cell division and expansion is highly coordinated by a number of molecular networks, which are triggered depending on the environment of the plant (Gonzalez et al., 2012) and therefore facilitate developmental programs that specify tissue and organ identity (Lastdrager et al., 2014). There were differences observed in the responses of the explants to the *in vitro* growth culture conditions, which was evidenced in the parameters measured such as number of leaves, roots, nodes and plant length. The findings indicate that the growth pattern of the plants given the parameters used, was better in nodal cuttings (NC) than shoot apical meristems (SAMs). This could be attributed to the fact that architectural development of nodal cuttings is already set therefore the explant utilizes the available sucrose in the media for energy and biomass, allowing for production of new cells and transition from a vegetative to a generative phase (Rolland et al., 2006; Lastdrager et

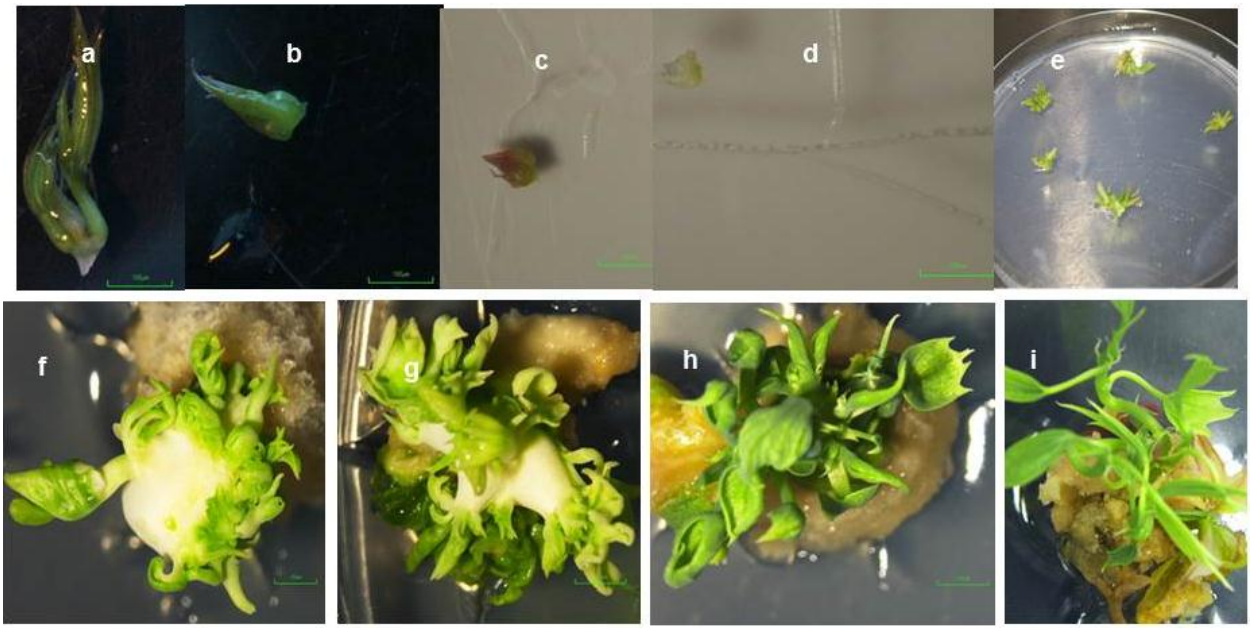

Figure 5. Different stages from collection of SAMs to regeneration into complete plants. a) The SAM explant excised from screen house-grown mother plant, b) SAMs to which leaf petioles have been removed c) 2-3 mm size of SAMs, d) 0.5-1 mm size SAMs, e) Sprouting SAMs, seven days after establishment, f & g) Progressive stages of germination and regeneration, g) A regenerated plant with defined leaves, h) A fully regenerated plant with well-defined stem and leaves.

al., 2014), while for the SAMs, cell division, cell differentiation and maturation into complete plants is possible in the presence of cytokinins and auxins (Murray et al., 2012).

Auxins are central to plant growth and development by moderating the activity of auxin response factors (ARFs), whose metabolism and transport are modulated by sugars, that induce phytochrome-interacting factors (PIFs) that promote growth (Leivar and Quail, 2011; Lastdrager et al., 2014). The study investigated the use of auxins like naphthalene acetic acid (NAA) and cytokinins like benzylaminopurine (BAP) and Kinetin (KIN) to promote growth and development of the SAMs despite the sizes used. The results show that for the different sizes of SAMs, the cytokinin BAP facilitated growth and development of the SAMs better than kinetin. A detailed study in *Arabidopsis thaliana*, indicated that the high cytokinin levels in SAMs, sustain the stem cell population (Shani et al., 2006; Murray et al., 2012), and can do so due to the SHOOT MERISTEMLESS (STM), homeodomain transcription factor which promotes cytokinin sysnthesis by increasing ISOPENTENYL TRANSFERASE (IPT) gene expression (Yanai et al., 2005; Murray et al., 2012). The larger sizes of SAMs (2-3 mm) established in BAP, responded better and faster with regard to growth than the smaller sizes (0.5-1 mm) grown in the same medium. This could be associated with the fact that when cytokinins are applied alone, they have the ability to increase the apical growth leading to initiation of primordia, indicating that cytokinins stimulate growth of SAMs (Yoshida et al., 2011; Murray et al., 2012). On the other hand, while there is a high level of auxins at the SAMs center, previous work suggests there is active repression of auxin responses alongside the high levels of cytokinin signaling (Bartrina et al., 2011). However, it is important to note that auxins play an important role in organ initiation and positioning at the SAM (Murray et al., 2012). This may explain the responses observed in the explants when the auxin NAA was used alone.

The auxins and cytokinins in these experiments were used singly; however, in each situation, depending on the concentration of the auxin and cytokinin, the SAMs were able to regenerate into complete plants by producing the leaves, roots and an increase in plant length was observed. The SAMs are indeterminate structures which continue to grow as long as the environmental conditions favour growth. They form phytomere which is a developmental unit consisting of many leaves, and a node to which the leaves are attached (Lazar, 2003). Similar observations were noted in the emergence of the SAMs despite the sizes used for the different genotypes. The development of leaves indicated the ability of the explants to photosynthesize; given that leaves are organs used by the plant to capture light energy, which further facilitates chemical reactions that sustain the life of the plant. For explants like nodal cuttings (NC), which are determinate, the emergence of leaves is genetically programmed (Lazar, 2003).

The findings indicate that the cytokinin BAP facilitated

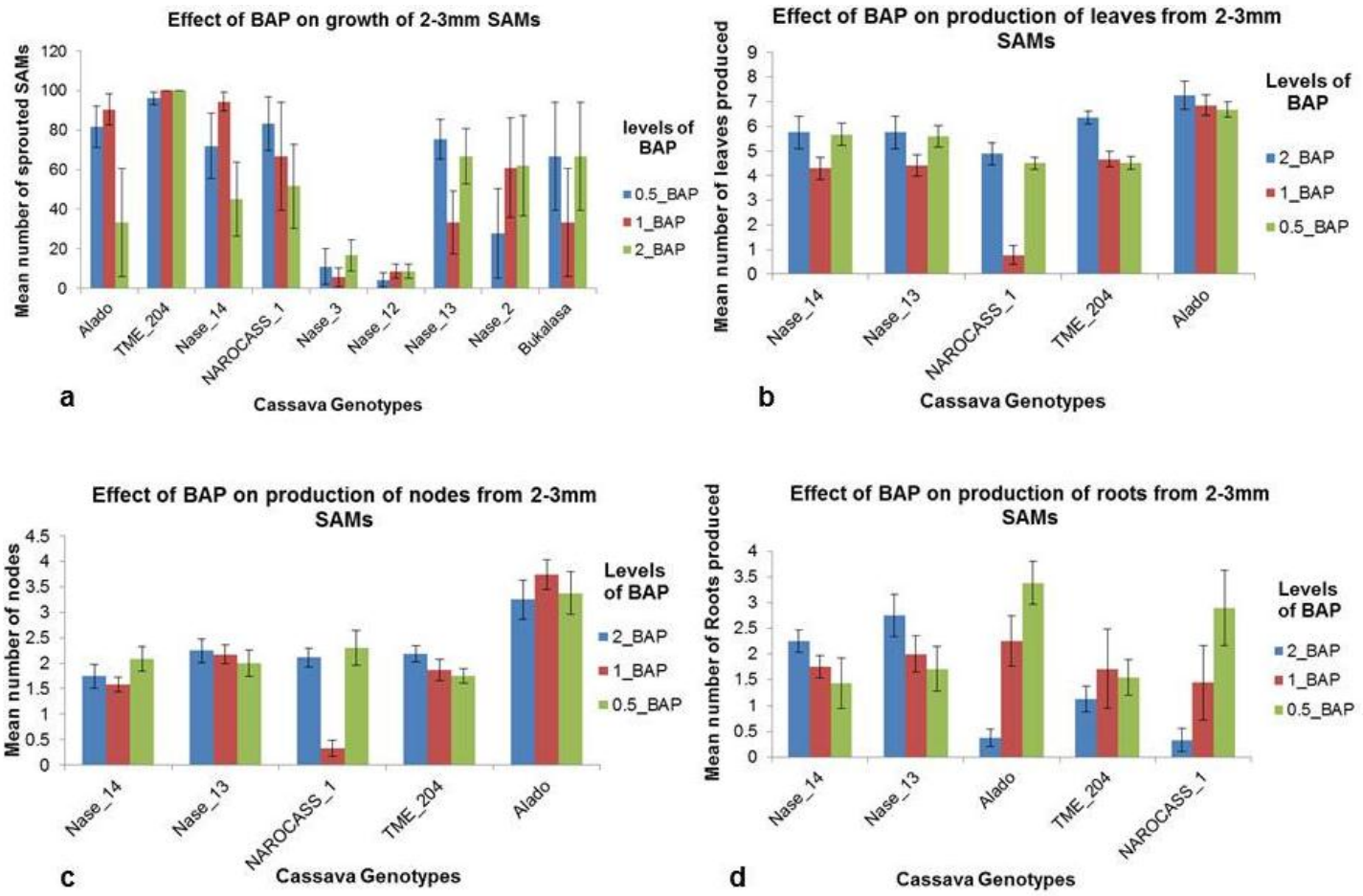

Figure 6. The regenerative ability of the Shoot Apical Meristems (SAMs) (2-3) mm sizes for eight cassava genotype in vitro. Values are means ± SD of two independent experiments.

Table 2. Percentage sprout of 0.5-1mm sized SAMs from cassava genotypes.

	*Sprouting (%) of SAMs					
Plant growth regulator levels	**Nase_19**	**Nase_12**	**NAROCASS_2**	**NAROCASS_1**	**Alado**	**TME_204**
2_BAP	100	25	41.7	8.3	41.7	25
4_BAP	58.3	0	58.3	79.2	0	25
2_NAA	16.7	16.7	8.3	8.3	0	25
4_NAA	100	0	33.3	8.3	0	25
2_KIN	33.3	25	0	8.3	0	0
4_KIN	58.3	58.3	8.3	8.3	16.7	0

*Mean values of sprouted 0.5 -1 mm SAMs from two experiments Values are mean ± SD of two independent experiments. Data was analysed using ANOVA at P≤0.05 using Genstat 12th Edition.

the regeneration of the SAMs into complete plants better than kinetin, despite the fact that similar concentrations were used. In the case of BAP, both concentrations allowed for regeneration at different percentages while in the case of kinetin, 4 ml/L favoured regeneration. This could be associated with the fact that different concentration levels of each cytokinin, may result in expression of different transcription factors that promote different developmental pathways (Bhatia, 2015), as noted in both cytokinins. The responses observed among the genotypes with regard to the number of leaves, nodes, roots produced and plant length noted, which

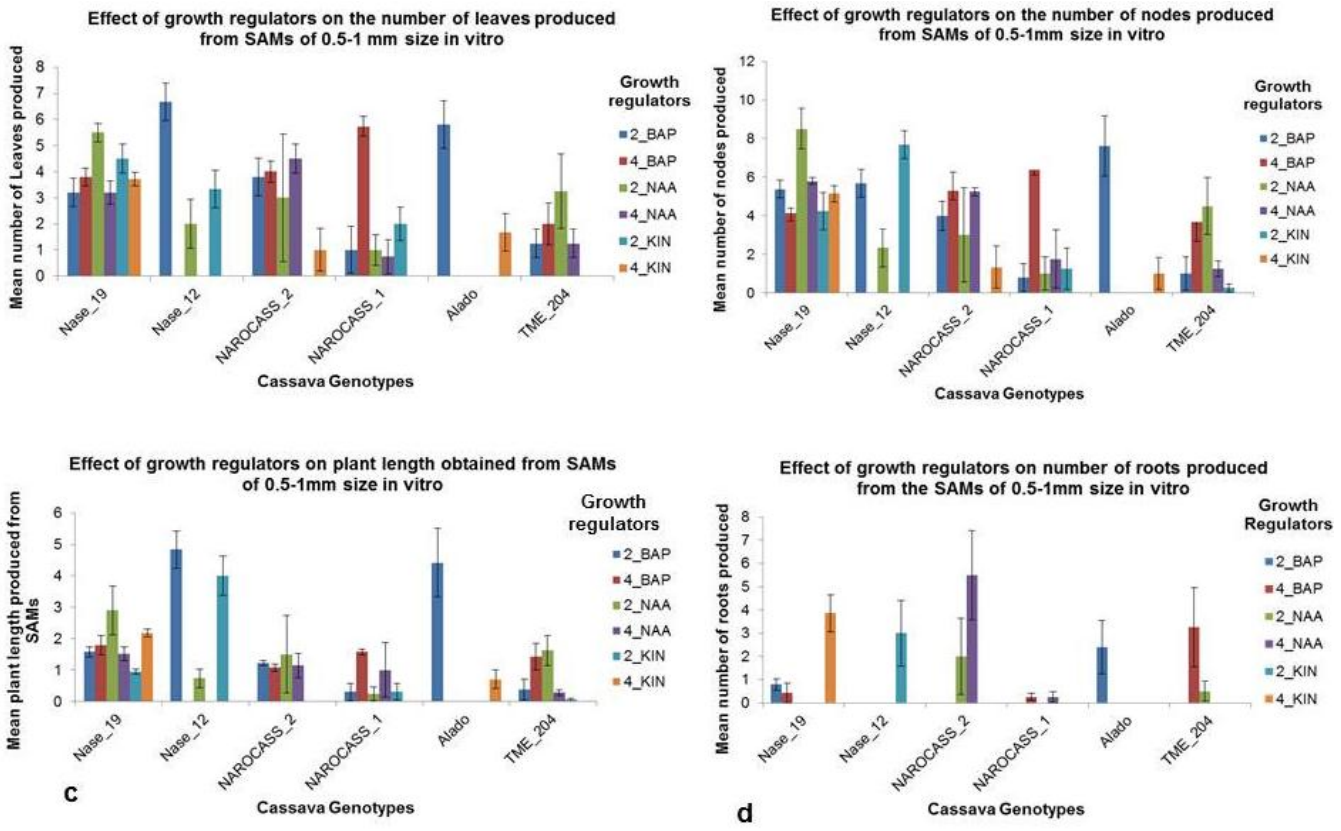

Figure 7. The average number of leaves, nodes, roots and plant length of six cassava genotypes established *in vitro* using SAMs (0.5-1 mm in size) on different concentrations of growth regulators. Values are means ± SD of two independent experiments.

were used to assess the growth and regenerative ability of the SAMs and NCs, suggest that the genetic differences for each cassava genotype, together with *in vitro* conditions like temperature and photoperiod (George et al., 2008) were crucial for regeneration.

The ability of the explants to produce roots plays an important role in absorption of water and minerals nutrients to facilitate plant growth and help the plant to be firmly anchored in the medium. In both explants used, it was noted that root emergence was observed 14 days after co-culture in the appropriate medium (Lazar, 2003). The findings obtained on the number of nodes and the plant height are important indicators as to how many explants can be generated, facilitating bulking up of plant materials all year round through micro propagation. SAMs can also be used for production of disease-free planting materials (George et al., 2008; Isah, 2015).

Conclusions

The number of leaves, roots, nodes and the plant length of cassava plants are reliable indicators of plant growth, development and establishment *in vitro*, for both the nodal cuttings (NC) and shoot apical meristems (SAMs). The auxin and cytokinin at the three concentrations, (2 and 4 ml/L BAP as well as 2 ml/L NAA) facilitated growth and development of the explants into complete plants singly. Plant growth regulators are essential for the development of SAMs, which is not necessary for the NCs. With the demand for certified materials on the rise, the information generated from this study, gives a clear picture of the vigor of different genotypes in vitro. These parameters can also be used to facilitate micro propagation and production of disease-free plants all year round.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Adenine sulfate and glutamine enhanced shoots multiplication in *Jatropha curcas* L. a potent biofuel plant

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Jatropha is a drought-tolerant plant producing seed oil for the biodiesel. Limitation to the development of jatropha is unavailability of high-yielding varieties and efficient *in vitro* regeneration system which is required for micropropagation. In this study, *in vitro* regeneration system from jatropha juvenile cotyledon was established. Firstly, concentrations of hormones in the MS medium were optimized and it was found that 1.5 mgL^{-1} benzyl adenine (BA) + 0.05 mgL^{-1} indole-3-butyric acid (IBA) + 0.5 mgL^{-1} thidiazuron (TDZ) turned out to be the best for shoot induction (3.82 ± 0.18 shoots/explant). Secondly, shoot induction medium was fortified with different concentrations of glutamine and adenine sulfate. It was found that 25 mgL^{-1} each of glutamine and adenine sulfate was the most effective, resulting to 9.09 ± 0.37 shoots/explant and 93.0% regeneration frequency. Regenerated shoots were cultured on medium containing 0.5 mgL^{-1} BA and different concentrations of gibberellic acid (GA_3), 0.5 mgL^{-1} GA_3 with 0.5 mgL^{-1} BA were found to be the best for shoot elongation (2.13 ± 0.18 cm). The highest frequency of root (40%) was observed on the medium with 0.5 mgL^{-1} IBA. The established procedures will be useful for the mass propagation and genetic transformation of elite jatropha genotypes.

Key words: Adenine sulfate (Ads), benzyl adenine (BA), indole-3-butyric acid (IBA), *in vitro* regeneration, glutamine (Gln)

INTRODUCTION

Jatropha *(Jatropha curcas*) is a potent biofuel crop native to Mexico and Central America and now is distributed throughout tropical and subtropical regions (Kumar and Tewari, 2015). Its seed contains high amount of oil in the concentration of 30 – 60% (Openshaw, 2000; Kumar and Sharma, 2008), which is non-edible (Chhetri et al., 2008) and suitable for the biodiesel (Heikal et al., 2015). Due to the depletion of fossil fuel reserves, increasing petroleum prices and global climate changes, jatropha has received considerable attention as renewable energy sources

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(Pandey et al., 2012). However, the low productivity under certain conditions restricts fuel usage of jatropha, mainly because it has not been domesticated for large-scale production. Therefore, increasing oil yield, growing ability under abiotic stresses and improvement of agronomic traits must have a priority. Jatropha plant is propagated through asexual methods and by seeds, and thus seed yield and oil content varies significantly (Jha et al., 2007). Furthermore, seeds of *J. curcas* have a limited viability and can only be stored for 15 months after which their viabilities are reduced by 50% (Kochhar et al., 2005). Cuttings propagation of jatropha can be carried out for maintaining true to type genotypes, but the produced plants do not have deep roots and the quality is not sufficient to meet the growing demand of *J. curcas* (Heller, 1996; Openshaw, 2000). Additionally, propagated plants exhibited lower longevity and resistance to drought and diseases (Sujatha et al., 2005). Generally, vegetative propagation methods have drawbacks such as sources of disease transmission (Fufa et al., 2019). Therefore, an efficient *in vitro* regeneration for mass production of disease free and true to type jatropha genotypes is desirable. It can propagate superior genotypes and contributes to plant improvement through the application of biotechnological techniques.

Recently, several studies have been reported on the regeneration of jatropha by using various explants and different combinations of phytohormones and additives (Chiangmai et al., 2015; Gangwar et al., 2015; Jadon et al., 2015; Liu et al., 2015; Mishra, 2018; Fufa et al., 2019). BA and IBA were found to be effective growth regulators for the induction of callus and shoot regeneration from various explants of *J. curcas* plant. Previously in the laboratory, the suitable concentration of TDZ (0.5 mgL^{-1}) for callus induction and combination of BA and IBA for shoot multiplication from juvenile of jatropha were established by Khemkladngoen et al. (2011). However, the low regeneration efficiency is a main obstacle to jatropha regeneration. Thus, this study—aimed to investigate the effect of adding adenine sulfate and glutamine in shoot regeneration media on shoots multiplication. Here, we have developed an efficient *in vitro* regeneration protocol for shoots induction, multiplication and plant regeneration from juvenile cotyledons explants.

MATERIALS AND METHODS

All experiments were conducted at Plant Bioengineering for Bioenergy Laboratory, Department of Biotechnology, Osaka University. Suita City, Osaka, Japan.

Plant materials and preparation of explants

Mature decorated seeds of jatropha (Thai line) were surface-sterilized with 70% (v/v) ethanol for 2 min followed by 40% (v/v) sodium hypochlorite and 0.01% Triton X-100 for 10 min, and then washed five times with sterile distilled water. After washing, surface-sterilized seeds were soaked overnight. The sterilized seeds were germinated *in vitro* on half strength hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose, 10 mgL^{-1} thiamine, 100 mgL^{-1} *myo*-inositol (pH 5.8) and 0.8% agar at 25°C for one week. The juvenile cotyledons (Figure 1a) were used to prepare explants as described in Khemkladngoen et al. (2011). They were cut into pieces (3 mm x 3 mm) and used as explants (Figure 1b).

Callus induction

Explants were cultured on a callus induction medium consisting of MS medium supplemented with different concentration sets of benzyl adenine (BA: 1.0, 1.5 and 3.0 mgL^{-1}) and indole-3-butyric acid (IBA: 0.5 and 0.05 mgL^{-1}), as well as 0.5 mgL^{-1} thidiazuron (TDZ) and 0.8% (w/v) agar. Cultures were incubated at 25 ± 2°C under 16-h lights (31-35 µmol photon m^{-2} s^{-1})/ 8 h dark photoperiod for two weeks. The experiment was replicated twice.

Shoot regeneration

Two approaches were used in this study. Firstly, calli induced from cotyledon explants in the callus induction media were subcultured on MS medium supplemented with the same concentration sets of BA and IBA as the callus induction media. Secondly, calli induced from cotyledon explants in the callus induction medium containing 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA and 0.5 mgL^{-1} TDZ were subcultured on MS medium supplemented with combination of 1.5 mgL^{-1} BA and 0.05 mgL^{-1} IBA in the presence of different concentrations of adenine sulfate and glutamine mixture (0, 5, 10, 15, 20 and 25 mgL^{-1} each). Cultures were incubated under the same condition as described above for 2 weeks and subcultured twice. The frequency of shooting response and shoots formed per explant were recorded. Each experiment was conducted twice.

Shoot elongation

The regenerated shoots were cultured on MS medium supplemented with 0.5 mgL^{-1} BA in combination with different concentrations (0.1 – 1.0 mgL^{-1}) of gibberellic acid (GA_3) for shoot elongation. Cultures were incubated under the same condition as described above for 4 weeks. The experiment was replicated twice.

Rooting and acclimatization

The elongated shoots were excised individually and transferred to rooting medium, which consisted of a half-strength B5 medium supplemented with 2% (w/v) sucrose, 10 mgL^{-1} thiamine, 100 mgL^{-1} *myo*-inositol, four concentrations of glutamine (0, 5, 10 and 15 mgL^{-1}) in combination with 0.5 mgL^{-1} IBA and 0.7% agar (pH 5.8). Cultures were incubated under the same condition as described above for 6 weeks. Plantlets with rooted shoots were transplanted into autoclaved soil in small pots covered with transparent plastic lids and maintained under high humidity for 7 days, and thereafter gradually exposed to the growth chamber condition. Established plantlets were then transferred to plastic pots containing soil and cultivated in a growth chamber. Each experiment was replicated twice.

Statistical analysis

Data of shoot number per explant, percentage of explant response,

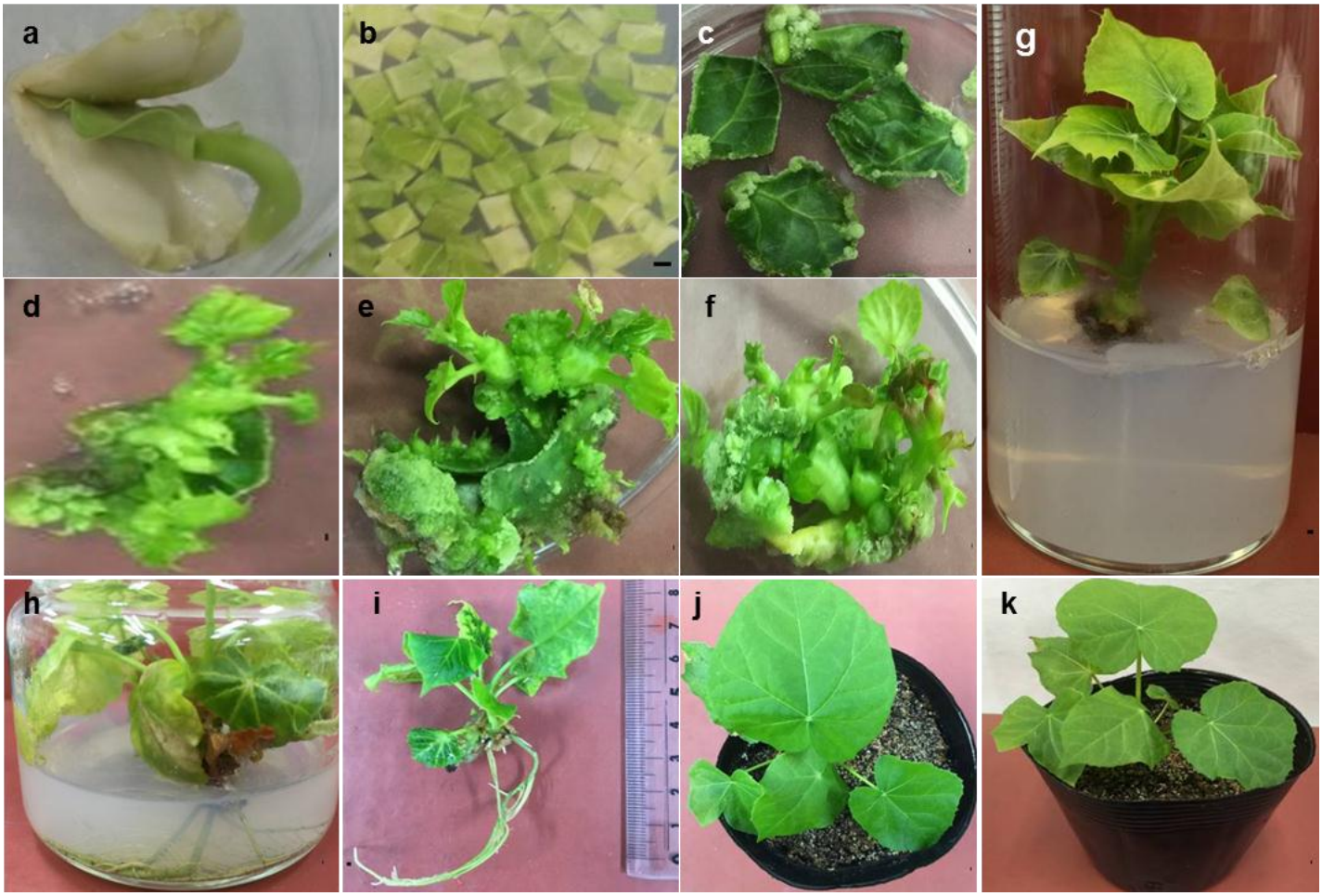

Figure 1. *In vitro* regeneration of jatropha, a) Juvenile cotyledons used as explants cultured on ½ MS basal medium; b) Incised cotyledons cultured on MS medium supplemented with 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA and 0.5 mgL^{-1} TDZ at 0 day; c) Callus induction on MS medium supplemented with 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA and 0.5 mgL^{-1} TDZ after 15 days; d) Shoot initiation on MS medium supplemented with 1.5 mgL^{-1} BA and 0.05 mgL^{-1} IBA after 30 days; e) Shoot initiation on MS medium supplemented with 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA and 25 mgL^{-1} Gln and Ads after 15 days; f) Shoot multiplication on MS medium supplemented with 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA and 25 mgL^{-1} Gln and Ads after 30 days; g) Shoot elongation on MS medium supplemented with 0.5 mgL^{-1}BA and 0.5 mgL^{-1} GA_3 after 30 days; h and i) Rooted plantlet in ½ B5 medium supplemented 0.5 mgL^{-1} IBA after 45 days; j and k) Plantlets after acclimatization. Bars = 1 mm.

shoot length, root number per explant and percentage of root shoot were collected. Recorded data were analyzed using one-way ANOVA and the mean separations were carried out using Tukey's HSD test at $P \leq 0.05$. All statistical analysis was performed using SPSS 22.0 (SPSS Inc. USA).

RESULTS AND DISCUSSION

Shoot induction and multiplication

The results showed that BA and IBA affected the shoot regeneration with 0.5 mgL^{-1} TDZ, and the combination of 1.5 mgL^{-1} BA and 0.05 mgL^{-1} IBA was the most effective in regenerating shoots from calli (3.82 ± 0.18 shoots/explant) (Table 1 and Figure 1c, d). This finding was similar to previous results reported by Chiangmai et al. (2015), Nanasato et al. (2015) and Fufa et al. (2019). The study was divergent from previous study in our lab by Khemkladngoen et al. (2011) which showed that the combination of 3 mgL^{-1} BA and 0.1 mgL^{-1} IBA produced the highest regeneration frequency from calli due to genotype difference.

Low shoot multiplication rate is a major constraint facing *in vitro* regeneration protocol of jatropha plant. Evaluation on the synergistic effect of glutamine and adenine sulfate on shoot regeneration and multiplication was further studied. The effectiveness of organic nitrogen source particularly glutamine for multiplication and maintenance of healthy *in vitro* tissue for long time periods have been reported in other plant species (Green et al., 1990; Ogita et al., 2001; Vasudevan et al., 2004; Sanjaya et al., 2005). The synergistic effects of adenine

Table 1. Effect of different combinations of BA and IBA on shoot regeneration from juvenile cotyledon

Concentration (mgL^{-1})*		Number of shoots/ explant**
BA	IBA	
1.0	0.5	2.50 ± 0.18^{ab}
1.0	0.05	2.06 ± 0.11^{ab}
1.5	0.5	2.54 ± 0.30^{b}
1.5	0.05	3.82 ± 0.18^{c}
3.0	0.5	2.46 ± 0.22^{ab}
3.0	0.05	1.92 ± 0.19^{a}

* Concentration of BA and IBA in the callus induction medium (with 0.5 mgL^{-1} TDZ), as well as in the shoot regeneration medium (without TDZ). **Values represent mean ± standard error of 25 - 30 explants per treatment. Means with different letters are significantly different ($P \leq 0.05$) by Tukey's HSD test.

Table 2. Effect of adenine sulfate (Ads) and glutamine (Gln) on shoot regeneration.

Concentration (mgL^{-1})*		Total number of explants	Explants response (%)	Number of shoots/ explant**
Ads	Gln			
0	0	139	80.58	4.08 ± 0.16^{a}
5	5	142	75.35	4.54 ± 0.20^{ab}
10	10	148	74.32	4.57 ± 0.18^{ab}
15	15	128	71.09	5.44 ± 0.30^{b}
20	20	116	74.14	4.77 ± 0.18^{ab}
25	25	115	93.04	9.09 ± 0.37^{c}

*Concentration of Ads and Gln in the callus induction medium (with 1.5 mgL^{-1} BA, 0.05 mgL^{-1} IBA, and 0.5 mgL^{-1} TDZ), as well as in the shoot regeneration media (with 1.5 mgL^{-1} BA and 0.05 mgL^{-1} IBA). ** Values represent mean ± standard error of 55 - 80 explants per treatment. Means with different letters are significantly different ($P \leq 0.05$) by Tukey's HSD test.

sulphate and cytokinin on stimulating cell growth and enhancing shoot formation of *Holarrhena antidysenterica* were observed by Raha and Roy (2001). The simulative role of adenine sulfate in shoot multiplication was emphasized in different woody species such as *Melia azedarach* (Husain and Anis, 2004), *Bauhinia vahlii* (Dhar and Upreti, 1999), and *Petrocarpus marsupium* (Husain et al., 2008). Several studies showed the enhancement of shoot multiplication of jatropha in MS medium containing BA and IBA fortified by adenine sulfate and glutamine (Maharana et al., 2012; Samson et al., 2013; Mishra, 2018; Hegazi et al., 2020). The inclusion of glutamine and adenine sulfate exhibited significant effect on shoot multiplication. Among the different concentrations evaluated, 25 mgL^{-1} each of glutamine and adenine sulfate was the most effective for shoot regeneration. When comparing with the media without glutamine and adenine sulphate (4.08 ± 0.16 shoots/explant), the addition of 25 mgL^{-1} each of glutamine and adenine sulfate resulted in more than two-fold increase in the shoot number (9.09 ± 0.37 shoots/explant) (Table 2 and Figure 1e, f). The enhancement of shoot multiplication might be due to synergistic effect of glutamine and adenine sulfate. Thus, the results were similar with previous reports by Maharan et al. (2012), Samson et al. (2013), Mishra (2018) and Hegazi et al. (2020) in jaropha plant which revealed that the addition of glutamine and/or adenine sulphate significantly enhanced shoot multiplication.

Shoot elongation

The physiological role of GA_3 is well known on shoot elongation and widely used for *in vitro* regeneration of different plants (Machado et al., 2011; Gonbad et al., 2014; Padrón et al., 2020). The results showed that GA_3 at the concentration of 0.5 mgL^{-1} significantly improved shoot elongation of jatropha (2.13 ± 0.18 cm) (Table 3 and Figure 1g). The result was consistent with the results reported recently by Amiri and Mohammadi (2021) when they used the combination of BA and GA_3 for *in vitro* regeneration of Sumac plant. The established regeneration protocol of jatropha in our lab by Khemkladngoen et al. (2011) did not examine the effects of GA_3 shoot elongation. The results of this study

Table 3. Effect of GA_3 on shoot elongation in the presence of 0.5 mgL^{-1} BA.

Concentration of GA3 (mgL^{-1})	Shoot length (cm)*
0.0	1.09 ± 0.17^{a}
0.3	1.29 ± 0.17^{a}
0.5	2.13 ± 0.18^{b}
1.0	1.25 ± 0.19^{a}

*Values represent mean ± standard error of 30 – 35 explants per treatment. Means with different letters are significantly different ($P \leq 0.05$) by Tukey's HSD test.

Table 4. Effect of Gln on rooting in the presence of 0.5 mgL^{-1} IBA.

Concentration of Gln (mgL^{-1})	No. of roots/shoot	Rooted shoot (%)*
0.0	3.17 ± 1.01^{a}	40
5	1.67 ± 0.47^{a}	18
10	1.75 ± 0.39^{a}	25
15	1.75 ± 0.40^{a}	40

*Values represent mean ± standard error of 15 -20 explants per treatment. Means with different letters are significantly different ($P \leq 0.05$) by Tukey's HSD test.

indicated that GA_3 remarkably improved shoot elongation of regenerated shoots.

Rooting and acclimation

The promontory effect of IBA on *in vitro* rooting of jatropha shoots was reported previously (Deore and Johnson, 2008; Singh et al., 2010; Sharma et al., 2011). Jatropha is recalcitrant for *in vitro* regeneration at exactly rooting and acclimatization (Pankaj and Divay, 2011). Recently, Liu et al. (2015) found that the addition of glutamine to the medium in the presence of IBA effectively stimulated the initiation and growth of roots in jatropha and 16 mgL^{-1} of glutamine exhibited the best rooting rate (51.72%). The highest rooting induction efficiency (40%) was observed in medium containing 15 mgL^{-1} of glutamine, as well as that without glutamine (Table 4; Figure 1h, i). This result showed that the addition of glutamine did not significantly affect rooting efficiency, which might be due to *in vitro* elongated shoots that were regenerated in medium containing glutamine. Rooted plantlets were transplanted and acclimatized successfully to the soil for 3 weeks. The acclimatized plants exhibited normal morphological growth (Figure 1j, k).

The study described the enhanced *in vitro* regeneration protocol of jatropha from juvenile cotyledon. Explants were cultured on shoot induction medium fortified with different concentrations of adenine sulfate and glutamine. The highest number of shoots per explant with high regeneration frequency was achieved in shoot induction medium fortified by 25 mgL^{-1} each of adenine sulfate and glutamine. The results also showed the highest shoot multiplication rate in jatropha plant that has been never done before. Enhanced *in vitro* regeneration protocol would be useful for large-scale production and *Agrobacterium*-mediated transformation of elite jatropha genotypes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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