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Full Length Research Paper

Involvement of class 1 and class 2 integrons in dissemination of *tet* and *cat*A1 resistance genes of *Salmonella enterica* from children with diarrhea in rural Burkina Faso

René Dembélé[1,2*], Wendpoulomdé Aimé Désiré Kaboré[1,3], Issiaka Soulama[4], Ali Konaté[1], Assèta Kagambèga[1,5], Oumar Traoré[1,2], Alfred S. Traoré[1], Awa Aidara-Kane[6], Amy Gassama-Sow[7]† and Nicolas Barro[1]

[1]Laboratory of Molecular Biology, Epidemiology and Surveillance of Bacteria and Viruses Transmitted by Food (LaBESTA)/Center for Research in Biological, Food and Nutritional Sciences (CRSBAN)/Graduate School of Science and Technology (EDST), University of Ouaga I, Professor Joseph KI-ZERBO, 03 BP 7021 Ouagadougou 03, Burkina Faso.
[2]Training and Research Unit, Applied Sciences and Technologies (TRU/AST), University of Dédougou, BP 176 Dédougou, Burkina Faso.
[3]Training and Research Unit in Health Sciences (TRU/HS), University of Ouaga I, Professor Joseph KI-ZERBO, 03 BP 7021 Ouagadougou 03; Burkina Faso.
[4]National Centre for Research and Training on Malaria (NCRTM), 01 BP 2208 Ouagadougou 01, Burkina Faso.
[5]Institute of Sciences, 01 BP 1757 Ouagadougou 01, Burkina Faso.
[6]Department of Food Safety and Zoonoses, World Health Organization, WHO-AGISAR, Switzerland.
[7]Unit of Experimental Bacteriology, Pasteur Institute of Dakar, 36 avenue Pasteur, BP 220, Dakar, Senegal.

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With high annual mortality rates among young children, antimicrobial resistant salmonellosis is considered a major public health concern worldwide. Antimicrobial resistant salmonellosis is a worldwide health issue, particularly in low income countries with high microbially-derived food contaminations. As a result, it is important to better understand the biological factors that may control these bacteria's dissemination low immunity individuals such as children. Thus, a sound epidemiological surveillance and control of salmonellosis (that is, *tet* and *cat*A1) requires a better understanding of the role that class 1, 2 and 3 integrons play in the spread of these antimicrobial resistant genes. A total of 275 stool samples of children suffering of diarrhea in rural Burkina Faso were collected and their *Salmonella* species were screened. The antimicrobial resistance determinants were investigated by Polymerase Chain Reaction, checking the presence of class 1, 2, 3 integrons, *tet* and *cat*A1 resistance genes. Seven of the nine confirmed *Salmonella* strains (78%) were multidrug resistant while 100% were resistant to amoxicillin. Antibiotic resistance genes *cat*A and *tet* were present in 11.1 and 22.2%, respectively. Integrons were detected as follows: *Int1* (44.4%) and *Int2* (22.2%). No class 3 integron was detected. A surveillance and control programme of antimicrobial drug resistant *Salmonella* species is of paramount importance for limiting spread of these pathogens among children.

Key words: Antibiotic resistance genes, Class 1 and 2 integrons, *Salmonella*, children.

INTRODUCTION

Salmonella enterica subspecies *enterica* is one of the most common foodborne pathogens (Olsen et al., 2001), causing more than 93 million illnesses and 155,000 deaths worldwide, 85% of which were related to contaminated food (Hendriksen et al., 2011; Majowicz et al., 2010). *Salmonella enterica* serovars are recognized as a common cause of childhood infections all over the world; particularly gastroenteritis, bacteremia, and typhoid (enteric) fever (Bula-Rudas et al., 2015). Indeed, *Salmonella* causes Salmonellosis, which can be characterized by diarrhea, fever, vomiting and abdominal cramps after 12 to 72 h of infection. *Salmonella* enteric serotype typhi is the common serotype of *Salmonella* that causes typhoid fever. Typhoid fever is a systemic disease with diarrhea and it is the major causes of morbidity and mortality worldwide in under the age of five children (WHO, 2008). A recent review indicates that *Salmonella* Enteritidis (*S.* Enteritidis) and *Salmonella* Typhimurium (*S.* Typhimurium) cause approximately 80% of Salmonellosis in children fewer than five years (Wen et al., 2017). Clinical treatment of severe salmonellosis is based on the prescription of antibiotics, including ampicillin, third and fourth generation cephalosporins and fluoroquinolones (Hohmann, 2001). However, *Salmonella* isolates with multidrug resistance (defined as resistance to three or more antimicrobials) have been found (Ameya et al., 2018) and had increased to 70% by early this century (Su et al., 2004). The spread of resistant *Salmonella* is a particular concern for pediatricians because of the limited therapy options available for infants and children. Moreover, antimicrobial resistance in multidrug-resistant (MDR) *Salmonella* serotypes may contribute to their virulence (Wannaprasat et al., 2011). Otherwise, it is known that both resistance and virulence determinants may be located on the bacterial chromosome, on transposons or on plasmids, clustered in resistance or pathogenicity islands and transferred by mobile genetic elements or phages (Rychlik et al., 2006). Of particular concern is the presence of both determinants on the same transposon or plasmid, which may be selected by antibiotic pressure resulting in more virulent and antibiotic-resistant *Salmonella* (Wannaprasat et al., 2011). Another factor that may promote Salmonella resistance is the presence of integrons as these DNA materials can capture and mobilize antibacterial genes among bacteria including *Salmonella*, and that play a central role in spreading antibacterial resistance genes (Leverstein-van et al., 2003; Randall et al., 2004). Among integrons, class 1 is by far the most abundant in clinical isolates of the Enterobacteriaceae in general and in *Salmonella* in particular (Wannaprasat et al., 2011).

Limited number of studies has been carried out on *Salmonella* serotyping and its susceptibility to antibiotics in urban and rural setting in Burkina Faso (Bonkoungou et al., 2013; Dembélé et al., 2014). There are no data on the prevalence of resistance bacterial genes among rural communities in the country. The objective of the present study was to assess the extent of class 1 and 2 integrons implication in propagation of *tet* and *cat*A1 resistance genes of *Salmonella* among children suffering from diarrhea in rural Burkina Faso.

MATERIALS AND METHODS

Salmonella isolates

Samples were collected in two rural medical centre (Boromo and Gourcy; Figure 1) by healthcare personnel and stored in iced sterile stood containers. In the laboratory, the standard method developed by Gillespie and Hawkey (2006) was used to identify bacterial isolates.

Antimicrobial susceptibility testing

The European Committee on antimicrobial susceptibility testing method (EUCAST, 2017) was used to conduct antimicrobial susceptibility testing. The antibacterial drugs used in the testing are listed in Table 1.

Antimicrobial resistance genes detection

To detect the molecular determinants of resistance, all the multidrug-resistant isolates of *Salmonella* were considered and PCR was carried out with specific primers for resistance genes including chloramphenicol (*cat*A1) (Letchumanan et al., 2015), tetracycline (*tet*) (Waters et al., 1983), and for integrons (*intI1*, *intI2* and *intI3*) (Ploy et al., 2000) as shown in Table 2. 2.5 µl of supernatant were added to 22.5 µl reaction mixture containing 5U of Taq DNA polymerase (Accu Power, Korea), deoxyribonucleic triphosphate (10 mM), buffer GC (10X), $MgCl_2$ (25 mM), and PCR primers (10 µM). The PCR conditions were as follows: 5 min at +94°C, followed by 35 amplification cycles of +94°C for 30 s, 59±4°C for 60 s and +72°C for 60 s with a final extension of +72°C for 10 min on a thermal cycler (Gene Amp 9700, Applied Biosystems). The reaction products were separated by electrophoresis in 1.5% weight/volume agarose gel, stained with a Redsafe solution (Prolabo, France) and visualized under ultraviolet (UV) light (Gel Logic 200).

Ethical considerations

The protocol of the present study has been approved by the Burkina Faso's National Ethical Committee for health Research, and a verbal consent was obtained from the parents or custodian of the children before sample collection.

*Corresponding author. E-mail: simavedemb@gmail.com. Tel: +226-70-05-13-42.

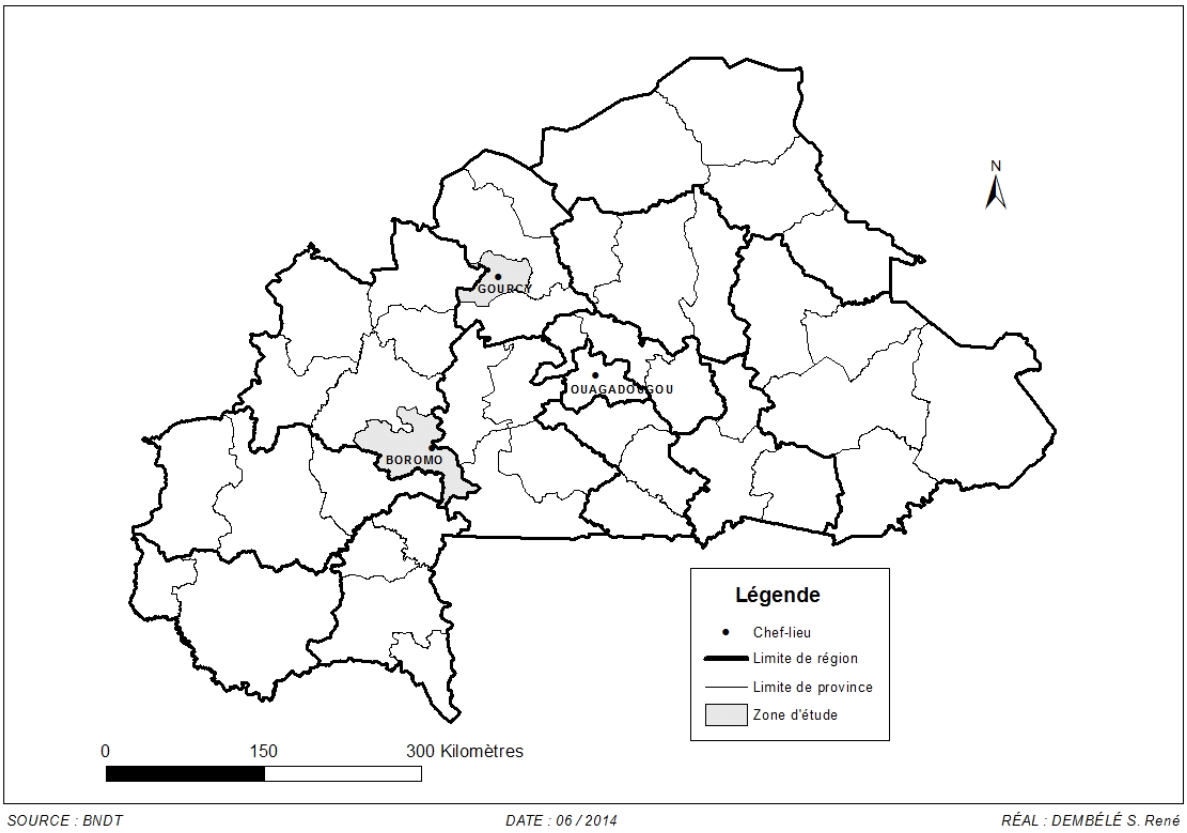

Figure 1. Administrative map of Burkina Faso showing the sampling sites in (Boromo and Gourcy) in grey colour.

RESULTS

Global prevalence of antibiotic resistance in *Salmonella*

Nine (9) isolates were confirmed positive for *Salmonella* by conventional method of serotyping with antiserum and genetic resistance to antibiotics by molecular methods. The result showed that the nine isolated *Salmonella* were resistant to at least three antibiotics. Likewise, seven isolates (~78%) appear to be MDR *Salmonella* that were resistant to three different antimicrobial drugs. Resistances to ciprofloxacin, nalidixic acid, piperacillin, trimethoprim and sulfametoxazo were found to be 11, 22, 33, 44 and 67% of the isolates, respectively. Resistance to gentamycin, chloramphenicol, cefotaxime and amoxicillin–clavulanic acid was detected in three (33%), four (44%), five (56%) and eight (89%) of the isolates, respectively (Table 3).

Resistance genes and integrons in *Salmonella* isolates

The PCR analysis indicated that about 11% of resistant *Salmonella* Poona strain to chloramphenicol was positive for the presence of cat*A*1 gene. One strain of *S.* Duisburg, one strain of *S.* Typhimurium, one strain of *S.* Ouakam and one strain of *S.* Poona (11.1% of rate each) harbored class 1 integron. Class 2 integron were reported in one strain of *S.* Poona (11.1%) and in one strain of *S*. Hvittingfoss (11.1%) (Table 3) whereas Class 3 integrons were not detected.

Coexistence of resistance genes and integrons in the same *Salmonella* isolates

The results showed that *cat*A1 gene and class 2 integron were simultaneouly harbored by the *S*. Poona strain. Similarly, there was a coexistence between the *tet* genes and class 2 integrons (in *S*. Hvittingfoss) and between the *tet* genes and class 1 integrons (in *S*. Poona) (Table 3). However, two multiresistant strains of *Salmonella* were found in which no *tet*, *Cat*A1 gene and integrons (*Int1, Int2, Int3*) were detected (22.2%).

DISCUSSION

The resistance of *Salmonella* to antibiotics observed in the current study is relatively greater than that reported in

Table 1. Zones of inhibition of the tested antibiotics.

Families		Antibiotics	[C] (µg)	Ø (mm)	
				R (Ø<)	S (Ø≥)
β-lactamins	Aminopenicillins	Amoxicillin- clavulanic acid (AMC)	30	19	19
		Amoxicillin (AMX)	25	19	19
		Piperacillin (PIP)	75	17	20
		Piperacillin-tazobactam (TZP)	100/10	17	20
	Cephalosporins C3G	Ceftriaxone (CRO)	30	20	23
		Cefixime (CFM)	10	17	17
		Cefotaxime (CTX)	30	17	20
	Cephalosporines C4G	Cefepime (FEP)	30	21	24
	Monobactam	Aztreonam (ATM)	30	21	24
	Carbapenemes	Imipenem (IPM)	10	16	22
Quinolones		Nalidixic acid (NAL)	30	14	19
Fluoroquinolones		Ciprofloxacin (CIP)	5	19	22
Cyclines		Tetracycline (TET)	30	15	18
Phenicols		Chloramphenicol (CHL)	30	17	17
Sulfamides		Trimethoprim-sulfamethoxazole (SXT)	1.25/23.75	13	16
Polymyxines		Colistin sulfate (CST)	50	15	15
Aminoglycosides		Gentamycin (GMI)	15 (10 IU)	14	17
		Netilmicin (NTM)	10	12	15
		Tobramycin (TMN)	10	14	17

[C] = Antibiotics' disc concentration ; **R** = Resistant ; **S** = Sensible; **Ø** = Zone of inhibition.

Table 2. Oligonucleotides primers used for PCR reaction.

Genetic resistance support	Genes	Primers sequence (5'to3')	Size (bp)	Reference
Chloramphenicol	*cat*A1	F : CGC CTG ATG AAT GCT CAT CCG R : CCT GCC ACT CAT CGC AGT AC	456	Letchumanan et al. (2015)
Tetracycline	*tet*	F : GCA GGC AGA GCA AGT AGA GG R : GTT TCG GGT TCG GGA TGG TC	956	Waters et al. (1983)
Integrons	*Int1*	F: ATT TCT GTC CTG GCT GGC GA R: ACA TGT GAT GGC GAC GCA CGA	600	Ploy et al. (2000)
	Int2	F : CAC GGA TAT GCG ACA AAA AGG T R : GTA GCA AAC GAC TGA CGA AAT G	806	
	Int3	F: GCC CCG GCA GCG ACT TTC AG R: ACG GCT CTG CCA AAC CTG ACT	600	

previous studies in three West-African countries (Burkina Faso, Mali and Niger) (Bawa-Ibrahim et al., 2016). Moreover, the majority of *Salmonella* strains isolated from children suffering from diarrhea exhibited MDR profile, suggesting that emergence of these types of *Salmonella* has become a public health concern. As a result, the monitoring programs of antibacterial resistance bacteria in food, animals and humans is urgently required so that decision-makers can foresee a better use of antimicrobial drug in both veterinary and human medicines (Cummings et al., 2013). Because of the central role played by antimicrobial drugs in controlling virulent and invasive human salmonellosis, the finding of the present study is highly important in clinical studies. This is particularly important as fluoroquinolones and third-generation cephalosporins are now commonly used in adults for

Table 3. Antibiotic resistance phenotypes and genes detected in *Salmonella* isolates from clinical samples.

Isolate	Antibiotic-resistance phenotype	Resistance genes	Integrons
084B	AMC, AMX, CTX, ATM, CRO, FEP, CFM, TET, SXT, CST, GMI, PIP, TMN	-	*Int1*
057B	AMC, AMX, FEP, TET, CHL, CST, GMI	*cat*A1	*Int2*
066B	AMC, AMX, TET, SXT, CIP, NAL, GMI	-	-
068B	AMX, CTX, ATM, CRO, FEP, CFM, SXT, CST	-	*Int1*
078B	AMC, AMX, TET, CST, TMN	-	*Int1*
063G	AMC, AMX, CTX, ATM, CRO, FEP, CFM, CHL, PIP	*tet*	*Int2*
087G	AMC, AMX, CTX, ATM, CRO, FEP, CFM, TET, CHL, CST, PIP, TMN	*tet*	*Int1*
112G1	AMC, AMX, CTX, CRO, FEP, CFM, TET, SXT, NAL, CHL	-	-
112G2	AMC, AMX, CFM	-	-

AMC, Amoxicillin- clavulanic acid; AMX, Amoxicillin; CTX, Cefotaxime; ATM, Aztreoname; CRO, Ceftriaxone; FEP, Cefepime; CFM, Cefixime; TET, Tetracycline ; CHL, Chloramphenicol; SXT, Trimethoprim-sulfametoxazole; CIP, Ciprofloxacin; NAL, nalidixic acid; CST, Colistin sulfate; GMI, Gentamicin; PIP, Piperacillin; TMN, Tobramycin.

treatment due to widespread resistance to chloramphenicol, ampicillin, and cotrimoxazole. Fluoroquinolones are often the last resort for treatment of children and are given by the World Health Organization as critically important antimicrobials for human health (Collignon et al., 2009). Because of their low cost and high availability, the studied antibacterial drugs are widely used in human medicines in most developing countries. Indeed, previous results had shown that antibiotics with and without prescription were the most common medicine used in Burkina Faso. For example, among children, the use of antibiotics with prescription was more common (23%); while 43.5% of the involved persons in this study lived in promiscuous animals (Dembélé et al., 2016). Consequently, MDR *Salmonella* isolates, that is susceptible to contaminate human through foodwebs, are likely to interfere with antibiotic treatment. In the present study, the presence of one tetracycline-resistant genes (*tet*), one chloramphenicol-resistant gene (*Cat*A1) and integron genes (*int1*, *Int2 Int3*) in all the MDR *Salmonella* isolates by PCR was investigated.

For studied isolates, the phenotypic expression of resistance in antibiogram was always accompanied by the presence of the corresponding gene encoding for the particular resistance determinant. One strain of *Salmonella* Poona (11.1%) that showed resistance to chloramphenicol was positive for the presence of *cat*A1 gene by PCR that requires further study so that interaction between bacteria and the antimicrobial drugs can be better understood. Furthermore, one isolate of *S*. Hvittingfoss, even if non-MDR and one isolate of *S*. Poona harbored *tet* resistance gene (22.2%). The results seem low comparatively to a study which previously reported the five type of tetracycline resistance genes as follows: 20 (100%), 6 (30%), 7 (35%), and 10 (50%) for *tet*A, *tet*B, *tet*C, and *tet*G, respectively (Adesiji et al., 2014). It is interesting to note that the tetracycline-resistant isolates did not contain more than a singles tet gene, indicating that the presence of just one *tet* can cause phenotypic resistance characteristics in *Salmonella* isolates (Jun et al., 2010).

Global prevalence of 44.4% of class 1 integrons and 22.2% of class 2 integrons was reported. These results are higher than 11.4% reported by Ahmed et al., (2005) and 25.9% reported by Huang et al., (2013) as far class 1 integrons are concerned. Otherwise, in contrast to the prevalences, 7.9% of class 1 integrons and 39.4% of class 2 integrons genes have been detected in Uruguay (Macedo-Viñas et al., 2009). The class 3 integrons were not detected in this study. Identification of these integrons has been limited in certain microorganisms such as *Acinetobacter* spp., *Alcaligenes*, *Citrobacter freundii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Salmonella* spp. and *Serratia marcescens* (Arakawa et al., 1995; Rowe-Magnus et al., 2001; Ploy et al., 2003), and their occurrence has been low in common bacteria associated with median IMP-1 metallo-beta-lactamse (Arakawa et al., 1995). About 9% of the sequenced bacterial genomes was integrons with the class 1 platform, the most ubiquitous integrons (Barlow et al., 2004; Labbate et al., 2009). Class 1 integrons are commonly found in clinical isolates, and most antibiotic resistance genes belong to this class. Class 1 integrons, consisting of a myriad of resistance gene cassettes, are likely to play a central role in propagation and maintenance of antibiotic resistance in *Salmonella* isolates in the presence or absence of selective pressure (Deekshit et al., 2012). A part from the cassettes that are different from nucleotide sequence by more than 5%, over 80% of different cassettes of class 1 integrons have been extensively described (Mazel, 2006). These elements confer resistance to all known β-lactams, all aminoglycosides, chloramphenicol, trimethoprim, streptothricin, rifampin, erythromycin, fosfomycin, lincomycin and antiseptics of the quaternary-ammonium-compound family (Rowe-Magnus and Mazel, 2002; Fluit and Schmitz, 2004).

In addition to class 1, class 2 integrons, that are

commonly reported in some Gram-negative organism species such as *Acinetobacter*, *Enterobacteriaceae*, *Salmonella* and *Psuedomonas* are considered as major contributor to widespread of antibiotic resistance in microorganisms (Machado et al., 2008; Ozgumus et al., 2009; Xu et al., 2011).

Coexistence between the *cat*A1 gene and *Int2* as well as between the *tet* gene and the two class of integrons (*Int1* and *Int2*) have been notified in the *Salmonella* isolates. Indeed, a recent study has further demonstrated that the potential presence of antibiotic resistance in *Salmonella* is chiefly attributed integrons (Zhao et al., 2017). However, two MDR *Salmonella* strains lacking *tet*, *Cat*A1 gene and integrons were detected in the present study. These strains were resistant to tetracycline and/or chloramphenicol.

Conclusion

Results illustrate the contribution of integrons in spreading antimicrobial resistance genes in Salmonella strains isolated in children. By far, antimicrobial resistance genes remain the leading public health concern in rural Burkina Faso because bacteria can acquire resistance genes through genetic mutation or through horizontal transfer of resistance genes. Therefore, surveillance and monitoring of antimicrobial drug resistance, including screening for class 1 and 2 integrons, are necessary steps in planning effective strategies for controlling MDR *Salmonella*.

CONFLICTS OF INTEREST

The authors have not declared any conflict of interests.

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Impact of transgenic conversion on the characteristics of Burkina Faso cotton

BOURGOU Larbouga[1*], HEMA S. Omer[1], KOULIBALY Bazoumana[1], DIANE S. Karamogo[1] and SAWADOGO Mahamadou[2]

[1]INERA, Programme Coton, BP 208, Bobo-Dioulasso, Burkina Faso.
[2]Université Ouaga1 Joseph Ki-Zerbo, Laboratoire de Génétique et Amélioration des Plantes, 09 BP 848, Ouagadougou 09, Burkina Faso.

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Genetically modified cotton (GMC) was compared with two conventional cultivars, using data from trials and commercial farms. Objectives were to highlight disparate agronomic and technological performance parameters and identify perspectives to Burkina Faso comeback to GMC. Results showed that compared materials are similar for most agronomic characteristics, except fiber percent. As this important parameter is concerned, GMC outperformed conventional cultivars in trials (+1.2 to +2.2%) and some commercial fields by +0.3, +0.5, +0.6 and +1% at Banfora, Koudougou, Houndé and Dédougou, respectively. For fiber quality, they did not show significant difference in maturity, micronaire, uniformity index, elongation, short fiber index, reflectance and yellowness. For two important parameters in cotton fiber marketing, Upper Half Mean Length and Strength, GMC was highly handicapped by -1.43 to -2.09 mm and -19.70 to -40.67 kN m Kg^{-1} compared to conventional cultivars, in commercial production. In trials, differences averaged -1.75 mm and -32.34 kN m Kg^{-1}. Differences between compared materials are genetic, due to failure in recovering important characteristics after the transgenic conversion. Local GMC cultivars could be an asset if more perfectly achieved, on site selected and pre-release evaluated, to take into account local production particularities.

Key words: Genetic conversion, *Bt* cotton, agronomic characteritics, fiber properties, Burkina Faso.

INTRODUCTION

Cotton is a strategic agricultural product of critical importance to many African countries, with small-scale producers in small acreages and low intensification (Hussein et al., 2005). For several decades, the application of chemical insecticides has been used to control pest attacks in commercial cotton production. In 1998, cotton production was strongly affected by the development of resistance of several pests, mainly *Helicoverpa armigera*, to efficient chemical products formerly known as pyrethroids (Martin et al., 2000; Omer et al., 2009). In the 2000s, to adopt genetically modified cotton (GMC) represents a solid alternative to chemical insecticides that had become less effective; that is still actual today (Vitale et al., 2008; James, 2017).

*Corresponding author. E-mail: diallabourgou@yahoo.fr.

At the request of the cotton interprofession of Burkina Faso (AICB), experiments were conducted from 2003 to 2007 by the Institute of Environment and Agricultural Research (INERA) to investigate the impacts of prospective adoption of GMC technology. The trials were initially conducted with *Bt* (*Bacillus thuringiensis*) transgenic cultivars of American origin (Coker 312 *Bt* and DP50 *Bt*), then during the 2006/2007 campaign with local GM cultivars. The indirect transformation route, by crossing with the already transformed cultivar Coker 312, was used to introgress two *Bt* transgenes (Cry1Ac-MON531 and Cry2Ab-MON15985 BGII™) into two cultivars from Burkina Faso and one introduction cultivar from Togo (Stam 59 A). The introgression of FK290 failed, and those which succeeded (FK37 and Stam 59 A) were two times backcrossed (BC2), followed by selection process conducted by Monsanto in the USA.

Preliminary studies reported a potential increase in field yield of around 30%, biological efficacy of transgenes on phyllophagous and carpophagous target insects which include *H. armigera*, and a reduction of four insecticide applications (from 6 to 2) in the case of an agreement to popularize *Bt* varieties in Burkina Faso (Cabanilla et al., 2005; Vitale et al., 2008). The evaluation of fiber properties from pre-release trials was not sufficiently considered. Results under research station conditions revealed no gain in terms of fiber length and strength into both converted cultivars compared to their near-isogenic conventional. There was a mitigated gain in fiber percent as FK37 BG2 was concerned (INERA, 2006). But, seeing in GMC a miracle solution to pest infestation with a hope of competitive advantage, Burkina Faso becomes in 2008 the second African country after South Africa to adopt GMC. Seed production is launched the same year, followed in 2009 by commercial cultivation on 418,200 ha. During 2015 campaign, GMC coverage rate reached 62.53% that is, 325,425 of the total 520,428 ha planted (SOFITEX, 2017; Vitale et al., 2010). By the first years of large-scale release coupled with unfavorable worldwide conjuncture of oversupply and price depression highlighted problems in Burkina Faso *Bt* cotton fiber technology (Fok, 2016). The venture was suddenly suspended in 2016; the cotton sector blaming GMC to underperform as expected, especially for fiber quality (AICB, 2015, 2019). However, it is not excluded even it is very probable that Burkina Faso returns back to GMC cultivation.

This study to assess the impact of the transgenic conversion on the characteristics of Burkina Faso cotton was based on data collected from producer farms of 2015 commercial production and from trials conducted in research station trials in 2016, 2017 and 2018. The objectives were to *i*) highlight disparate agronomic and technological performance parameters between conventional cotton cultivars and *Bt* ones and *ii*) identify perspectives in the eventuality of Burkina Faso's returning back to GMC.

MATERIALS AND METHODS

Plant material

The plant materials consisted of three varieties that is, Burkina Faso transgenic cultivar (FK95 BG2 or FK37 BGII) and its conventional near-isogenic recurrent parent (FK37) plus a new conventional cultivar designated FK64. In 2006, FK37 was used to introgress *Bt* transgenes conferring resistance to cotton pests (MON15985). Conversion was done by crossing with an already *Bt* introgressed American cultivar Coker 312, follow by two backcrosses with FK37, that gave rise to FK95 BG2. FK64 is in the process of being launched in commercial production because of its improved fiber characteristics above the current cultivars, especially fiber lengh.

Data recording from commercial fields

In December 2015, one kilogram (1 Kg) seed cotton samples of FK95 BG2 and FK37 were collected at producers' farms from 34 districts in seven cotton regions of SOFITEX (Société Burkinabè des Fibres Textiles) which covers 80% of Burkina Faso cotton belt (Figure 1). Three cotton producer groups (CPG) were randomly selected by district and then three producers by CPG. Within CPG, fields of producers growing the two types of cotton were sampled preferentially and if any case, producers having complementary types were considered.

Collected samples were ginned with a 20-saw laboratory gin to record one agronomic variable that is, fiber percent (FP). Thereafter, 100 g of cotton fiber per sample were evaluated on an integrated measurement chain (IMC), following ISO/DIS 139 laboratory conditioning and testing conditions. Commonly used major fiber properties that is, micronaire (Mic), maturity (Mat), length (UHML), uniformity (UI), short fiber content (SFI 12.7 mm), strength (Str), elongation (Elo), degree of reflectance (Rd) and yellowness (+b), were recorded as variables.

Data recording on trials at research station

Three trials were conducted (2016, 2017 and 2018), at Farako-Bâ research station to compare FK95 BG2 to FK37 and FK64. This station is located in the western cotton belt of Burkina Faso, between isohyets 800 and 1200 mm, at 405 m of altitude, 4°20'W of longitude and 11°06'N of latitude. Soils are lexisols with low clay and organic matter content, with a notable deficiency in nitrogen and phosphorus (Bado, 2002). Climate is South Sudanian type with a cumulative rainfall of 924 mm in 57 rainy days, 766.5 mm in 52 rainy days and 1303.8 mm in 70 rainy days in 2016, 2017 and 2018, respectively.

Trials were a Fisher block design with 3 replications. Two meters alley was separating replications and each elementary plot was composed of three rows of 20 m. Distance was 0.40 m between seedlings holes and 0.80 m between rows. A thinning to 2 plants/hill was done two weeks after emergence, and then all fertilization and phytosanitary practices were carried out, from sowing to harvest, in accordance with recommendations for cotton production in Burkina Faso.

Five major agronomic variables were measured i.e. the first flowering date (FFD)/first boll opening date (FBOD) that is, the date by which 50% of the plants in each elementary plot produced at least one flower/one open boll; the cotton seed yield (RDT) deduced using harvested cotton from central row of each elementary plot; the seed index (SI) or an average weight of 5 samples of 100 undelinted seeds and the fiber percent (FP) measured after ginning 200 g seed cotton per elementary plot. For fiber properties, as on samples from commercial fields, the same commonly used major fiber properties (Mic, Mat, UHML, UI, SFI

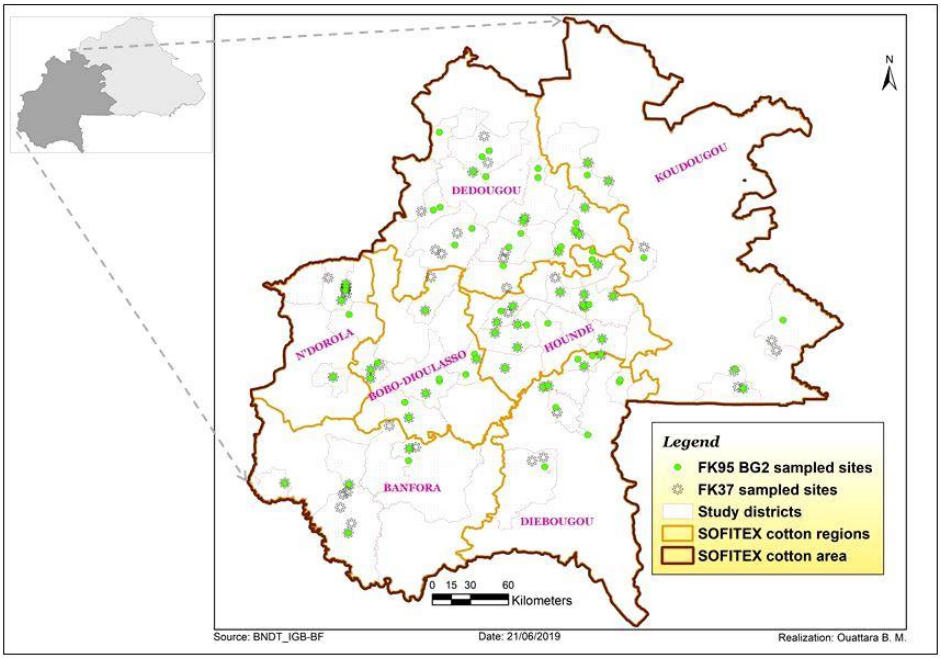

Figure 1. SOFITEX cotton zone, regions and departments including study sampled sites.

12.7 mm, Str, Elo, Rd and +b) were recorded in the same IMC, following the same laboratory conditions.

Data analysis

From data collected via producers' fields, so-called GMC samples expressing less than 95% of presence of both transgene proteins Cry1Ac and Cry2Ab, and conventional samples with more than 5% contamination by *Bt* transgenes were eliminated. Then, remaining data were subject to descriptive statistics analysis, using R software. Data from trials have been analysed through an analysis of variance (ANOVA), using the SISVAR 5.1 Build 72 software. The averages were compared by the Scott-Knott test at 5% threshold level.

RESULTS

Results concerning certain fiber properties that is, Mat, SFI, Elo and reflectance never showed statistical difference between the compared materials, in trials as well as in commercial production (Results not presented in this article).

Agronomic characteristics to compare GMC cultivar to conventional ones

The results of descriptive analysis on fiber percent (FP), the single agronomic characteristic recorded in commercial production, revealed differences between the compared cultivars. As showed in Table 1, the GMC compared to FK37 presented positive margins in 4 cotton regions (+0.3% in Banfora, +0.5% in Koudougou, +0.6% in Houndé, +1% in Dédougou) and negative ones in 3 regions (-0.1, -0.1 and -0.5%). When we considered SOFITEX zone as a whole, the margin remains positive for GMC of +0.1% (Table 2).

From trials, the ANOVA results showed significant differences for FP ($p = 0.02$ and $p = 0.00$) to the advantage of the GMC compared to FK37 and FK64 in 2016 as well as in 2017 and 2018 (Table 3). On the other hand, there are no significant differences between the GMC and conventional cultivars for the opening of the first flower (FFD) or first boll (FBOD). Seed index (SI) in 2016 and cotton seed yield in 2018 revealed significant difference, with better value for FK64 while FK37 and FK95 BG2 were similar in terms of SI, and with the lowest value for FK64 while FK37 and FK95 BG2 gave similar values in terms of yield that is, RDT (Table 3). It can also be noted that in 2017, the values of RDT, SI and FP were in order of magnitude lower than those of 2016 and 2018 as a long drought affected the western area of Burkina Faso during cotton maturation in 2017; all studied materials had been affected similarly and interaction Year*Cultivar effect was never significant (Table 3).

Fiber properties to compare GMC cultivar to conventional ones

Analysing fiber properties data from commercial farms and by cotton region (Table 1) revealed that GMC exhibited net negative margins compared to FK37 for

Table 1. Descriptive analysisof agronomic characteristics and fiber properties of samples collected in 2015 in commercial farms from SOFITEX cotton regions.

Var.	Desc.Par.	KG		HD		DD		ND		DB		BO		BA	
		Conv	***Bt***	**Conv**	***Bt***	**Conv**	***Bt***	**Conv**	***Bt***	**Conv**	***Bt***	**Conv**	***Bt***	**Conv**	***Bt***
Pf	nb	20	38	26	42	18	43	24	36	10	25	36	46	23	35
	min	43.0	41.7	40.3	42.7	39.3	40.3	42.3	41.0	42.3	42.0	42.3	42.7	43.3	44.3
	max	47.0	46.7	46.3	48.0	45.3	46.7	48.0	48.3	47.0	46.3	48.0	48.0	47.7	47.3
	aver	44.6	45.1	44.2	44.8	43.3	44.3	44.8	44.7	44.9	44.4	45.2	45.1	45.5	45.8
	marg		**+0.50**		**+0.60**		**+1.00**		**-0.10**		**-0.50**		**-0.10**		**+0.3**
	cv	0.02	0.03	0.03	0.03	0.03	0.03	0.04	0.04	0.03	0.03	0.03	0.03	0.03	0.02
Mic	min	3.4	4.1	3.8	3.8	3.9	4.2	3.6	3.5	3.7	3.5	3.4	3.6	3.7	4.2
	max	5.2	5.3	5.1	5.0	5.1	5.2	4.8	5.2	4.9	5.3	4.7	5.8	4.8	5.1
	aver	4.4	4.7	4.3	4.5	4.5	4.7	4.1	4.4	4.3	4.5	4.1	4.4	4.2	4.7
	marg		**+0.24**		**+0.15**		**+0.15**		**+0.3**		**+0.13**		**+0.32**		**+0.5**
	cv	0.10	0.06	0.09	0.07	0.06	0.06	0.07	0.10	0.09	0.09	0.07	0.08	0.06	0.06
UHML	min	27.1	24.9	26.4	25.3	26.9	24.0	27.0	25.3	28.1	25.4	26.2	24.9	26.6	23.9
	max	30.6	30.0	30.3	29.6	30.0	29.4	30.5	30.7	31.0	29.3	30.2	29.5	31.0	28.5
	aver	28.9	27.5	28.7	27.3	28.8	27.1	29.0	27.3	29.3	27.2	28.5	26.6	28.5	26.6
	marg		**-1.47**		**-1.43**		**-1.65**		**-1.8**		**-2.09**		**-1.87**		**-1.9**
	cv	0.03	0.04	0.03	0.03	0.03	0.04	0.03	0.04	0.03	0.03	0.03	0.04	0.03	0.04
Str	min	260.7	247.0	259.7	225.4	260.7	223.4	264.5	230.5	286.2	248.0	271.5	225.4	260.6	239.6
	max	383.2	329.3	337.1	345.9	385.1	331.2	327.3	315.2	374.4	296.0	331.2	314.6	344.0	307.9
	aver	310.7	291.1	301.8	271.5	313.6	277.3	299.0	263.6	312.6	272.4	293.0	266.6	291.8	266.6
	marg		**-19.7**		**-30.5**		**-36.6**		**-35.4**		**-40.7**		**-26.8**		**-25.3**
	Cv	0.1	0.07	0.07	0.1	0.1	0.07	0.05	0.07	0.07	0.05	0.05	0.07	0.07	0.06
UI	min	80.4	77.2	76.2	76.9	78.9	76.0	79.47	76.98	79.2	78.3	76.7	77.9	78.20	77.65
	max	84.2	83.6	83.4	82.6	83.9	83.3	84.98	83.44	84.6	82.3	83.4	82.7	83.25	81.97
	aver	82.6	81.0	81.2	80.6	82.0	80.8	82.00	80.85	82.0	80.4	80.9	80.2	80.94	79.69
	marg		**-1.64**		**-0.57**		**-1.17**		**-1.16**		**-1.58**		**-0.66**		**-1.25**
	cv	0.01	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.01	0.02	0.01	0.01	0.01
b+	min	7.5	7.1	7.3	6.8	7.1	6.7	7.00	7.16	6.7	7.0	7.2	7.0	6.99	6.12
	max	10.9	10.1	9.8	10.7	9.6	9.4	10.47	10.44	10.3	10.0	9.9	10.1	9.52	9.42
	aver	8.7	8.7	8.5	8.3	8.4	8.0	8.90	8.55	8.1	8.4	8.3	8.5	8.33	8.01
	marg		**+0.01**		**-0.16**		**-0.32**		**-0.35**		**+0.26**		**+0.17**		**-0.33**
	cv	0.08	0.07	0.07	0.09	0.08	0.07	0.11	0.10	0.16	0.11	0.08	0.08	0.08	0.10

Cotton regions are KG = Koudougou, HD = Houndé, DD = Dédougou, ND = N'Dorola, DB = Diébougou, BA = Banfora and BB =Bobo; Vegetal materials are *Bt* = FK95 BG2 and Conv = FK37; Descriptive analysis parameters (Desc. Par.) are nb = number of individuals, min = minimum, max = maximum, aver = average, marg = margin, cv=variation coefficient. Var = studied variable.

length (-1.43 to -2.09 mm), strength (-19.7 to -40.7 kN m Kg^{-1}) and uniformity (-0.57 to -1.64%). The margin was positive for Mic (+0.13 to + 0.53) whereas fluctuating for +b that is, positive in the cotton regions of Koudougou, Diébougou and Bobo-Dioulasso and negative in the remaining regions (Table 1). In overall analysis, with all cotton regions taken together, trends are maintained. In other words, in SOFITEX zone, the GMC fibers had a short length (-1.7 mm), low tenacity (-28.4 kN m Kg^{-1}), low uniformity (-0.95%) compared to those of FK37 (Table 2). GMC fibers had an advantage for Mic (+0.28) and yellowness index +b (-0.10).

At trials level, the results showed statistically significant differences only in fiber length (UHML) and strength (Str). Differences were highly significant for UHML (p = 0.00) in 2016 as well as in 2017 and 2018, whereas for Str they

Table 2. Aggregate Sofitex zone descriptive analysis.

Var.	Desc. Par.	SOFITEX zone	
		Con	***Bt***
Pf	nb	155	247
	min	39.3	40.3
	max	48.0	48.3
	aver	44.7	44.8
	marg		**+0.1**
	cv	0.0	0.0
Mic	min	3.4	3.5
	max	5.2	5.8
	aver	4.3	4.5
	marg		**+0.2**
	cv	0.1	0.1
UHML	min	26.2	23.9
	max	31.0	30.7
	aver	28.8	27.1
	marg		**-1.7**
	cv	0.0	0.0
Str	min	259.7	223.4
	max	385.1	345.9
	aver	301.8	273.4
	marg		**-28.4**
	cv	0.1	0.1
UI	min	76.2	76.0
	max	85.0	83.6
	aver	81.6	80.6
	marg		**-1.0**
	cv	0.0	0.0
b+	min	6.7	6.1
	max	10.9	10.7
	aver	8.5	8.4
	marg		**-0.1**
	cv	0.1	0.1

Vegetal materials are *Bt* = FK95 BG2 and Conv = FK37; Descriptive analysis parameters (Desc. Par.) are nb = number of individuals, min = minimum, max = maximum, aver = average, marg = margin, cv=variation coefficient. Var = studied variables.

were significant (p = 0.03) in 2016 and highly significant (p = 0.00) in 2017 and 2018 (Table 4). FK95 BG2 was below FK64 and FK37 by 2.7 and 1.5 mm and by 2.0 and 0.8 mm in 2016 and 2017, respectively, while in 2018 the deficits were 2.7 mm compared to FK64 and 0.6 mm compared to FK37. As regard to strength, FK95 BG2 was below FK37 with 22.5 and 33.3 kN m Kg^{-1} and with 47.0 and 26.5 kN m Kg^{-1} in 2016 and 2017, respectively; in 2018 FK64 outperformed it by 55.7 kN m Kg^{-1} while FK37 was better by 28.2 kN m Kg^{-1}. For the other properties, namely Mic, UI and +b, differences were insignificant both in 2016, 2017 and 2018 years of experimentation

Table 3. Anova of cultivars agronomic characteristics recorded in trials (2016, 2017 and 2018).

Cultivar	Agronomic characteristics														
	FFD[z] (jas)			FBOD (jas)			RDT (kg/ha)			SI (g)			FP (%)		
	2016	2017	2018	2016	2017	2018	2016	2017	2018	2016	2017	2018	2016	2017	2018
FK 37	68.2	74.7	61.3	112.7	121.3	113.7	990.6	581.8	2379.3 a_2	6.8 a_1	4.9	7.7	41.9 a_1	39.5 a_1[y]	42.0 a_1
FK 64	68.2	74.0	61.0	112.2	117.0	113.3	1090.6	524.4	1924.4 a_1	7.6 a_2	5.5	7.8	41.9 a_1	39.9 a_1	42.5 a_2
FK 95 BG2	68.5	73.3	60.3	111.5	117.7	112.0	1150.0	785.9	2389.5 a_2	7.0 a_1	4.6	6.9	43.1 a_2	41.7 a_2	44.6 a_3
Average	68.3	74	60.9	112.2	118.7	113.0	1077.1	630.7	2231.07	7.2	5	7.47	42.3	40.4	43.01
Cv (%)	1.58	1.35	3.51	0.87	1.93	0.98	14.70	24.42	8.36	5.14	11.74	7.33	1.28	1.40	0.29
Probability	0.93 (NS)[x]	0.33 (NS)	0.8 (NS)	0.24 (NS)	0.18 (NS)	0.23 (NS)	0.39 (NS)	0.17 (NS)	0.04 (S)	0.04 (S)	0.30 (NS)	0.19 (NS)	0.02 (S)	0.00 (S)	0.00 (S)
Year*Variable		0.00 (HS)			0.00 (HS)			0.00 (HS)			0.00 (HS)			0.00 (HS)	
Year*Cultivar		1.00 (NS)			0.86 (NS)			0.97 (NS)			0.95 (NS)			0.14 (NS)	

z FFD = first flowering date, FBOD = first boll opening date, RDT = seed cotton yield, SI = Seed Index, LP = lint percent; **y** Means within a column followed by the same letter (a) and index number (a_1 or a_2 or a_3, etc.) are not significantly different according to Scott-Knott test at p = 5% and form a same group. **x** Difference between groups is significant (S) to highly significant (HS). Means with any letter (a) are not significantly different (NS). NS = not significant, S = significant at p = 0.05, HS = high significant at p = 0.01.

Table 4. Anova of studied cultivars major fiber properties analyzed from trials samples (2016, 2017 and 2018).

Cultivar	Fiber properties														
	Mic[z]			UHML (mm)			UI (%)			Str (kN m Kg-1)			+b		
	2016	2017	2018	2016	2017	2018	2016	2017	2018	2016	2017	2018	2016	2017	2018
FK 37	3.3	2.5	4.0	27.6 a_2[y]	26.1 a_2	28.0 a_2	81.1	78.7	80.6	195.0 a_2	232.3 a_2	270.5 a_2	12.5	9.2	8.4
FK 64	3.6	2.8	3.9	28.8 a_3	27.3 a_3	30.1 a_3	81.5	79.4	82.4	283.8 a_2	252.8 a_3	298.0 a_3	13.5	9.4	8.6
FK 95 BG2	3.5	2.3	3.6	26.1 a_1	25.3 a_1	27.4 a_1	80.3	75.97	80.5	261.6 a_1	205.8 a_1	242.3 a_1	12.8	8.8	8.5
Average	3.5	2.5	3.8	27.5	26.2	28.5	81.0	78.0	81.2	280.3	230.3	270.27	13	9.1	8.5
Cv (%)	7.93	9.74	6.23	2.58	0.27	0.96	1.31	2.53	1.37	5.33	3.02	1.76	5.93	6.2	4.5
Probability	0.51 (NS)[x]	0.12 (NS)	0.20 (NS)	0.00 (S)	0.00 (HS)	0.00 (HS)	0.33 (NS)	0.17 (NS)	0.14 (NS)	0.03 (S)	0.00 (HS)	0.00 (HS)	0.22 (NS)	0.41 (NS)	0.77 (NS)
Year*Variable		0.00 (HS)			0.00 (HS)			0.00 (HS)			0.00 (HS)			0.00 (HS)	
Year*Cultivar		0.92 (NS)			0.96 (NS)			0.80 (NS)			0.77 (NS)			1.00 (NS)	

z Mic = micronaire, UHML = upper half mean length, UI = uniformity index, Str = fiber bundle strength, +b = yellow index.
y Means within a column followed by the same letter (a) and index number (a_1 or a_2 or a_3, etc.) are not significantly different according to Scott-Knott test at p = 5% and form a same group.
x Difference between groups is significant (S) to highly significant (HS). Means with any letter (a) are not significantly different (NS). NS = not significant, S = significant at p = 0.05, HS = high significant at p = 0.01.

(Table 4).

DISCUSSION

At least two advantages on agronomic level (insect resistance, FP) for GMC compared to conventional cultivars

Seed cotton yield was an important parameter in our investigation. During 3 years trials, GMC exhibited between 5.44 to 35.1% yield surplus compared to FK64 and FK37, but differences were statistically significantive. In pre-release evaluation prior to adoption, 30% yield gains were expected (Vitale et al., 2008), in addition to four insecticide pulverizings saving as biological efficacy of GMC against main targeted phyllophagous and carpophagous pests (Cabanilla et al., 2005; Omer et al., 2009). However, in real years of cultivation, these gains were adjusted around 15% and then served as baseline of royalties sharing calculation (Vitale et al., 2010).

Transgenes introgressed into Burkina Faso GMC are not specifically yield genes, so observed surplus in yield is a repercussion of their efficacy to control cottons pests, which are harmful in cotton production. According to Monsanto, adoption of *Bt* cotton enhanced yield in Burkina Faso by 17.2% annual average. Moreover, during seven years of production, many growers had become familiar with and faithful to *Bt* technology, then exhibiting production increase, as small as expected combined with reduction in insecticide contact and spraying to be notable assets of GMC (Vitale et al., 2011).

In commercial production, margin between FK95 BG2 and FK37 for FP was positive, to the advantage of GMC. Even better, in trials, it was significantly higher by 1.17 to 2.2% compared to those of FK37 and FK64 in 2016, 2017 and 2018. These results are in agreement with FK37 annual FP averages reported from the ginning tests conducted yearly by SOFITEX and INERA Cotton Research Program that is, 43.19 and 43.77% prior to GMC release, in 2007 and 2008 respectively (INERA, 2007, 2008). During years of GMC production[1], FK95 BG2 and FK37 yielded annual FP averages of 43.16 and 43.12% in 2009, 43.57% versus 42.79% in 2010, 42.79% versus 42.61% in 2011, 43.03% versus 43.51% in 2012, 42.28% versus 42.42% in 2014 and finally 42.24% versus 42.19% in 2015. Taking randomly into account two years of transgenic production and two of no transgenic, it is reported national FP averages of 43.52 and 42.73% in 2013 and 2015, respectively (when the cultivated varieties were FK37 and FK95 BG2) against 42.68 and 42.62% in 2016 and 2017, respectively (when the cultivated varieties became FK37 and FK64) (SOFITEX, 2017). To finish, pre-adoption trials conducted by INERA in 2006 in research stations, reported that FK95 BG2 and FK37 recorded 44.06 versus 43.82% respectively at Farako-Bâ in western cotton belt, and 43.56 against 42.93% at Kouaré in the eastern cotton belt (INERA, 2006).

Assertions that FK95 BG2 had a FP below FK37 by AICB (2015) or Fok (2016) may have been amalgamated; it was FK96 BG2 which presented FP below its conventional near-isogenic Stam59 A as well as FK37, then partly justifying its hasty withdrawal from commercial production by 2013. It is not explainable this advantage of FK95 BG2 in FP to come from the American cultivar donor of transgenes as it is known that Coker 312 is obsolete and below African cotton cultivars for this characteristic (Kuraparthy and Bowman, 2013; Fok, 2016). The recovery of agronomic characteristics, like FP, being easier around 3 back-crosses (Stiller et al., 2006), a hypothesis of a recovery obtained by BC2 can be issued if it was not beyond the expected value of FK37. More likely hypothesis is beneficial random recombinations of genes caused by crossings or backcrossings, transgressive segregation, or heterotic effect but the latter is not usual in cotton (Shankar et al., 2018), even depressive effect for FP after hybridization is reported (Mendez-Natera et al., 2007).

FP is an intrinsic characteristic of African cotton cultivars, especially in French-speaking countries, expressing the highest level in the world (Mergeai, 2006; Dessauw and Hau, 2007; Fok, 2016). In the case of Burkina Faso GMC, this advantage in FP conferred by the transgenic conversion process has not been sufficiently highlighted or sufficiently exploited to its true value; maybe because it was coupled with a major fiber handicaps, reduced length and strength, which resulted in at least temporary rejection of the entire GMC technology.

A major fiber length disability played a critical role in stopping GMC commercial production in Burkina Faso

Results of our investigation showed net significant differences between GMC and FK37 then FK64 as regard to fiber properties that is, length and strength, in trials as well as in commercial production. These results are in the same trends to those reported by the cotton comapanies grading office during years of commercial cultivation, from 2009 to 2015. According to these data, there were no significant differences between GMC and FK37 for Mat, Mic, Ui, Elo, +b, Sfi and Rd; these characteristics are known to be more influenced by production conditions than by cultivar (Darawsheh, 2010). For UHML and Str, the differences between compared materials are considered consistent. As exemples,

[1]INERA-SOFITEX ginning tests are conducted annualy in order to keep ginners in good conditions. Results reported here were based on tests on FK95 BG2 vs. FK37 i.e. 32 vs. 22 in 2010; 46 vs. 19 in 2011; 49 vs.27 in 2012; 45 vs. 31 in 2013; 54 vs. 23 in 2014; 36 vs.19 in 2015.

analysing 6,875 fiber samples[3] from FK95 BG2 and 5,821 fiber samples from FK37, the following differences appeared between the two varieties, respectiveley, in 2012: 4.26 vs. 4.07 for Mic, 80.16 vs. 80.93% for Ui, 84 vs. 84% in Mat, 11.14 vs. 10.02 in Sfi, 8.85 vs. 7.99 in Elo, 78.34 vs. 77.83 in Rd, 9.30 vs. 9.27 in +b, 26.74 mm vs. 28.25 mm in UHML and 270.77 kN m Kg^{-1} vs. 290.47 kN m Kg^{-1} in Str. In the last year of GMC cultivation in 2015, from analysis based on 3,263 FK95 BG2 fiber samples and 1,615 FK37 fiber samples, it appeared the following differences: Mic (4.37 vs. 4.17), Ui (79.82 vs. 80.51%), Mat (87 vs. 87%), Sfi (10.06 vs. 9.52), Elo (5.35 vs. 4.75), Rd (79.19 vs. 78.82), +b (9.05 vs 9.06), UHML (27.40 vs 28.65 mm) and Str (289.00 vs. 308.60 kN m Kg^{-1}). In other words, comparing two years of transgenic production and two year of no transgenic, it is reported that 54.45 and 51.75% of all entire cotton fiber were sold as ordinary short staple (1.1/16" to 1.3/32") in 2014 and 2015, respectively. After GMC withdrawal, Burkina Faso cotton fiber was already marketed in superior short staple classification (1.1/8" to 1.7/32") at 99.2 % by 2017 and 93.25 % in 2019 with FK37 and FK64 as commercially cultivars (AICB, 2019).

All the aggregate results clarify and abound in the sense of no complete equivalence between FK95 BG2 and FK37 for certain fiber properties, UHML and Str, at the origin of dissatisfaction of the cotton sector and which led to suspension of GMC in Burkina Faso (AICB, 2015). Diference is very presumably genetic effect and was already suspected during the first pre-release experiments in 2006 at Faroko-Bâ and Kouaré; it was showed that FK95 BG2 (or FK37 BGII) fibers were shorter and less tenacious than those of FK37. As FK96 BG2 (Stam 59A BGII) is concerned, in addition to handicaps in fiber length and strength, its ginning turn-out (FP) was lower compared to Stam 59A from which it is derived (INERA, 2006). At this time, used GM seeds were judged as low-end (BC2), sent only for biological effectiveness demonstration purposes. Higher-performing, more advanced backcrossing seeds expressing all expected *Bt* transgenes and all qualities found in conventional cultivar were promised to Burkina Faso as soon as an agreement is signed for large-scale production (D. Sanfo, INERA, personal communication).

The commercial production was launched in 2009 with FK95 BG2 and FK96 BG2, however and more likely, the same low-end BC2 seeds. By 2010, problems in fiber length were highlighted on all GMC cultivars in large-scale production by the cotton companies. According to Monsanto, these shortcomings are related to the growing conditions of Burkina Faso, arguing that Cooke et al., (2001) reported that any variation in characteristics between GMC and their conventional isogenics is most often attributable to environmental factors and production conditions. On the international fiber market, the reputation of Burkina Faso for providing high quality cotton is negatively affected; cotton from Burkina Faso was even discounted on shorter fibers from *Bt* cotton and a penalty was promulgated on all export sales of cotton produced from 2010 (Fok, 2016). During a tripartite consultation (AICB-INERA-MONSANTO) in 2011, INERA reasserted that GMC fiber handicaps in length and strength are more of varietal origin than environmental effect, suggesting advancement of backcrosses in hope to solve the problem. Monsanto agreed and assumed an obligation to provide more advanced back-crossed GMC cultivars by 2014 (D. Sanfo, INERA, personal communication).

[3]SOFITEX grading office data. Data are from a compilation individual FK95 BG2 and FK37 bales fiber analysis grading by HVI

Bt transgenes (Cry1Ac and Cry2Ab) have been introgressed in local cultivar to create the GMC, but imperfectly

In the GMC path, similar to Australia, Burkina Faso wanted to take into account the specificity of its varieties, well adapted to local conditions with good yield and meeting fiber market requirements (Constable et al., 2001; ICAC, 2013). Because direct transgenic introgression is tedious and expensive (May et al., 2003), crossing route with the already easily transformed Coker 312 variety was used to obtain Burkina Faso *Bt* cultivars. It seems that the donor parent was inappropriate as Kuraparthy and Bowman (2013) reported Coker 312 to be an old and obsolete variety, out of commercial production in USA since a couple years. Its fiber properties are far different i.e. shorter at least two millimeters and lower in FP compared to French-speaking African countries' varieties (Kuraparthy and Bowman, 2013; Fok, 2016). By stopping the backcross process at two only (BC2), the genetic background of Burkina Faso GMC was theoretically composed of 87.5% from local cultivars genetic value versus 12.5% from American donor cultivar; this amount of remaining undesired genetic background from Coker 312 was high enough to affect the quality of converted varieties. An advancement of the backcrossing process up to BC6, to raise GMC cultivars' genetic background to 99.22% of genetic value from local cultivars, could have drastically reduced probability of undesirable Coker 312 trait effects. However, Stiller et al. (2006) pointed out difficulty even impossibility of recovering all desired characteristics in a transgenic conversion process in cotton. According to these authors, at most 3 backcrosses can recover major agronomic traits, and for any other trait, 3 to 5 backcrosses followed by a rigorous selection should be sufficient.

In 2011, during a tripartite consultation (AICB-INERA-MONSANTO), Monsanto endorsed the idea to continue the backcross process but for work that could have been done on site, the process (backcrosses and selections) were conducted outside Burkina Faso. Since 2012, eight

cotton lines called BC3 hastily developed have been delivered to AICB. After 3 years of trials evaluation on dozens of sites conducted by INERA, no new line was found combining more both agronomic and technological achievements of FK37, being able to substitute FK95 BG2 (Bourgou et al., 2014).

Conclusion

The present study comparing FK95 BG2, FK37 and FK64 highlights a genetic effect on differences between GMC and conventional; production condition, year or site would be non-significant. Indeed, GMC presented equivalences with the conventional cotton for certain agronomic characteristics and fiber properties. It even had some asset, sometimes significant compared to conventional cultivars, especially FP and seed cotton yield to a lesser extent (not significant). But GMC was carrying major handicaps in fiber length and strength, pointed out in trials as well as in large-scale production. Although conversion was not focused on fiber properties, entire recovery in fiber length and strength was crucial as these characteristics are, like FP, intrinsically linked to African cotton cultivars. They are also reflecting the reputation of countries on the fiber market, so difference in fiber lengh was an important shortcoming, which led to GMC withdrawal.

Problems observed in Burkina Faso GMC were, more likely, a consequence of imperfect conversion caused by certain precipitation and lack of rigor in the process conducted outside Burkina Faso. It was a great opportunity for Burkina Faso to benefit GMC of introgressed local cultivars as adoption of non-local transgenic cultivars could result in unsuitability to local production conditions. However, it should be of more advanced (at least 6) backcrossing level and paired with an effective selection process to recover as much as possible very specific characteristics linked to varieties from which they are made. Rigorous selection after backcrossing is critical when traits differ signficantly between donor and recurrent parent. The revocation of GMC means a return back to conventional cultivation and FK64 a new launching variety confirmed potentials. But, taking into account all known benefits demonstrated by GMC in Burkina Faso and elsewhere, and the increasing parasitic pressures, temptation of Burkina Faso to return back into GMC is high.So, the following recommendations must be made and rigorously followed to ensure irreproachable quality of conversion process and transformed cultivars to be used:

(i) Always develop GMC via local cultivars, already adapted to local production conditions, expressing regional or even national specificities in terms of cotton sector requirements and good fiber quality for fiber market;
(ii) Run on the national territory the entire process to develop local transgenic cultivars, by fully empowering national breeders, especially if the introgression is by crossing. This approch allows continuous adaptation of materials in creation to local conditions;
(iii) Carry out pre-release tests on GMC biological efficacy and also a special focus on conversion effects on agronomic characteristics as well as fiber properties, in research stations and in large-scale commercial production environments.

These recommendations are effective for any other country attempted by GMC adventure.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of two agro-pedo-climatic zones, drying methods and pelleting processes on chemical composition of *Manihot esculenta* (sp.), *Leucaena leucocephala* and *Cajanus cajan*

Nathalie MINATCHY[1], Harry ARCHIMÈDE[1], Dingamgoto Jesse BARDE[1], Liza DAHOMÉ[1], Fernand LABIRIN[2], Brigitte CALIF[1] and Carine MARIE-MAGDELEINE[1*]

[1]INRA, URZ Recherches Zootechniques, INRA, 97170, Petit-Bourg (Guadeloupe), France.
[2]INRA UE PTEA, Plateforme Tropicale d'Expérimentation sur l'Animal, INRA, 97170, Petit-Bourg (Guadeloupe), France.

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In tropical and subtropical countries, livestock productivity may be affected by the availability of food resources and the high prevalence of gastrointestinal pathogenic nematodes. The classical method of control using anthelmintic drugs is becoming decreasingly efficient because of a generalised resistance of the gastrointestinal nematodes suppress (GIN) to most of the drugs. In small farms, protein-rich biomasses with significant amounts of condensed tannins (CT), which are known to have anthelminbtic properties, might be good candidates to produce nutraceuticals. This experiment was conducted to determine the feasibility of producing nutraceutical pellets from *Manihot esculenta* sp., *Cajanus cajan* and *Leucaena leucocephala*, considering the influence of agro-pedo-climatic conditions plant species and technological factors, such as drying and pelleting. The samples were harvested in two different agro-pedo-climatic zones and sundried under shelter (at 25 to 35°C) or in a ventilated oven (45°C) before pelleting. Chemical analysis on crude protein and condensed tannins were conducted. The chemical composition of the plants did not vary significantly with agro-pedo-climatic conditions. Sun-drying and oven-drying decreased the CT content of the plants. No effect of pelleting was recorded on crude protein and CT contents, except for *C. cajan*, for which a small decrease in CT content was observed. Protein-rich foliage types with CT contents above 50 g/kg of dry matter are potentially good candidates to produce nutraceutical pellets if they are dried using mild drying conditions, like sun-drying under shelter.

Key words: Condensed tannins, nutraceuticals, drying, pelleting processes.

INTRODUCTION

Livestock productivity can be affected by gastrointestinal-induced pathologies that cause almost 45% mortality in sheep and goats before weaning. The classical method of control using anthelmintic drugs is becoming

*Corresponding author. E-mail: carine.marie-magdeleine-chevry@inra.fr. Tel : +590 25 59 32. Fax: +590 25 59 36.

decreasingly efficient because of a generalised resistance of the gastrointestinal nematodes (GIN) to most of the drugs, especially in tropical areas (Mahieu, 2014). Many plants from tropical areas could be used as sources of nutraceuticals due to their composition of primary and secondary metabolites, and thus constitute part of an alternative to the use of anthelmintic drugs within integrated pest management systems against GIN (Cei et al., 2018; Santos et al., 2019).

Condensed tannins (CT) are of particular interest because they exert direct and indirect actions on pathogens (Hoste et al., 2012). These polyphenolic compounds could reduce the worm burden by impacting different steps of the development cycle of the nematode, as they have well-known actions on egg hatching rate, larval exsheathment and female fecundity (Hoste et al., 2012; Waghorn, 2008). Condensed tannins could also impact on the nutritional balance for the animals, given their influence on the quantity and the profile of available amino acids. By aiding to increase the bypass of dietary proteins in the intestine, condensed tannins protect proteins from degradation in the rumen (ruminal escape), causing increased lactation, wool growth and live weight gain, without changing voluntary feed intake (Piluzza et al., 2014).

Condensed tannin activities are known to depend on their concentration and nature (size, structure and profile) in the plant (Mueller-Harvey et al., 2019; Waghorn, 2008). Previous analysis shows that CT concentrations between 20 and 50 g/kg of the dry matter (DM) of the plant generate nutraceutical properties in the plant (Piluzza et al., 2014).

The CT composition depends on the plant species (Mueller-Harvey et al., 2019), plant growth stage and organs (Piluzza et al., 2014), as well as on the harvesting area, soil composition (Barry and Forss, 1983), humidity rate and weather conditions (Lees et al., 1994; Frutos et al., 2002). Moreover, the availability of a simple technology, like pelleting, suitable for the farmers and respectful of plant properties can improve the use of CT to manage GIN infections (Gaudin et al., 2016).

However, in order to produce plant pellets with an effective CT content against GIN infection, the sensitivity of CT to temperature has to be considered, as well as the variability of CT concentration in plants.

The global aim of this study was to assess the feasibility of pelleting condensed tannins-rich plants for their use as nutraceuticals at the farm level. For this purpose, experiments were undertaken to investigate the impact of natural conditions (soil, temperature and humidity) on the chemical composition, as well the influence of practices for preservation (drying and pelleting processes), on the CT content of four tropical plants.

MATERIALS AND METHODS

Foliage sampling: Collection and preparation

Four distinct types of plant foliage containing CT were chosen: *M. esculenta* sp.1; *M. esculenta* sp.2; *C. cajan* and *L. leucocephala*. *M. esculenta* sp.1 has lower levels of cyanhydric acid in its leaves and tubers compared to *M. esculenta* sp.2. The samples were harvested from two zones: (i) Grande–Terre that is characterised by a vertisol soil and humid tropical climate with a long dry season (3 to 5 months), 83% humidity and a mean temperature of 25°C; (ii) Basse–Terre that is characterised by a ferralitic soil and humid tropical climate with a short dry season (less than 2 months), 88% humidity and a mean temperature of 25°C. For each zone, the sampling was done from three sites, during the middle and the end of the dry season. Thirty to forty kilograms of stems of *L. leucocephala*, *C. cajan* and *M. esculenta*, aged 6, 8 and 12 months, respectively, were harvested.

These samples were mixed by site and then divided into three sub-samples that were dried under different conditions: Freeze-dried; sun-dried under shelter (at 25 to 35 °C); dried at 45°C for 2 days in a ventilated oven. These three sub-samples were used for chemical analysis, in triplicate. The freeze-dried and the sun-dried samples were pelleted without additives in a GR 150 E system (Oligotechnology, Wissembourg, France).

Chemical analysis and analytical procedures

The DM content was determined using a forced-air oven at 60°C until constant weight is achieved.

Foliage samples were milled through a 1 mm screen (Reich hammer mill, Haan, Germany) prior to analysis. Organic matter (OM) and nitrogen (N) were analysed according to the AOAC methods 923.3 and 992.15, respectively (AOAC, 1990). Crude protein (CP) content was estimated as N × 6.25. Cell wall components (neutral detergent fibre [NDF], acid detergent fibre [ADF] and acid detergent lignin [ADL]) were determined as described by Van Soest et al., (1991). CT content was determined on freeze-dried, sun-dried under shelter and 45°C ventilated-oven samples, respectively, using the vanillin-H_2SO_4 method reported by Laurent (1975). For improved accuracy, the CT concentration of the plant was determined using the CT extract of each plant as a standard for the individual calibration curves. The CT were extracted using a 70% (v/v) aqueous acetone solution (Giner-Chavez et al., 1997) and then isolated with Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO). Only N and CT were measured in the pellets. Each analysis was done in triplicate, and the means were calculated.

Statistical analysis

Statistical analysis was performed using the mixed general linear model procedure in SAS 9.2 (2008). The global model used to analyse the soil, the foliage and the drying process effects was:

$$Y_{ijkl} = m + F_i + Z_j + D_k + (F \times Z)_{ij} + (F \times D)_{ik} + S_l + e_{ijkl},$$

where m is the mean, F_i is the foliage fixed effect (i=1,2,3,4), Z_j is the zone fixed effect (j=1,2), D_k is the drying fixed effect (k=1,2,3), $(F \times Z)_{ij}$ is the interaction between the foliage and the zone effects, $(F \times D)_{ik}$ is the interaction between the foliage and the drying effects, S is a random effect associated with harvesting site and e_{ijkl} is the residual term.

The global model to analyse the pelleting process effect was:

$$Y_{ijk} = m + F_i + P_j + (F \times P)_{ij} + S_k + e_{ijk},$$

where m is the mean, F_i is the foliage fixed effect (i=1, 2, 3, 4), P_j is the processing fixed effect (j=1, 2, 3), $(F \times P)_{ij}$ is the interaction between the foliage and the processing effects, S is a random effect associated with harvesting site, and e_{ij} is the residual term.

Table 1. Chemical composition of *Manihot esculenta, Leucaena leucocephala* and *Cajanus cajan* foliage harvested from two different agro-pedoclimatic zones in Guadeloupe, France.

Item	Plant species				SEM	*p*-value	
	M. esculenta sp.1	*M. esculenta* sp.2	*L. leucocephala*	*C. cajan*		Agro-pedo-climatic zone[1)]	Zone × species
OM (g/kg DM)	894[a]	904[b]	910[bc]	922[c]	0.92	0.4991	0.0001
NDF (g/kg DM)	445[a]	422[ab]	378[b]	511[c]	2.51	0.4597	0.1157
ADF (g/kg DM)	247[a]	263[b]	189[c]	356[d]	1.21	0.4043	0.0001
ADL (g/kg DM)	109[a]	116[a]	91[c]	189[d]	0.73	0.4335	0.0003
CP (g/kg DM)	189[a]	189[a]	262[b]	217[c]	1.33	0.4337	0.0001
Ash (g/kg DM)	106[a]	96[b]	90[bc]	78[c]	0.92	0.4991	0.0001
CT (g/kg DM)	73[a]	60[b]	157[c]	170[c]	1.08	0.9546	0.0001

M. esculenta sp.1 with low cyanhydric acid content. *M. esculenta* sp.2 with high cyanhydric acid content.[1)] Vertisol and long dry season *vs*. ferralitic soil and short dry season. SEM, standard error of the mean; OM, organic matter; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; CT, condensed tannins. [a–d] Means within a row with different superscript letters differ significantly (p<0.05).

The values were expressed as least square means and standard error of the mean. Statistical differences were declared significant at p<0.05.

RESULTS

The harvested *C. cajan* and *L. leucocephala* seemed relatively homogeneous. Conversely, *M. esculenta* sp.1 and sp.2 constituted a more heterogeneous population, based on the size, the colour and the shape of the leaves, and the length and the colour of the petioles.

The chemical composition of the samples collected in this study is presented in Table 1. The comparisons of CP contents indicated significant differences between the plants (p<0.05), except for the two *Manihot* sp. (p=0.9502). For all foliage types, the CP content varied from 189 to 262 g/kg DM. Plants were also different based on the different components in the plant cell walls (NDF, ADF and ADL), excluding the two *Manihot* sp, for which the concentrations of NDF and ADL were similar. Regarding ash, no significant differences were observed between *L. leucocephala* and *M. esculenta* sp.2, and between *L. leucocephala* and *C. cajan. Manihot esculenta* sp.1 was significantly different from the other plants. CT concentrations were significantly different between the foliage types, except for *L. leucocephala* and *C. cajan*, which had similar concentrations with each other.

No significant effect of harvesting zone was registered on plant composition. However, interactions were observed between plants and harvesting zones. Although the differences were not significant, the plants harvested from Basse–Terre had NDF contents higher than those harvested from Grande–Terre. Similar observations were made for ADF, but the difference tended to be significant only for *C. cajan* (p=0.08). CP differences were not significant between harvesting zones for *L. leucocephala* and *C. cajan*, whereas, CP was significantly higher in *M. esculenta* sp.2 harvested from Basse–Terre (p=0.008), and higher, but not significantly, in *M. esculenta* sp.1 harvested from Basse–Terre (p=0.18). Although the differences were not significant, CT content was higher for the two *Manihot* sp. and *L. leucocephala* from Basse-Terre. In contrast, *C. cajan* CT levels were significantly higher in the plants harvested from Grande-Terre than Basse-Terre (p=0.0377).

The drying process significantly impacted on the CT content of the plants (Table 2). Sun-drying and oven-drying reduced the CT concentrations in plants. The most significant losses were observed with oven-drying at 45°C.

There was no significant influence of the pelleting operation on the N contents irrespective of the foliage and drying process considered (Table 3).

Pelleting noticeably affected the CT contents of *C. cajan*, only. The entire process (sun-drying then pelleting) significantly altered the CT contents of *M. esculenta* sp.2 and *C. cajan*. Comparing the sun-dried and pelleted plant to the freeze-dried plant, abnormally high CT contents were reported for *M. esculenta* sp.2, leading to a significant effect of the sun-drying and pelleting process.

DISCUSSION

General considerations

In this study, soil and rainfall were the parameters that had the most variation between the two areas. Temperature and humidity were quite the same. Globally, the chemical composition of leaves concurs with values reported in the literature (Table 4). The CP and CT values reported in literature vary in the ranges 168 to377 and 4 to 92 g/kg DM for *M. esculenta*; 153 to 403 and 9 to 181 g/kg DM for *L. leucocephala*; and 185.6 to

Table 2. Effect of drying technology on condensed tannin content in *M. esculenta, L. leucocephala* and *C. cajan* foliage.

Plant species	Condensed tannin content (g/kg DM)			SEM
	Freeze-dried	Sun-dried	Oven-dried	
Manihot esculenta sp.1	68.3[a]	40.8[b]	37.5[b]	0.871
Manihot esculenta sp.2	64.6[a]	26.4[b]	33.8[b]	0.858
Leucaena leucocephala	154.8[a]	135.6[b]	58.7[c]	0.831
Cajanus cajan	172.9[a]	152.8[b]	51.4[c]	0.926

DM, Dry matter; SEM, standard error of the mean. *M. esculenta* sp.1 with low cyanhydric acid content. *M. esculenta* sp.2 with high cyanhydric acid content. [a–c] Means within a row with different superscript letters differ significantly (p<0.05).

Table 3. Effect of pelleting technology on nitrogen and condensed tannins contents in *M. esculenta sp., L. leucocephala* and *C. cajan* foliage.

Species	Treatment			SEM
	Freeze-dried	Freeze-dried and pelleted	Sun-dried and pelleted	
Nitrogen (g/kg DM)				
Manihot esculenta sp.1	196.2	185.5	187.2	1.019
Manihot esculenta sp.2	194.8	176.2	187.2	0.974
Leucaena leucocephala	268.4	275.5	270.9	1.019
Cajanus cajan	228.2	221.5	225.5	1.096
Condensed tannins (g/kg DM)				
Manihot esculenta sp.1	66.3	59.9	57.5	0.7381
Manihot esculenta sp.2	93.1[a]	79.7[a]	19.2[b]	0.705
Leucaena leucocephala	138.8	156.9	137.9	0.7381
Cajanus cajan	172.4[a]	148.8[b]	129.1[b]	0.7934

M. esculenta sp.1 with low cyanhydric acid content. *M. esculenta* sp.2 with high cyanhydric acid content. [a–c] Means within a row with different superscript letters differ significantly (p<0.05).

236 and 47 to 77.1 g/kg DM for *C. cajan*, respectively. Considering the accuracy of the method for CP determination, it can be hypothesise that the results reported for these components are mainly due to natural variations in the plants. On the contrary, because of variations in methods, procedures and standards used for the analysis of CT (Frutos et al., 2002), it can be surmised that a large part of the variation reported in the literature is linked to those factors.

The high CP contents in the three distinct types of foliage evaluated, confirm their potential as feed resources. However, the digestibility and the amino acid profiles have to be taken into account to determine their nutritive value. The CP content of *L. leucocephala*, being the highest one, favours it as a good potential candidate in the development of a nutraceutical. *C. cajan* had the highest lignin content, which depresses digestibility. This plant cell wall component must be kept in mind when discussing the quality of foliage as a nutraceutical. Both *Manihot* sp. were similar in chemical composition, and so their method use can be the same for both species.

Effect of agro-pedo-climatic conditions on chemical composition

In the present study, significant effect of the agro-pedo-climatic conditions on the primary compounds (CP, cell wall) was not observed. This result can be explained by the low temperature and humidity variations and the absence of nutrient deficiencies in the soil of the two harvesting zones. This outcome is a classic result because, to the researchers' knowledge, no author has reported any significant effect under similar conditions. Even under adverse conditions, effects on primary components were low (Minson, 1990). Similarly, the effect of temperature only appears for large ranges of variation (Minson, 1990).

A variable effect of the agro-pedo-climatic conditions on the CT content, depending on the plant; hence, the

Table 4. Crude protein and condensed tannin contents in *C. cajan*, *L. leucocephala* and *M. esculenta* reported in the literature.

Species	CP (g/kg DM)	CT (g/kg DM)	Method for CT analysis	Reference
	236.0	-		Journal of Agricultural Science Research. 2016, 5(2):035-9.
	206.1	77.1	Vanillin–HCl–methanol; Price et al. (1978)	Journal of Biology, Agriculture and Healthcare. 2016, 6
	199.8	-		Journal of Animal Science. 2012, 41(3):717-25
C. cajan	199.8	-		Journal of Animal Science. 2000, 29(3):871-9.
	193.8	-		Tropical Grasslands. 1995, 29(4):263-9.
	185.6	-		Journal of Animal Feed Science and Technology. 1994;46:343.
	-	47.0	Butanol–HCl; Bate-Smith (1975) and Porter et al. (1986)	Journal of Range Management. 1994, 47(5):398-404.
Mean	**203.5**	**62.1**		
	403.0	181.0	Vanillin-HCl; Butler (1982)	Agroforestry Systems. 2003, 59(3):231-41
	306.0	18.0	Butanol-HCl–iron; Makkar (2003)	Animal Feed Science and Technology. 2011, 163(2-4):231-43.
	268.0	16.0	Butanol-HCl–ferric ammonium sulphate; Porter et al. (1986)	Asian-Australasian Journal of Animal Sciences. 2012, 25(10):1404-10.
	266.0	75;0	Vanillin-H_2SO_4; Laurent (1975)	Journal of Animal Physiology and Animal Nutrition. 2016, 100(6):1149-58.
	254.5	-		Brazilian Journal of Animal Science. 2012, 41(3):717-25.
	252.7	-		Journal of Animal Feed Science and Technology. 1994;46:343-8.
L. leucocephala	250.0	-		Animal Feed Science and Technology. 1998, 70(4):305-14.
	222.0	18.1	Vanillin–HCl–methanol; Price et al. (1978)	Global Journal of Animal Scientific Research. 2015;3(2):419-22.
	193.8	-		Tropical Grasslands. 1995, 29(4):263-9.
	193.0	9.0	Butanol–HCl; Porter et al. (1986)	Livestock research for rural development. 2008, 20 (11)
	153.0	12.7	Butanol–HCl; Makkar (2003)	Animal Feed Science and Technology. 2005, 119(3-4):345-61.
	-	134.0	Butanol–HCl; Makkar (1995)	Animal Feed Science and Technology. 2001, 91(1-2):95-106.
	-	129.5	Butanol–HCl; Terrill et al. (1992)	Journal of the Science of Food and Agriculture. 2004, 84(4):291-4
Mean	**251.1**	**65.9**		
	377.0	4.0	Butanol–HCl–iron; Makkar (2003)	Animal Feed Science and Technology. 2011, 163(2-4):231-43
	376.3	-		Brazilian Journal of Animal Science. 2012, 41(3):717-25.
	300.0	-		Animal Feed Science and Technology. 2013, 180(1-4):44-54.
	224.0	40.0	Vanillin–HCl; Nakamura et al. (2003)	Small Ruminant Research. 2010 Sep;93(1):10-8.
	208.0	92.0	Vanillin–H_2SO_4; Laurent (1975)	Journal of Animal Physiology and Animal Nutrition. 2016, 100(6):1149-58.
Manihot esculenta	208.0	21.6	Butanol–HCl; Porter et al. (1986)	Livestock Science. 2010, 129(1-3):24-30.
	200-300	43.0	-	Asian-Australasian Journal of Animal Sciences. 2003, 16(3):463-72.
	198.0	-		Animal Nutrition. 2016, 2(4):253-61.
	197.0	-		Asian-Australasian Journal of Animal Sciences. 2012, 25(12):1691-700.
	168.0	-		Livestock Science. 2010, 128(1-3):166-72.
	-	145.4	Butanol–HCl; Giner-Chavez et al. (1997)	Journal of the Science of Food and Agriculture. 1997, 74:359-68.
	-	81.6	Butanol–HCl; Terrill et al. (1992)	Journal of the Science of Food and Agriculture. 2004, 84(4):291-4.

Table 4. Contd.

	-	33.4	-	Journal of Agricultural and Food Chemistry. 1989, 37(3):712-6.
Mean	**250.6**	**45.1**		

DM, Dry matter; CP, Crude protein; CT, Condensed tannins.

absence of an overall effect was shown. The presence of a plant-dependent agro-pedo-climatic effect can be postulated. Some authors have shown a significant soil effect based on fertility and acidity. Low soil fertility or acid soil may lead to an increase in the rate of CT found in *Lotus* (Barry and Duncan, 1984; Kelman and Tanner, 1990). For some authors who have worked on contrasting agro-pedo-climatic conditions, the differences between CT concentrations varied from 70 to 400 g/kg DM, depending on the age of the plant (Muir et al., 2014), and from 50 to 20 g/kg DM, depending on the variety (Kelman and Tanner, 1990).

CT content may also vary with temperature and drought conditions. Lees et al. (1994) found an overall increase of 24% for big trefoil grown at 20 or 30°C. However, the effect of temperature could be age-dependent, as indicated in this same study. Indeed, the difference in temperature-related concentration decreased steadily from 36 to 9% for regrowth ages from 14 to 81 days.

When investigating *Lotus* grown on slightly acidic soils with a low-to-medium phosphorus content, Acuña et al. (2008) reported variations in CT due to water stress and temperature rather than to soil conditions. Similar results were found by Malisch et al. (2016) for sainfoin (*Onobrychis viciifolia* Scop.).

In this study, the effects of soil acidity and rainfall were mixed. Drought is more pronounced in basic soils than acid soils. It is not excluded that in addition to the problem of acidity, the issue of drought arises. The specific response of *C. cajan* on CT, compared with the two *Manihots* and *L. leucocephala* may be explained by its higher sensitivity to drought and not to acidity.

Effect of the plant species on the variation of chemical composition

In this study, the species was the main factor of variation for primary and secondary metabolites. According to the results, *C. cajan* and *L. leucocephala*, which are leguminous, are logically richer in N than the two *M. esculenta*. The NDF levels were relatively low compared with grasses because leaves analysed (Minson, 1990; Archimede et al., 2018).

As mentioned by Malisch et al. (2016), although the CT content of the plant is sensitive to external factors, the main factors of variation are of genetic and physiological origin.

In addition, the chemical characteristics of the plants are consistent with those found in the literature (Table 4). Moreover, intra-plant variations was not observe because the biomasses were harvested at similar ages and following the same procedures. The intra-plant variations reported were related to different varieties, or different ontogenetic stages (Malisch et al., 2016).

Effect of the drying and pelleting process on pellet composition

The process to produce the pellets was constituted by two operations: Drying and pelleting. CT are the components most sensitive to drying. The effect of different drying methods on CT: freeze-drying, sun-drying and oven-drying were first evaluated. In a second step, the type of process on the levels of CP and CT, which are important components from a nutraceutical perspective, was evaluated. In regards to drying, the results showed that oven-drying severely depreciated the CT content of the plants, whereas sun-drying had a more moderate effect when compared with the freeze-dried samples (control). This is a classical result. Indeed, depressive effects are reported only for temperatures above 55°C and for durations longer than 48 h.

Dzowela et al. (1995) and Hove et al., (2003), working on some tropical fodder shrubs, reported depressive effects for drying at 55 and 65 °C, respectively, for 48 h. Muetzel and Becker (2006) dried temperate plants at 60 °C for 2 h and specified that the effect of temperature could be plant-dependent.

Except for C. cajan, wherein the decrease was significant but relatively low (13%), pelleting did not have a depressive effect on the CP and CT contents compared with the corresponding values of freeze-dried non-pelleted samples and pellets obtained from freeze-dried plants. This behaviour can be explained by the technical parameters of the pelleting process used. During the pelleting, the temperature reaches 70 °C but the residence time of the plant particles in the apparatus is not sufficient to damage CP and CT. Indeed, since the pelleter has a capacity of 500 kg of forage per

hour, it was estimated that a plant particle stays in the pelleter for less than 1 min.

Conclusion

This study did not show any major effects of agro-pedo-climatic conditions on the variation of the chemical composition of the targeted plants for pelleting. This result can be explained by the absence of major stress to the plant. The main factor of variation was drying. Consequently, under a mild drying condition, like sun-drying under shelter, the main recommendation would be to select forages with CT content above 50 g/kg DM, to ensure post processing nutraceutical properties.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Bacterial and vermi-remediation of soil contaminated with chlorpyrifos insecticide

Nagla Abdelmoniem Mohamed Ahmed[1], Sulieman Hammad Nasser Ali[2], Ahmed Mohammed Ali Hammad[3], Abd Elaziz Sulieman Ahmed Ishag[3*] and Azhari Omer Abdelbagi[3]

[1]Soil and Water Resources Department, Environment and Natural Resources and Desertification Research Institute, National Center for Research, Khartoum, Sudan.
[2]Soil and Environment Sciences Department, Faculty of Agriculture, University of Khartoum, Khartoum, Sudan.
[3]Crop Protection Department, Faculty of Agriculture, University of Khartoum, Khartoum, Sudan.

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This study aimed at investigating the effect of consortia of three types of indigenous bacteria (*Corynebacterium* sp., *Sphingobacterium gobiense* and *Kocuria flava*) and earthworm (at 5 and 10 earthworms/kg soil) and their combination on the percentage removal of chlorpyrifos from chlorpyrifos contaminated soil in Sudan. Silt soil (Gerf soil) samples were mixed with known concentration of chlorpyrifos (450 mgkg^{-1}) and incubated for various exposure periods (3, 7, 15 and 45 days) with the bacterial consortia alone, low and high densities of earth worm and their combinations under laboratory conditions. Remaining chlorpyrifos residues were measured by gas chromatograph equipped with flame ionization detector. Degradation rates and half-lives were found to follow biphasic model with an initial fast rate of disappearance followed by a second phase of slow disappearance. All treatments caused significant ($P<0.05$) effect on the degradation rates of chlorpyrifos compared to the control. The bacterial consortia alone induced the highest effect (73.83%) on the percentage removal of chlorpyrifos, followed by the bacterial consortia plus high density of earth worm (71.22%). Earth worm alone induced the least effect on the rate degradation of chlorpyrifos (64.27 and 66.49% for the high and low concentrations respectively). Based on this finding indigenous bacterial consortia represent a promising bioremediation agent for treatment of chlorpyrifos contaminated soil and therefore may deserve further investigation under different experimental conditions as well as validation of results under real contaminated soil conditions.

Key words: Sudan, earthworm, indigenous bacteria, chlorpyrifos.

INTRODUCTION

Pesticides used in Sudan started in the late 1940s; the irrigated cotton and sugarcane schemes are the major sectors that use pesticides in Sudan. The annual consumption of pesticides in Sudan had changed over time from an average amount of 5000 Metric Ton (MT) before the 1990s to an average of 2000 to 3245 MT after that for many reasons; changes in agriculture policy, reduction in total area of production of cotton, the

*Corresponding author. E-mail: a.aziz30@gmail.com. Tel: +249915378530.

adoption of Integrated Pest Management (IPM), and other reasons (Abdelbagi et al., 2000, 2003). The poor or substandard storage facilities and management practices of pesticide stores led huge amounts of the stored pesticides to become obsolete. According to the last inventory, the total amount of the obsolete pesticides in Sudan was estimated at 666 tons. In addition there is also about 6459 cubic meters of heavily contaminated storage soil scattered over 43 major and minor sites in the country (Butrous, 1999). Horizontal and vertical movements of contaminants have been reported (Babiker, 1998; Abubaker, 2008). Until now there is no update of the previous inventory (inventory carried out in 1999) of pesticides obsolete stocks in the Sudan. However the concerned governmental departments and national experts estimated the current amounts at 1000 tons in addition to 10,000 tons of pesticides heavily contaminated storage soils. FAO with other stakeholders are currently engaged in formulating a project for the disposal of the obsolete stocks in the country (PIF, Project Identification Phase, unpublished). If such project was launched it will most likely focus on disposing of the obsolete stocks and the empty containers leaving the contaminated storage soil behind. Therefore remediation of these sites seems to be the only feasible and attractive method available. Remediation can be done by various methods: chemical, biological and photochemical. Investigations of the potential bioremediation and photodegradation methods using indigenous microbes and sunlight photolysis are underway with promising results (Elsaid et al., 2010; Elsaid and Abdelbagi, 2010; Shaer et al., 2013; Abdurruhman et al., 2015; Ishag et al., 2016, 2017; Abdelbagi et al., 2018; Ishag et al., 2019). However, it might be of relevant importance to assess other biological remediation methods (include the earthworms and bacteria) of the affected sites. Therefore, bioremediation using earthworms and consortia of bacteria should be evaluated.

One of the bioremediation technologies is the use of earthworms (Vermi-remediation). Earthworms represent a major part of the soil fauna and biomass. They live in the certain soil types and contribute fundamentally to the nutrient cycling in the soil ecosystems. Furthermore, earthworms feed on different organic materials and minerals by diffusion from interstitial water throughout the membrane followed by digestion of soil particles and organic materials in the gut and finally come up in form of casts (Shang et al., 2013). Earthworm contributes in the fate of organic pollutants in soil, improves soil aeration and water infiltration, increase microorganisms density and diversity, and enhances the nutrition status of soil (Hickman and Reid, 2008a; Lemtiri et al., 2014). The approaches for earthworm assisted bioremediation could include, direct application of earthworms into contaminated soils (Schaefer et al., 2005), co-application of earthworms to contaminated soil with other organic media such as compost (Ceccanti et al., 2006), application of contaminated media to earthworms as feeding system (Getliff et al., 2002) and indirect use of earthworms through the application of vermidigested materials (Alvarez-Bernal et al., 2006).

Based on the promising results of removal of soil contaminants by earthworms reported elsewhere (Hickman and Reid, 2008b; Gevao et al., 2001; Njoku et al., 2018) and promising results obtained with indigenous bacterial (native bacteria) biodegradation of pesticides in Sudan (Elsaid et al., 2010; Elsaid and Abdelbagi, 2010; Shaer et al., 2013; Abdurruhman et al., 2015; Ishag et al., 2016; 2017; Abdelbagi et al., 2018), it might be of interest to evaluate the combined effect of earthworms and consortia of bacteria in removal of some of the commonly reported pesticide contaminants in storage soils and hot spots in Sudan. One of the frequently reported pesticide contaminants, chlorpyrifos, was selected for the current study. Chlorpyrifos has also been registered under different trade names in Sudan with annual imports exceeding 50 tons representing 17-25% of the total annual import of pesticides in Sudan (PPD, report 2006-2010). Chlorpyrifos was reported as a major contributor to soil contamination in storage soil and hot spots in Sudan (Ishag et al., 2016, 2019). Based on the above, the current study was initiated to investigate the potential of vermi-remediation combined with consortia of indigenous bacteria in removal of chlorpyrifos from contaminated storage soils and hot spots in Sudan.

MATERIALS AND METHODS

Study area

Laboratory experiments were conducted at the Faculty of Agriculture, University of Khartoum, to study the degradation of soil contaminated with chlorpyrifos insecticide by two densities of earthworms (*Lurbicus teristris* L.) and consortia of three types of bacteria.

Chemicals and reagents

Chlorpyrifos insecticide Tricel (SL 48%) and analytical standard of chlorpyrifos (96.5% pure) obtained from Agricultural Research Corporation, Sudan were used. For extraction and GC analysis, the *n*-hexane (95% pure) and acetone (99.9% pure) obtained from Fisher company, U.K.; sodium sulfates anhydrous (99%) and sodium chloride (99.5%) purchased from Lab. Line company, Sudan were used.

Soil sampling

Soil samples were collected from the River Nile first terrace (Gerf) in Shambat area (15.6587° N, 32.5302° E), Khartoum North, Sudan. The samples were air-dried at room temperature, cleaned from debris (to avoid any interaction during chemical analysis), gently crushed, passed through a 2 mm sieve and then sterilized in an oven at 180°C for 3 h according to Ishag et al. (2019). One kilogram of sterile soil was mixed thoroughly with the chlorpyrifos as Tricel SL 48%. The properties of the native soil are illustrated in

Table 1. Some chemical and physical properties of Gerf soil.

Parameter	Value
pH	7.3
Electrical conductivity (dS m^{-1})	0.58
N (%)	0.11
P (%) as P_2O_5	0.43
K (%) as K_2O	0.13
Total organic carbon (%)	0.18
Organic matter (%)	0.31
Clay (%)	31.7
Silt (%)	53.6
Sand (%)	14.7

Table 1. The study was conducted during the period of 2018 to 2019.

Preparation of bacterial growth media

Nutrient Agar (NA) and Meat Peptone Agar (MPA) media were prepared according to the methods described by Tepper et al., (1993).

Biological materials

One liter of MPA was prepared and three quantities (250 each) were taken into three flasks, and each flask was inoculated with one strain of bacteria under study (see below) using sterilized loop. Flasks were tightly closed, shaked using a reciprocating shaker machine for 15 min at 150 rpm, and placed in an incubator at 25°C for 48 h. About 15 ml of inoculums were mixed with 30 ml of distilled water and added to the soil of each container which was pre-treated with bacteria. Earthworms were collected from along the White Nile, Jabal Awlia area (15.2286°N, 32.465260°E), Khartoum, Sudan and were kept in soil rich in organic matter for the experiment.

Bacteria used in this experiment were consortia of *Sphingobacterium gobiense, Kocuria flava,* and *Corynebacterium* sp. which were previously isolated from contaminated soils with pesticides and identified by Osman (2019) at the Pesticide Research Centre of the Department of Crop Protection, Faculty of Agriculture, University of Khartoum. Bacteria were cultured separately in a nutrient agar media and were allowed to grow in an incubator. Then a mixture of the three types of bacteria was prepared. Pre-experiment was conducted to select a concentration of chlorpyrifos not harmful for earthworm existence. Two different earthworm densities were used namely low density (5 individuals per kg soil) and high density (10 individuals per kg soil).

Experimental design

Six different treatments were designed namely: untreated control soil, consortia of native bacteria alone (mixture of *Sphingobacterium gobiense, Kocuria flava* and *Corynebacterium* sp.), earthworm low density (5 individuals per kg soil), earthworm high density (10 individuals per kg soil), mixture of bacteria and low density earthworm, and mixture of bacteria and high density earthworm. Polyethylene containers capacities of 3 kg (17 × 18 cm id) were used as containers for each treatment. One kg of silty clay loam soil artificially contaminated with chlorpyrifos was added to each container. Chlorpyrifos commercial product Tricel (SL 48%) (Equivalent to 450 mg chlorpyrifos per kilogram soil) was diluted with distilled water and thoroughly mixed with soil of each treatment. The container with their contents were wrapped with aluminum foil, and left for three days before adding the bacterial consortia and/or earthworms to protect the organisms from lethal direct effects of the insecticide. The application volume was adjusted so that it did not exceed the soil field capacity. Each treatment was replicated three times and the experimental units were arranged in completely randomized design. Soil samples were taken after 3, 7, 15 and 45 days of incubation (in thermostatic incubator, Austria at 25°C) to determine the remaining concentration of chlorpyrifos and consequently the rate of degradation.

Extraction of chlorpyrifos from the treated soil

At each sampling time, residual chlorpyrifos were determined according to the method described in AOAC-International (1996). Hundred grams of soil were taken from each treatment, placed in clean bottles (capacity 250 ml) and wetted with 30 ml of distilled water. 100 ml of solvent mixture (n-hexane: acetone mixture, 1:1 v/v) was added to each bottle. Each bottle was tightly closed and placed firmly in a rotary shaker for 3 h and then were left to stand for a while to allow the soil particles to settle down and then filtered through 24 cm WHATMAN filter paper No. 1 (Qualitative A1) in round bottom flask (capacity 500 ml). The round bottle flask and its content were placed in a rotary evaporator under vacuum at 40°C to reduce the filtrate volume to 80 ml. The content of each treatment was then transferred to separatory funnel (capacity 1000 ml) then 300 ml distilled water and 10 ml of saturated sodium chloride solution were added and shaken for one min (the cork was opened several times to release pressure) (Ishag et al., 2019). The upper organic phase layer was transferred to another 500 ml separation funnel. The aqueous phase was re-extracted with 30 ml n-hexane, and the n-hexane fraction was transferred to the 500 ml separation funnel. The combined extracts were washed twice with 100 ml of saturated sodium chloride solution (5%) which was then discarded. The organic phase was filtered through glass filter covered with 25 g of anhydrous sodium sulfate, and the solvent was evaporated by a rotary evaporator (Buchi, Postfach, Switzerland) at 40°C until dryness. The residues were reconstituted in 10 mL of n-hexane and kept in 10 ml vials, tightly closed with Teflon lined screw caps and stored in the refrigerator at 4°C for GC analysis (Ishag et al., 2019).

GC- analysis

Gas liquid chromatographic analysis was done according to Ishag

Table 2. Average concentration of chlorpyrifos (mg/kg) after incubations of soil with bacterial. consortia and earth worms.

Treatment	Incubation time (days)			
	3	7	15	45
Untreated control	430.6± 0.03^{A}	426.8± 0.08^{A}	425.5± 0.05^{A}	424.5± 0.45^{A}
Consortia of bacteria	421.8± 0.40^{B}	373.4± 1.26^{B}	342.6± 0.17^{B}	110.1± 0.06^{F}
Earthworm low density	415.5± 0.03^{C}	384.2± 0.7^{B}	252.8± 0.26^{C}	152.0± 0.1 ^{B}
Earthworm high density	374.2± 0.02^{D}	312.6± 0.26^{C}	245.6± 0.16^{D}	148.8± 0.03^{C}
Consortia of bacteria and earthworm low density	334.0± 0.20^{E}	285.6± 0.06^{D}	230.4± 0.39^{E}	147.9± 0.02^{D}
Consortia of bacteria and earthworm high density	322.3± 0.029^{F}	251.1± 0.07^{E}	229.0± 0.55^{E}	129.8± 0.81^{E}
LSD	**2.9223**	**0.3328**	**2.4654**	**0.7513**

Means followed with the same letter (s) in the same column are not significantly different (P<0.05) according to LSD= Least Significant Difference. Means± SD=Standard Deviation.

et al. (2016, 2017). The analysis was done by gas chromatography (GC) (SHIMADZU, Kyoto, Japan model GC-2010) equipped with Flame Ionization Detector (FID) and DB-5 fused silica capillary column of 30 m and 0.25 µm id. The stationary phase is 5% phenyl methyl polysiloxane, 0.25 mm thickness. The detector and injector temperatures were 300 and 280°C, respectively. Nitrogen was used as carrier gas at a flow rate of 1.13 ml minute^{-1}. The oven temperature was programmed as follows: initial temperature was 50°C and increased at 5°C minute^{-1} until 75°C, increased again at 10°C minute^{-1} until 160°C, increased by 5°C minute^{-1} until 180°C and finely increased by 3°C minute^{-1} until the final temperature of 240°C at which it was held for 2 min. Flow rates of the makeup (Nitrogen), Hydrogen and Air were 30, 40 and 400 ml minute^{-1}, respectively. Samples were analyzed in triplicates at an injection volume of 1 µl with spilt injection mode (Spilt Ratio was 2). Four concentrations 5, 50, 100 and 200 ppm of the analytical standard of chlorpyrifos (99.9% pure) were injected under the same conditions and the response was used for the construction of the standard curve. The limit of detection (LOD) was 45.278*10^{-2} mg kg^{-1}. The recovery of the method ranged from 84 to 98%.

Analysis of variance (ANOVA) was used to test if there are significant differences between treatments, and means were separated by Least Significant Difference (LSD) using SAS 9.0 for windows (SAS Institute, 2004) software. The constant rate of chlorpyrifos degradation was calculated by exponential regression analysis. A biphasic model was assumed to calculate the half-lives according to the following equation describe by Ishag et al. (2019):

$$R= A_0e^{-\alpha t}+B_0e^{-\beta t}$$

Where R = the amount of the chlorpyrifos at t days, A_0 and B_0 are concentrations of chlorpyrifos at t=0, α and β are the disappearance rate constant for first and second phase models, respectively.

The half-life of the exponential decay was calculated according to equation:

$$t_{1/2} = (2.303 \log 2)/ \text{constant rate}.$$

RESULTS AND DISCUSSION

Generally, all the treatments showed significant differences from the untreated control (Table 2). Results showed that the degradation rate increases with increasing the incubation periods. A similar finding was reported by Njoku et al. (2018) on their study of vermiremediation of dichlorvos insecticide using *Lumbricus terrestris* and *Eudrilus euginae* species of earthworm. They found that the activities of the earthworms caused a decrease in pesticide level and the total amount of pesticide in the soil was higher in the initial day than that in the final days. Further, Gevao et al. (2001) applied earthworms to the soil contaminated with non-extractable pesticides residues. They found that the physical activities of earthworms (burrowing actions) caused a release of bound pesticides residues compared to those without worms. This may be explained by the increase in microbial activity which may lead to the increase in microbial biomass (Meharg, 1996). Earthworms may enhance the interaction of soil microorganisms with burrow linings through earthworm intestine (Brown and Doube, 2004), mucus, urine, glucose, cast deposition on burrow walls, and other organic carbon sources transferred through the burrow systems. All these may promote the increase and distribution of microorganisms in earthworm burrows (Farenhorst et al., 2001) and subsequently increases the microbial biomass (Scheu, 1987). Further Meharg (1996) mentioned that increases in microbial biomass is linked to increased microbial catabolic activity. Moreover, it was reported that such increased activity, is linked with potential increases in bioavailability due to earthworm actions upon the soil within earthworm guts which could potentially increase compound losses via microbial mineralization (Gevao et al., 2001; Barois et al., 1993). Earthworms were reported by several researchers to accelerate the removal of contaminants from soil, and also facilitate and increase the contact between contaminant and soil microorganism (Hickman and Reid, 2008b).

The degradation rates by earthworm high density, Consortia of bacteria and earthworm low density and Consortia of bacteria and earthworm high density followed a biphasic model with an initial faster rate in the first phase of degradation followed by a second phase of slower rate (Table 3), while the biphasic model did not properly describe on the other two treatments (Consortia

Table 3. The half-lives of chlorpyrifos after incubation with the consortia of bacteria and earthworms (low and high densities) in soil.

Biological material\days	Half-lives (days)			
	3	7	15	45
Control	47.187^{B}	91.66^{A}	185.722^{A}	534.692^{A}
Consortia of bacteria	32.131^{C}	26.003^{C}	38.129^{B}	22.155^{F}
Earthworm low density	26.069^{D}	30.693^{B}	18.030^{C}	28.738^{B}
Earthworm high density	11.273^{E}	13.3179^{D}	17.17^{D}	28.1857^{C}
Consortia of bacteria and earthworm low density	6.975^{F}	10.672^{E}	15.531^{E}	28.032^{D}
Consortia of bacteria and earthworm high density	6.230^{A}	8.317^{F}	15.391^{F}	25.088^{E}
LSD	**0.0028**	**0.0219**	**0.0263**	**0.0043**

Means followed with the same letter (s) in the same columns are not significantly different at p = 0.05 according to LSD= Least Significant Difference.

Table 4. Mean lifetime and decay constant of chlorpyrifos after incubation with the consortia of bacteria and earthworms (low and high densities) in soil.

Biological material\Days	Mean lifetime (days)				Decay constant (d^{-1})			
	3	7	15	45	3	7	15	45
B	46.356	37.514	55.009	31.963	0.022	0.0267	0.018	0.031
W1	37.61	44.280	26.012	41.461	0.027	0.023	0.038	0.024
W2	16.264	19.214	24.771	40.664	0.061	0.052	0.040	0.024
BW1	10.064	15.396	22.407	40.442	0.099	0.065	0.0446	0.025
BW2	8.988	11.999	22.205	36.195	0.111	0.083	0.045	0.028
Control	68.077	132.244	267.94	771.398	0.015	0.007	0.003	0.001

Where; C: Untreated control; B: Consortia of bacteria; W1: Earthworm low density; W2: Earthworm high density; BW1: Consortia of bacteria and earthworm low density; BW2: Consortia of bacteria and earthworm high density.

of bacteria and Earthworm low density). This is clearly reflected in the half-life values obtained (Table 4).

This phenomenon of biphasic biodegradation in soil is common in many pesticides (Shaer et al., 2013; Abdurruhman et al., 2015; Ishag et al., 2016, 2017, 2019; Abdelbagi et al., 2018). The relative importance of the phases depends upon the availability of the pollutants,hydrophobicity, and affinity for organic matter (Rigas et al., 2007). After 45 day the removal of chlorpyrifos from the soil treated with consortia of bacteria alone exceeded 73.83%, whereas the corresponding value for mixtures of earthworms low density and/or high density with the consortia of bacteria did not exceed 71.22%. On the other hand the percentage of removal of chlorpyrifos by earthworms alone (whether high or low density) did not exceeded 66.49% (Figure 1).

Reduction in the levels of chlorpyrifos was substantial and increased with the increase in the length of incubation period. The half-lives, mean lifetimes and decay constants have shown decreased with the time approaching that is clearly manifested in the treatments of earthworm high density; Consortia of bacteria and earthworm low density; Consortia of bacteria and earthworm high density (Tables 3 and 4). Pesticides were reported to be degraded in soil by many microorganisms including *Pseudomonas aeruginosa* (Geetha and Fulekar, 2008; Shaer et al., 2013), *Sphingomonas* sp. (Li et al. 2007), Kocuria sp. (Nagavardhanam and Vihnuvardhan, 2012; Neti and Zakkula, 2013) and Corynebacterium sp. (Dhanya, 2014), B. safensis strain FO-36bT, *B. subtilis* subsp. inaquosorum strain KCTC 13429T and B. cereus strain ATCC14579T (Ishag et al., 2016; 2017). The biodegradation of chlorpyrifos by bacteria was confirmed by (Nagavardhanam and Vihnuvardhan 2012) who reported that the bacteria *Sphingomonas* sp., and *Kocuria flava* and the the bacteria *B. safensis* strain FO-36bT, *B. subtilis subsp. inaquosorum* strain KCTC 13429T and *B. cereus* strain ATCC14579T (Ishag et al., 2016, 2017) can degrade these compounds and have the ability to utilize the chlorpyrifos as source of carbon. Results indicate that treatments with bacteria have the highest degradation after 45 days of incubation compared with other treatments and this may be explained by the possible inhibitory effect of chlorpyrifos on the soil microbial population caused by the transformation of chlorpyrifos into 3, 5, 6-trichloro-2-pyridinol (TCP) which is known to have antimicrobial properties (Chu et al., 2008; Sasikala et al., 2012; Ishag et al., 2016, 2019).

Earth worms were reported to degrade or help in degradation of many soil pollutants (Hickman and Reid,

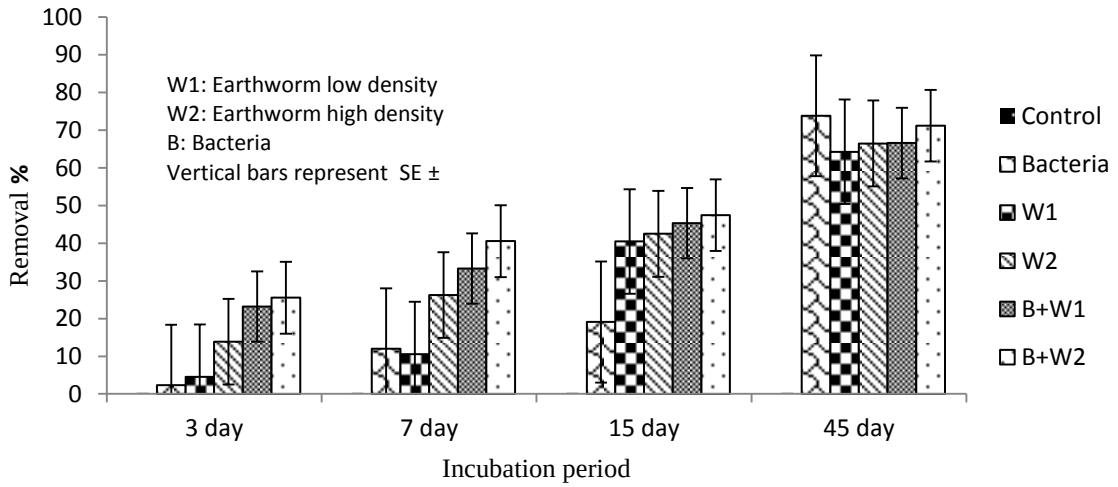

Figure 1. Removal (%) of chlorpyrifos after 45 days of incubation period over all treatments.

2008a; Lemtiri et al., 2014; Njoku et al., 2018). Earthworms can tolerate many chemical contaminants like heavy metals and organic pollutants in soil and also can accumulate them in their tissues. Earthworm species of *Eisenia fetida*, *Eisenia tetraedra*, *L. terrestris*, *L. rubellus* and *Allobophora chlorotica* were reported to remove heavy metals, pesticides and lipophilic organic micropollutants such as Polycyclic aromatic hydrocarbons from soil (Sinha et al., 2008). Microorganisms were the main cause of the biochemical degradation of organic matters by earthworm which play a critical role in this process through fragmentation of the substrates, increase in the surface area for growth of microorganisms and aeration (Aira and Dominguez, 2011). Further, partial bioremediation of polychlorinated biphenyl (PCB)-contaminated soils using bio-augmentation with PCB degrading bacteria and earthworms was reported by Luepromchai et al., (2002) who found that earthworms could facilitate PCB bioremediation by providing environmental conditions favorable for the growth and activity of indigenous PCB-degrading bacteria as well as accelerating the dispersal of PCB-degrading bacteria in bio-augmented columns (Luepromchai et al., 2002).

Kersante et al. (2006) assessed the impact of earthworm on atrazine mineralization in representative soil microsites of earthworm activities. Generally, earthworms enhance the degradation of organic compounds to about 30%, through a mechanism not clearly understood (Blouin et al., 2013).

Conclusion

(i) Both earthworms and bacteria have shown promising potential in enhancing the degradation rate of chlorpyrifos in contaminated soils and therefore may deserve further investigation and validation of results under real contaminated soil conditions.
(ii) Degradation rates and half-lives were found to follow biphasic model in three treatments; earthworm high density, Consortia of bacteria and earthworm low density and Consortia of bacteria and earthworm high density.
(iii) The bacterial consortia alone induced the highest effect on the percentage removal of chlorpyrifos, followed by the bacterial consortia plus high density of earthworms while earthworms alone induced the least effect on the rate degradation of chlorpyrifos.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Studies on some major yield responsive genes in selected rice (*Oryza* species) cultivars grown in Nigeria using candidate gene SSR-based markers approach

Zaharaddin Halilu[1*], Rabi'u Aliyu Umar[1], Riskuwa Arabu Shehu[1], Adamu Aliyu Aliero[2], Aliyu Abdullahi Turaki[3], Ahmed Hamza Balarabe[4], Abubakar Muhammad Gumi[2] and Sha'aya'u Shehu[1]

[1]Department of Biochemistry, Faculty of Science, Usmanu Dan fodiyo Universty, Sokoto. Nigeria.
[2]Department of Biological Sciences, Faculty of Science, Usmanu Dan fodiyo Universty, Sokoto Nigeria.
[3]Department of Biochemistry and Molecular Biology, Faculty of Science, Federal University of Birnin Kebbi, Kebbi Nigeria.
[4]Medway Centre for Pharmaceutical Sciences, Faculty of Engineering and Science, University of Greenwich, Medway Campus, Chatham Maritime, Kent, UK.

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This study was aimed at determining the presence of five major yield responsive genes shown to be among key determinants of rice yield in ten rice cultivars grown in Nigeria using candidate gene SSRs markers (Cg-SSRs). DNAs were extracted from young leaf samples using optimized cetyltrimethylammonium bromide (CTAB)-extraction method. An established gene sequences for high yielding rice Nipponbare cultivar for the five genes were retrieved from NCBI database and used to mine for SSRS using SSR identification tool. A pair of primer were designed manually from the nucleotide sequences flanking the selected SSRS for each gene. The designed primers were used to screen for the presence of the genes. Three pair of primers (for *Gif1*, *Gn1a* and *Gw2*) were able to amplify the targeted regions. The remaining two (for *Gs3* and *Dep1*) did not amplify even the positive control. Two representative polymerase chain reaction (PCR)-products for the PCR-positive genes were sequenced and compared with established gene sequences of high yielding cultivars on NCBI database and their percentage sequence identities were determined. Phylogenetic trees (dendrograms) were generated using neighbor joining method. Overall, all the ten cultivars have both *Gif1* and *Gn1a* genes while only eight cultivars have *Gw2* gene. *Gs3* and *Dep1* need further optimization of PCR-conditions.

Key words: Polymerase chain reaction, SSRs markers, primer.

INTRODUCTION

Rice is one of the most important staple food crops that is cultivated worldwide. Rice is safe and crucial to food security for more than one-half of the world population (Miura et al., 2010). Rapid increase in population coupled

*Corresponding author. E-mail: Zh119ahmad@gmail.com.

with urbanization continuously decreased our farmlands, creating ever increasing demand for rice. Thus, there is the need to improve rice varieties with high yield, high quality and stress tolerance.

Information on genetic diversity and relationships within and among crop species as well as their wild relatives is essential for the efficient utilization of plant genetic resource collections (Chan and Sun, 1997; Govindaraj et al., 2015). Breeding and genetic conservation programmes aimed at developing new improved rice cultivars can boost rice production. Grain yield is a complex trait determined by Quantitative Trait Loci (QTLS). Rice grain yield is mainly determined by three components; number of panicle per plant, grain number per panicle and grain weight (Cheung, 2014). For rice breeders, identification of suitable genotypes containing these agronomic traits for grain yield determination should be the first critical step toward breeding high yield hybrid rice.

In the last few decades, many QTLS related to rice grain yield were identified. Amongst which include *grain number 1a (gn1a)*, the first gene to be isolated that controls rice grain number reported by Ashikari et al. (2005). *Grain size 3* (*Gs3*) was the first major gene to be isolated that determine rice grain size (Fan et al., 2006), *Grain weight and width2* (*Gw2*) influences rice grain width and weight (Song et al., 2007), *Grain incomplete filling 1* (*Gif1*) regulates rice grain filling (Wang et al., 2008), *Dense and erect protein 1* (*Dep1*) determines rice panicle architecture (Huang et al., 2009; Wang et al., 2009; Zhou et al., 2009), *Osspl14* promotes panicle branching and higher grain yield in rice (Miura et al., 2010). Other genes identified include *Gs5* (Li et al., 2011), *Gs6* (Sun et al., 2013), *DST* (Li et al., 2013), *Gs2* (Hu et al., 2015), Osspl13/*Glw7* (Si et al., 2016).

Despite many policies of the Federal Government of Nigeria in the rice sector, rice production has not matched the growing domestic demand. In Nigeria, rice production has marginally increased from 5.5 million metric tons (MT) in 2015 to 5.8 million MT in 2017. The current consumption rate is 7.9 million MT (Punch Newspaper, 2017). Thus, a deficit of about 2.1 million MT is been met by importation. Nigeria spent 356 billion naira yearly on rice importation, amounting to about one billion naira per day (Vanguard Newspaper, 2013). Figures available with the Central Bank of Nigeria (CBN) showed that from January 2012 to May 2015, Nigeria had spent over 2.41 billion dollars on rice importation (Premium Times Newspaper, 2015). If Nigeria want to meet up with rice demand and save more money for other infrastructural projects. There is, therefore, the need to improve the yielding potentials of the local rice as part of the measures to increase rice production.

Understanding genetic structure and status of genetic variation of different rice varieties cultivated in Nigeria are of paramount importance as it is the first stage in a crop breeding programme. Three key elements are used in the determination of grain yield, viz: panicle size, grain number and grain size. Some QTLS have been identified as the major yield responsive genes and/or QTLS in rice plant. However, no research on these genes and/or QTLS to the best of our knowledge was conducted on rice cultivars grown in Nigeria. This informed our decision to screen for the presence of five major yield responsive QTLS in ten cultivars grown in Nigeria. These five genes are: Dense and erect protein 1 (*Dep1*), Grain number 1a (Gn 1a), Grain size 3 (*Gs3*), Grain weight and width 2 (*gw2*) and Grain incomplete filling 1 (*Gif1*). The rice Dense and erect protein 1 (*dep1*) is the major rice grain yield QTL that determines the panicle architecture. The locus was first identified by two independent research groups with quantitative trait locus analysis to control grain yield, grain number per panicle and panicle morphology (Huang et al., 2009; Wang et al; 2009; Zhou et al., 2009). *Dep1* encodes plant-specific gamma (ɣ) subunit of GTP-binding protein (Chakravorty et al., 2011). Grain number is one of the most important traits in the determination of crop productivity. A pioneering study by Ashikari et al. (2005) on the molecular analysis of grain number determination in rice identified grain number 1a (*Gn1a*) as a major QTL of grain number using the backcross inbred lines (BILs) of Koshihikari (lower grain number) and Habataki (higher number). *Gn1a* encodes an enzyme, namely cytokinin oxidase/dehydrogenase (Osckx2), which degrades the phytohormone Cytokinin (Galuszka et al., 2001). Grain size 3 (*Gs3*) was the first major QTL in this category to be isolated, and it has been found that it contributes to both grain length and weight. *Gs3* was identified in progeny derived from a cross between Minghui 63 (large grain) and Chuan 7 (small grain) alleles (Fan et al., 2006). Grain weight and width (*Gw2*) influences grain width and weight. This QTL was identified in progeny from a cross between WY 3 (large grain) and Fengaizhan-1 (small grain). *Gw2* encodes the ring-type protein with E3 ubiquitin ligase activity that localizes to the cytoplasm and is constitutively expressed in various tissues, which is known to be involved in the degradation processes of the ubiquitin-proteasome pathway (Song et al., 2007). Grain incomplete filling 1 (*Gif1*) encodes a cell wall invertase required for carbon partitioning during early grain filling (Wang et al., 2008).

This study was aimed at determining the presence of five major yield responsive genes (*Gif1*, *Gn1a*, *Gw2, Gs3* and *Dep1*) shown to be among key determinants of rice yield in ten rice cultivars (Faro 44, Faro 45, Faro 60, Faro 61, Dan-boto, Dan-kaushi, Jan-iri, Dan rai-rai, Bakin-iri and Walkin-kambari) grown in Nigeria using candidate gene SSRS markers (Cg-SSRs).

MATERIALS AND METHODS

Samples

A total of ten rice genotypes including four improved rice (high yielding) genotypes obtained from National Cereal Research

Table 1. SSRs found in *Dep1*, *Gn1a*, *Gs3*, *Gw2* and *Gif1* with minimum of five (5) di- and tri- motif repeats.

S/N	Gene	Motif	Number of repeats	SSR start	SSR end
		CT	5	9	18
1	*Dep1*	GC	5	181	190
		TC	5	2204	2213
		CA	5	105	114
		CA	5	3613	3622
2	*Gn1a*	CGC	5	375	389
		TCC	5	1205	1219
		TAA	5	4070	4084
		AT	5	4243	4252
3	*Gs3*	TCC	6	5797	5814
		CTC	6	5865	5882
		GCA	9	155	181
4	*Gw2*	GGA	5	494	508
		CGC	5	735	749
5	*Gif1*	TAG	6	956	973

The primer pair (forward and reverse) were designed on the sequences flanking at least one of the following motif repeats for each gene (SSRs gene markers). *Dep1 = dense and erect protein 1*; *Gif1 = grain incomplete filling 1*; *Gn1a = grain number 1a*; *Gs3 = grain size 3*; *Gw2 = grain weight* and *width*; SSR = simple sequence repeats.

Institute (NCRI), Badeggi and six local rice (low yielding) genotypes obtained from rice farmers within Argungu and Suru towns of Kebbi State were collected. The improved rice genotype samples included: Faro 44, Faro 45, Faro 60, and Faro 61. The local rice genotype samples included: Dan-boto, Dan-kaushi, Jan-iri, Dan rai-rai, Bakin-iri and Walkin-kambari. The seeds were stored in 9' ×4' brown envelops that allowed for aeration and stored in refrigerator (4°C) before used.

Samples planting

The seeds were soaked for 2 h prior to planting, then an average of ten seeds was each transferred into individual sterilized petri-dishes bedded with filter-papers. The seeds were watered everyday by way of soaking the filter-papers with few drops of water. This was maintained for seven days after which they were harvested and stored in the refrigerator (-20°C) before use for DNA extraction.

Genomic DNA extraction using cetyltrimethylammonium bromide (CTAB)

The extraction protocol used in this study was adopted from Turaki et al. (2017), but with modification that involves the removal of phenol:chloroform: isoamylalcohol (25:24:1) step and replace the step with chloroform: isoamylalcohol (24:1). The removal of phenol is associated with its toxicity and difficulty associated with disposing of its waste.

Estimation of quantity and quality of isolated genomic DNA using UV/Visible Nano-drop Spectrophotometer

The re-suspended total nucleic acid was allowed to stand for at least 12 h in refrigerator (-20°C) to allow it dissolve evenly in the solution, then diluted in molecular grade water in a ratio of 1:10 before taking the absorbance readings. The quantity and purity of the extracted total nucleic acid were analysed using Nano-drop Spectrophotometer, as total nucleic acid purity is the ratio of spectrophotometric absorbance of total nucleic acid at λ=260 nm and protein at λ=280 nm. First, 2 µl of molecular graded water was used to set a reference value (blank) then absorbance of the samples was measured. 260/280 absorbance ratio of the samples was recorded.

SSRs mining

The gene fasta format reference sequences retrieved from NCBI database were copied and pasted into SSR identification tool (SSR IT) according to Temnykh et al. (2001). The criteria for the search were that only SSRs with di- and tri-nucleotide motifs, having minimum of five number of repeats were mined (Table 1).

Primers design

Both forward and reverse primers were designed manually, one set for each gene. The primers were designed to sit on the flanking sequences of the identified SSRs region. Vector NTI (version 11.5 advanced) (http://thermofisher.com/ng/en/home/life-science/cloning/vector-nti-advance-download.html) software and oligocalc northwestern (http://basicbiotools.nubic.northwestern.edu/oligocalc.html) software were used to test for the following parameters: primer length 18 to 25 base pairs (bp), melting temperature (T_m), 50 to 60°C, forward and reverse primers T_m difference of not more than 4°C, percentage GC content 45 to 65 and expected amplicon size of 150 to 500 bp (Table 2).

Table 2. Yield responsive gene, respective primers with %GC content and Tm, expected band sizes, motif(s) with repeat number and location of motifs within gene sequence.

Gene	Forward primer, 5'→3' (%GC/Tm)	Reverse primer, 5'→3' (%GC, Tm)	Expected (Amplicon size, bp)	Motif repeats (captured)	Location of motifs (Within gene)
Dep1	CTCCTCATCGCATCGCAT (55.6, 51.5)	GCCAACTCAGAACCACCC (61.1, 49.7)	236	$(GC)_5$	5'UTR
Gn1a	GCCTTCCATCGTCAGCAC (61.1, 50.9)	GCAGTTGAGCATGAGGAG C (57.9, 50.5)	185	$(CA)_5$	5'UTR
Gw2	CCTCCATCTCCACCACCGTA (60.0, 54.5)	GGAGGAAGTGAGGAAGAG GG (60.0, 51.7)	408	$(GGA)_5$ and $(CGC)_5$	5'UTR and CDS
Gs3	CAAGTGCGTGCTGCCTCA (61.1, 53.9)	AGCGGCACGAGCATCAC (64.7, 52.5)	390	$(TCC)_6$ and $(CTC)_6$	CDS (both)
Gif1	GCTGCTGCGTCATCAACATT (50.0, 53.6)	GTGGTCATCAGCCGTGGT AG (60.0, 52.7)	285	$(TAG)_6$	Intron

Bp, Base pair; CDS, coding sequences; *Dep1*, *dense and erect protein 1*; *Gif1, grain incomplete filling 1*; *Gn1a, grain number 1a*; *Gs3, grain size 3*; *Gw2, grain weight and width*; Tm melting temperature; UTR, untranscribed region.

Constitution of primers

A set of five primers (forward and reverse) obtained from Sigma-aldrich® (sigma-life sciences, UK) were used for the PCR analysis. The tubes containing the primers were first centrifuged to pull down primers that might have been displaced from the bottom of the tubes during shipment. Primer stock solutions were prepared according to manufacturer's instruction with nuclease free distilled water. The stock solution was divided into smaller 25 µl aliquots for long term storage to avoid frequent freeze-thaw cycles and accidental cross contamination.

Polymerase chain reaction (PCR)

The PCR reaction was set using a total of 40 µl reaction mixture containing 4 µl 10x dream Taq green buffer (20 mm $MgCl_2$, Thermo Scientific, UK), 1.0 µl of dNTPs (2 mm of each dNTP, Thermo scientific, UK), 2.0 µl of each primer (0.1 µm of each primer, Sigma-life Sciences, UK), 0.4 µl of dream Tag total nucleic acid polymerase (1.25 U, Thermo Scientific, UK), 2 µl total nucleic acid template and 28.6 µl of molecular grade water. Thermal-cycling was performed in a supercycler (SC300, kyratec, Queensland, Australia) programmed as follows: 95°C/3 min initial denaturation, 30 cycles of 94°C/20 s, 58°C/30 s (for *Gw2*, *Gif1* and *Gs3*), 52°C/30 s (for *Dep1* and *Gn1a*) and 72°C/1 min and final cycle of 72°C/5 min.

Agarose gel electrophoresis

Amplification products were resolved on 1% agarose gel, stained with 5 µl ethidium-bromide in a horizontal electrophoresis tank system (Flowgen biosciences, UK) containing 1x TBE buffer. About 6 µl of 100 bp ladder (promega, UK) were used at both edges. The electrophoresis run was programmed as follows: time/75 min, current/80 milliamps (mA) and voltage/120 V. The gels were visualized and their snap shots taken in a gel documentation system (desktop gel imager, scope 21).

Sequence analysis

A total of four PCR-amplicons were sequenced by Inqaba biotech, South Africa (www.inqababiotec.co.za) using Sanger's sequencing method (Sanger et al., 1977). Two amplicons each from the two gels that gave strong and targeted bands (Figure 1a and c) were chosen, one amplicon each from a local cultivar and an improved cultivar. The obtained sequences (chromatograms) were viewed using FinchTV (www.digitalworldbiology.com/finchtv). Molecular evolutionary genetic analysis (mega7.0) software (www.megasoftware.net/mega.php) was used to edit the obtained sequences. The edited fasta format sequences were used for similarity searches using the basic local alignment search tool (BLAST) program of Molecular Evolutionary Genetic Analysis (MEGA7.0) (Kumar et al., 2016) software in the National Centre for Biotechnology Information (NCBI) genbank databases (www.ncbi.nlm.nih.gov/blast.cgi). The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004). All positions containing gaps and missing data were eliminated.

RESULTS

Spectrophotometric absorbance

The ratio of spectrophotometric absorbances of the extracted total nucleic acid at λ=260 nm and protein at λ=280 nm were between 1.90 and 2.20, with a mean value of 2.05. The quantity of the extracted ranged between 125.2 and 253.5 ng/µl, with a mean value of 179.8 ng/µl.

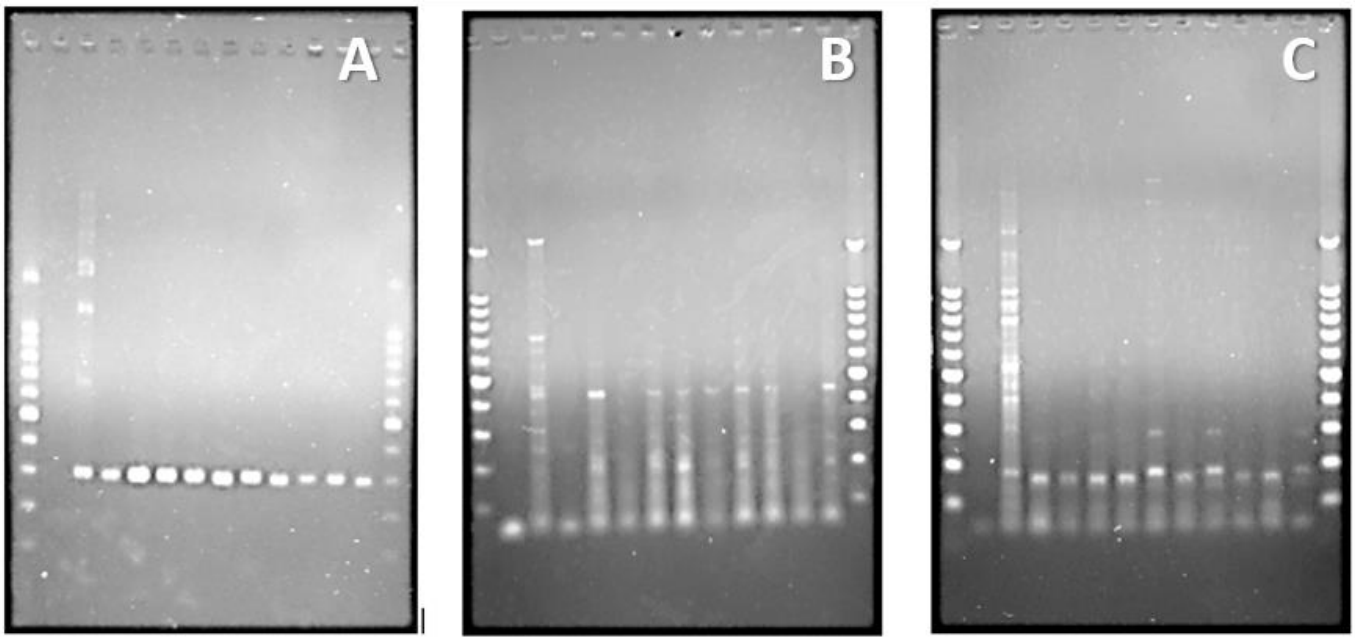

Figure 1. (A) Electrophoresis of rice *Gif1* gene. Lane M = 100 bp total nucleic acid ladder (Promega; USA); lane 1 = negative control (no template control); lane 2 = positive control (Nipponbare cultivar); lane 3 = Faro 44; lane 4 = Faro 45; lane 5 = Faro 60; lane 6 = Faro 61; lane 7 = Dan-boto; lane 8 = Bakin-iri; lane 9 = Jan-iri; lane 10 = Dan-rairai; lane 11 = Walkin-kambari; lane 12 = Dan-kaushi. **(B)** Electrophoresis of rice *Gw2* gene. Lane M = 100 bp total nucleic acid ladder (promega; USA); lane 1 = negative control (no template control); lane 2 = positive control (Nipponbare cultivar); lane 3 = Faro 44; lane 4 = Faro 45; lane 5 = Faro 60; lane 6 = Faro 61; lane 7 = Dan-boto; lane 8 = Bakin-iri; lane 9 = Jan-iri; lane 10 = Dan-rairai; lane 11 = Walkin-kambari; lane 12 = Dan-kaushi. **(C)** Electrophoresis of rice *Gn1a* gene. Lane M = 100 bp total nucleic acid ladder (Promega; USA); lane 1 = negative control (no template control); lane 2 = positive control (Nipponbare cultivar); lane 3 = Faro 44; lane 4 = Faro 45; lane 5 = Faro 60; lane 6 = Faro 61; lane 7 = Dan-boto; lane 8 = Bakin-iri; lane 9 = Jan-iri; lane 10 = Dan-rairai; lane 11 = Walkin-kambari; lane 12 = Dan-kaushi.

SSRs mining

The SSRs mined using SSR identification tool (SSR IT) satisfying the di- and tri-nucleotide motifs with minimum of five number of repeats are shown in Table 1.

Primers designing

Five set of primers, one each for a gene on the flanking sequences of at least one SSR were designed. The details of the genes, their respective primers, percentage GC content (%GC), melting temperature (T_m), expected band sizes, motif(s) with repeat number and location of motifs within gene sequence are shown in Table 2.

PCR analysis

In this study, 1% agarose gels were used to analyze the amplified PCR products and the expected band sizes were in the range of 185 to 408 bp. The amplified total nucleic acid product were stained with a chemical dye (0.1% ethidium bromide) which intercalates between the two strands of the duplex facilitating a large increase (up to 1,000 times) in their capacity to fluoresce under UV light for visualization. Three out of the five gels showed PCR products of expected band sizes. The remaining two showed no bands on the sample lanes while the positive controls showed multiple bands on both gels but not of the expected sizes:

(1) *Gif1* gene: The positive control (lane 2) and all the samples (lanes 3 to 12) showed single, clear and expected band sizes of approximately 285 bp. The negative control (lane 1) showed no band as expected (Figure 1a).
(2) *Gw2* gene: It can be seen from Figure 1b that the positive control lane (2) together with samples lanes 4, 6, 7, 8, 9, 10, 11 and 12 all showed expected band sizes of approximately 400 bp on the gel. But lanes 3 and 5 show no band. The negative control (lane 1) showed no band as expected (Figure 1b).
(3) *Gn1a* gene: The positive control (lane 2) and all the samples (lanes 3 to 12) showed single, clear and expected band sizes of approximately 185 bp. The negative control (lane 1) showed no band as expected (Figure 1c).
(4) *Gs3* gene: All the sample lanes showed no band (lanes 3 to 12). The positive control showed multiple

bands and not of the expected band sizes of approximately 390 bp. The negative control (lane 1) showed no band as expected (not shown).
(5) *Dep1* gene: All the sample lanes (lane 3 to 12) showed no band. The positive control (lane 2) showed multiple bands and not of the expected band sizes of approximately 236 bp. The negative control showed no band as expected (not shown).

Sequence analysis

The edited fasta format of *Gif1* (Faro 44 and dan boto cultivars) and *Gn1a* (Faro 60 and Dan rairai cultivars) partial gene sequences are as follows:

(1) Faro 44 - *Gif1* partial gene sequence:

TTTATAATATATCCTGTCACGAATCTCTGTCTACTAGT
AGTAGTAATAGTACTAGAACTTTTATGCCTTGCAACTT
GCAATTTCGTTGTACGGGAGAGAACTGTAGTTAGTG
ACGCCTTTCATGGTACGATTAAAGGTTCAAAGCACAT
TTTAGCACGAAAATGGTAGGCGCACTGGGACTCCAC
ATGCAGGCTTGCTTGTCGACCGTGGGGTACCTAGCC
CCTACCACGGCTGATGACCAC

(2) Dan boto - *Gif1* partial gene sequence:

TTTATAATATATCCTGTCACGAATCTCTGTCTACTAGT
AGTAGTACTAGAACTTTTATGCCTTGCAACTTGCAAT
TTCGTTGTACGGGAGAGGACTGTAGTTAGTGACGCC
TTTCATGGTAGGATTAAAGGTTCAAAGCACATTTTAG
CACGAAAATGGTAGGCGCACTGGGACTCCACATGCA
GGCTTGCTTGTCGACCGTGGGGTACCTAGCCCCTAC
CACGGCTGATGACCACAAC

(3) Faro 60 - *Gn1a* partial gene sequence:

CACCTTGTCCCTTCTACAATGGTGCAAGAACACACAA
ATTCACACACACACTGACACACACAAACCGATCGATT
GATTGATTGATAATGAAGCAAGAGCAGGTCAGGATG
GAGTGCTCCTCATGCTCAACTGC

(4) Dan rairai - *Gn1a* partial gene sequence:

TGTCCCTTCTAAATGGTGCTCGAACACACAAATTCAC
ACACACACTGACCACACAAACCGATCGATTGATTGAT
TGATAATGAAGCAAGAGCAGGTCAGGATGGCAGTGC
TCCTCATGCTCAACTGC

BLAST search

For *Gif1* gene, Faro 44 (an improved cultivar) and Dan-boto (a local cultivar) were sequenced. The BLAST search for *Gif1* sequence from Faro 44 shows 100% sequence identity with *Oryza sativa* indica (cultivar: RP bio 226) and Bac clone: Osigb0134p10 sequences. The sequence also shows 98% sequence identity with *O. sativa* japonica (cultivar: Nipponbare), *O. sativa* indica (cultivar: Shuhui 498) and *O. sativa* Bac clone: Oj000126_13. Comparing the obtained sequence from Dan-boto shows 97% sequence identity with *O. sativa* indica (cultivar: RP bio 226) and Bac clone: Osigb0134p10 sequences. The sequence also shows 96% identity with *O. sativa* japonica (cultivar: Nipponbare), *O. sativa* indica (cultivar: Shuhui 498) and *O. sativa* Bac clone: Oj000126_13. For *Gn1a*, Faro 60 (an improved cultivar) and Dan-rai-rai (a local cultivar) were sequenced. Comparing the obtained sequence from Faro 60 with *O. sativa* japonica cytokinin dehydrogenase 2 mRNA (loc 4327333), *O. sativa* japonica (cultivar: Nipponbare), *O. sativa* japonica Bac clone: B1046g12 and *O. sativa* japonica Pac clone: P0419b01 shows 99% sequence identity. The sequence, however, shows 87% sequence identity with *O. sativa* indica Shuhui 498 and *O. sativa* indica RP bio 226. Comparing the obtained sequence from Dan-rai-rai with *O. sativa* japonica cytokinin dehydrogenase 2 mRNA (loc 4327333), *O. sativa* japonica (cultivar: Nipponbare), *O. sativa* japonica Bac clone: B1046g12 and *O. sativa* japonica Pac clone: P0419b01 shows 96% sequence identity. An 86% sequence identity was obtained upon comparing the sequence obtained from Dan-rai-rai with that of *O. sativa* indica Shuhui498 and *O. sativa* indica RP bio 226.

Phylogenetic analysis

Phylogenetic analysis of Gif1 gene

Phylogenetic analysis of the seven partial nucleotide sequences of rice *Gif1* gene showed two major clusters (A and B). Bac clone: Oj000126_13 cluster separately (cluster B) while the remaining six cultivars (Faro 44, Dan boto, RP bio-226, Nipponbare, Shuhui 498, Bac clone: Osigb0134p10) cluster together (cluster A). Cluster A was sub-grouped into two clusters. Five of the six cultivars cluster together (cluster I) while Nipponbare cultivar clusters differently. Cluster I was equally divided into two clusters. Shuhui 498 forms an independent cluster while the remaining four cultivars cluster together (cluster II). In the subsequent grouping, Dan boto (a local cultivar) forms a cluster of its own while the remaining three forms cluster III. Lastly, RP bio 226 and Bac clone: Osigb0134p10 cluster together (cluster IV) while Faro 44 (an improved cultivar) forms a different cluster (Figure 2A).

Phylogenetic analysis of Gn1a gene

Phylogenetic analysis of the seven partial nucleotide

sequences of rice *Gn1a* gene shows two major clusters (cluster A and B). Cluster A has RP bio-226, Nipponbare, Shuhui 498, Faro 60, Dan rairai and Pac clone: P0419b01 while Bac clone: b1046g12 cluster differently (cluster B). Cluster A was grouped into two clusters (I and II), each cluster having three cultivars. Cluster I was re-grouped into two clusters; cluster I (RP bio-226 and Shuhui 498) while Faro 60 forms a different cluster. Cluster II was equally re-grouped into two clusters; cluster II (Pac clone: P0419b01 and Dan rairai) while Bac clone: b1046g12 forms a different cluster (Figure 2b).

DISCUSSION

Extraction of pure total nucleic acid free from contaminants is the first critical step for molecular biology experiments. Contaminants such as polyphenols and polysaccharides are often co-precipitated with total nucleic acid (Mumford and Seal, 1997). In the present study, the extraction method was based on modified CTAB method reported by Turaki et al. (2017). Young fresh leaf tissues were used for the total nucleic acid extraction to avoid the potentials of nucleic acid contamination by plant metabolites that interfere with the solubilization of precipitated nucleic acids (Puchooa, 2004). Another advantage of using young leaf is the ease of the disruption of cell wall and membranes to release nuclear materials.

The simplest and fastest method of determining total nucleic acid concentration and purity is the spectrophotometric method (Teare et al., 1997). The best test for total nucleic acid quality is its ability to be amplified in downstream analysis. It is a common knowledge that total nucleic acid absorbs maximally at 260 nm while proteins absorb at 280 nm wavelength. Total nucleic acid purity is determined by its absorbance ratio of 260/280 nm wavelength (Glasel, 1995). By calculating absorbance ratio of 260/280 nm wavelengths, it can be determined whether total nucleic acid preparations are contaminated with protein or not. Extracted total nucleic acid with protein contamination absorb strongly at 280 nm due to aromatic rings on amino acid side chains and will lower the ratio to <1.8. If the ratio is greater than or equal to 1.8 (≥ 1.8) the total nucleic acid can be considered as pure total nucleic acid (William et al., 1997). Pure RNA has a ratio of approximately 2.0.

The ratios in the present study fall within a range of 1.90 and 2.20. Thus, the total extracted total nucleic acid can be considered good for downstream analysis (that is, 2.0±0.20). The total nucleic acid concentrations of the diluted samples were of reasonable quantity (yield range between 15.50 and 25.35 ng/µl).

In this study, 1% agarose gels were used to analyze the amplified PCR products and the expected band sizes were in the range of 185 to 408 bp. The amplified total nucleic acid products were stained with a chemical dye (0.1% ethidium bromide) which intercalates between the two strands of the duplex facilitating a large increase (up to 1,000 times) in their capacity to fluoresce under UV light for visualization (Srinivasan et al., 1993). The band size of ~285 bp PCR products for *Gif1* and ~185 bp *Gn1a* genes on the gels (Figure 1a and c, respectively) shows single and clear bands for all the rice genotypes screened. This indicates that all the genotypes have both *Gif1* and *Gn1a* genes. This is supported by the expected band sizes of 285 and 185 bp for *Gif1* and *Gn1a* PCR positive results for the positive control genotype. The PCR assay did work because the no total nucleic acid-template control (negative control) lanes showed no band as expected.

In the present study, *Gw2* gene was also studied. Eight genotypes (Faro 45, Faro 61, Dan-boto, Bakin-iri, Jan-iri, Dan-rai-rai, Walkin-kambari and Dan-kaushi) show weak bands of approximately 400 bp. The weak bands may be due to small copy numbers of the targeted DNA region within the extracted genomic total nucleic acid. Lorenz (2012) pointed out that it is not the concentration of the extracted total nucleic acid that actually counts rather the number of copies of the region targeted. He posited that copies of 10^4 to 10^7 of the targeted total nucleic acid region are required for PCR with total reaction volume of 50 µl. The two other sample genotypes (Faro 44 and Faro 60) show no bands at all. This indicates that the two genotypes have no *Gw2* gene or have a different variant of the gene resulting in primer mis-match with the complementary sequences. The puzzles can be solved by using degenerate primer sets which have the ability to amplify different variants of a gene instead of the sequence specific primers used in this research (Gahoi et al., 2013). Furthermore, primer sets used to screen for *Gs3* and *Dep1* (not shown) require further optimization because none of the genotypes screened was amplified and the positive controls showed multiple unexpected bands. These indicate that both the primer sets may be having regions of preference on the genomic total nucleic acid different from the targeted sequence on the positive control genotype. Moreover, success of PCR is critically dependent on the design of an effective primer pair (Kalendar et al., 2009). PCR failures could be as result of polymorphisms such as SNP, indels and copy number variations (Piriyaponga et al., 2009). Unexpected SNP in a designed primer, in particular in the 3' end (SNP-in primer), primers designed within the intron/exon boundaries or within repetitive DNA elements are possible reasons for PCR failures (Piriyaponga et al., 2009).

The best standard in PCR product analysis is direct sequencing of the amplicon to determine its actual nucleotide composition. For the Faro 44 sequence (obtained in the present study), 100% sequence identity was obtained with *O. sativa* indica (cultivar: RP bio 226) and Bac clone: Osigb0134p10 sequences. This shows no nucleotide deletion/insertion or mismatching between

the sequences. The difference (98%) observed in the sequence identity with *O. sativa* japonica (cultivar: Nipponbare), *O. sativa* indica (cultivar: Shuhui 498) and *O. sativa* Bac clone: Oj000126_13 is as a result of triplet base deletion of 'TAG' in the Faro 44 sequence at the positions 956, 957 and 958. The 'TAG' deleted is the first of the six 'TAG' motif SSR repeats in the gene. For the Dan-boto sequence, the difference in the sequence identity (97%) with *O. sativa* indica (cultivar: RP bio 226) and Bac clone: Osigb0134p10 sequences is as a result of two triplet base deletion of 'TAG' in the Dan-boto sequence at positions 956, 957, 958, 960, 961 and 962. The 'TAGs' deleted are the first and second of the six 'TAG' motif SSR repeats in the gene. The sequence also shows 96% identity with *O. sativa* japonica (cultivar: Nipponbare), *O. sativa* indica (cultivar: Shuhui 498) and *O. sativa* Bac clone: Oj000126_13. The difference was also consequent to deletion of the first three 'TAGs' of the six 'TAG' motif SSR repeats. For *Gn1a* gene, the 99% sequence identity of Faro 60 with *O. sativa* japonica cytokinin dehydrogenase 2 mRNA (Loc4327333), *O. sativa* japonica (cultivar: Nipponbare), *O. sativa* japonica Bac clone: B1046g12 and *O. sativa* japonica Pac clone: P0419b01 is as a result of deletion of 'C' nucleotide at 176 position in the Faro 60 obtained sequence. The sequence, however, shows 87% sequence identity with *O. sativa* indica Shuhui498 and *O. Sativa* indica RP bio 226. The lower identity score is as a result of 16 nucleotide deletion ("CCGATCGATTGATTGA" at 129 to 144 positions) in the sequences of the two accessions (*O. sativa* indica Shuhui498 and *O. sativa* indica RP bio 226) which are conserved in the Faro 60 sequence. The obtained sequence from Dan-rai-rai shows 96% sequence identity with *O. sativa* japonica cytokinin dehydrogenase 2 mRNA (Loc4327333), *O. sativa* japonica (cultivar: Nipponbare), *O. sativa* japonica Bac clone: B1046g12 and *O. sativa* japonica Pac clone: P0419b01. The percentage identity obtained is as a result of insertion of an 'A' nucleotide at position 64, deletion of 'C' at position 82 and another deletion of 'A' nucleotide at position 120 within the sequence obtained from Dan-rai-rai. An 86% sequence identity was obtained upon comparing the sequence obtained from Dan-rai-rai with that of *O. sativa* indica Shuhui498 and *O. sativa* indica RP bio 226. This lower identity score is as a result of insertion of an 'A' nucleotide at position 64, deletion of 'C' at position 82, deletion of 'A' nucleotide at position 120 and another 16 nucleotide deletion ("CCGATCGATTGATTGA" at 129 to 144 positions) in the sequences of the two accessions (*O. sativa* indica Shuhui498 and *O. sativa* indica RP bio 226).

The two dendrograms (Figure 2a and b) demonstrate clearly all the eleven cultivars (positive control cultivar inclusive) shared ancestral relationships. The dendrograms also show both genetic similarities and differences abound among the rice cultivars. However, a dendrogram from complete gene sequences would have been more reliable because it will take into account the differences in the entire gene sequences. Notwithstanding, the similarities observed in the dendrograms indicate genetic, structural and functional relationships. The differences indicate the genetic diversity of both *Gif1* and *Gn1a* genes among the rice cultivars. The genetic diversity indicates possibilities for genetic improvement of rice yield through appropriate selection and cross breeding.

Conclusion

In this research work, five sequence specific candidate gene SSRs based (CgSSRs-based) primers have been designed. Genomic total nucleic acids of good quality and concentration were extracted using optimized CTAB method. PCR products revealed the presence of *Gif1* and *Gn1a* genes in the ten rice cultivars screened. Eight rice cultivars (Faro 45, Faro 61, Dan boto, Dan kaushi, Dan rai-rai, Jan iri, Bakin-iri and Walkin-kambari) have *Gw2* gene while the remaining two cultivars (Faro 44 and Faro 60) do not have the gene. The primers for *Gs3* and *Dep1* need further PCR optimization or new degenerate primers be designed. It can be deducted from the two dendrogram that both genetic similarities and differences are abound in both *Gif1* and *Gn1a* genes. Based on the aforementioned findings, it can be concluded that candidate gene SSRs-based (CgSSRs-based) markers are valuable molecular markers for the identification of major yield responsive genes in rice.

RECOMMENDATIONS

Based on the findings of this study, the following recommendations are made:

(1) Research should be conducted to ascertain the presence or otherwise of other yield responsive genes not covered by this research (such as *Gs5*, *Gs6*, *Gs2*, *Gw5*, *Ipa1*, *DST*, *Glw7*) using candidate gene SSRs (CgSSRs) and other molecular markers.
(2) Those yield responsive genes found to be positive should be cloned and sequenced in order to know the level of variation throughout the genes.
(3) Quantitative RT-PCR should be carried out to know the level of expression of the genes in those cultivars found to be positive of the genes screened and those found to be robustly expressed be transferred to other accessions for their yield improvement.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

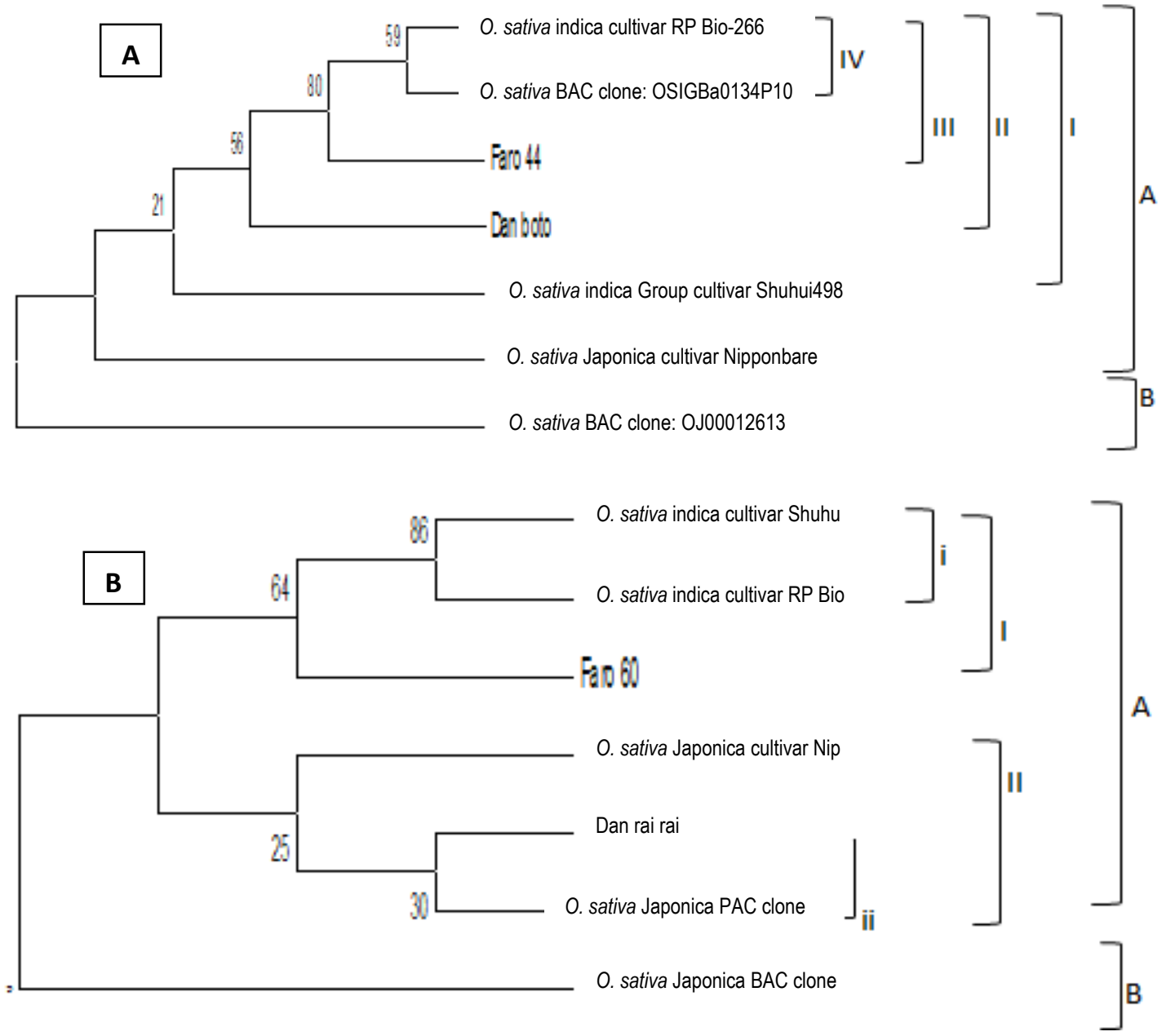

Figure 2. (A) Bootstrap consensus neighbor-joining phylogenetic tree (dendrogram) generated from nucleotide sequences of 7 rice *Gif1* partial gene sequences. **(B)** Bootstrap consensus neighbor-joining phylogenetic tree (dendrogram) generated from nucleotide sequences of 7 partial *Gn1a* gene sequences.

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Amino acyl tRNA synthetase inhibitors is therapeutics for gliomas at sub nanomolar concentrations

Peace C. Asuzu and Chidiebere U. Awah*

Feinberg School of Medicine, Northwestern University, Chicago Illinois, United States of America.

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Glioblastoma is the most malignant and fatal of all primary brain tumors. Each year in the United States, about 22,000 cases of glioma are diagnosed and about 70% of these patients die. The current treatment modality options available for glioma patients are limited to temozolomide, radiation, and surgery. The poor outcome with these treatment options has necessitated the search for a better chemotherapeutic strategy to improve patient outcomes. In a previous study, a genome-scale clustered regularly interspaced short palindromic repeats (CRISPR) screen in glioblastoma was performed under human type II topoisomerase (TOP2) poison selection and found that various aminoacyl tRNA synthetases (AARS) were the most enriched in our screen. This high expression of AARS was validated by western blot and the gliomas were treated with sub-nanomolar doses of AARS inhibitors. Our results showed that AARS inhibitor treatment effectively killed 75 to 90% of the tumors within 72 h and that this killing is independent of DNA damage repair machinery. Taken together, the findings suggest that application of AARS inhibitors might be curative for glioma, but more experimental *in-vivo* tests will be needed to validate this.

Key words: Borrelidin, mupirocin.

INTRODUCTION

According to surveillance epidemiology end result programme (SEER) (Ostrom et al., 2018), about 23,820 cases of brain tumor are diagnosed annually in the US. 17,760 of the affected patients die, which is approximately 70% of the cases, making glioblastoma a lethal tumor, and among brain tumors, the most malignant. The current modality for treating these patients includes radiation and chemotherapy and tumor treating field and survival is 14 months, at best (Mehta et al., 2018).

Aminoacyl tRNA synthetase is a very important enzyme which ensures fidelity in the transmission of genetic information. They play a key role in the loading of amino acids unto the charged tRNA, which then transfers it to ribosomes for decoding of the information (Walter, 2017; Carter and Wolfenden, 2015). Aminoacyl tRNA synthetases (AARS) is conserved across the phyla of life. Comparative evolutionary studies have shown that AARS is conserved from fungi to yeast to apicomplexa and humans. Evidence has shown that non-ribosomal peptides of fungi show conservation to AARS from eukaryotes (Luque et al.,

*Corresponding author. E-mail: chidiebere.awah@gmail.com.

2008). AARS are broadly classified into two groups: Classes I and II, based on the position of their active site. Class I AARS show Rossman's fold and form active sites at the interface between parallel β strands, while class II AARS, have their active sites on the anti-parallel β strands (Cavarelli and Moras, 1993). Orthogonal AARS and tRNA pairs have enabled the expansion of the genetic code (Normanly et al., 1990; Wang et al., 2001; Hancock et al., 2010; Anderson and Schultz, 2003; Sakamoto et al., 2002; Zhang et al., 2004) via the introduction of unnatural amino acids into the genome.

AARS has been shown to be highly over-expressed in tumors such as glioblastoma (Awah et al., 2019; Kim et al., 2012), prostate cancer (Vellaichamy et al., 2009), and other tumors. Furthermore, various small molecules have been shown to specifically inhibit different types of amino acyl tRNA synthetases. Both natural and synthetic AARS inhibitors have been described (Fang et al., 2015). These inhibitors are broadly classified into: (1) mimetics binding to ATP pockets or amino acids, (2) mimetics binding to tRNA-binding sites, (3) mimetics binding to tRNA editing sites, and (4) all substrate-binding blocking drugs by geometry fitting.

Mupirocin and borrelidin are potent inhibitors of amino acyl tRNA synthetase. They belong to categories 1 and 4, respectively and have been used in treating different diseases. Mupirocin is a topical skin ointment which inhibits isoleucine tRNA synthetase at IC50 of 2.5 - 32 nM (Fang et al., 2015). Borrelidin is a threonine tRNA synthetase inhibitor (IC50 = 0.8 - 7 nM) (Fang et al., 2015), and is very effective in treating malaria at the very low concentration of 0.93 ng/ml. It is effective in the treatment of both drug-sensitive and drug-resistant malaria (Sugwara et al., 2013). In addition, some studies have shown that borrelidin possesses angiogenic, antiviral and antifungal activities. Since borrelidin is effective against many diseases, efforts are being made to use it to treat cancers such as metastatic breast cancer (Jeong et al., 2018), oral cancer (Sidhu et al., 2015), and acute lymphoblastic leukaemia (Habibi et al., 2012).

To repurpose a well-known DNA damaging agent, etoposide for glioblastoma, we performed a genome-scale CRISPR knockout screen in a glioma cell line under etoposide selection (Awah et al. 2019). It was found that the most enriched themes were genes in protein translation (Awah et al., 2019), which belonged to amino acyl tRNA synthetase and ribosomal protein subunits. In addition, using the genome-scale CRISPR, we also found that glioblastoma expresses high levels of AARS and inhibition of AARS with borrelidin and mupirocin specifically kills the glioma cell lines *in-vitro* and at a very low concentration.

Therefore, the aim of this study was to investigate *in-vitro* if the inhibition of AARS is a potential therapy for glioblastoma.

METHODOLOGY

Genome scale CRISPR screen knockout

Briefly, as previously described (Awah et al., 2019), we obtained Brunello SgRNA library from Addgene (73179-LV), this library contains 70,000 sgRNA. 60,000 of which target the 20,000 genes at 3-4 guides per gene and 10,000 non-targeting controls. The guides were transduced into lentivirus. Using lentiviral particles, we spinfected 70,000 SgRNA Brunello library into 0.5 billion of SNB19 cells and selected them with puromycin at 0.6 µg/ml for 96 h, to achieve an MOI of 21%. About 140 million cells were harvested to ascertain the initial representation of the library after puromycin selection. The cells were then expanded to 200 million cells; 100 million of each were treated with etoposide (5 µM) and DMSO, respectively for 14 days. At the end of 14 days, the cells were harvested and the genomic DNA from the DMSO and the etoposide treated groups were harvested using Zymo Research gDNA kit and the SgRNA were amplified by a unique set of barcodes, then using NextSeq, the SgRNA abundance was determined. The data was analyzed as described (Awah et al., 2019) using CRISPRAnalyzer (Winter et al., 2017). We used 8 statistical methods from the CRISPRAnalyzer to call a guide a hit using a very stringent $p<0.001$ and an FDR of 0.01.

Western blot

To determine the protein expression level of AARS genes in various GBM, we extracted the proteins using M-PER (Thermoscientific: 78501) and a cocktail of phosphatase and protease inhibitors. The cells were lysed using a water bath ultrasonicator for 4 min. Subsequently, we spun down the cells and collected the supernatant. We measured the concentration and denatured the proteins in laemmli SDS buffer at 100°C for 14 min and directly loaded them unto 4 to 20% Tris-glycine gels (Novex), before separation at 180 V for 2 h. The gels were transferred unto a PVDF membrane by semi-dry blotting for 1 h. We blocked the membrane in 5% non-fat milk TBST for 30 min and incubated with primary antibodies AlaRS (Santa Cruz) (1:500) and IleRS, ThreRS (Santa Cruz) (1:500) and/or ACTB (Cell Signalling) (1:1500) in 5% BSA, respectively, with overnight shaking at 4°C. Primary antibodies were removed and we added the secondary polyclonal HRP (1:20,000) in TBST and incubated, with shaking for 2 h at room temperature. The membrane was washed 6X in TBST and developed with ECL (Cat No: 1705061), then band-imaged on a Biorad Chemi-doc imaging system.

Cell viability assay

GBM PDX and glioma cells were seeded at 4,000 cells per well in a 96 well plate and treated with 0.5 to 30 nM Borrelidin or DMSO. The plate was incubated for 72 h, after which we added cell titre glo (Cat No: G7572) and then incubated the cells while shaking for 5 min. We read the viability by measuring the luminescence. We normalized the intensity against DMSO treated cells of each cell line or PDX or the edited cell and then determined the survival.

Kaplan Meier survival analysis

To ascertain if high levels of AARS expression conferred survival

benefit or not to different cancers, we used the KM plotter online software platform (Nagy et al., 2018) to make a determination if patients whose tumors expressed high levels of AARS survived better or not. The algorithm from KM plotter calculates the KM survival based on the gene expression profiles based on RNA seq or tiled microarray probe and calculates the FDR for the p values of the survival.

GBM patient-derived xenografts

GBM patient-derived xenografts (MES83, GBM43, GBM6, GBM12) were originally from Dr Ichiro Nakano (University of Alabama, USA), they were a kind gift to me from Dr Peng Zhang (Northwestern University, USA). The cells were all grown in 1% fetal bovine serum in DMEM media with antibiotics, while SNB19, KS1, and GB1 (commercially obtained from ATCC) were all grown in 10% fetal bovine serum in MEM with antibiotics. All cells were grown to 80% confluency before they were harvested with trypsin for further analysis.

RESULTS

AARS is expressed in glioblastoma and its high expression is associated with poor survival outcome

Since AARS has been shown to be expressed in other tumors, we explored if it is also highly expressed in glioblastoma. First, we interrogated our CRISPR screen and found that many aminoacyl tRNA synthetases were enriched in glioblastoma (Figure 1A and B and Table 1). Chief amongst these were CARS (p = 1E-6), AARS (p = 2E-5), LARS (p = 0.00071), GARS (p = 0.001), TARS (p = 0.05), VARS (p = 0.006), MARS (p = 0.008), and HARS (p = 0.01). To verify the high expression of aminoacyl tRNA synthetase observed in the CRISPR screen, we performed a western blot analysis on three glioblastoma patient-derived xenografts (MES83, GBM6, GBM43) and on a glioma cell line SNB19. We found that alanyl tRNA synthetase was markedly enriched in some of the cell lines (Figure 1B). To ascertain, if the high expression of AARS confers any prognostic values to patients' survival, we used the KM-plotter (Nagy et al., 2018), an online survival algorithm that calculates the survival based on the mRNA expression obtained by RNA seq and microarray probe expression to test 13 different cancers, breast carcinoma, lung squamous cell carcinoma (Figure 1C), bladder carcinoma, cervical squamous cell carcinoma, testicular germ cell tumor, head and neck squamous cell carcinoma, kidney renal clear cell carcinoma, and liver carcinoma (Supplementary Figure 1A and B). We found that high expression of AARS in these 13 different cancers confers an unfavorable survival prognosis from the tumors. This indicates that tumors over-express AARS in order to usurp translational fidelity and outgrow normal tissue. In addition, inhibition of AARS might represent a therapeutic target for gliomas.

Borrelidin and mupirocin specifically inhibit classes I and II of AARS in gliomas at nanomolar concentration

As AARS has been established to be over-expressed in many tumors, we explored if the inhibition of AARS might be lethal for the glioblastoma PDX and glioma cell lines. We acquired two glioblastoma patient-derived xenografts (PDX-MES83, GBM6) and two glioma cell lines (Figure 2A) and performed a viability assay by treating with Borrelidin for 72 h. We found that at nanomolar concentrations (7 - 18 nM), borrelidin kills 99% of the glioma (Figure 2A). Next, to ascertain if treating gliomas with AARS inhibitors specifically targets aminoacyl tRNA synthetase (AlaRS, isoleucine and threonine tRNA synthetase), we treated cell lines: three patient-derived xenografts (GBM6, GBM12, GBM43) and three glioma cell lines (SNB19, GB1, KS1), with borrelidin for 36 h and performed a western blot with antibodies against alanyl, isoleucine and threonine tRNA synthetase and GAPDH as loading control. it was found that borrelidin and mupirocin (Figure 2B and C) inhibit AARS expression.

Inhibition of AARS does not impact DNA damage repair nor anti-apoptotic machinery

To determine if inhibition of AARS impacted other pathways such as DNA damage repair machinery or the anti-apoptotic pathway, we performed a western blot against FANCB, a DNA damage repair protein that has been validated previously (Awah et al., 2019), among others, to be involved in response to chemotherapy. We found that gliomas treated with inhibitors of AARS do not show a reduction nor an increase in the expression level of FANCB (Figure 2D). Likewise, we explored if the anti-apoptotic machinery were impaired upon AARS inhibition of glioma cells and found no changes in the expression of BCL2L2 (Figure 2D), which have been shown to be triggered in the presence of chemotherapeutics. Taken together, these findings suggest that AARS inhibition is very specific and targets the unique pathway of amino acid loading unto tRNAs for ribosomal translation.

DISCUSSION

A cure for glioblastoma remains elusive. The current therapies are limited to temozolomide, radiation and electric field treatment. So far, these regimens have remained inefficient, which has called for reevaluation into the strategies for curing this disease. The revelation of AARS as a gene involved in carcinogenesis has opened new possibilities for targeting gliomas and many other cancers. Evidence showing the expression of

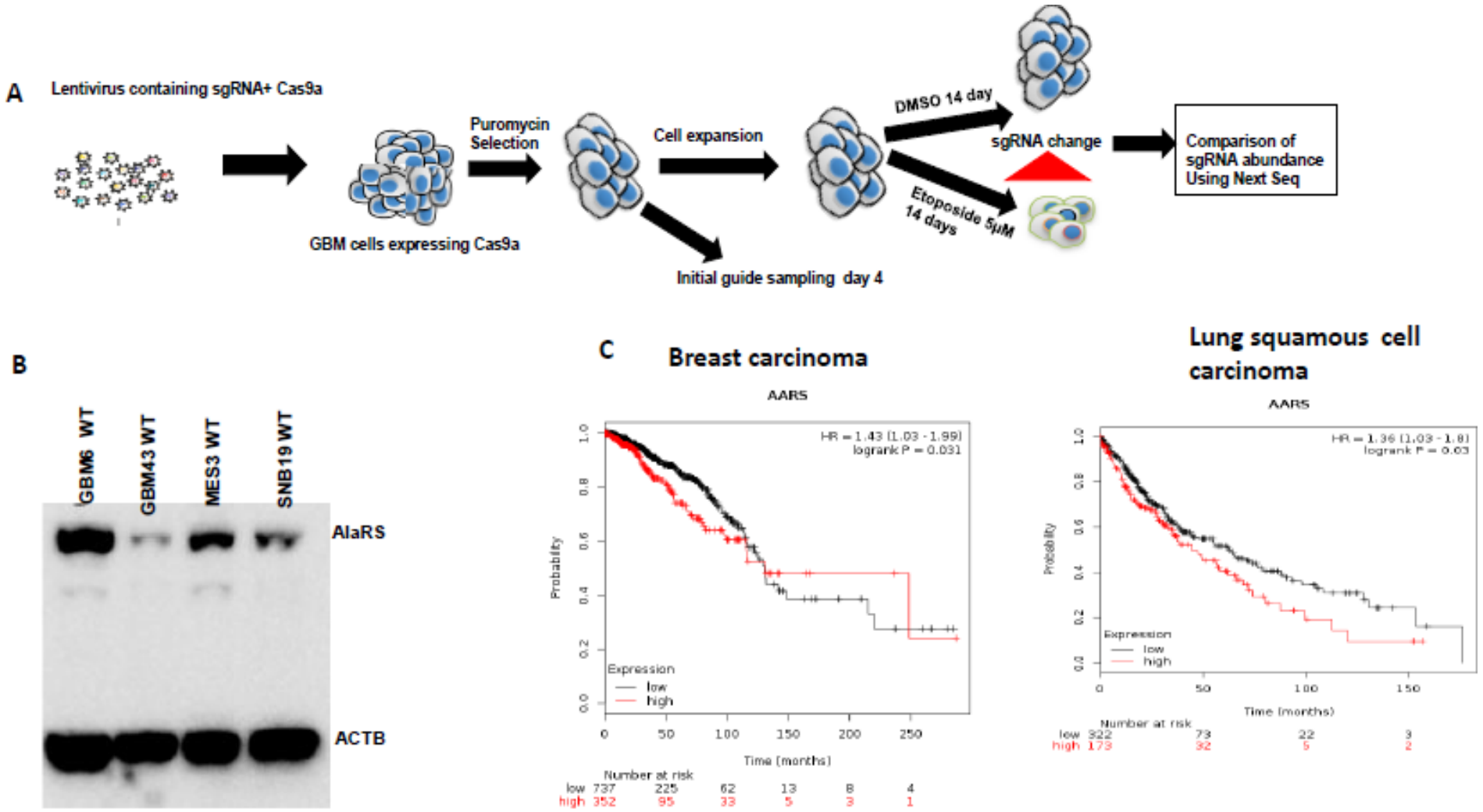

Figure 1. Genome wide CRISPR identifies Aminoacyl tRNA synthetase in glioblastoma. A. Schematic depiction of CRISPR screen in glioblastoma, the numbers ''x'' represents the coverage of sgRNA library in that experimental condition after sequencing. Briefly, as previously described (Awah et al., 2019), we spinfected 70,000 SgRNA Brunello library into 0.5 billion of SNB19 cells and selected them with puromycin at 0.6 µg/ml for 96 h. We achieved an MOI of 21%. About 140 million cells were harvested to ascertain the initial representation of the library after puromycin selection. The cells were then expanded to 200 million cells; 100 million of each were then treated with etoposide (5 µM) and DMSO, respectively, for 14 days. At the end of 14 days, the cells were harvested and the SgRNA were amplified by a unique set of barcodes and using NextSeq, the SgRNA abundance was determined. The data was analyzed as described in (Awah et al., 2019) using CRISPRAnalyzer (Winter et al., 2017). B. Western blot shows the expression of AlaRS in different glioma patient derived xenografts (GBM6, 43, MES83 and SNB19). C. Kaplan Meier survival curve shows high expression of AlaRS is a poor prognostic marker for breast cancer and lung squamous cell carcinoma.

Table 1. Table shows different AARS that were enriched and the p values showing enrichment of the guide.

Etoposide vs DMSO	P value
CARS	1.05E-06
AARS	2.51E-05
LARS	0.00071
GARS	0.001855
TARS	0.005013
VARS	0.006298
MARS	0.008141
HARS	0.010263

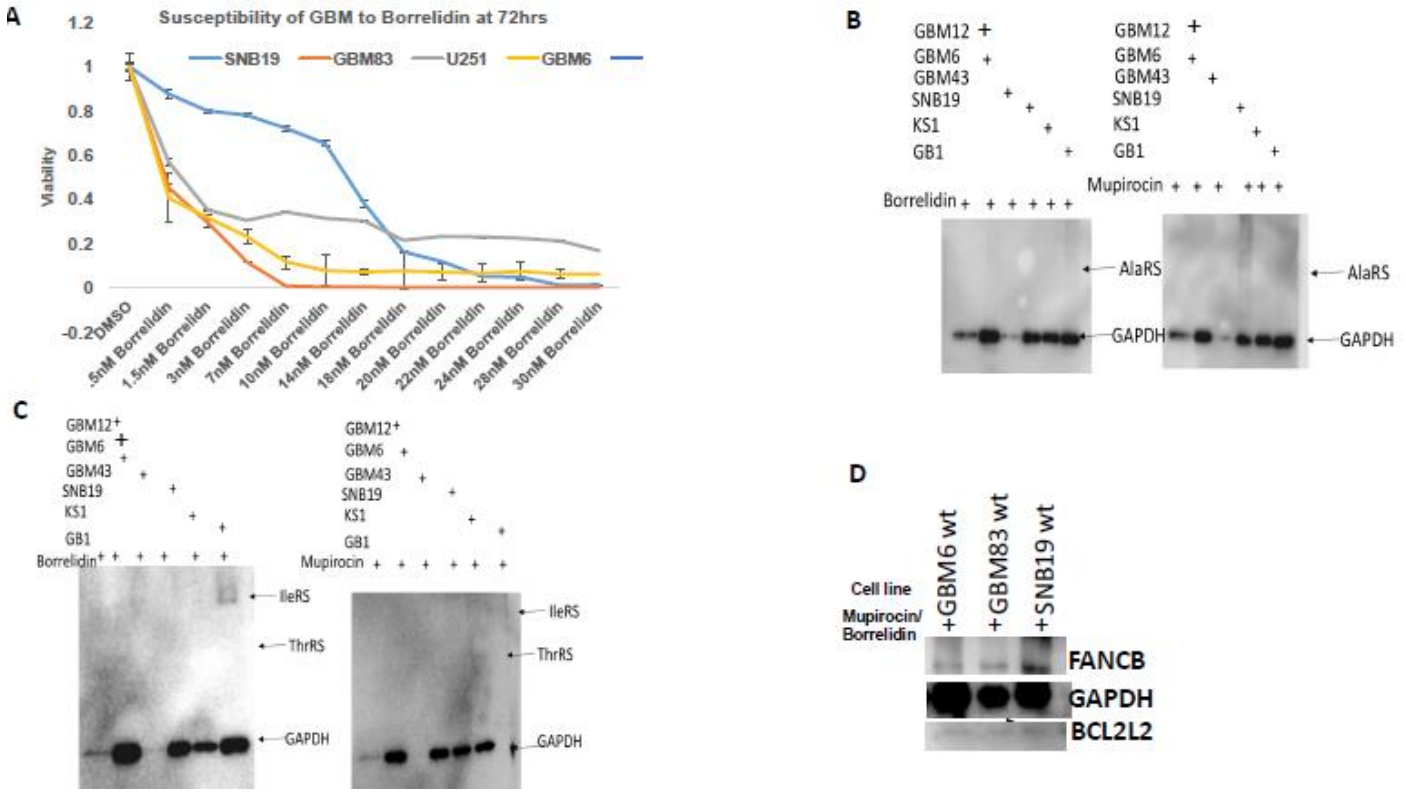

Figure 2. Borrelidin and mupirocin inhibits class II AARS and I and does not impair FANCB and BCL2L2. A. Viability assay shows dose dependent killing of glioma by AARS inhibitor borrelidin for 72 h normalized against DMSO. B. Western blot shows the inhibition of AlaRS by both Borrelidin and Mupirocin across different glioma. C. Western blot shows inhibition of IleRS and ThrRS by Borrelidin and Mupirocin across different glioma. D. Western blot shows expression of FANCB and BCL2L2 on gliomas treated with Mupirocin or Borrelidin.

AARS in glioblastoma is beginning to emerge. Kim et al., (2012), demonstrated through gene expression analysis that AARS were expressed in glioblastoma. We leveraged an unbiased genome-scale CRISPR knockout (Awah et al., 2019) and could show that AARS are indeed, highly expressed in some glioma cell lines and in some glioblastoma patients derived xenografts, with validation by western blot. The results presented here show that AARS plays a key role in glioma malignancy.

AARS have been characterized and broadly classified into classes I and II based on the position of their ATP binding, editing, amino acid, and tRNA A76 active

sites (Carter and Wolfenden, 2015; Fang et al., 2015). Various inhibitors are also classified based on their ability to inhibit each specific sites. Borrelidin and mupirocin are specific inhibitors of AARS. Studies have demonstrated their effectiveness in treating malaria and other cancers (Sugwara et al., 2013; Jeong et al., 2018; Sidhu et al., 2015; Habibi et al., 2012). We repurposed the AARS inhibitor into treating glioma. Using borrelidin, we demonstrated *in-vitro*, that AARS inhibitors are indeed specific and can target AARS without impact on DNA damage and on anti-apoptotic machinery; our observation under the microscope shows cells blebbing and bursting from the nuclei when treated with borrelidin. A key concern for the use of AARS inhibitors is toxicity, it has been demonstrated that this can be alleviated using niacin and tryptophan supplement treatment (Cooperman et al., 1951). AARS inhibition is lethal to cancers (Sugwara et al., 2013; Jeong et al., 2018; Sidhu et al., 2015; Habibi et al., 2012), when compared against cancer resistance to most chemotherapeutics. The AARS inhibitors kill glioma cell lines and patient derived xenografts at sub-nanomolar concentration compared to etoposide, which we demonstrated kills gliomas at micromolar concentration (Awah et al., 2019). Cancers and indeed the human genome, have not been exposed to AARS inhibitors in general, except for mupirocin, which is a skin ointment. Thus, the feasibility of AARS inhibitors being very effective is very plausible.

The fundamental function of AARS is to load amino acids unto tRNA for translation of the genetic code into proteins via the ribosome (Walter, 2017). The over-expression of AARS across cancers points to the fact that cancers usurp translation fidelity to gain evolutionary advantage over normal cells. AARS has extensively been used for genetic code expansion (Normanly et al., 1990; Wang et al., 2001; Hancock et al., 2010). The overexpression of AARS in cancers offers the unique opportunity to use the genetic code expansion technique to encode unnatural amino acids, drugs, and diagnostic compounds into glioma, for the purpose of treating them. This approach can be broadly applied to many cancers that over express AARS, presenting a general platform to address untreatable, undruggable and even chemo resistant cancers. Taken together, our *in-vitro* findings present evidence that AARS inhibitors can be applied to treat gliomas. However, this finding requires further validation *in-vivo* in an animal model to prove the effectiveness of this drug, how much of it can be tolerated and its toxicity in the animal model.

Conclusion

It was concluded that AARS is over-expressed in gliomas and that the inhibition of AARS might be a potential therapy for gliomas. This finding will require further *in-vivo* investigation.

LIMITATION

This study represents preliminary findings and would require validation in mouse models to confirm the therapeutic benefits of AARS inhibitors in a glioma model.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

CRISPR, Clustered regularly interspaced short palindromic repeats; **AARS,** aminoacyl tRNA synthetase; **Mupirocin AlaRS,** alanine tRNA synthetase; **TheRS,** threonine tRNA synthetase; **IleRS,** isoleucine tRNA synthetase; **GAPDH,** glyceradehyde 3-phosphate dehydrogenase; **SEER,** surveillance epidemiology end result programme; **Glioma cell line,** SNB19. KS1, GB1; **Glioblastoma PDX,** MES83, GBM6, GBM43, GBM12.

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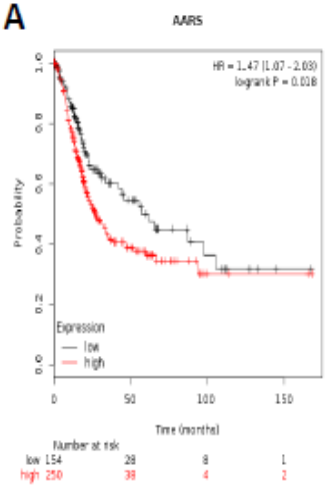
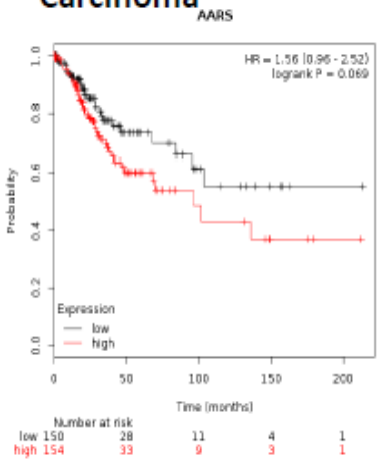
Supplementary Fig 1. High expression of Alanyl tRNA synthetase is a poor prognostic factor for many cancers.

A

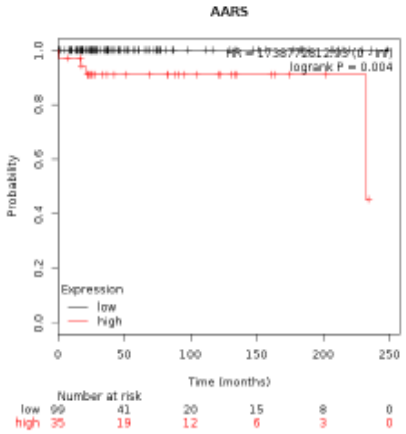
Bladder carcinoma



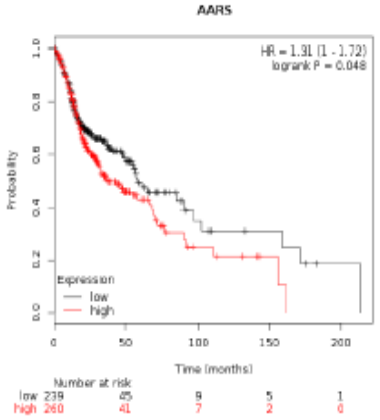
Cervical Squamous cell Carcinoma



Testicular Germ cell tumor

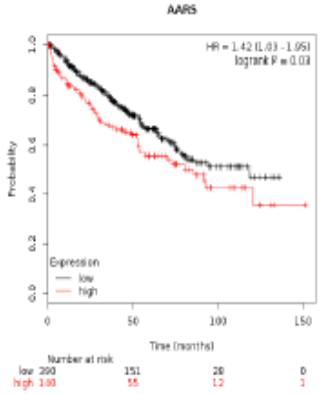


Head and neck Squamous cell Carcinoma

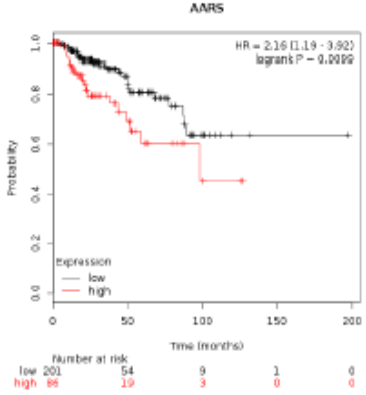


B

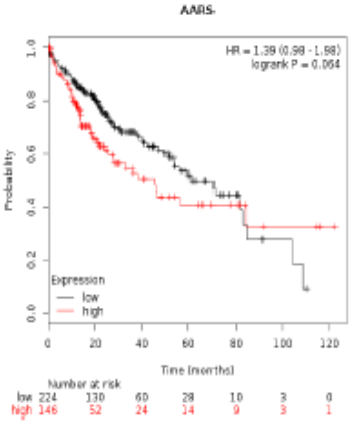
Kidney Renal Clear carcinoma



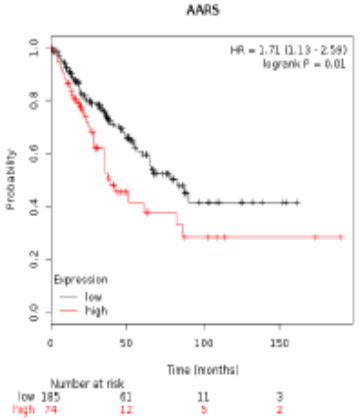
Kidney Renal papillary cell carcinoma



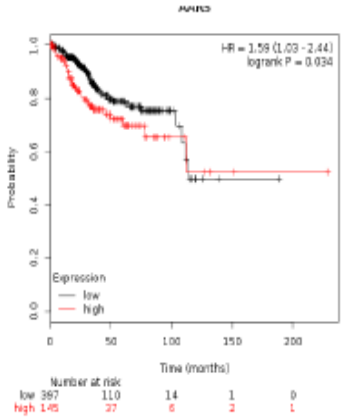
Liver carcinoma



Sarcoma



Uterine carcinoma



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Lack of association of toll-like receptor 2 rs3804100 polymorphism with paediatric tuberculosis in South Africa

Inyang Udosen

Department of Biological Sciences, Akwa Ibom State University, Mkpat-Enin, Akwa Ibom State, Nigeria.

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Toll-like receptor 2 (TLR 2) genetic polymorphisms are important factors that are involved in the development of clinical tuberculosis. In this study, a single nucleotide polymorphism of the immune response protein molecule, toll-like receptor 2 designated as rs3804100 involving T/C polymorphism was carried out by genotypic analysis of DNA obtained from blood samples of the paediatric population in South Africa consisting of 151 cases and 82 controls for tuberculosis. Genotypic analysis of tuberculosis cases for T/C rs3804100 polymorphism showed genetic frequency of 5% for TT, 90% for CT and 5% for CC genotypes while those of controls were 2% for TT, 91% for CT and 7% for CC genotypes. Statistical analysis of the polymorphic genotypes in cases and controls gave no association with the disease. The result from this study showed that this T/C single nucleotide polymorphism of TLR 2 was not associated with the development of tuberculosis (p= 0.34) in the paediatric population.

Key words: Tuberculosis, toll-like receptor 2, single nucleotide polymorphisms.

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are variation in DNA sequence that occur when a single nucleotide is altered among individuals of the same species or between paired chromosomes of an individual. They are usually defined as sites having least frequent common variants at a frequency of not less than 1% in the population (Brookes, 2005).

SNPs are one of the forms of sequence variants that occur in the human genome but accounts for greater than 90% of all differences found (Brumfield et al., 2003) and are the most abundant. Although most of the SNPs are diallelic: there are only four different types of diallelic forms- two transitions (T/C and A/G) and two transversions (T/A and C/G).

SNPs are useful molecular tools now used to study genes that might cause diseases; some SNP alleles cause disease by introducing difference in gene function or by regulation. However, many SNPs have little or no effect on disease but are useful in identifying marker SNP and functional SNP in disease association.

Single nucleotide polymorphisms in toll-like receptor 2 (TLR2) gene have been implicated in blunted immune response of TLR2 to pathogens (Lorenz et al., 2000; Kang and Chae, 2001; Yim et al., 2006). Lorenz et al. (2000)

E-mail: inyangudosen@aksu.edu.ng.

demonstrated the mutation in the TLR2 gene- Arg753Gln polymorphism- showed less response to bacterial peptides derived from *Borrelia burgdoferi* and *Treponema pallidum*. In addition, they reported that subjects with TLR2 Arg735Gln polymorphism had staphylococcal infections especially septic shock. They also suggested that the carboxyl terminus of TLR2 which is involved in signaling might be affected by this polymorphism because Arg753Gln is necessary for the receptor function. However, a study by Ryu et al. (2006) reported that both TLR2 Arg677Trp and Arg753Gln polymorphisms were not responsible for host susceptibility to non-tuberculous mycobacterial lung diseases in the South Korean population but another report by Yim et al. (2006) showed association of guanine-thymine repeat polymorphisms in intron II of the TLR2 gene and the predisposition to clinical tuberculosis in the same population. Furthermore, Kang and Chae (2001) had earlier reported the implication of TLR2 Arg677Trp polymorphism in the development of lepromatous leprosy.

Studies involving the association of TLR2 with tuberculosis in African populations have been very few in the literature. In one study by Ben-Ali et al. (2004) in investigating the role of TLR2 Arg677Trp polymorphism in tuberculosis in Tunisian patients, they reported involvement of this polymorphism in predisposing individuals to tuberculosis infection. Another study in Turkey by Ogus et al. (2004) reported the association of Arg753Gln polymorphism of the TLR2 gene to be a risk factor in the development of clinical tuberculosis.

The TLR2 polymorphism under study was the T/C base substitution which does not result in amino acid change and it occurred in the coding region of exon 2. This study was undertaken in a paediatric population of 233 individuals in South Africa to assess if there is association of toll-like receptor 2 rs3804100 polymorphism with development of tuberculosis infection.

MATERIALS AND METHODS

Study population

The study population samples consisted of cases and controls which were obtained from Capetown in Western Cape Province which is the region with the highest incidence of tuberculosis in South Africa.

The study population samples were made up of 233 individuals consisting of 151 paediatric patients and 82 controls. The age range of paediatric cases and controls ranged from 6 months to 14 years having median ages of 69 and 74 months for paediatric cases and controls respectively.

Samples were obtained from two ethnic groups in South Africa: Xhosa and Coloureds. The study populations were made up of 198 Xhosa and 33 Coloureds. Patients recruited for the study were diagnosed as having had tuberculosis while some were past history of the disease. They were diagnosed for the presence of tuberculosis by clinical and radiological test to confirm findings associated with the disease, reactivity test or Mantoux test for *M. tuberculosis* together with pathological findings of tuberculosis disease in lymph node, lungs and associated organs. Genomic DNA used for genotyping was isolated from whole blood using QIAGEN DNA purification kit. The genomic DNA of these case and control study samples were the original source of DNA.

Patients who were HIV positive were excluded as well as those that did not have definitive evidence for the disease. The control group consisted of unrelated subjects that had been diagnosed as not having any history of the disease. The data for all the paediatric patients and paediatric controls including sex and ethnic groups were collated from their medical records for this study. The cohorts for this were recruited from different areas of Capetown. The cases were obtained from Red Cross Children's Hospital in Capetown and the controls were the contacts of cases.

The Xhosa ethnic group used in the study is the second largest ethnic group of the Black South Africans. The coloured ethnic groups are a distinct population that could be considered as mixed population that are different from Black, White or Asian. The study group included one Caucasian case as well as an Indian case.

Samples were obtained in accordance with guidelines and approval from Ethics Committee of the Red Cross Children's Hospital.

TLR2 genotyping

For TLR2 SNP rs3804100, genotyping was performed by ARMS PCR (Newton et al., 1989) using 5'ATCCAGCACACGAATACACAGT 3' and 5' ATCCAGCACACGAATACACAGC 3' as forward primers for T and C allele respectively with 5' ATGGAAACGGTGGCACAGGAC 3' as reverse primer. The PCR was performed in 25 µl under the following conditions: 2 min of denaturation at 94°C followed by 30 cycles of denaturation for 30s at 94°C, annealing of primers to the template for 30s at 53°C; and extension at 72°C for 30s. A final extension was carried out at 72°C for 5 min. PCR products obtained were separated by subjecting them to electrophoresis in a 2% agarose gels and visualized under UV fluorescence for identification of the bands. The genotypes of the various individuals for the polymorphic genotypes were determined as follows: Individuals with TT genotypes which is the wild type will only show PCR fragments on gels when only oligonucleotide forward primer 5'ATCCAGCACACGAATACACAGT 3' is used along with the reverse primer; those with CC genotypes have the mutant allele which will only form PCR fragments on gel with oligonucleotide forward 5' ATCCAGCACACGAATACACAGC 3' in combination with reverse primers while those with heterozygous genotype CT will form PCR fragments with the two forward primers in PCR.

RESULTS

The SNP in the TLR2 gene rs3804100 was genotyped using ARMS PCR to generate 520-base pair fragment (Figures 1 and 2).

In the 233 paediatric samples consisting of 151 cases and 82 controls, rs 3804100 TLR2 were genotyped in 149 of cases and in all controls (Table 1). The allele frequency of rs3804100 was 150 (50%) for C and 148 (49%) for T allele in the paediatric cases while controls had the frequency of 86 (53%) and 76 (47%) for C and T allele, respectively.

The control groups of the study population deviated from Hardy-Weinberg equilibrium (p=0). In the coloured population, the genotype occurrence for cases was 1(5%) for TT and 18 (95%) for CT as there was no CC

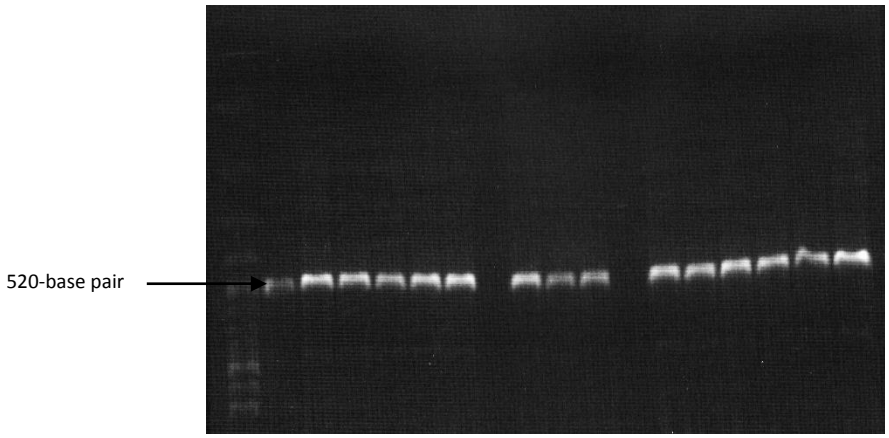

Figure 1. A 520 base pair PCR fragment of the wild type (T) allele from samples of the study population.

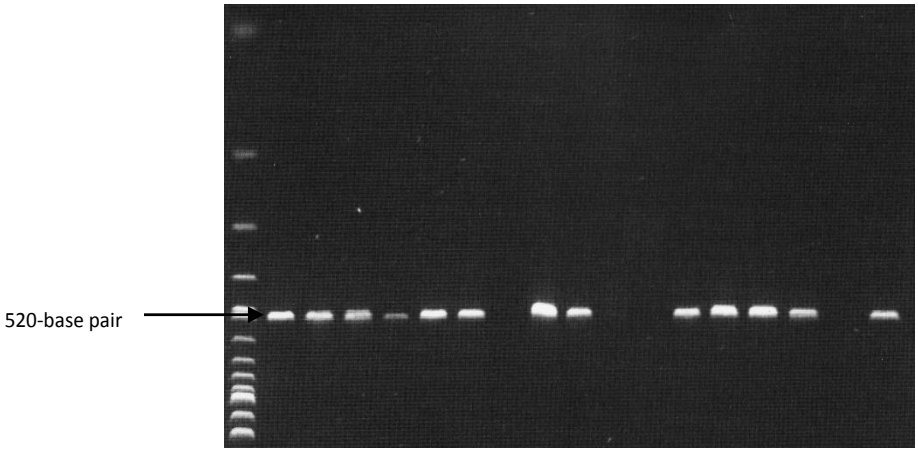

Figure 2. A 520 base pair amplicon product of PCR showing the mutant (C) allele from samples of the study population.

Table 1. Genotype distribution of rs3804100 TLR2 in population study of cases and controls.

	TT	CT	CC
Cases	7 (5%)	134 (90%)	8 (5%)
Controls	2 (2%)	74 (91%)	6 (7%)

genotype. Its controls had only CT genotype (100%). The study population had a predominance of CT genotype in both cases and controls while the homozygotes accounted for 9% in both cases and controls, respectively. The study population genotype was found to be statistically not significant ($\chi 2$= 2.18 at p= 0.34). This was also replicated for the allele frequency of the study population which showed that it was not significant ($\chi 2$= 0.328 at p= 0.57).

The Xhosa population cases had the genotype frequency of 6 for TT genotype, 110 for CT and 8 for CC genotype while its control population had 1, 64 and 6 for TT, CT and CC genotypes, respectively. The analysis of the Xhosa ethnic population genotype showed that it was statistically not significant ($\chi 2$= 1.742 at p= 0.42). The allele frequency distribution in the Xhosa ethnic group were 126 and 122 for C and T alleles, respectively with its controls having frequency of 76 for C allele and 66 for

Table 2. Genotype distribution of rs3804100 TLR2 in the gender study population.

Gender	TT	CT	CC
Male	2	56	3
Female	5	75	5

Table 3. Genotype frequency of rs3804100 TLR2 in extra-pulmonary and pulmonary tuberculosis.

Tuberculosis type	TT	CT	CC
Extra-pulmonary	5	64	3
Pulmonary	2	67	3

T allele. Comparison of the allele frequency between Xhosa paediatric cases and controls gave no significant difference ($\chi2$= 0.267 at p= 0.61).

The heterozygote genotype was also predominant in both genders (Table 2). The data showed that heterozygote genotype (CT) had a higher occurrence than homozygotes (CC and TT).

Statistical analysis of the severity of the disease between gender showed it was not significant ($\chi2$= 0.613 at p=0.74). Allele frequency in the gender population was 62 and 60 for C and T allele respectively in males. However, the female population had the same allele frequency of 85 for both C and T alleles. Comparison of the allele frequencies of the male and female population showed no significant difference ($\chi2$= 0.019 at p= 0.89).

The Xhosa gender population had the genotype frequency of 2, 46 and 3 for TT, CT and CC genotypes in males while its females had 4, 64 and 5 for TT, CT and CC genotypes respectively. Its statistical analysis showed it was not significant ($\chi2$= 0.216 at p= 0.93). The allele frequency of the gender population of Xhosa paediatrics showed occurrence of C and T allele as 52 and 50 respectively in males and females having frequency of 72 for C and 54 for T allele. Statistical analysis of males and female allele frequency gave no significant difference ($\chi2$= 0.067 at p= 0.80).

The occurrence of the genotypes between extra-pulmonary and pulmonary tuberculosis in the study population also showed a predominance of heterozygotes over homozygotes (Table 3). However, no pulmonary tuberculosis was recorded for the coloured population and the genotype distribution was 1 for TT and 19 for CT genotype for extra-pulmonary tuberculosis. The allele frequency distribution of rs3804100 in the study population showed allele frequency of 70 and 74 for C and T allele, respectively in extra-pulmonary cases; pulmonary tuberculosis cases had allele frequency of 73 for C allele and 71 for T allele. Analysis of the alleles between pulmonary and extra-pulmonary tuberculosis showed no significant difference ($\chi2$= 0.125 at p= 0.72).

Analysis of data for tuberculosis type of the study population showed it was not statistically significant ($\chi2$= 2.151 at p= 0.34).

The Xhosa population extra-pulmonary cases had genotype frequency of 4, 33 and 3 for TT, CT and CC genotypes respectively. Statistical analysis of its genotypes showed it was not significant ($\chi2$= 4.028 at p= 0.13).

DISCUSSION

In the study of the polymorphism rs3804100 of TLR2, it was observed that it had no association with tuberculosis in this study population and even within ethnic groups. No significant difference was found neither in the gender population nor Xhosa ethnic group as well as between pulmonary and extra-pulmonary tuberculosis both in its genotype distribution as well as its allelic distribution. The rs3804100 TLR2 T/C polymorphism was found in 149 out of 151 cases while it was observed in 81 of its controls. The polymorphism in the study population showed a genotype distribution having a predominance of heterozygotes across all the groups studied.

In addition, the genotype distribution of rs3804100 TLR2 polymorphism in the control paediatrics used for this study was outside Hardy-Weinberg equilibrium. This deviation could be due to various factors that might include migration, population sample consisting of subpopulation that do not completely interbreed, selection pressure in the population study in favor of heterozygote genotype as well as environmental factors which could be a direct effect of the geographical location as well as small population size of the study.

Toll-like receptor 2 polymorphisms have been associated with differences in susceptibility to different disease infections in some populations (Ioana et al., 2012; Skevaki et al., 2015). Indeed, a toll-like receptor 2 polymorphism rs765641 has been implicated in influencing susceptibility to tuberculosis infection in a

Sudanese population (Zaki et al., 2018).

The data from rs3804100 TLR2 polymorphism in the population study does not contribute to clinical tuberculosis which contradicts other TLR2 polymorphisms studies reported in other populations such as Arg677Trp in Tunisian population (Ben-Ali et al., 2004) and Arg677Trp polymorphism in Korean population (Kang and Chae, 2001; Kang et al., 2002). The result from this study shows similarity with a previous study involving TLR2 rs3804099 polymorphism in the same population (Udosen, 2019). The reason for this could be that other polymorphisms could be a contributing factor to the development of tuberculosis with the exception of the one under study.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Antiproliferative effect of *Amburana cearensis* seed extracts on human cancer cell lines

Aline Aires Aguiar[1*], Ernane Gerre Pereira Bastos[2], Ilsamar Mendes Soares[1], Ivana Grivicich[2], Jéssica Machado Miri[2], Sérgio Donizeti Ascêncio[1] and Raimundo Wagner de Souza Aguiar[1]

[1]Federal University of Tocantins (UFT), Palmas, Tocantins, Brazil.
[2]Lutheran University of Brazil (ULBRA), Canoas, RS, Brazil.

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Amburana cearensis **A. C. Smith (Fabaceae) is a plant of Brazil, popularly known as umburana or cumaru, and is widely used in folk medicine. The population uses its bark and seeds against many pathologies including cancer. The aim of this study was to examine the antiproliferative effect of *A. cearensis* extracts on human tumor cell lines of HT-29 colon adenocarcinoma and HepG2 hepatocellular carcinoma, as well as on normal mouse fibroblasts L929. This study consists of *in vitro* tests with extracts obtained from *A. cearensis* seeds with solvents of increasing polarity against neoplastic and normal cell lines. Knowing that *A. cearensis* extracts are rich in coumarins and have high antioxidant activity, aspects related to antineoplastic activity and cytotoxicity of four different extracts was evaluated. *A. cearensis* extracts were analyzed in cancer cells using the MTT assay. Cytotoxicity data demonstrated that methanolic extract (MEA) has activity against HT-29 cell line (IC_{50} 18.8 ± 0.4 µg mL^{-1}) while normal fibroblasts L929 and HepG2 cells were not affected by the extract. The crude seed extracts of *A. cearensis* did not demonstrate a cytotoxic effect against the cancer cell used in this study; however, the MEA extract can also be promising for this purpose.**

Key words: *Amburana cearensis,* cytotoxicity, antiproliferative, tumor cell, coumarin.

INTRODUCTION

The carcinogenesis process results from changes that accumulate progressively in the genetic material (DNA) of a normal cell. This process is related to exposure to chemical, physical and/or biological carcinogens present in the environment, to the lifestyle of a given individual or population, and to genetic and epigenetic factors (Parmigiani and Camargo, 2010; Vineis et al., 2010). Cancer is an epidemic disease that affects all regions, races and socioeconomic classes worldwide (Linsalata and Russo, 2008).

Colorectal cancer (CCR) is the second most important cause of cancer death in North America and Western Europe (Jemal et al., 2008) and it has a higher incidence in western countries (Linsalata and Russo, 2008). Diet is an important factor in their prevention (Block et al., 1992). In Brazil, according to INCA - 2018 (National Cancer Institute), the incidence varies according to the region evaluated, being higher in the south and southeast regions. Hepatocellular carcinoma (HCC) is the most common form of liver cancer, accounting for 85 to 90% of

*Corresponding author. E-mail: alineairesaguiar@hotmail.com.

all liver cancers (El-Serag and Rudolph, 2007). The HCC is associated to infection by the hepatitis virus and contact with carcinogens, such as aflatoxins (Idrees et al., 2009).

Plants have a long history of use in the treatment of cancer, serving as a source of therapeutic agents for many centuries and are used or served as the basis for the development of synthetic drugs (Cragg and Newman, 2005). Medicinal plants are traditionally used for the treatment of malignant neoplasms and have attracted considerable attention (Johnson et al., 2010). Because they exhibit pharmacological effects and may be potent chemotherapeutic agents (Hwang et al., 2007). Many studies have demonstrated that several secondary metabolites have antiproliferative activity, either by inducing apoptosis, or by their antioxidant capacity (Aguiar et al, 2017, Ezekiel et al., 2013; Feng et al., 2005). For example, polyphenols have been shown to inhibit cancer in various ways, acting as antioxidants, affecting signal transduction pathways, inducing apoptosis and inhibiting angiogenesis (Yang et al., 2009). Drugs such as vincristine and vinblastine, isolated from *Catharantus roseus* (Carvalhaes et al., 2002) are examples of plant metabolites used in chemotherapy. Currently, the chemistry of natural products is one of the main lines of research in the search for new anticancer agents (Silva et al., 2003). Sixty percent of antitumor drugs approved in the United States have a natural origin, and among these, many are secondary compounds of higher plant metabolism (Graham et al., 2000). The aim of the present study was to evaluate the *in vitro* antiproliferative activity of the *Amburana cearensis* seeds crude extract and fractions against three different cell lines: colorectal adenocarcinoma cells (HT-29), hepatocellular carcinoma cells (HepG2) and normal fibroblast cells (L929).

MATERIALS AND METHODS

This study was carried out in Natural Products Research Laboratory (LPPN) of Federal University of Tocantins (UFT), Palmas, Tocantins, Brazil and in Laboratory of Cancer Biology - Lutheran University of Brazil (ULBRA), Canoas, RS, Brazil.

Plant

The seeds of *A. cearensis* were obtained from popular commerce in the city of Palmas, Tocantins, Brazil, and their authenticity recognized by EMBRAPA Herbarium, voucher specimen (CPAP 5948). The seeds were dried in drying oven at 45°C and powdered using knives mill (Start FT 50 - Fortinox). The powder obtained was stored in glass bottles and kept at room temperature and sheltered from light and moisture (Al-Marby et al., 2016).

Seed extract preparation

To prepare the extracts, 15 g of pulverized seeds were extracted for 4 h using the Soxhlet apparatus with different solvents, *n*-hexane, methanol, ethanol 80% and water, from the least to the highest polarity according to the methodology of Soares et al. (2017). All solutions obtained had the solvents removed on a rotary evaporator at -600 mm Hg (Fisaton 804) and 45°C. They were then dried in the exhaust hood and stored in an amber bottle and kept at 4°C. The hexanic, methanolic, 80% ethanolic and water extracts were respectively named as, HEA, methanolic extract (MEA), EEA and AEA.

Phytochemical screening

The plant material was subjected to qualitative chemical screening for the identification of the major classes of active constituents. The phytochemical profile of *A. cearensis* seeds was determined according to methodology described by Matos (2009). The method consists in colorimetric reactions for qualitative detection of flavonoids, tannins, alkaloids, saponins and coumarins.

Cell lines used

The human colon adenocarcinoma cell line HT-29 was obtained from American Type Culture Collection (Rockville, MD, USA), and the mouse normal fibroblast cell line L929 and hepatocellular carcinoma cell line HepG2 were purchased from the RJCB Collection (Rio de Janeiro, RJ, Brazil).

Cytotoxic assay

Cell cultures were maintained in 25 cm^2 culture flasks with Dulbecco's Modified Eagle's medium culture medium (DMEM) containing 10% heat-inactivated fetal bovine serum (v/v) and antibiotic (1% penicillin/streptomycin) at 37°C in an atmosphere of 5% CO_2 and humidity of at least 95%. For the experiments, cell cultures were inoculated into 96-well microplates at a density of 5×10^4 cells/well/100 µL in triplicates. After stabilization for 24 h, the cultures were treated with serial concentrations (0 to 100 µg/ml) of the compounds. Cytotoxicity was assessed using the MTT colorimetric assay ((3- (4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide)). Cultures in triplicates were exposed for 24 h to the compounds. After the treatments, the cells were incubated with 100 µL of MTT solution (1 mg/mL MTT) in fetal bovine serum free culture medium without phenol-red at 37°C for 4 h. After incubation, the supernatant was carefully removed, and the violet formazan crystals were solubilized in 200 µl of Dimethyl sulfoxide (DMSO) and quantified in a microplate reader (Multiskan, UNISCIENCE) at 540 nm optical density. The 10% DMSO was used as positive control. Each experiment was performed 3 times independently.

For compounds that exhibited cytotoxicity according to ISO10993-5 guide (ISO, 2009) (that is, cell viability less than 70%), the IC_{50} value (amount of compound required to inhibit growth/induce death of 50% of cells) was determined. IC_{50} values of 20 to 30 µg/mL were considered by the National Cancer Institute (USA) to be of interest to crude extracts with anticancer potential (Suffness and Pezzuto, 1990).

The extracts concentration of 25 µg/mL was used as a parameter to evaluate the cytotoxic potential of the compound. The extracts concentration of 12.5 µg/ml (half the previous dose) was also analyzed in order to see if reducing the dose would also have cytotoxicity.

Gas chromatography-mass spectrometry (GC-MS) analysis

Qualitative analyses were performed through GC-MS using the

Table 1. Phytochemical profile of *A. cearensis* extracts.

Extract	Saponins	Alkaloids	Flavonoids	Coumarins	Tannins
CEA	+		+	+	
HEA			+	+	+
MEA		+	+	+	+
EEA			+	+	
AEA		+	+	+	+

+ indicates the presence of the compound.
Crude extract (CEA); hexanic extract (HEA), methanolic extract (MEA), 80% ethanol extract (EEA) and water extract (AEA).

Shimadzu GC-2010 model equipped with selective detector for the mass Model QP2010Plus, with the equipment operated under the following conditions: fused silica capillary column RTX-5MS (30 m × 0.25 mm × 0.25 µm film thickness), with the following schedule of temperature in the column: 60 to 240°C (3°C min-1), temperature of the injector 220°C, helium gas carrier, injection with rate of split (1:100) with injected volume of 1 µL of a solution 1:1000 in hexane. For the mass spectrometer (MS), the following conditions were used: impact energy of 70 V and temperature of the source of ions and the interface 200°C. A homologous series of *n*-alkanes (C_9H_{20} … $C_{26}H_{54}$) were injected under the same conditions as samples. The constituents were identified by comparing their spectra of masses with those from the databases from the Nist and Wiley 229 libraries and by comparing between their rates of retention calculated using those reported in the literature (Adams, 2007). The quantification of the levels of the compounds, expressed as a percentage based on the standardization of areas, was obtained by using a gaseous chromatograph equipped with a detector flame (DIC), using a diagnostic Shimadzu GC-2010, in the following experimental conditions: a capillary column RTX-5MS (30 m × 0.25 mm × 0.25 µm film thickness); temperature of the injector 220°C; temperature of the DIC 300°C; programing the column: initial temperature of 60°C with a heating rate of 3°C min^{-1} up to 240°C, then increasing to a heating rate of 10°C min^{-1} up to 300°C and remaining at this temperature for 10 min; nitrogen drag gas (1.18 mL min^{-1}); rate of split 1:50; pressure in the column of 115 kPa, and injected volume of 1 µL, diluted in hexane (1:100 v/v). The calculated retention index was performed according to Mühlen (2009).

Statistics

All the experiments were conducted in triplicates. The differences in means were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. $P \leq 0.05$ was taken to indicate statistical significance.

RESULTS AND DISCUSSION

Phytochemical analyses

Phytochemical analyses of *A. cearensis* seeds indicated the presence of saponins, alkaloids, flavonoids, coumarins and tannins (Table 1). Lopes (2003) have shown that secondary metabolites are frequently associated with cytotoxic potential that is associated with increased oxidative damage. This data was also reported by Rattmann et al. (2005) in *C. roseus*, which has alkaloids as vinblastine and vincristine, that are cytotoxic.

Cytotoxic assay

Evaluation of cell proliferation was performed using the MTT assay, where it is reduced to formazan purple crystals by living cell mitochondria. Increased numbers of cells are detected by increased metabolism of MTT, and decrease in the number of cells is reflected by decreased metabolism of the same (Seoane et al., 2010). *A. cearensis* seeds crude extract (CEA) and fractions (HEA, MEA, EEA and AEA) were assayed against three different cells: HT-29; HepG2 and L929. The cytotoxicity was measured and the IC_{50} values were determined. The HEA and AEA extracts showed cytotoxic action against L929 and HepG2 cells at concentration 25 µg mL^{-1} (Figure 1). Meanwhile, HEA, MEA and AEA extracts showed cytotoxic action against HT-29 cells at concentration 25 µg mL^{-1} (Figure 1). At concentration of 12.5 µg mL^{-1} the extract AEA showed cytotoxicity against L929 and HT-29 cells (Figure 2).

Toxic concentrations of the 3 extracts that reduce the viability of HT-29, HepG2 and L929 cells by 50% (IC50) were determined. The HEA, MEA and AEA have demonstrated to be active in the HT-29 cell line (IC_{50} 46.2 ± 0.2, 18.8 ± 0.4 and 41.7 ± 4.6 µg mL^{-1}, respectively) (Table 2). Moreover, the MEA demonstrates a great antiproliferative effect in this cell line.

National Cancer Institute (USA) considers extracts with IC_{50} values of 20 to 30 µg mL^{-1} good candidates to anticancer compounds (Suffness and Pezzuto, 1990). Based on this, the MEA extract has been shown to be the most promising extract of *A. cearensis* seeds. In addition, this extract has no cytotoxic action against L929 cells from mice, inferring that it is not cytotoxic against non-cancerous cells. To the best of our knowledge, this is the first study to examine the effect of *A. cearensis* seeds in cancer cell lines.

CG-MS analysis

Many compounds were identified in *A. cearensis* HEA,

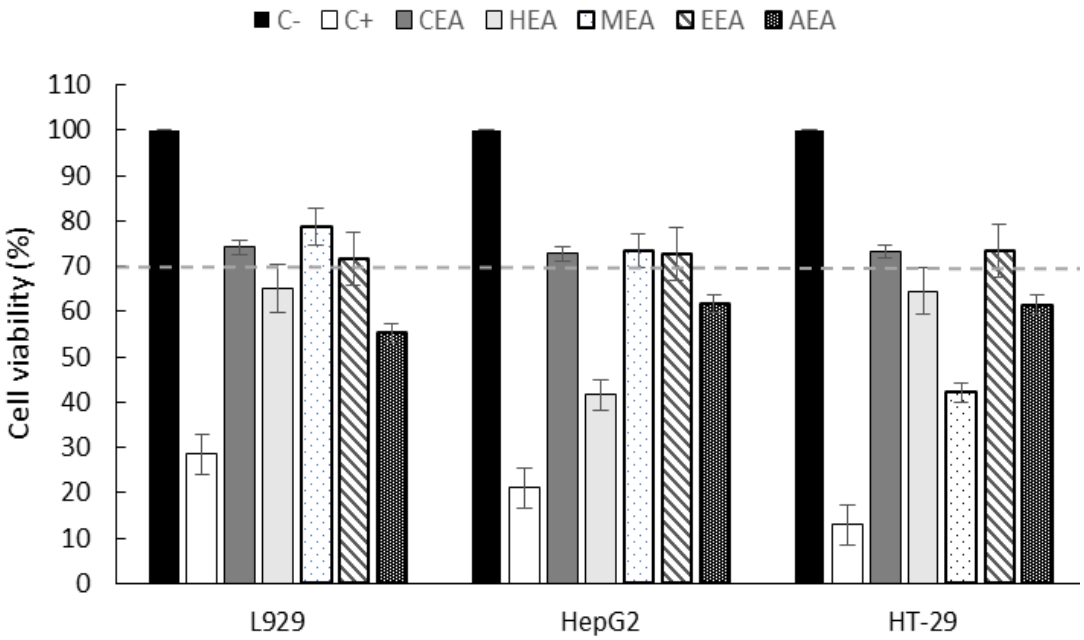

Figure 1. Cell viability expressed as a percentage (mean ± standard deviation), with the untreated control (C-) considered as 100% viability. Extract concentration (25 µg mL^{-1}); (C+) = positive control (10% DMSO).

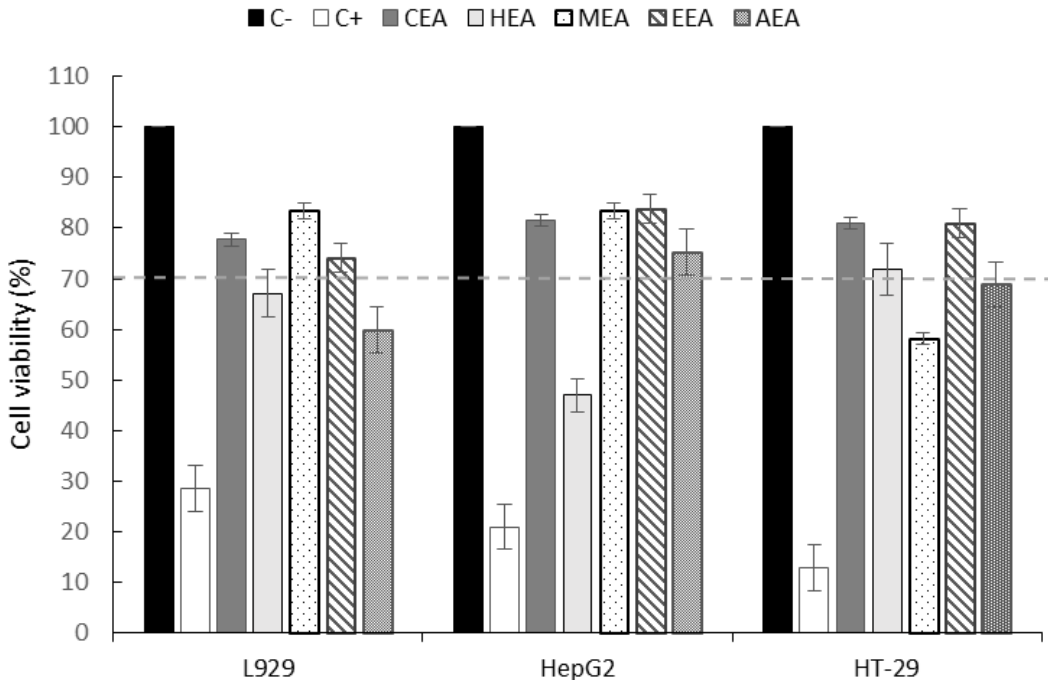

Figure 2. Cell viability expressed as a percentage (mean ± standard deviation), with the untreated control (C-) considered as 100% viability. Extract concentration (12.5 µg mL^{-1}); (C+) = positive control (10% DMSO).

MEA and AEA extracts (Table 3), such as 2,3-dihydro-benzofuran, catechol, hydrocoumarin, coumarin; the predominant molecule is coumarin with 14.41% of area for HEA extract (Figure 3, Table 4), 55.62% of area for MEA (Figure 5, Table 6) and 62.75% for AEA extract (Figure 4, Table 5). Recent data suggests that coumarin

Table 2. IC_{50} values of *A. cearensis* extracts (µg mL^{-1}: mean ± SD, *n* = 6) that presented viability less than 70%.

Extract	Cell lines (IC_{50})		
	HT-29	HepG2	L929
HEA	46.2 ± 0.2	87.4 ± 8.5	65.0 ± 6.1
MEA	18.8 ± 0.4	ND	ND
AEA	41.7 ± 4.6	44.2 ± 1.9	59.1 ± 3.7

Hexanic extract (HEA), methanolic extract (MEA) and water extract (AEA).
ND: Values could not be determined in the concentrations tested.

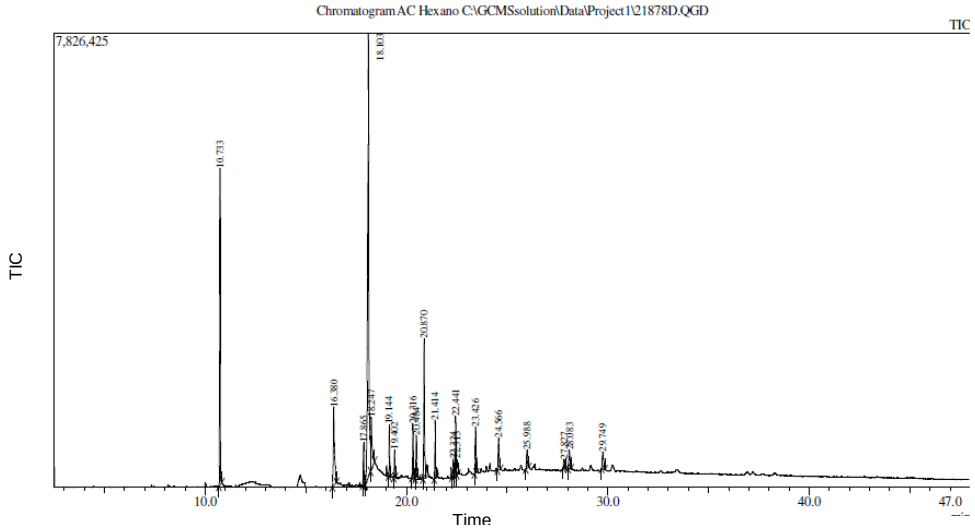

Figure 3. CG-MS of HEA extract of *A. cearensis* seeds.

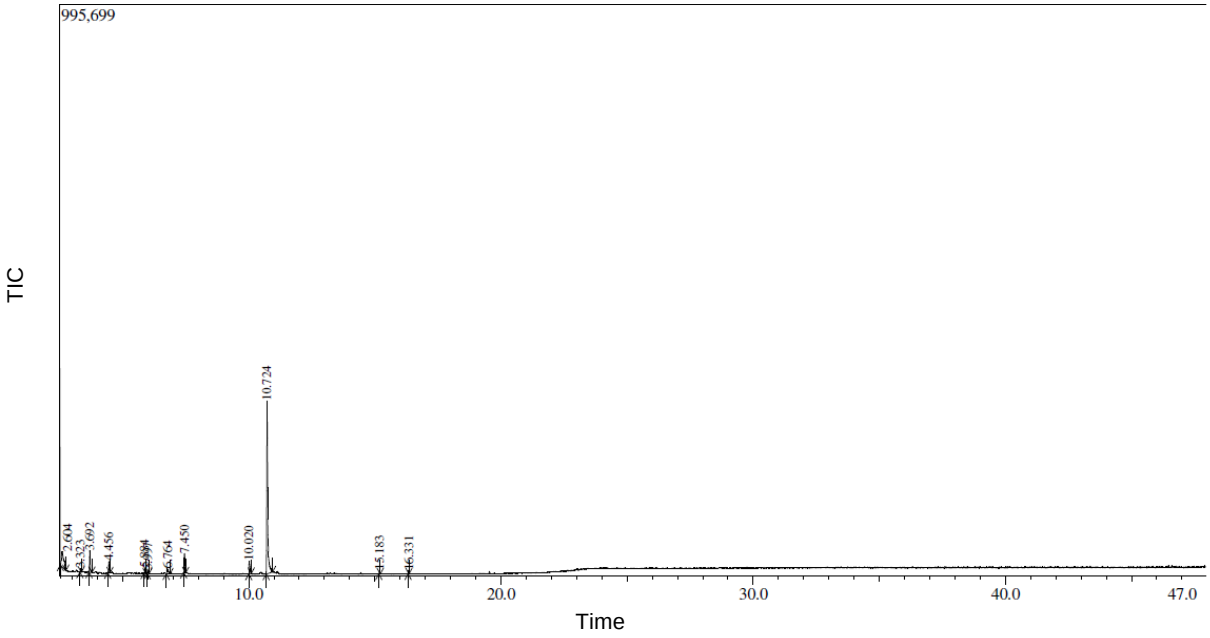

Figure 4. CG-MS of AEA extract of *A. cearensis* seeds.

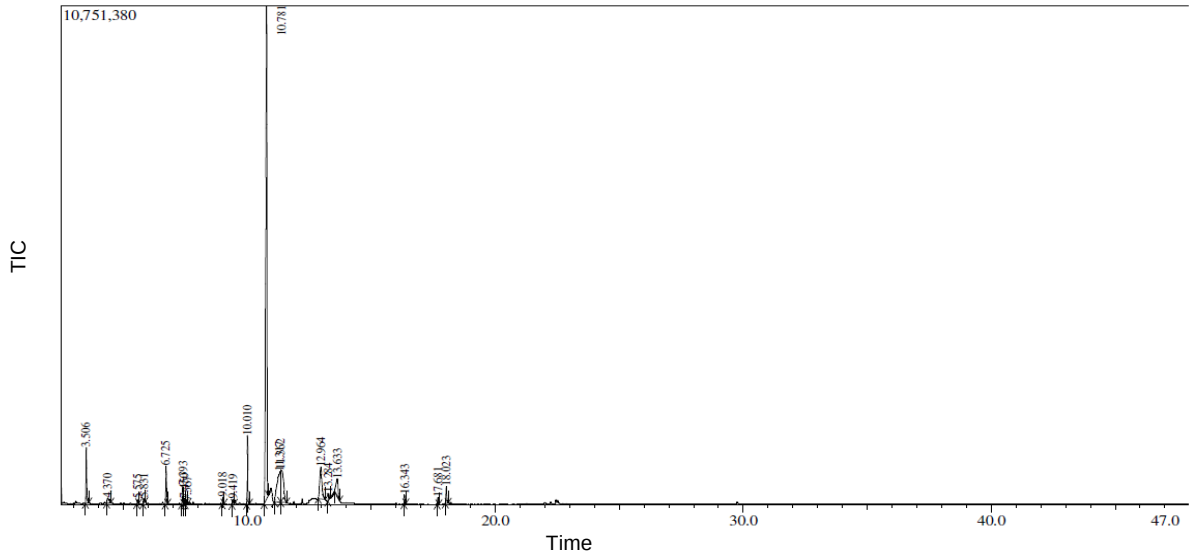

Figure 5. CG-MS of MEA extract of *A. cearensis* seeds.

Table 3. Chemical constituents of *A. cearensis* seed extracts in GC-MS analysis.

Compound	Extracts		
	HEA	MEA	AEA
l-(+)-Ascorbic acid 2,6-dihexadecanoate		+	
2,3-dihydro-Benzofuran	+	+	
1,6-anhydro-beta-D-Glucopyranose		+	+
Catechol		+	+
Coumarin	+	+	+
D-Allose		+	
Decanal		+	+
2-Hydroxy-gamma-butyrolactone		+	
Hydrocoumarin		+	+
Oleic Acid	+	+	
1,3,5-Triazine-2,4,6-triamine		+	

Hexanic extract (HEA), methanolic extract (MEA) and water extract (AEA).
+: Presence of the compound in extract.

may exert significant antineoplastic effects in several systemic malignancies and of clinical benefit in mammary malignancies (Kapoor, 2013). Coumarin also attenuates the extent of extremity lymphedema following breast cancer surgery and radiation therapy. In a study, almost 12.5% decline in the volume of upper extremity lymphedema was noticed. As a result, quality of life is significantly improved, and attenuation of tumor growth has been seen in pulmonary malignancies (Liu et al., 2012).

Choi et al. (2015) have demonstrated that the 2,3 dihydrobenzofuran molecule is cytotoxic for following cancer cell lines: HCT-15 (colon), NUGC-3 (gastric), and NCI-H23 (lung). In addition, this compound also inhibited NF-κB transcriptional activity. The GC-MS analysis has shown this compound in MEA extract (Table 3). Arulmurugan and Kavitha (2013) have shown that the molecule 1,3,5-Triazine-2,4,6-triamine has potent cytotoxicity against HT-29 and MCF-7 (breast cancer) cells. MEA extract has also this molecule, corroborating the cytotoxicity potential of this extract showed in this study. Purushothaman et al. (2013) demonstrated that *Hibiscus rosa-sinenis* extract is rich in 1,6-anhydro-beta-D-glucopyranose as in MEA extract, and in their study, it induces a significant reduction of tumor multiplicity and tumor volume in a defined experimental rat mammary carcinogenesis model mammary carcinoma in rats.

D-Allose, also present in MEA extract, is a rare

Table 4. Chemical composition of HEA extract of *A. cearensis*.

Peak #	Name	RT	Area (%)
1	Coumarin	10.733	14.41
2	*n*-Hexadecanoic acid	16.380	6.28
3	Cyclooctasiloxane, hexadecamethyl-	17.865	1.98
4	Oleic Acid	18.103	40.07
5	Octadecanoic acid	18.247	4.44
6	Cyclononasiloxane, octadecamethyl-	19.144	2.21
7	Glycidyl palmitate	19.402	1.06
8	Cyclodecasiloxane, eicosamethyl-	20.316	2.51
9	Oleoyl chloride	20.484	2.07
10	Glycidyl oleate	20.870	7.33
11	Tetracosamethyl-cyclododecasiloxane	21.414	2.63
12	Petroselinic acid, TMS derivative	22.324	0.84
13	Tetracosamethyl-cyclododecasiloxane	22.441	3.10
14	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	22.513	0.82
15	Tetracosamethyl-cyclododecasiloxane	23.426	2.45
16	Tetracosamethyl-cyclododecasiloxane	24.566	2.26
17	Tetracosamethyl-cyclododecasiloxane	25.988	1.47
18	Tetracosamethyl-cyclododecasiloxane	27.827	0.82
19	Heptacosanal	28.083	1.55
20	gamma.-Sitosterol	29.749	1.69

RT = Retention time.

Table 5. Chemical composition of AEA extract of *A. cearensis*.

Peak#	Name	RT	Area (%)
1	2-Hydroxyisocaproic acid, methyl ether, methyl ester	2.604	13.34
2	4-Ethylbenzoic acid, tridec-2-ynyl ester	3.323	0.69
3	2-Cyclopenten-1-one, 2-hydroxy-	3.692	5.46
4	Decane, 6-ethyl-2-methyl-	4.456	3.15
5	3-Pentanone, 2-methyl-	5.884	1.18
6	Oxalic acid, butyl propyl ester	5.997	0.61
7	N,N-Dibenzylethylenediamine diacetate	6.764	2.69
8	Decanal	7.450	4.05
9	1H-2-Benzopyran-1-one, 3,4-dihydro-	10.020	4.94
10	Coumarin	10.724	62.75
11	2,2'-Bifuran, 2,2',5,5'-tetrahydro-	15.183	0.49
12	Butanoic acid, 2-hydroxy-2-methyl-, methyl ester	16.331	0.65

RT = Retention time

monosaccharide known to exert anti-proliferative effects on cancer cells. The effects of D-Allose on the cellular membranes of hormone-refractory prostate cancer cell line (DU145), prostate cancer cell (LNCaP), and normal prostate epithelial cells (PrEC) were shown by Jeong et al. (2011).

Recently, the interest in phenolics has increased due to its protective role, through the ingestion of fruits and vegetables, against oxidative damage diseases such as coronary heart disease and cancer (Fernandes et al., 2010). In this line, it was demonstrated that the presence of phenolic compounds in different plants has antitumor effect (El-Hawary et al., 2012; Bhatt et al., 2013; Hasibuan et al., 2013).

The natural products are frequently associated to anticancer properties, inferring that bioactive compounds present in *A. cearensis* seeds extracts might be responsible for their cytotoxic activity against the cells

Table 6. Chemical composition of MEA extract of *A. cearensis*.

Peak#	Component	RT	Area (%)
1	1,2-Cyclopentanedione	3.506	3.50
2	2-Hydroxy-gamma-butyrolactone	4.370	0.82
3	1,3,5-Triazine-2,4,6-triamine	5.575	0.29
4	2-methoxy-Phenol	5.831	0.31
5	2,3-dihydro-Benzofuran	6.725	2.36
6	Catechol	7.393	1.49
7	Decanal	7.450	0.35
8	1,4:3,6-Dianhydro-alpha.-d-glucopyranose	7.567	3.89
9	1-(2-hydroxy-5-methylphenyl)-Ethanone	9.018	1.74
10	4-hydroxy-Benzenemethanol	9.419	2.58
11	Hydrocoumarin	10.010	4.29
12	Coumarin	10.781	55.62
13	D-Allose	11.317	9.20
14	1,6-anhydro-beta-D-Glucopyranose	11.362	7.78
15	Ethyl .alpha.-d-glucopyranoside	12.964	6.25
16	Silane	13.284	2.28
17	4-O-Methylmannose	13.633	3.31
18	l-(+)-Ascorbic acid 2,6-dihexadecanoate	16.343	0.54
19	6-Octadecenoic acid, methyl ester	17.681	0.39
20	Oleic Acid	18.023	1.22

RT = Retention time.

studied in this work.

Conclusion

This study was the first to investigate the antiproliferative properties of *A. cearensis* seeds on cancer cells. It was verified that the MEA extract has active principles with relevant antiproliferative potential. The results obtained are promising for a chemical and medicinal point of view, and stimulate the continuity of this study for the search of compounds with potential antiproliferative activity, with the aim to discover new and effective anticancer therapeutic agent.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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