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ARTICLES

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Full Length Research Paper

Genetic diversity of *Sorghum bicolor* (L.) Moench landraces from Northwestern Benin as revealed by microsatellite markers

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The understanding of genetic diversity within local crop varieties constitutes an important step in the preservation of their genetic potential. The objective of this study was to assess the genetic diversity of sorghum (*Sorghum bicolor* (L.) Moench) cultivated in the Northwest of Benin and to reveal certain fundamental evolutionary mechanisms. A total of 61 accessions of sorghum landraces belonging to the four identified races in Benin were estimated using 20 microsatellite markers. For all the loci analyzed, 140 polymorphic alleles were detected with a mean value of 7.00 per locus and polymorphic information content (PIC) average value was 0.33 for all the 20 simple sequence repeats (SSRs), suggesting an important genetic diversity within the cultivated sorghum germplasm used. An unweighted pair group method arithmetic average (UPGMA) clustering and principal coordinate analysis (PCoA) based on DICE coefficient revealed three major genetic groups supported by two main components: the botanical race and the morpho-physiological characteristics of the grains (colour and degree of bitterness). It was thus recommended that further research on genetic diversity of sorghum should integrate these genetic parameters for a better preservation of the genetic resources of this important crop in Benin.

Key words: Genetic diversity, simple sequence repeats (SSRs) markers, *Sorghum bicolor*, Benin.

INTRODUCTION

Knowledge of the level of genetic diversity and structure in crop plants constitutes a very important aspect in selection, conservation and/or genetic improvement programmes. It is necessary for the development of sustainable preservation programmes of plant genetic resources (Adoukonou-Sagbadja et al., 2007).

Consequently, these last decades, an important basic activity in the management of genetic resources has been the assessment of genetic diversity and its structure within cultivated plants (Brown, 1989; Bhosale et al., 2011). For instance, these works largely focused on economically important cereals such as maize (*Zea mays*

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L.) (Perales et al., 2003; Bracco et al., 2009), rice (*Oryza sativa* L.) (Barry et al., 2007; Wang et al., 2014), wheat (*Triticum aestivum* L.) (Reif et al., 2005), barley (*Hordeum vulgare* L.) and sorghum (*Sorghum* spp.) (Bhosale et al., 2011).

Sorghum (*Sorghum bicolor* (L.) Moench) is one of the most important staple cereal crops in the semi-arid regions around the world. In sub-Saharan Africa, it is the second most important cereal crop after maize. *S. bicolor* is a monocotyledon plant of tropical origin, belonging to *Poaceae* family and the genus of *Sorghum* which includes 20 to 30 species. It is an annual plant, preferentially autogamous (Ollitrault et al., 1997), diploid (2n = 20 chromosomes) (Harlan and de Wet, 1972). Using panicle, grain and spikelet morphology, Harlan and de Wet (1972) classified cultivated sorghum (*Sorghum bicolor* ssp. *bicolor*) into five basic races (*bicolor*, *caudatum*, *durra*, *kafir* and *guinea*) and 10 intermediate ones. In many African countries, the crop is dominated by sorghum landraces which were traditionally selected and maintained by indigenous farmers. In most producing countries including Benin, the great majority of the production is consumed locally. This is in accord with the strategic role of sorghum for local household in order to ensure food security (Kayode et al., 2006).

The genetic diversity of sorghum at global scale has been studied extensively by using different markers including nuclear DNA markers. Moreover, relatively few studies have been specifically conducted on genetic variation in some Beninese sorghum. For instance, amplified fragment length polymorphism (AFLP) markers were used on genetic variation of Beninese sorghum landraces (Kayode et al., 2006). This study showed that the genetic diversity of local varieties of the three studied regions was similar and no correlations could be established between the genetic features and the culinary characteristics of varieties. However, the factors shaping the genetic diversity of varieties (ecogeographic distribution, racial membership, farmers' agromorphological and organoleptic preferences, ethnic and other social factors, etc.) were not considered in the former investigations. But, the understanding of these factors is an important asset for the management, selection and genetic improvement of this crop. What is the state of the genetic diversity estimated with the molecular genetics tools (simple sequence repeats (SSRs) markers) of the sorghum landraces in Benin? What are the major factors which structure this diversity?

Microsatellite markers have been developed and largely used in sorghum during more than a decade (Brown et al., 1996; Taramino et al., 1997; Schloss et al., 2002; Barnaud et al., 2007; Barro-Kondombo et al., 2010). They are multi-allelic, codominantly inherited, locus specific, easily detected by polymerase chain reaction (PCR) and require only a small amount of starting DNA (Powell et al., 1996). In this study, microsatellite polymorphism was determined to estimate the genetic diversity in farmers' varieties or ''sorghum landraces'' grown in the north-west of Benin. Specifically, this work (1) assessed genetic diversity in collection using SSR markers and (2) determined the major factors which structure this diversity.

MATERIALS AND METHODS

A survey was conducted in Donga department (9°10'0" N and 1°49'60" E) (Figure 1) for the collection of plant materials in December, 2010 (Missihoun et al., 2012a). A total of 61 accessions of sorghum landraces (Table 1) was collected and then subjected to molecular analysis. The study was carried out at the Department of Genetics and Biotechnologies, University of Abomey-Calavi in Benin.

Genomic DNA extraction and SSRs genotyping

Young fresh leaves from bulked plant samples of each accession were harvested in the greenhouse and then brought to the laboratory for genotyping. DNA was extracted using the mixed alkyl tri-methylammonium bromide (MATAB) extraction protocol previously described for sorghum (Missihoun et al., 2012b). 0.2 g of leaf material was ground in a porcelain mortar with 2 mL of Tris-Sorbitol EDTA buffer. The mixture in Eppendorf tubes was centrifuged at 10.000 rpm for 10 min at 4°C. After centrifugation, the supernatant was taken out and discarded. 750 µL of 4% MATAB buffer preheated at 65°C were added to the pellet in the Eppendorf and placed in water bath at 65°C for 1 h 30 min while stirring up at each 10 min. Then, 750 µL of chloroform/Isoamyl alcohol (24:1) were added to the mixture cooled at ambient temperature and shaked by gently inverting for 2 min and then centrifuged at 10.000 rpm for 15 min at 4°C. The supernatant was taken out in a separate tube and then an equal volume of iso-propanol was added to it. The tube was carefully mixed to obtain DNA pellet. After centrifugation the supernatant was carefully discarded and the pellet was purified with 70% ethanol. The pellet was dried then sterile ultra-pure water was added to each tube containing the pellet.

To be sure of the success of the DNA extraction, 2 µL of genomic DNA extract were visualized on a 1% agarose gel. After this confirmation, DNA samples were kept in a freezer at -20°C for the genotyping.

Genomic sorghum SSR markers used in this study belonged to the linkage maps of sorghum published by Taramino et al. (1997), Bhattramakki et al. (2000) and Kong et al. (2000) which were recently used by Shehzad et al. (2009), Barro-Kondombo et al. (2010) and Bhosale et al. (2011). Thirty six (36) SSR primers were initially tested on four sorghum accessions and finally, 20 genomic sorghum SSR markers were selected based on their high polymorphic information content (PIC) value observed on the previous studies on African sorghum Landraces. These SSR markers are distributed across the sorghum genome with an average of two (2) SSRs per chromosome (Table 2).

Polymerase chain reaction (PCR) amplifications using the 20 selected SSRs were carried out in a Peltier-Effect Cycling's thermocycler in a volume of 25 µL containing 3 µL of genomic DNA (approximately 3 ng/uL), 0.2 µM of each primer (F and R), 2.5 µl of PCR buffer (10X), 200 µM dNTP, 1.25 µM $MgCl_2$, 0.1 U/uL Taq DNA-polymerase and sterile ultra-pure water (H_2O). The cycle of amplification included a pre-denaturation at 94°C for 4 min followed by 35 cycles, each cycle consisted of a denaturation at 94°C for 30 s, hybridization in the appropriate temperature (50°C or 60°C, Table 2) for 1 min and an elongation at 72°C for 1 min. A final incubation
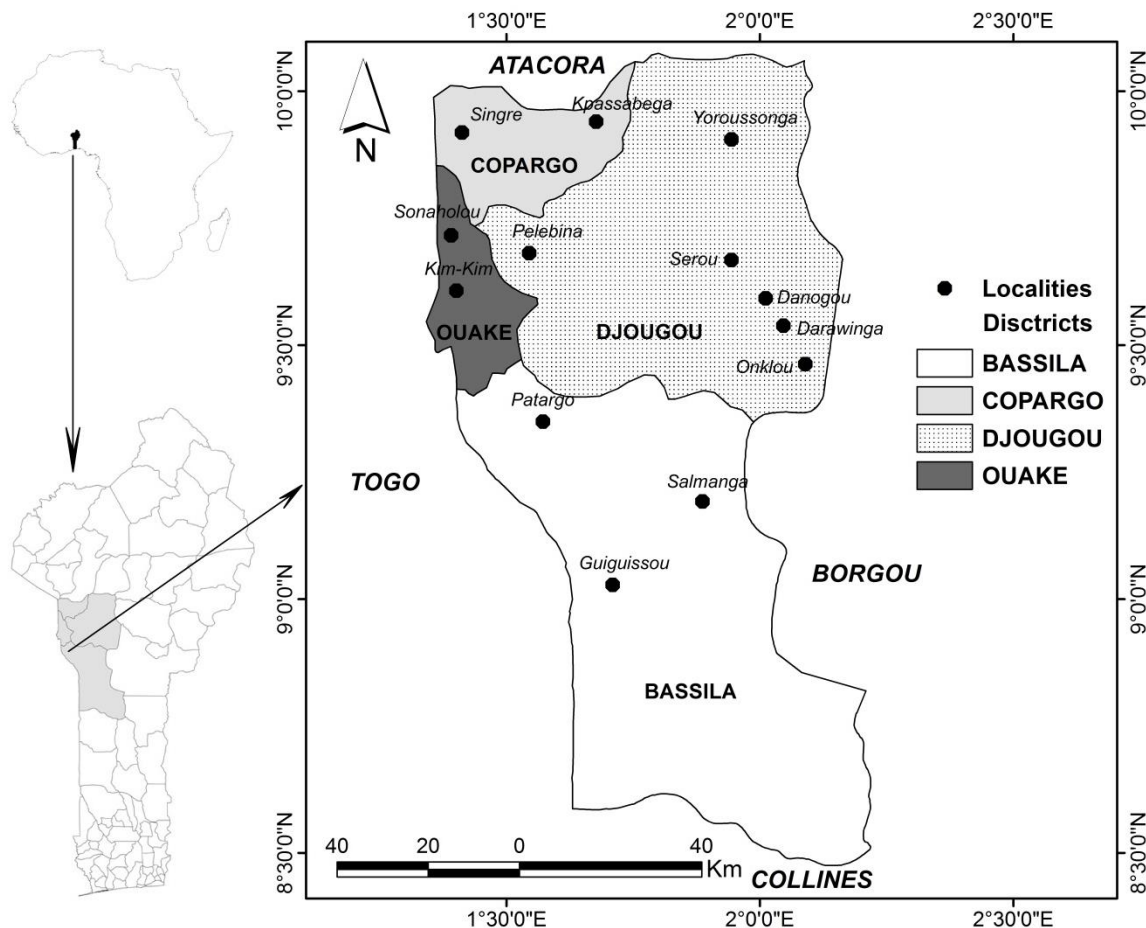
Figure 1. Maps showing the geographical locations of thirteen selected and surveyed villages.

at 72°C for 8 min ended the program. The effectiveness of the amplification was tested by electrophoresis on 2% agarose gel in 0.5X TBE buffer. Gels were run in horizontal gel system at 100 V for 30 min and later photographed under UV light. Afterwards, PCR amplification products were migrated by electrophoresis in 5% denaturing polyacrylamide gel of 305 × 385 mm (5% acrylamide-bisacrylamide (19:1), 8 mol urea in Tris-borate-EDTA/L (TBE) buffer, pH 8) at constant power of 60 W for 1 h 30 min to 2 h, depending on the expected product size. The detection of electrophoretic plates was carried out with silver nitrate according to Creste et al. (2001).

Data analysis

Fifty nine (59) accessions were considered for final analysis as 2 samples were eliminated for lack of amplification. NTSYS pc software 2.20q (Rohlf, 2000) was used for data analysis. Similarity coefficient of DICE based on the proportion of common alleles (Nei and Li, 1979) was calculated to measure genetic similarities between accessions. The diversity of each accession was analyzed based on three genetic diversity parameters: the rate of polymorphism (P), number of alleles per locus (Na) and polymorphism information content (PIC) which provided an estimation of the discriminatory power of a locus by taking into account not only the amount of alleles expressed but also the relative frequency of each allele (Botstein et al., 1980; Smith et al. 2000). The values of PIC were

calculated according to the algorithm:

$$PIC = 1 - \Sigma f_i^2$$

Where, f_i was the frequency of the ith allele; PIC value ranged from 0 (monomorphic locus) to 1 (very highly discriminative, with many alleles of which each was in equal and low frequency).

Genetic structure was assessed by a dendrogram construction using unweighted pair group method arithmetic average (UPGMA) method following the Sequential agglomerative hierarchical nested method (SAHN) procedure of NTSYS software version 2.21f (Rohlf, 2000). Besides, to confirm the inferred groupings of the analyzed accessions and to better estimate the genetic differentiation between the groups, DCENTER and EIGEN procedures of this software were also used to conduct a principal coordinate analysis (PCoA) on the basis of the same matrix of genetic distances.

RESULTS

Genetic polymorphism and allele's distribution

The analysis of SSRs revealed a high allelic polymorphism. As a matter of fact, 100% of the loci investigated were polymorphic and each of them exhibited at least two alleles. Overall, the twenty SSR markers used in this study

Table 1. Code of the accession, vernacular name, site location, racial group, grain type and main characteristics to make distinction.

Code of the accession	Vernacular name	Location/Village	District	Racial group	Grain type Colour	Grain type Nature	Main characteristics to make distinction
1a	Zokaram nini (Da) 1	Danogou	Djougou	G	Reddish	No bitter	High yield of grain, intermediate-maturing
3a	Zobomdjouha (Da) 1	Danogou	Djougou	G	white	No bitter	High yield of grain and easy to shell
4a	Zomoaha (Se) 2	Serou	Djougou	G	Red	No bitter	Early-maturing
5a	Agbani (On)	Onklou	Djougou	G	White	No bitter	Very high yield of grain but late-maturing
6a	Kèmnin piha	Kpassabega	Copargo	G	White	No bitter	High yield of grain
7a	Zomoaha (Se) 1	Serou	Djougou	G	Red	No bitter	Early-maturing
8a	Narabeunzo	Danogou	Djougou	B	blackish	No bitter	To be associated with''Wild Sorghum''
9a	Agbani (Yo)	Youroussonga	Djougou	G	White	No bitter	Very high yield of grain but late-maturing
10a	Zoténtérém (Se)	Serou	Djougou	DK	Red	No bitter	Cultivated only for his red leaves which have used for traditional medicine
12a	Zomoora	Youroussonga	Djougou	D	Yellow	Bitter	Bitter yellow grain
13a	M'ssée	Youroussonga	Djougou	C	Yellowish	No bitter	Twin grain
14a	Zobomdjouha (Se)	Serou	Djougou	G	White	No bitter	High yield of grain
15a	Zokpénaï	Serou	Djougou	D	Yellowish	No bitter	Very early-maturing (two harvest by year) but cause itching during of harvest
16a	Zoniniléti	Serou	Djougou	G	Whitish	No bitter	High yield of grain
17a	Lamnéza	Kpassabega	Copargo	G	Reddish/ Whitish	No bitter	High yield of grain but late-maturing
18a	Zobomdjouha (Yo)	Youroussonga	Djougou	G	white	No bitter	High yield of grain and easy to shell
20a	Vèmah (Yo) 1	Youroussonga	Djougou	DG	White	No bitter	Very high yield of grain but poor quality of food
22a	Moussii	Pélébina	Djougou	D	Yellowish	No bitter	Early-maturing
23a	Zoumbouara	Onklou	Djougou	G	Red	No bitter	Pure red grain, Very early-maturing
24a	Zoumboua	Onklou	Djougou	G	Red	No bitter	Early-maturing
25a	Zooga	Pélébina	Djougou	G	White/Red	No bitter	High yield of grain but late-maturing
26a	Moukoulikouté	Sonaholou	Ouaké	C	Reddish/ Yellowish	No bitter	Low yield of grain, require a wet ground of sandbank, late-maturing
27a	Koussèm (So)	Sonaholou	Ouaké	G	Red	No bitter	Very early-maturing
29a	Koussèm (Da)	Darawinga	Djougou	G	Red	No bitter	Very early-maturing
30a	Moussema (So)	Sonaholou	Ouaké	G	Red	No bitter	Early-maturing
31a	Zobomdjouha (Da) 2	Danogou	Djougou	G	White/Red	No bitter	High yield of grain
32a	Kouhloumè (So)	Sonaholou	Ouaké	G	White	No bitter	Early-maturing
33a	Zomoaha (Yo) 1	Youroussonga	Djougou	G	Red	No bitter	Early-maturing
34a	Kouhloumè (K-K)	Kim-Kim	Ouaké	G	White	No bitter	Early-maturing
35a	Talèm'la (So)	Sonaholou	Ouaké	G	Whitish	No bitter	Very high yield of grain but late-maturing
36a	Zogawa	Danogou	Djougou	G	Whitish	No bitter	High yield of grain, intermediate-maturing

Table 1. Contd

37a	Zoténtérém (Da)	Danogou	Djougou	DK	Red	No bitter	Cultivated only for his red leaves which have used for traditional medicine
38a	Agbani (Da)	Danogou	Djougou	G	Whitish	No bitter	Very high yield of grain but late-maturing
39a	Talèm'la (K-K)	Kim-Kim	Ouaké	G	White	No bitter	Very high yield of grain but late-maturing
40a	Zopira B	Danogou	Djougou	G	White	No bitter	High yield of grain but late-maturing
41a	Zomouara	Danogou	Djougou	G	Red	No bitter	Early-maturing
42a	Zopéra	Pélébina	Djougou	G	White	No bitter	High yield of grain but late-maturing
43a	Zomoora	Pélébina	Djougou	D	Yellow	Bitter	Bitter yellow grain
44a	Koulom	Darawinga	Djougou	G	White	No bitter	High yield of grain but late-maturing
45a	Agbani (Se)	Serou	Djougou	G	White	No bitter	High yield of grain but late-maturing
46a	Sèmoutchè	Sonaholou	Ouaké	G	White	No bitter	Pure white grain, very loose panicle, cultural
47a	Zotihou	Danogou	Djougou	D	Yellow	Bitter	Bitter yellow grain
48a	Moussèma (Da)	Darawinga	Djougou	G	Red	No bitter	Early-maturing
49a	Zokaram nini (Da) 2	Danogou	Djougou	G	Reddish	No bitter	High yield of grain
51a	Zopiha 2	Kpassabega	Copargo	G	White	No bitter	High yield of grain but late-maturing
52a	Lam'za moaha	Kpassabega	Copargo	G	Whitish	No bitter	Medium yield of grain, intermediate-maturing
53a	Sèmgnin moaha	Kpassabega	Copargo	G	Whitish	No bitter	Medium yield of grain, intermediate-maturing
54a	Sèmgnin piha	Kpassabega	Copargo	G	Whitish	No bitter	High yield of grain, intermediate-maturing
55a	Zowémoaha 1A	Kpassabega	Copargo	G	Red	No bitter	Early-maturing
56a	Zowémoaha 1B	Kpassabega	Copargo	G	Red	No bitter	Early-maturing
57a	Zowémoaha 2B	Kpassabega	Copargo	G	Red	No bitter	Early-maturing
58a	Zopiha 1	Kpassabega	Copargo	G	White	No bitter	High yield of grain, late-maturing, demanding fertile soil and good pluviometry
59a	Zomoala	Kpassabega	Copargo	D	Yellow	No bitter	Bitter yellow grain
60a	Za (Si) 1	Singre	Copargo	G	Whitish	No bitter	High yield of grain, late-maturing, demanding fertile soil and good pluviometry
61a	Lamza	Kpassabega	Copargo	G	Whitish	No bitter	Medium yield of grain, intermediate-maturing
62a	Zomoaha	Singre	Copargo	G	Red	No bitter	Early-maturing
63a	Za (Si) 2	Singre	Copargo	G	Whitish	No bitter	High yield of grain, late-maturing, demanding fertile soil and good pluviometry
64a	Zomoila	Singre	Copargo	D	Yellow	Bitter	Bitter yellow grain
67a	Zowémoaha 2A	Kpassabega	Copargo	G	Red	No bitter	Early-maturing
69a	Aka foufou kékéré	Patargo	Bassila	G	White	No bitter	Medium yield of grain, intermediate-maturing
71a	Anoro kin'ka	Salmanga	Bassila	G	Reddish	No bitter	Medium yield of grain, intermediate-maturing

yielded 140 alleles which allowed the classification of all the 59 accessions of sorghum collected from different villages. The number of alleles per locus range from 2 to 14 with an average of seven alleles. The most polymorphic markers were Xtxp 295 and Xtxp 274 (14 alleles) and the least polymorphic ones were Xtxp 59, Xtxp 60 and Xtxp 65 (two alleles). The discriminant power of each SSR markers assessed on this study by the PIC

Table 2. List of SSRs used with their chromosome location, primer sequences (forward and reverse), repeat motif type, number of alleles recorded per locus and PIC values.

SSR locus and (chromosome location)	Forward (F) and reverse (R) primer sequences (5' to 3')	Type of SSRs	T°a	Na	PIC
Xtxp 149 (1)	F = AGCCTTGCATGATGTTCC R = GCTATGCTTGGTGTGGG	$(CT)_{10}$	60	12	0.34
Xtxp 284 (1)	F = CCAGATTGGCTGATGCATACACACT R =AAGGGTAATTTATGCACTCCAAGGTAGGAC	$(AAG)_{19}$	60	10	0.51
Xtxp 201 (2)	F = GCGTTTATGGAAGCAAAAT R = CTCATAAGGCAGGACCAAC	$(GA)_{36}$	60	5	0.08
Xtxp 197 (2)	F = GCGTCAATTAATCCAAACAGCCTC; R = GAGTTCCTATTCCCGTTCATGGTGAT	$(AC)_{10}$	60	4	0.45
Cba (3)	F = AAAGCTCGGCGTTAGAAATA; R = CGCTTAACAACTCCTACCATC	$(TA)_{18}$	60	3	0.25
Xtxp 59 (3)	F = GAAATCCACGATAGGGTAAGG; R = GACCCAGAATAGAAGAGAGG	$(GGA)_5$	60	2	0.47
Xtxp 51 (4)	F = TCTCGGACTCAAGAGCAGAGG;R = GGACAGCAGCGGCTTCAG	$(TG)_{11}$	60	6	0.25
Xtxp 60 (4)	F = GCTAGCTGACGCACGTCTCTG; R = TGCAACCGAGCGGTGACTA	$(GT)_4$ GC$(GT)_5$	60	2	0.45
Kaf2 (5)	F = TCGGCGAGCATCTTACA; R = TACGTAGGCGGTTGGATT	$(CAA)_9$	60	5	0.26
Xtxp 65 (5)	F = CACGTCGTCACCAACCAA; R = GTTAAACGAAAGGGAAATGGC	$(ACC)_4+$ $(CCA)_3$ CG$(CT)_8$	60	2	0.29
Xtxp 145 (6)	F = GTTCCTCCTGCCATTACT R = CTTCCGCACATCCAC	$(AG)_{22}$	60	13	0.14
Xtxp 274 (6)	F = GAAATTACAATGCTACCCCTAAAAGT R = ACTCTACTCCTTCCGTCCACAT	$(TTC)_{19}$	60	14	0.51
Xtxp 278 (7)	F = GGGTTTCAACTCTAGCCTACCGAACTTCCT; R = ATGCCTCATCATGGTTCGTTTTGCTT	$(TTG)_{12}$	60	5	0.21
Xtxp 295 (7)	F = AAATCATGCATCCATGTTCGTCTTC; R = CTCCCGCTACAAGAGTACATTCATAGCTTA	$(TC)_{19}$	60	14	0.21
Xtxp 47 (8)	F = CAATGGCTTGCACATGTCCTA; R = GGTGCGAGCTAGTTAAGTGGG	$(GT)_8(GC)_5 + (GT)_6$	60	3	0.19
Xtxp 273 (8)	F = GTACCCATTTAAATTGTTTGCAGTAG R = CAGAGGAGGAGGAAGAGAAGG		60	8	0.30
Xtxp 258 (9)	F = CACCAAGTGTCGCGAACTGAA; R = GCTTAGTGTGAGCGCTGACCAG	$(AAC)_{19}$	60	8	0.43
Xtxp 10 (9)	F = ATACTATCAAGAGGGGAGC R = AGTACTAGCCACACGTCAC	$(CT)_{14}$	50	4	0.23
Xtxp 217 (10)	F = GGCCTCGACTACGGAGTT R = TCGGCATATTGATTTGGTTT	$(GA)_{23}$	60	8	0.41
Xtxp 270 (10)	F = AGCAAGAAGAAGGCAAGAAGAAGG; R =GCGAAATTATTTTGAAATGGAGTTGA	$(GAA)_{12}(GAAA)_6 + (GAA)_{21}$ $+ (GTA)_5 + (GTA)_3 + (GTA)_3$	60	12	0.65

SSRs polymorphism rate: 100%; Amount of alleles recorded in the population: 140 alleles; mean alleles/locus = 7.00.

value ranged from 0.08 to 0.65 with a mean value of 0.33 for all the 20 SSRs analyzed. Among the markers, Xtxp 270 was the most discriminant whereas Xtxp 201 and Xtxp 145 were poorly discriminant.

Among the alleles recorded in this study, 24 (17.14%) were "rare" (their frequency of occurrence range between 0.01 and 0.05), 50 (35.71%) had average frequencies ranging from 0.06 to 0.19; 41 (29.29%) were frequent(occurrence frequencies ranging from 0.20 to 0.50) and finally 25 (17.86%) were highly frequent with their occurrence frequencies ranging from 0.51 to 0.98 (Figure 2). With regards to their geographic distribution,
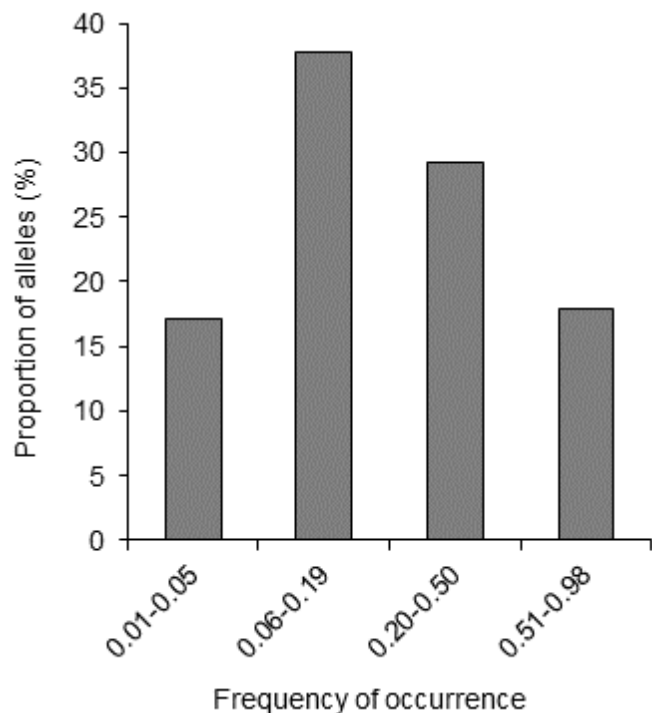
Figure 2. Amount of alleles SSRs in connection with their occurrence frequencies in the analyzed accession of sorghum.

97.17% of identified alleles in this study were present in the samples collected in Yom tribal area from Djougou district, 75.47% were present in the accessions sampled in Yom tribal from Copargo district and only 51.87% alleles were present in the accessions sampled in Lokpa tribal area living in Ouaké and Djougou districts.

Genetic relationship and samples' structure

Genetic distance estimated by dice genetic similarity (GS_D) for all accessions under investigation varied from 0.00 [landraces zooga (25a) vs. zogawa (36a)] to 0.93 [landraces Zokaram nini (1a) vs. Zoténtérém (37a)] with an overall mean of 0.47.

Genetic relationships between the different accessions of sorghum were analyzed by means of dendrogram using 140 alleles obtained from 20 SSR markers. This dendrogram obtained according to UPGMA hierarchical classification model using DICE coefficient allowed the classification of sorghum accessions into three main groups (Figure 3). Group I was subdivided into two sub-groups (Ia and Ib) containing sorghum accessions from guinea race and an accession from bicolor race. In terms of grain coloration, the sub-group Ia mainly consisted of accessions with red grains and subsidiary a few white grains accessions. In this sub-group, zooga (25a) and zogawa (36a), collected respectively at Pélébina and Danogou, two villages geographically distant in Djougou

district were shown to be genetically identical, suggesting that the two accessions belong probably to the same variety although they were called differently by farmers. On the contrary, two accessions of same variety "Zowémoha" (55a and 57a) were genetically identical, attesting their good identity as indicated by farmers. The sub-group Ib was mainly accessions with white grains and few red grains. The group II was made of accessions of durra race (12a, 47a, 59a, 64a, 15a and 22a) and one accession of *caudatum* race (13a). On the basis of grain bitterness and coloration, we could distinguish two sub-groups: the first consisted of sorghum accessions with yellow and bitter grains (12a, 47a, 59a and 64a) and sorghum with yellowish and twin grains (13a) and the second was composed of accessions with yellowish and no bitter grains. Finally, the group III contained two accessions of intermediate *durra-kafir* race (10a and 37a). These were staining sorghum landrace. The samples analyzed in this study seemed to be very well structured according to botanical race and morpho-physiological characteristics of the grains such as colour and degree of bitterness.

This classification of accessions into three major groups was confirmed by PCoA. The two major axes accounted for 24% of the total variation with axis 1 representing 13.99% and axis 2 accounting for 10.01% of the variation (Figure 4). The two sub-groups (Ia and Ib) in group I obtained from the genetic analysis correspond, with only few exceptions of the three morphotypes primarily identified in guinea race accessions in the same collection during the agro-morphological characterization (Missihoun, 2013).

The analysis of accessions from genetic groups revealed 34 accessions of group Ia collected from Yom farmers in Djougou and Copargo districts and 11 accessions collected from Lokpa farmers belonged to the group Ib. Eight accessions of group II (IIa and IIb) were collected from Yom farmers in Djougou and Copargo districts, whereas the 2 accessions of group III were collected from Yom in Djougou (Table 3).

DISCUSSION

Before the advent of GBS-based single nucleotide polymorphism (SNP) markers and additionally other whole-genome profiling markers (for example DArT), microsatellite markers are the most used worldwide in the characterization of sorghum genetic resources and particularly in genetic diversity analysis (Smith et al., 2000; Folkertsma et al., 2005; Barnaud et al., 2007; Deu et al., 2008; Barro-Kondombo et al., 2010; Bhosale et al., 2011). In Benin, although AFLP markers were used in genetic characterization of sorghum local varieties (Kayodé et al., 2006), it is the first time SSR markers were used for sorghum germplasm analysis. Microsatellite markers when compared with AFLP markers, they are specific, codominant and multi-allelic and well known to allow a good discrimination of closely related sorghum
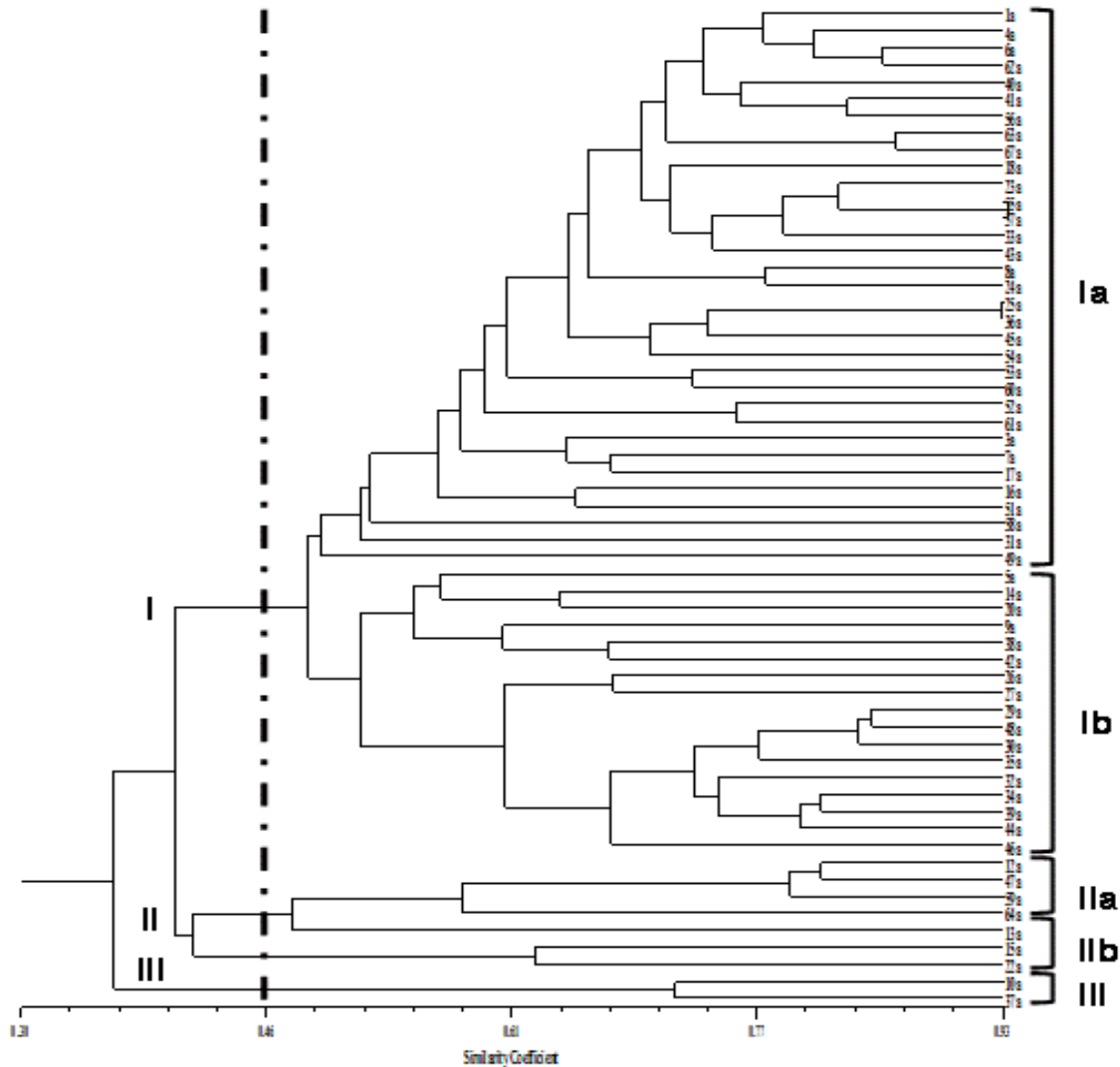
Figure 3. Dendrogram showing genetic connection between sorghum accessions by UPGMA analysis using DICE coefficient on SSRs data.

accessions (Djè et al., 1999; Smith et al., 2000; Ghebru et al., 2002).

Overall genetic diversity

The maintenance of crop genetic resources for evaluation and use in breeding programmes is crucial for the improvement of agricultural production (Sow et al., 2013). In this study, there was genetic diversity as revealed by different microsatellite markers. The average number of alleles recorded per locus (7.00 in average) in the present study was higher than that recorded by Agrama and Tuinstra (2003), Kudadjie (2006), Barro-Kondombo et al. (2010) which were 4.5 , 3.7 and 4.9 respectively on *Sorghum* ssp. and with the SSRs markers. Nonetheless, our results are similar to that of Sagnard et al. (2011)

(7.48 alleles in average per locus) who worked on 455 sorghum accessions from Mali and Guinea using 15 SSR markers. However, the average number calculated in the present study was lower than that reported by Deu et al. (2008) and Bhosale et al. (2011), which were 10.43 and 19, respectively from 472 accessions of sorghum from Niger and 219 accessions of sorghum cultivated in West Africa and assessed by means of 28 and 27 SSR markers, respectively.

The comparison of the level of allelic diversity per locus from various studies seems different but there were reasons that could explain the discrepancies observed as compared to the present study. First, the size of the population used could be one of the reasons. The number of accessions (59) was higher than that of Agrama and Tuinstra (2003) (22 accessions), Kudadjie (2006) (42 accessions) but lower than that of Deu et al. (2008)
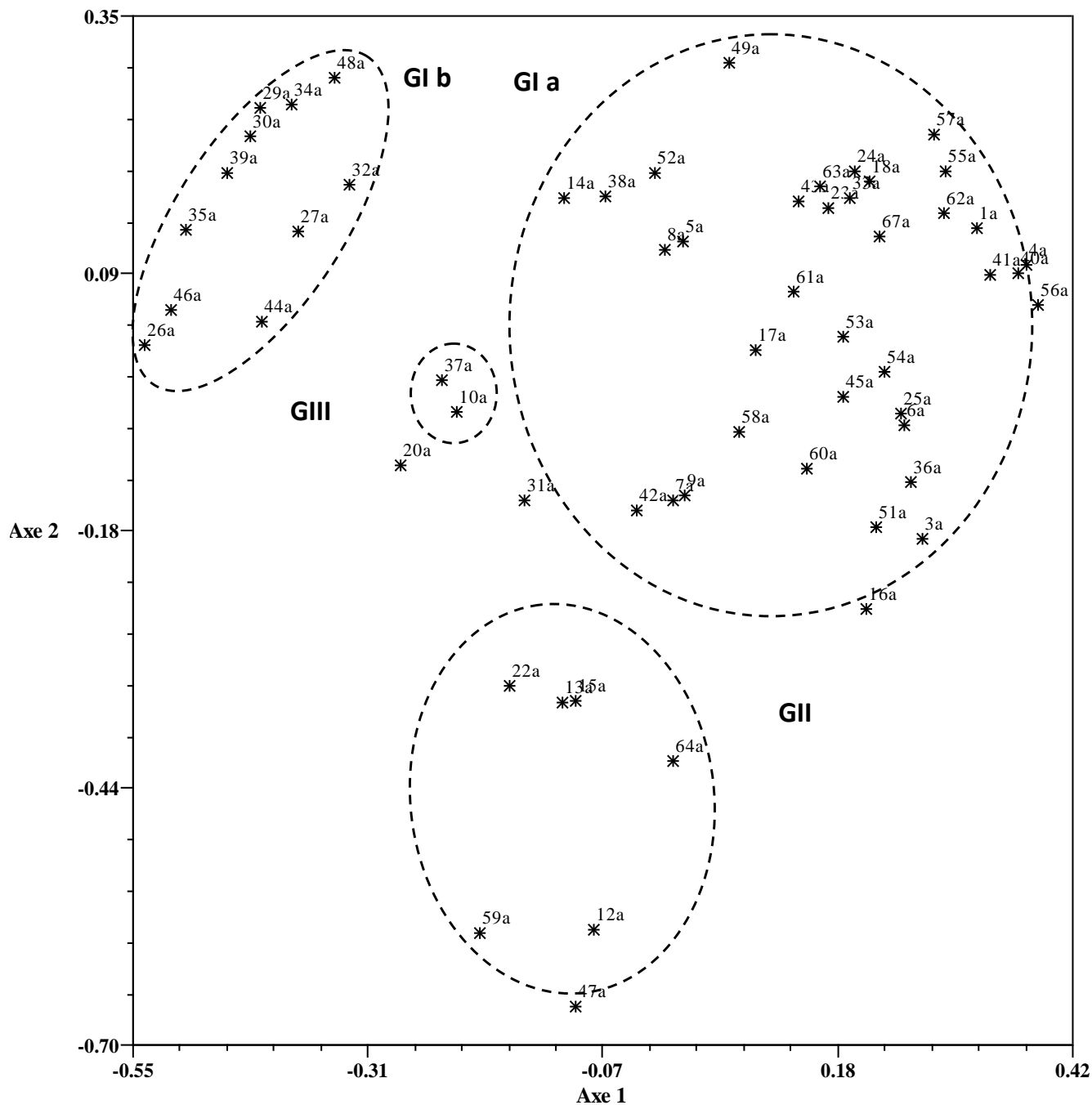
Figure 4. Principal coordinate analysis (PCoA) based on data of twenty SSRs polymorphic loci of 59 accessions of local varieties of sorghum.

(484 accessions), Barro-Kondombo et al. (2010) (124 accessions), Bhosale et al. (2011) (219 accessions) and Sagnard et al. (2011) (455 accessions). Moreover, the level of racial diversification of the samples analyzed could be taken into consideration. The samples used in the present study were more diversified in botanic terms (4 races on 5 represented in *Sorghum bicolor* ssp. *bicolor*) than that of Barro-Kondombo et al. (2010) (3

races on 5 are represented in *Sorghum bicolor* ssp. *bicolor*) who studied a larger population (120 accessions) but identified the lowest allelic diversity. Moreover, the analysis of the number and types of SSR markers used could be the reason. In this study, most of the markers used on samples from West African sub-region were polymorphic especially in Burkina-Faso (Barro-Kondombo et al., 2010), Niger (Deu et al., 2008) and Mali

Table 3. Distribution of accessions analyzed in the genetic groups individualized as compared to ethnic groups of producers.

Ethnical groups of farmers	Individualized genetic groups					Total of accessions
	Ia	Ib	IIa	IIb	III	
Lokpa		11				11
Yom/C*	14	1	2			17
Yom/D*	20	3	3	3	2	31
Total of accessions	34	15	5	3	2	59

*Yom/C: Yom de Copargo, Yom/D: Yom de Djougou.

(Sagnard et al., 2011). Furthermore, SSRs used belong to Xtxp series defined as non-coding regions and usually more polymorphic (Casa et al., 2005; Bhosale et al., 2011).

The proportion of "rare" alleles (17.14%) recorded in this study was lower as compared to that obtained in other studies (64% in Casa et al., 2005 and 64% in Deu et al., 2008). This could explain the fact that the samples used were from common ancestral origin with rare introduction relating to human migration. The case of Aka inodjo (*Ano manka*) and early-maturing varieties with red grains were not introduced (Missihoun et al., 2012a). Moreover, the restricted geographical origin of the samples as well the intense exchanges of materials between farmers among ethnical groups of populations belonging to the same geographic areas could justify the presence of shared alleles. Finally, the reduction of varietal pool during the evolution due to changes of pedological (poor and inadequate soils for cultivation) and agro-climatic (irregularity of rains and droughts), constraints reported by farmers (Kayodé et al., 2006; Missihoun et al., 2012a) could be a factor.

Another factor involved in allele's reduction during evolution is the method of selection of seeds by farmers, which preferred the best, attractive, long panicles and endowed with healthy grains (Missihoun et al., 2012a). Allelic discrimination of such a population appears to be difficult and showed by relatively low mean value of PIC (0.33). The structure of the population as observed in the present study, that is, the separation of the accessions according to the botanical race and morpho-physiological characteristics of the grains (colour, size and degree of bitterness of the grains) and particularly the separation of guinea race from others races (*caudatum* and *durra* precisely) was already reported in previous studies (Djè et al., 2000; Folkertsma et al., 2005; Barnaud et al., 2007; Deu et al., 2008; Barro-Kondombo et al., 2010; Bhosale et al., 2011).

Population structure and distribution

The main evolutionary forces shaping the genetic diversity in the populations of cultivated plants are among others gene flows, selection in connection with

environmental heterogeneity and/or preference criteria of farmers-consumers and genetic variation due to randomness (genetic drift) (Neal, 2004; Mutegi et al., 2011). In the present work, the first factor of the structuration of sorghum genetic diversity identified was the racial parameter. The results are consistence with those of Deu et al. (2008), Kondombo et al. (2010) and Sagnard et al. (2011) respectively in Niger, Burkina-Faso and Mali on samples from world banks of genes (Deu et al., 2006).

The second factor identified was morpho-physiological characteristics of sorghum grains. This findings are similar to our results on agro-morphological characteri-zation in which yellow grains of sorghum were different from bitter and no bitter accessions (Missihoun, 2013) and also farmers' classification using grain features as main criteria for varieties (Missihoun et al., 2012a). For instance, according to farmers from Koura ethnic group, all the local native varieties were named based on grain characteristics: *Aka kpankpan lako* (red sorghum with big grain), *Aka kpankpan kékéré* (red sorghum with small grain), *Aka foufou lako* (white sorghum with big grain) and *Aka foufou kékéré* (white sorghum with small grain). These results obtained in Benin are similar to those of Barro-Kondombo et al. (2010) recorded with SSRs in Burkina-Faso.

Finally, bicolor race was not supported by molecular analysis because the only one accession of this race was found in the group I of guinea race. Absence of differentiation of bicolor race from a genetic group according to molecular data has already been reported in previous studies (Deu et al., 1994; Perumal et al., 2007; Brown et al., 2011).

Implications for sorghum resources conservation and breeding programmes

Conservation genetics aim at identifying and understanding the evolutionary forces that have shaped the observed distribution of genetic diversity within a species on different scales, and identify populations or landraces that deserve priority conservation (Deu et al., 2008). In Benin, previous studies on genetic resources of

cultivated sorghum did not identify these evolutionary forces. In this study, two main factors determining the genetic structuring were identified: racial membership and morpho-physiological characteristics of the grain including grain color. This result is very important for genetic improvement of sorghum genetic resources in Benin. Beninese farmers characterized their local varieties based on grain colour (Missihoun et al., 2012a). In addition, racial classification of all accessions collected in Northwestern region were grouped into four races (*guinea*, *durra*, *caudatum* and *bicolor*) (Missihoun, 2013). At present, climatic fluctuations characterized by the reduction of raining period compel farmers to prefer red grains and low-yielding varieties to white grains and high yield potential varieties (Missihoun et al., 2012a). It is therefore important to conduct hybridization between varieties with white grains and long vegetative cycles, and varieties with red grains and short vegetative cycles to identify in the offspring of individuals adapted to the new growing conditions, that is, varieties with relatively short vegetative cycles and high yield potential. Moreover, it would also be interesting to conduct marker assisted selection exploiting natural hybridization that occurs in farmers' fields to identify hybrids that better meet current growing conditions. This hypothesis is supported by the very high rate (5-40%) of free hybridizations observed on-farm (Barnaud et al., 2008).

The results obtained in this study from the molecular genetics analysis are very important on the plant breeding programmes of varieties adapted to the current climatic fluctuations in Benin. Besides, the results are shown to be important to develop useful *in situ* conservation programs on sorghum in Benin.

Conclusion

The present study used microsatellite markers in estimating genetic diversity of Beninese sorghum landraces for the first time. The results reveal high genetic variability among the studied samples. This important genetic diversity was clearly structured following two important parameters: the racial group and morpho-physiological characteristics of grains (colour, bitterness degree).

The genetic partitioning of botanical races was obvious but guinea group included bicolor accession. It could be assumed that genetic proximity between the two races is due to domestication or lack of genetic support for differentiating the two races. Strategies for conservation and sustainable use of sorghum genetic resources in Benin should take into account the observed genetic components.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Applications of inter simple sequence repeat (ISSR) rDNA in detecting genetic variations in *Lymnaea natalensis* snails from certain Egyptian Governorates

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Inter-simple sequence repeat (ISSR)-PCR technique was used to assess genetic variation and phylogenetic relationships between *Lymnaea natalensis* collected from Giza, Ismailia, Damietta, and Beheira governorates in Egypt and compared with lab-bred snail in addition to characterization of watercourses from these sites. Five ISSR primers generated 47 amplified bands, of which 63.63% showed high polymorphism. All tested primers detected the common band (approximately 455 bp) in all *L. natalensis* studied. Three bands (318, 782 and 2013 bp for primers HB8, HB12 and HB13, respectively) are characteristic for *L. natalensis* collected from Ismailia, El Behira and Damietta governorates, respectively. These markers were used to estimate genetic similarity among the varieties using Jaccard's similarity coefficient. The similarity matrix was used to construct a dendrogram. The most abundant snails *Physa acuta* (56.0%) and the lowest abundant snails *Planorbis planorbis*, and *Melanoides tuberculata* (0.5) were found associated with *L. natalensis* in many governorates. *Eichhornia crassipes* is the only aquatic plant which grows and is found associated with *L. natalensis* in all canals of governorates. Also, dragon fly is the only macroinvertebrate collected from all canals, then shrimps and true bugs. Heavy metals were detected in water samples of all tested governorates with different significant differences. In conclusion, the present study used for the first time the ISSR PCR technique for studying genetic variations of *L. natalensis* snails in Egypt. *L. natalensis* snails can survive when associated with other snails, plants, and insects and can tolerate the heavy metals in water.

Key words: *Lymnaea natalensis*, inter-simple sequence repeat (ISSR)-PCR, dendrogram, heavy metals, macroinvertebrates.

INTRODUCTION

Fascioliasis is considered to be one of the most important parasitic diseases transmitted by freshwater lymnaeid snails and caused by *Fasciola hepatica* (*Lymnaea columella*) and *F. gigantica (Lymnaea natalensis*) (Mas-Coma et al., 2009). The Distribution of *Lymnaea* species in a given area is concerned with temperature and types of water body (De Kock et al., 2003). In South Africa, De Kock et al. (1989) reported that the most successful colonist of all freshwater snail species is *L. columella*, found associated with *Bulinus tropicus* and *L. natalensis*, and it is considered

the most widely distributed freshwater snail species in this region. In Egypt, Ibrahim et al. (1999) stated that *Lymnaea natalensis* is the most common species prevalent in Egypt compared to other species such as *L. columella*, *L. truncatula* and *L. stagnalis*. *L. natalensis* is found to live together with *Biomphalaria alexandrina* in most irrigation regions. Snail vectors were distributed according to different degrees of aquatic plants reflecting the degree of species preference plants for snails' life (Kader, 2001). In addition, Ashour et al. (2008) recorded that, morphometric analysis of *Lymnaea* spp. snails collected from different gover-norates revealed that the height, width, aperture and number of whorls of snails' shells belong to *L. natalensis* in all the surveyed Egyptian Governorates. The highest number of *Lymnaea* snails/site was collected from Giza (38 snails) while Kafr El-Sheikh has low number (8.8 snails). It was reported that Fayoum, Gharbiya, Damietta and Qaluobiya Governorates had the highest percentage of snail infection with *Fasciola.* They also found that *Lymnaea* snails were correlated with some factors such as prevalence of other snails and aquatic plants.

Molecular techniques such as random amplified polymorphic DNA- polymerase chain reaction (RAPD-PCR) analysis and DNA sequencing (Puslednik et al., 2009) have been extensively used as diagnosis tools as well as for the study of genetic structure, variations or relationships between populations of *Lymnaea* snails. Restriction fragment length polymorphism (PCR-RFLP) techniques were targeted to the first and second internal transcribed spacers (ITS1 and ITS2) rDNA and to the mitochondrial 16S ribosomal gene (16S rDNAmt) (Carvalho et al., 2004).

Inter-simple sequence repeat polymerase chain reaction (ISSR)-PCR technique was chosen for assessing genetic variation in the populations of *L. natalensis* snails. ISSRs are used as simple sequence repeats anchored at the 5'- or 3'-end by a short arbitrary sequence as PCR primers (Zietkiewicz et al., 1994). They are considered as powerful tool for genetic mapping and assessment of genetic diversity between closely related species and also to detect similarities between and within species as well (Moreno et al., 1998; Ghariani et al., 2003).

The main advantage of ISSRs is that no sequence data for primer construction are needed; only low quantities of template DNA are required. Among the techniques that can be used to ascertain population structure, PCR-ISSR has proven to be outstanding in the analysis of natural populations of many plant (Taheri et al., 2012), fungus (Priyanka et al., 2013), insect and vertebrate species

(Wolfe, 2005).

The main objectives of the present study were to assess genetic variation and genetic pattern of *Lymnaea* snails collected from four different Governorates and to compare them with laboratory breeding snails using ISSR markers, with the characterization of environmental parameters on their collecting sites.

MATERIALS AND METHODS

Study area and sampling

The freshwater canals in the geographical areas; Giza (Talkhaneya canal), Ismailia (Bort-Said canal), Damietta (Al-Sinania canal) and El- Beheira (El-Kandak El Sharky canal) governorates in Egypt were investigated for their biological and ecological parameters. Biological samples included macroinvertebrates and aquatic vegetation were collected from water canals starting from April to November (2011).

Ecological survey

An ecological survey was carried out in a total of 14 sites representing the four examined canals. Aquatic plants and macroinvertebrate samples including *L. natalensis* snails were collected from the examined sites by using a net, 15 cm deep and a mesh size of 3 mm. For each examined site five consecutive dips were taken according to Takougang et al. (2008). At each site during collection, water temperature, conductivity and pH were recorded and the aquatic plants were gathered. The collected macroinvertebrates and water samples were transferred to the Environmental Research and Medical Malacology Department at Theodor Bilharz Research Institute (TBRI). The medically important snails were examined immediately for any evidence of infection and the water samples were filtered and prepared for chemical analysis.

Biological assessment

In the laboratory, the macroinvertebrate organisms were sorted and taxonomically identified to the lowest possible taxonomic level according to Bouchard (2004) and Leska (1998). The water quality of the canals was biologically assessed using macroinvertebrates' information according to Sullivan (2007). Six comparable matrices were adopted, species richness (equals the total number of individuals represented within the sample), *Ephemeroptera, Plecoptera*, and *Trichoptera* (EPT) index (equal to the total number of individuals represented within these three pollution sensitive insect orders in the sample), ratio of EPT to Chironomidae (is calculated by dividing EPT index by the total number of individuals classified as Chironomidae the more tolerant, ratio of the scraper and filtering collector index) (this index is independent of taxonomy and is calculated by dividing the total number of individuals classified as scrapers by the total number of individuals classified as filtering collectors within the sample), contribution percent of major macroinvertebrate group (equals the abundance of the numerically dominant major group relative to the total number of

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organisms in the sample) and the sixth one was Hilsenhoff Biotic Index (HBI). Hilsenhoff Biotic Index (HBI) developed by Hilsenhoff (1982) and modified by Hilsenhoff (1987) was used to summarize the organic pollution tolerance of water body critters; values of HBI range of 2-4 indicate slightly enriched, 4-7 enriched, and 7-10 polluted. It is calculated by dividing the sum of multiplies of the number of taxa of the same taxonomical group by their tolerance value (values ranging from 0 to 10 based on tolerance to organic pollution) then dividing by the total number of organisms within the sample. The bio-assessment was compared between macroin-vertebrate matrices of each site and the community of the reference site, which was carefully selected. Percent of matrices similarity of each site to the reference one was calculated and awarded 6, 4, 2, 0 points and total points were determined.

Analysis of water samples

The prepared water samples from different sites of the canals were analyzed by graphite furnace and flame of an atomic absorption spectrophotometer (AVANTA, Pal 3000) to determine the concentration of the elements: cadmium (Cd), lead (Pb), copper (Cu), manganese (Mn), iron (Fe), nickel (Ni), sodium (Na), potassium (K) and calcium (Ca).

DNA extraction

Snail feet of the collected *L. natalensis* from each canal in different governorates and lab-bred *L. natalensis* snails were collected from Giza Governnerate and maintained in the Medical Malacology laboratory for several years were dissected, fixed in 70% ethanol and maintained at 4°C till used. Genomic deoxyribonucleic acid (DNA) was extracted from one snail using phenol / chloroform method as described by Vidigal et al. (1994). The foot of lymnaeid snail specimens were suspended in 400 µl of lysis buffer (10 ml Tris-HCl, pH 8.0, 100 ml EDTA, 100 ml NaCl, 1% sodium dodecyl sulfate SDS) containing 500 µg/ml Proteinase K (Promega, Madison, WI, USA) and digested for 1 h at 37°C with alternate shaking each 15 min. The extraction was then performed with phenol-chloroform and DNA was precipitated with ethanol. The pellet was dried and resuspended in 30 µl sterile TE buffer (pH 8.0). This suspension was stored at -20°C until use. DNA integrity and concentrations were estimated by comparison with molecular weight standard on 0.7% agarose gel electrophoresis. Genomic DNA of different groups was subjected to polymerase chain reaction (PCR) using primers (HB8, HB10, HB11, HB12, and HB13). Primers sequencing were: HB8: $(GA)_6$ GG, HB10:$(GA)_6$ CC, HB11:$(GT)_6$ CC, HB12:$(CAC)_3$ GC, HB13:$(GAG)_3$ GC.

PCR conditions

A total PCR reaction volume of 50 µl contained the following: 5 µl DNTPs (2.5 mM), 5 µl $MgCl_2$ (25 mM), 5 µl Buffer (10 x), 4 µl Primer (10 pmol), 0.5 µl Taq DNA polymerase (250 U) (Promega), 5 µl template DNA (250 ng) and 25.5 µl H_2O (d.w). The amplification was carried out in a DNA PTC100TM system using the following cycling conditions: predenaturation of DNA at 94°C for 1 min followed by 30 cycles of denaturation at 94°C for s, annealing at 55°C for 1 min, and extension at 72°C for 1 min and a final extension of 20 min at 72°C. PCR reactions were analyzed through electrophoresis using 1.5% agarose gel electrophoresis.

Data analysis

The data are presented as mean ± standard deviation for the calculation concentration of heavy metals. The heavy metals, temperature, conductivity, HBI and total points parameters were compared statistically with one way ANOVA test by using SPSS computer program, version 20 for Microsoft Windows, 2007.

ISSR-DNA fragments were scored as 1 or 0 for the presence or absence of bands, respectively. The obtained data were subjected to analysis with GelAnalyzer3 (Egygene) software. The level of similarity between species was established as the percentage of polymorphic bands, and a matrix of genetic similarity was compiled using Jaccard's similarity coefficient (Jaccard, 1908). Similarity coefficients were used to construct the dendrogram using the unweighted pair group method with arithmetic average (UPGMA). All calculations were performed with the NTSYS-pc 2.02 software package (Rohlf 2000).

RESULTS

Results of distribution of bio specimens and their association with *L. natalensis* are represented in Table 1. A total of 221 freshwater snail specimens were collected from 14 different sampling sites along four canals, each represents one governorate. Snails were identified as 125 *Physa acuta* (56% of the total snail sample), 28 for each *Lanistes carinatus* and *Cleopatra bulimoides* (12.7%), 17 *L. natalensis* (7.7%), 9 *Theodoxus niloticus* (4.1%), 7 *Bulinus truncatus* (3.2%), 3 *Succinea cleopatra* (1.4%), 2 *Bellamya unicolor* (0.9%) and 1 for each of *Planorbis planorbis*, and *Melanoides tuberculata*, (0.5%). The spatial distribution of snails showed that *L. carinatus* had the higher distribution and was collected from 9 sites (64.0 %), followed by *L. natalensis* that was collected from 6 sites (42.9%), *P. acuta* and *C. bulimoides* were collected from 5 sites (35.7%), *B. truncatus* was collected from three sites (21.0%), *B. unicolor*, *P. planorbis* and *T. niloticus* were collected from 2 sites with 14.3% and *S. cleopatra* was collected from one site with 7.0%. The most abundant snails *L. carinatus* was found in association with *L. natalensis* in many governorates, while in El-Behira Governorate, three species: *Physa acuta,Theodoxus niloticus* and *Cleopatra bulimoides* were found associated with *L. natalensis* snails. In relation to aquatic plants, *Eichhornia crassipes* the only aquatic plant which grows and is found in associations with *L. natalensis* in all canals of governorates, followed by *Jussiaea repens* which found in all Governorates except El-Behira governorate (Table 1). Also, Dragon fly is the only macroinveertebrate collected from all canals, followed by shrimps, true bugs and fishing spider was not collected from Damietta governorate (Table 1). In contrast, Water boatman, *Plecoptera Stoneflies* and *Corbicula consobrina* were collected only from Damietta governorate. El-Beheira governorate possessed the highest amount of species of collected macroinverte-brates (15 species) while the lowest were Ismailia and

Table 1. Distribution of biospecimens in the examined canals represented Egyptian Governorates and their association percentage with *Lymnaea natalensis* (frequency of bio-specimen coexistence with *L. natalensis* / No. of sites of *L. natalensis*) x100).

Bio-specimen		Governorates (no. of sites)	% of Distribution					% of Association
			Giza (4)	Damietta (3)	Beheira (4)	Ismailia (3)	Total (14)	
Biospecimens	Aquatic plants	*Eichhornia crassipes*	50	33	50	100	57	83 (5/6)
		Jussiaea repens	50	100	0	100	57	67 (4/6)
		Ceratophyllum demersum	0	67	0	33	21.4	33 (2/6)
		Bermuda grass	0	0	0	0	7.1	17 (1/6)
		Myriophyllum spicatum	0	0	50	0	14.3	0/6
		Typha angustata	0	0	75	0	21.4	17 (1/6)
		Cyperus alopecuroides	25	0	0	0	7.1	17 (1/6)
		Potamogeton sp.	25	0	0	33.3	7.1	0/6
	Snails (total number)	*Lymnaea natalensis*	75(4)	33 (1)	25(11)	33(1)	43(17)	
		Cleopatra bulimoides	0	100 (20)	50(8)	0	36(28)	17 (1/6)
		Lanistes carinatus	25(2)	100 (17)	50(2)	100(7)	64(28)	67 (4/6)
		Physa acuta	50(112)	67(11)	25(2)	0	21(125)	67 (4/6)
		Bulinus truncatus	0	67(4)	0	33(3)	21(7)	33 (2/6)
		Bellamya unicolor	25(1)	33(1)	0	0	14(2)	33 (2/6)
		Planorbis planorbis	0	33(1)	0	0	7(1)	0/6
		Succinea cleopatra	25(3)	0	0	0	7(3)	17 (1/6)
		Theodoxus niloticus	0	0	25(2)	33 (7)	14 (9)	17 (1/6)
		Melanoides tuberculata	0	33(1)	0	0	7 (1)	17 (1/6)
	Macroinvertebrates (total number)	Snails(Scraper, 7)	67(122)	100(56)	67(25)	100(18)	86(221)	
		Stoneflies (predator, 1)	0	33.3{1}	0	0	7.1 (1)	17 (1/6)
		Dameslfly (predator, 5)	0	0	50(6)	0	78.6 (6)	0/6
		Dragon fly (predator, 5)	25(1)	100 (5)	0	33 (1)	35.7(7)	50 (3/6)
		Riffle beetle (scraper, 4)	0	0	25(4)	100 (7)	28.6(11)	17 (1/6)
		Water boatman (predator, 6)	0	33.3(1)	0	0	7.1(1)	17 (1/6)
		Water strider (predator, 6)	50(5)	0	0	0	14.3 (5)	33 (2/6)
		Water scorpion (predator, 7)	25 (1)	0	0	33(2)	14.3(3)	33 (2/6)
		Back swimmer (predator,9)	0	0	25(1)	0	7.1 (1)	33 (2/6)
		True bugs (predator,8)	75(12)	0	100(14)	100 (14)	71.4 (40)	67 (4/6)
		Corbicula consobrina (collector filtering, 5)	0	33.3(2)	50(9)	33(1)	21.4 (12)	0/6
		Shrimps (collector filtering, 4)	25(6)	0	50 (2)	100 (20)	35.7 (28)	17 (1/6)
		Cray fish (predator,5)	25(3)	0	25 (2)	0	14.3 (5)	0/6
		Fishing spider (predator, 4)	25(5)	0	50(3)	33.3(1)	28.6 (9)	17 (1/6)
		Oligochaeta (collector gathering, 8)	25(1)	0	50 (22)	0	21.4 (23)	33 (2/6)
		Chironomidae (collector gathering, 6)	0	0	0	33.3(1)	7.1 (1)	0/6
		Leeches (Predator,9)	0	0	25 (2)	0	7.1 (2)	17 (1/6)
		Maggot larvae (shredder, 10)	25(2)	0	0	0	7.1 (2)	17 (1/6)

Table 2. Physico-chemical parameters and biological assessment of the examined canals represented Egyptian Governorates.

Heavy metal	Giza	Ismailia	Damietta	El Behira
Cd (ppp)	0.52±0.11	0.22±0	1.31±1.04	0.68±0.33
Pb (ppp)	14.66±2.29	6.10±1.02	152.95±242.36	35.37±37.03
Cu (ppp)	22.39±14.27	133.56±142.48	26.81±8.34	36.32±22.06
Na (ppm)	21.61±6.69^C	108.51±21.83^B	38.29±20.26^C	27.47±6.08^C
K (ppm)	6.29±0.24^C	3.84±0.15^C	9.89±3.42^B	6.55±0.43^C
Ca (ppm)	12.43±0.53^C	9.36±0.44^C	12.86±0.33^C	17.41±3.74^B
Mn (ppp)	10.16±6.63	21.55±18.93	6.51±1.71	14.94±13.91
Fe (ppp)	16.63±17.30	23.90±14.29	11.25±5.47	73.05±67.97
Ni (ppp)	9.73±2.16^C	25.44±7.24^B	6.07±0.33^C	6.53±1.73^C
Temperature	32.50±1.29^A	23.00±0.00^C	31.33±1.53^A	25.33±0.58^B
Conductivity	309.33±38.40 ^{C}	433.33±2.89^C	630.00±160.93^B	320.00±9.85^C
HBI	6.80±0.32 Enriched	5.90±0.82 Enriched	6.70±0.26 Enriched	6.60±0.88 Enriched
T points	9.50±4.43 moderately impaired	12.67±6.43 moderately impaired	13.33±8.08 moderately impaired	17.50±11.12 Slightly impaired

B, C= highly significant at p<0.01 (ANOVA), ppp= part per billion, ppm= part per million.

Damietta governorates (12 species).

Levels of the examined heavy metals (mean ± SD), the recorded physico-chemical parameters and biological evaluation in all water courses sites are displayed in Table 2. The analysis of variance recorded high significance differences (p<0.01) in the mean concentrations of sodium (Na), potassium (K), calcium (Ca) and nickel (Ni) among the tested governorate samples (Table 2). On the other hand, the heavy metals cadmium (Cd), lead (Pb), copper (Cu), manganese (Mn) and Fe (Ferrous) with no significant changes at p>0.05, Damietta Governorates showed the highest level of Cd and Pb (1.31±1.04 and 152.95±242.36, respectively) while Ismailia showed the highest level in Cu and Mn (133.56±142.48 and 21.55±18.93, respectively). Also, the temperature and conductivity recorded highly significant variation between the four mentioned governorates.

According to the present macroinvertebrate information, El-Tawfikia site in El-Khandak El-Sharky canal, Beheira governorate was determined as reference site and awarded 34 points. So, values of total points of other sites were calculated relative to reference site as values greater than 28 were evaluated as non-impaired, between18-28 slightly impaired, 7-17 moderately impaired and less than 7 points were evaluated as severely impaired. HBI and total points showed no significant changes at p>0.05 (Table 2).

The levels of different physicochemical parameters were compared in sites of *L. natalensis* and those free of

them (Table 3). The present results show that there was no relation between the level of heavy metals, temperature and HBI and the distribution of *L. natalensis* as its sites were characterized in some cases by the higher levels: Giza sites. Results of conductivity reveled that all the free sites were characterized by the higher levels. Also, the biological evaluation using macroinvertebrate information showed that *L. natalensis* tolerated moderately impaired water quality till total points equal to 8.3 and did not tolerate severely impaired habitats (<7 points).

Genetic diversity, a similarity coefficients and dendrogram tree among the *L. natalensis* snails collected from 4 governorates (Giza, Ismailia, Damietta and El Behira) and compared with Laboratory snails, were investigated by ISSR-PCR technique. All of the tested primers successfully amplified products from genomic DNA. The number of amplified, monomorphic and polymorphic bands generated by each primer is shown in Table 4.

A total of 47 amplification fragments, ranging from 188 to 2103 bp in size was detected from five ISSR primers depending on the origin of the collected snails and the primer tested (Figure 1). The highest total number of bands (11) was obtained using primers HB10 and HB13, while the lowest number (7) was obtained using primer HB12. By using all primers and according to the total number of amplified bands in each snail group, Ismailia and Damietta snails showed the highest number of bands

Table 3. Physico-chemical parameters and biological assessment (expressed by HBI, organic pollution (OP), and water quality evaluated by macroinvertebrate information (WQ) of sites that included *Lymnaea natalensis* and those free from them in the examined canals represented Egyptian Governorates.

Site		Cd (ppp)	Pb (ppp)	Cu (ppp)	Na (ppm)	K (ppm)	Ca (ppm)	Mn (ppp)	Fe (ppp)	Ni (ppp)	Temp.	Cond.	HBI	OP	T. points M	SD	WQ
Giza	*L. natalensis*	1.7	44.6	53.4	74.7	19.2	37.7	32.0	62.5	27.1	33.0	309.3	6.9	E	10.7	2.4	M
	Free	0.4	14.0	36.2	11.7	6.0	12.0	8.7	4.1	11.8	31.0		6.5	E	6***	2.4	S
Damietta	*L. natalensis*	0.9	15.3	26.1	19.2	10.0	12.5	7.5	13.6	6.0	31.0	450.0	6.8	E	12	3.1	M
	Free	1.5	221.8	27.2	47.8	9.8	13.0	6.0	10.1	6.1	31.5	720.0	6.7	E	9.0*	2.7	M
Beheira	*L. natalensis*	0.5	90.8	21.4	26.7	6.7	18.6	22.7	25.0	4.3	25.0	331.0	7.7	P	10	2.7	M
	Free	2.2	50.6	1.5	83.2	19.5	51.0	37.1	267.2	21.8	25.0	629.0	7.1	P	10.3ns	2.7	M
Ismailia	*L. natalensis*	0.0	7.1	298.1	84.5	3.7	8.9	15.2	32.3	33.2	23.0	430.0	7.1	P	9.8	3.0	M
	Free	0.1	5.6	51.3	120.5	3.9	9.6	24.7	19.7	21.6	23.0	435.0	7.1	P	10.1ns	2.3	M
Total	*L. natalensis*	0.8	39.5	99.8	51.3	9.9	19.4	19.4	33.4	17.7	28.0	380.1	7.1	P	10.6ns	4.8	M
	Free	1.1	73.0	29.1	65.8	9.8	21.4	19.1	75.3	15.3	34.1	594.7	6.9	E	8.9	9.6	M

E = Enriched, P = polluted, M = moderately impaired, S = severely impaired. T= temperature. *,***Significant difference between sites with *L. natalensis* and those free of them, at p<0.05 and 0.001, respectively. ppp= part per billion, ppm= part per million

(11), while the lowest number (seven bands) was detected in El Behira groups. All tested primers revealed the percentage level of polymorphisms between *L. natalensis* snails collected from four governorates and laboratory snails ranging from 0.1% for primer HB11 to 63.63% for primer HB13. Three monomorphic bands were detected (318, 782 and 2013 bp) for primers HB8, HB12, HB13 respectively. These bands are characteristic for *L. natalensis* collected from Ismailia, El Behira and Damietta governorates respectively. All tested primers (HB8, HB10, HB11, HB12, and HB13)

detected approximately the common band found in all groups of snails (452, 455, 463, 495 and 466 bp, respectively). This band was considered to be characteristic bands for *L. natalensis* snails.

The similarity coefficients estimated among the *L. natalensis* snails groups ranged from 0. 6-0.95. The highest value (0.95) was recorded between the Ismailia group and the highest value between Ismailia and Giza (0.95) while the lowest (0. 6) was between Giza and laboratory snails groups (Table 5). The UPGMA dendrogram (Figure 2) showed three main clusters. The first one

including two groups Damietta and El Behira, the second one included two groups Giza and Ismailia and the third major group included only the laboratory snails group.

DISCUSSION

The evaluation of the freshwater surface using biological assessment depending on macro-invertebrate information was more efficient in defining the polluted sites (El- Khayat et al., 2011a).

Table 4. Total number of amplicons and the level of polymorphism among the five snail populations as revealed by ISSRs.

Primer	Total number of amplified bands	Polymorphic amplified bands	Percentage of polymorphism bands
HB8	8	3	37.5
HB10	11	2	18.18
HB11	10	1	0.1
HB12	7	3	42.85
HB13	11	7	63.63
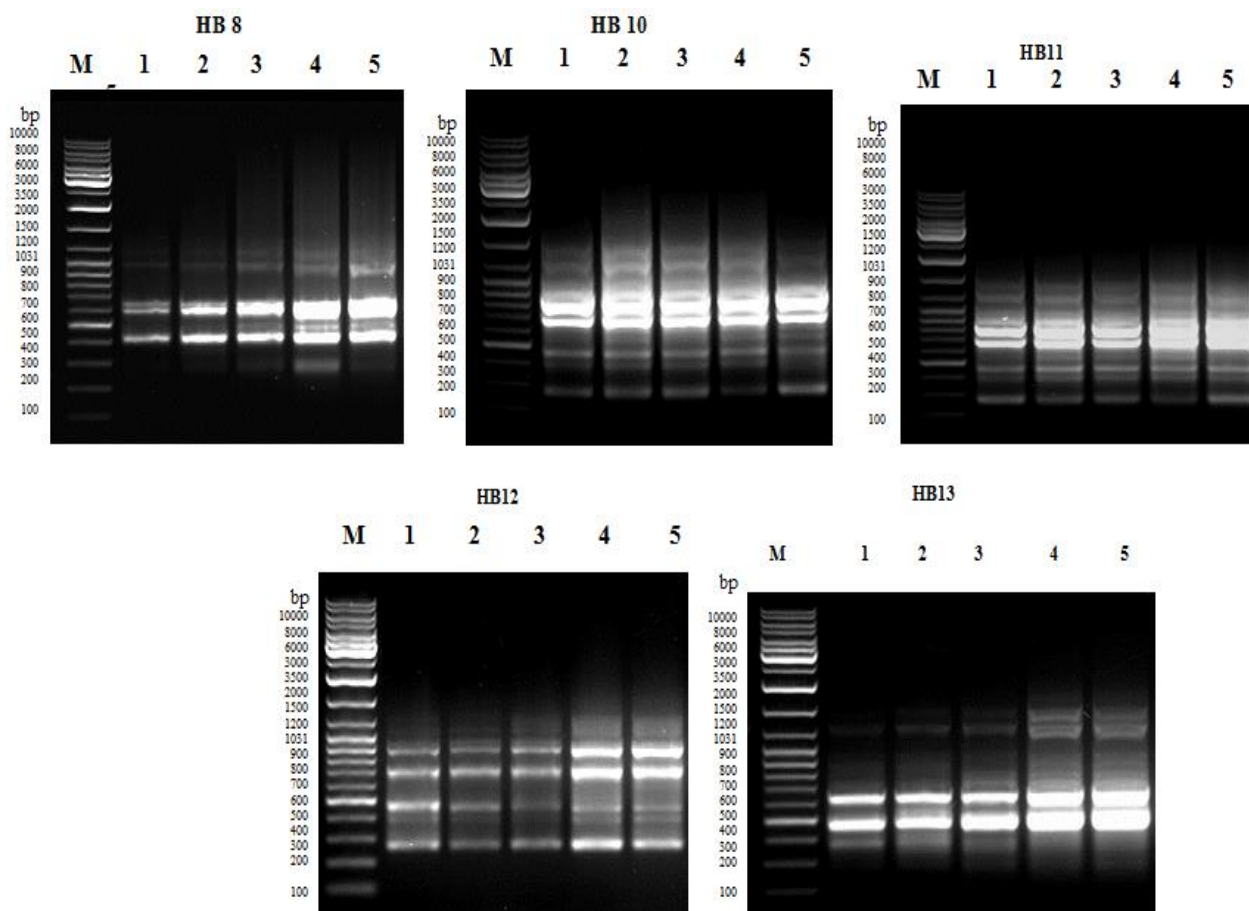
Figure 1. PCR products of genomic DNA from *Lymnaea natalensis* snails collected from 4 Governorates with ISSR primers. Lane M, DNA marker; lane 1, laboratory snails; lane 2, Giza; lane 3, Ismailia; lane 4, Damietta; lane 5, El Behira Governorates.

This was in accordance with EPA (2011), Mandaville (2002), Karr and Chu (2000) and Bartram and Balance (1996) who indicated that the biological assessments provide direct measures of the cumulative response of the biological community to all sources of stress to which the organisms were exposed over a period while

chemical assessments are designed to protect the biological community of a water body from different toxic levels of pollutants, is valid only for the instance in time when the sample was collected.

In this study, the toxigenic cadmium (Cd), lead (Pb), copper (Cu), cobalt (Co), sodium (Na), potassium (Ka)

Table 5. Genetic similarity indices between *Lymnaea natalensis* snails collected from four Governorates and Laboratory snails based on ISSR fragment analysis.

Collection	Laboratory snails	Giza	Ismailia	Damietta	El Behira
Laboratory snails	--	0. 6	0.75	0.82	0.77
Giza		--	0.95	0.91	0.83
Ismailia			--	0.84	0.86
Damietta				--	0.91
El Behira					--
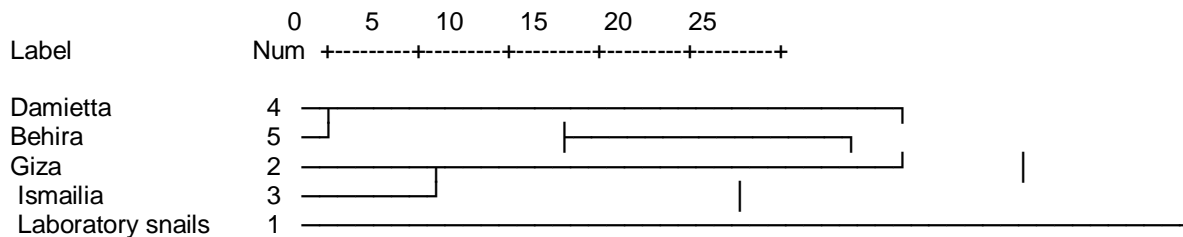
Figure 2. Dendrogram demonstrating the diversity and relationships among *Lymnaea natalensis* snails collected from 4 Governorates and Laboratory snails based on ISSRs

manganese (Mn), ferrous (Fe) and nickel (Ni) were found in all tested canals, Giza (Upper Egypt), Ismailia (Canal Cities), Damietta and Beheira governorates (Lower Egypt). Witt (1982) assumed that in the developing countries 80% of all human illness is associated with polluted water. The pH values play an important role in the interaction between heavy metals and many parameters. Toxicity with heavy metals can increase with basic pH values (Mandour and Azab, 2011). Cadmium is found in surface water as a pollutant from industries (Mandour and Azab 2011). This agreed with the same results found in Damietta governorate which is famous for wood industries and recorded high levels of Cd. Also, Damietta branches receive the water from a number of agricultural drains, which are heavily polluted by industrial and domestic sewage (Abdel Wahaab and Badawy, 2004).

Recently, freshwater snails and bivalves have been used frequently as bioindicator organisms and in several studies of chemical contaminants (Mostafa et al., 2013). In this study, in spite of the accumulations of heavy metals in canals, *L. natalensis* snails could survive. El-Khayat et al. (2011a) found that *L. natalensis* were more tolerant to Cd, Fe, Ni and Mn and more sensitive to Cu than other snails while all snails could live at approximately the same concentrations of Na, K and Ca. Siwela et al. (2010) reported that at elevated levels of heavy metal accumulation, *L. natalensis* snails could

survive through some physiological modifications. They found that *L. natalensis* responded to pollution by increasing the activity of catalase and selenium-dependent glutathione peroxidase specific activity in an effort to detoxify peroxides produced as a result of metal-induced oxidative stress.

In this study, the conductivity was measured in all sites and all the free sites were characterized by the higher levels indicating that it may be a limiting factor in *Lymnaea* distribution. Also, the biological evaluation using macroinvertebrate information showed that *L. natalensis* tolerated moderately impaired water quality till the total points equal to 8.3 and did not tolerate severely impaired habitats (<7 points). El-Khayat et al. (2011b) determined by macroinvertebrate matrices that *L. natalenthsis* showed intermediate pollution tolerance between *Biomphalaria alexanrina* and *Bulinus truncates*; severely impaired sites constituted 23% of *B. alexandrina* habitats, 14% of *L. natalensis* and 9.4% of *B.truncatus* sites. Hilsenhoff Biotic Index (HBI) showed the same tolerance to organic pollution.

To analyze various species of *Lymnaea* snails, there are many published reports on the use of molecular marker techniques such as RAPD (Puslednik et al., 2009; Bin Dajem, 2012; Koneva, 2013), RFLP (Carvalho et al., 2004) and SSU rDNA (Bargues et al., 1997). Moreover, Yang et al. (1994) reported that ISSR assay can provide more informative data than other techniques. The present

investigation clearly demonstrated that *L. natalensis* snails collected from different governorates could be distinguished by ISSR primers for the first time in Egypt. Segments of DNA from *L. natalensis* can be reproducibly amplified by short primer range of 10-20 bp nucleotides with the ISSR technique, showing a high level of polymorphism (63.63% for primer HB13). Seven polymorphic bands and the total number of amplified bands were 11; obtained with primer HB13. Thus, this primer may be useful in differentiating between different *L. natalensis* snails throughout the governorates.

In another related study on *B. glabrata* snails, Larson et al. (1996) reported that the identification of polymorphic bands was based on a comparison of banding patterns on the same gel for each of the six snail groups investigated. This suggests that ISSR markers were polymorphic markers suitable to detect the genetic diversity of snails at the DNA level. All tested primers (HB8, HB10, HB11, HB12, and HB13) detect approximately the common band found in all groups of snails (452, 455, 463, 495 and 466 bp). The apparent of this band was considered to be genetic characteristics bands for *L. natalensis* snails and lymnaeid snails from only one source.

In conclusion, the present study considered for the first time the use of ISSR PCR technique in studying genetic variations of *L. natalensis* snails in Egypt. ISSR primers showed a high level of polymorphism between collected snails.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Developmental response of tropical warehouse moth, *Ephestia cautella* (Walker) (Lepidoptera: Pyralidae)'s larvae to stored cocoa beans fermented at varied degrees

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Developing larvae of major insect pests like *Ephestia cautella* rapidly degrade stored cocoa beans through feeding and other biological activities on the beans. Completion of developmental stages of insects may be influenced by a number of biochemical factors triggered by the primary post-harvest processing of cocoa beans prior to storage. Hence, this study assessed the development of *E. cautella* from first instar larval stage to adult stage on cocoa beans fermented at varied degrees. Fresh ripening pods of Amelonado variety (N38) were harvested and fermented variedly at 0 - 7 days, and sun-dried to about 5-6% moisture content. The variedly fermented cocoa beans (300 g/treatment) were infested with newly hatched first instar larvae of *E. cautella* (N=10) in a completely randomized design with four replications at 28±2°C and 70±5% relative humidity (RH). Days to adult emergence of the larvae of *E. cautella* on the cocoa beans varied significantly (P>0.05) between 0 day and 76.50 days in 4 days and 7 days-fermented beans, respectively. Significant variations occurred (P>0.05) in the total adult emergents of *E. cautella*, ranging between 0 and 8.25 in 4 days and 1 day fermented beans, respectively. This study reveals that the fermentation period of cocoa beans prior to drying and storage influenced adult emergence of *E. cautella* on stored cocoa beans, with 0 - 3 days fermented beans highly susceptible to *E. cautella*'s development to adult stage. Cocoa beans need to be fermented adequately for 4 to 7 days before drying to prevent insects' damage. Notably, 4-days fermented cocoa beans did not support *E. cautella* development to adult stage at 90 days post larvae-introduction. The 4-days fermented cocoa beans require further investigations for its nutritional suitability and importance in human diets.

Key words: Varied fermentation, *Ephestia cautella*, total adult emergents, days to adult emergence.

INTRODUCTION

Cocoa is a major export and source of income in some countries in West Africa including Nigeria (Tah et al., 2011) and is widely cultivated in the tropical rain forest and adjoining rain-fed ecologies in Nigeria (Opeke,

2005). Nigeria is the fourth largest producer of dried cocoa beans in the world after Cote d'Ivoire, Ghana and Indonesia with an average output of about 225,000 - 245,000 metric tons between 2011 and 2013

(The Statistics Portal, 2014). It is the major component used in production of chocolate, cocoa butter and mucilage, cocoa wine and beverages, cosmetics and many other products (Opeke, 2005). Insect pests infestation is an important production constraint of cocoa on the field and in the store. Over 1500 species of insects are known to feed on cocoa on the field but only 2% is of economic importance (Entwistle, 1972; Wood and Lass, 1989). Fermentation is an essential post-harvest processing of freshly harvested cocoa beans which precedes drying (Hii et al., 2009), in order to attain the desired flavour, physicochemical and/or industrial properties for its acceptability by consumers. Fermentation periods of freshly harvested beans affects the physiological and chemical compositions of the dried cocoa beans in store either positively or negatively, which in turn determines the frequency of development of biotic factors that can degrade and/or reduce the quality and quantity of stored cocoa beans.

There is a microbial succession of wide range of yeasts, lactic acid, and acetic-acid bacteria during which high temperature (about 50°C) and microbial products such as ethanol, lactic acid and acetic acid kill the embryo of the beans (Schwan and Wheals, 2004; Nielsen et al., 2005; Nielsen et al., 2007; Nielsen et al., 2008). In this process of microbial succession, there are flavor precursors production (Schwan and Wheals, 2004; Camu et al., 2008). The fermentation periods of freshly harvested cocoa beans is influenced by the microbial activities on the beans as the epicatechin and theobromine levels decrease, which in turn affects the colour, flavor (aroma) of the beans (Lagunes et al., 2007; Rodriguez-Campos et al., 2011) and the organoleptic properties of the dried cocoa beans in store (Camu et al., 2008). Freshly harvested cocoa beans require fermentation for 5 - 7 days before drying in order to have the desired chocolate flavor (Fowler 1999; Beckett, 2000), but field survey of farmers in some communities in Southwestern Nigeria revealed that some farmers have not been fermenting the cocoa beans for the recommended period of 5 - 7 days before drying, which suggests why insect infestation rate and other degrading biotic agents might be on the increase in stored cocoa beans from such localities. This in turn pollutes other cocoa beans of good quality pooled together with those that were not properly fermented (Oyedokun, 2013). Without proper fermentation, there is no chocolate flavor because during fermentation, precursor compounds for chocolate flavor are formed which will react together during roasting at optimum temperature of 150°C for 30 min (Ramil et al., 2006; Lambert, 2008).

Insect infestations affect the quality and quantity of stored cocoa beans through bioactivity processes, thereby stripping the beans of the desired flavor and other quality parameters that might have been built up during fermentation process. Inadequate fermentation of cocoa beans could be a factor or bio-indicator that is contributing mainly to insect development and eventual infestation of the produce in store. Hence, this study evaluated the influence of fermentation periods on nutritional contents of the freshly harvested cocoa beans and its impacts on some aspects of developmental biology of *E. cautella* by determining the relationships between fermentation periods, some chemical constituents of dried cocoa beans of varying fermentation periods and days to adult emergence of *E. cautella* on cocoa beans at 90 days in store.

MATERIALS AND METHODS

Freshly harvested, ripening cocoa pods of Amelonado variety (genotype-N38) were collected from the Common Fund for Commodities (CFC) Plot at Cocoa Research Institute of Nigeria, Ibadan, Nigeria during the main crop harvest season of 2010. The pods were broken with the aid of a small wooden club and were subjected to different fermentation periods (DFP) of F0, F1, F2, F3, F4, F5, F6 and F7 days using box fermentation method. The wooden boxes (7 cm height, 25 cm width and 35 cm length) were lined with fresh plantain leaves before the fresh beans were poured into the boxes. The whole beans removed from the pods were pooled and divided into eight batches of equal fresh weight (2 kg) and each batch was fermented at different days. Zero-day fermented beans (F_0) were collected from the batches as batch 1, rubbed in fine sawdust and later rinsed thoroughly with clean water to remove the pertinacious mucilage covering the beans and thereafter sun-dried for ten days on a raised platform till well-dried. One-day fermented beans (F_1) were collected after one day of fermentation and subsequent fermentation periods were collected following the number of days assigned per batch up till seven-days. The fermented beans were sun-dried to about 6% moisture content at 10 days of drying. Each fermentation day's batch was replicated three times in completely randomized design in the three fermentation boxes.

Homogenous samples of *E. cautella* Walker (n=10) that were raised on cocoa-wheat-soya diet were collected, sexed, paired for mating and placed in pairs in plastic oviposition cages (18.5 cm top diameter, 13.5 cm base diameter and height of 19.5 cm) lined with black filter papers which provided the contrast needed to collect the milky-white laid eggs. The eggs laid after 24 h were collected and placed in 90 mm diameter Petri dishes lined with black filter paper. The lid of the Petri dishes were perforated to allow for aeration and the Petri dishes were placed on a table with its legs dipped in water polluted with fresh engine oil so as to prevent ants from predating on the eggs. Ten day-old, freshly hatched first instar larvae of *E. cautella* were carefully introduced into each rearing cage (12.5 cm top diameter, 11.5 cm base diameter and height of 6.5 cm) containing 300 g of dried cocoa beans fermented at different days.

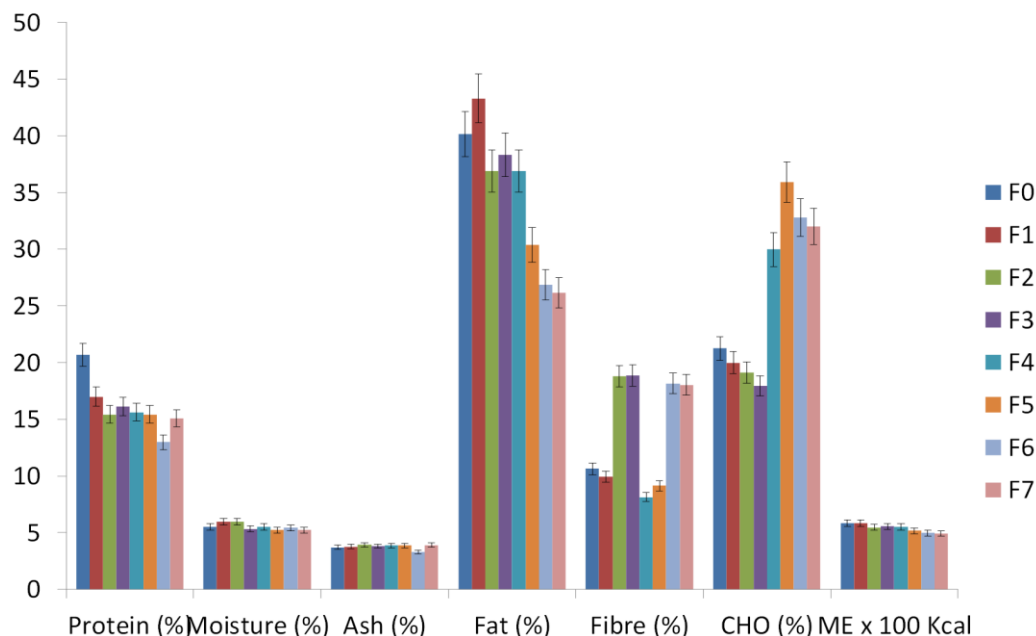*Corresponding author. E-mail: gokman.dee@gmail.com

Figure 1. The proximate composition of dried cocoa beans fermented for different days. F0 to F7, fermentations at 0 to 7 days respectively.

Prior to introduction of the day-old larvae onto the beans, the beans were sterilized in a Gallenkamp Oven at 40°C for 4 h to kill any insect eggs that might have been laid on the beans during outdoor drying of the cocoa beans. After cooling, the larvae were introduced into the cages containing sterilized cocoa beans fermented at varied degrees using a soft, pointing-tip camel hair brush dipped in sterile water and adequately drained. The experiment was replicated four times. The rearing cages (cut at the sides) containing the dried cocoa beans and day-old larvae had its sides covered with muslin cloth and were kept undisturbed and observed daily in the laboratory at the ambient tropical temperature (28 ± 2°C) and relative humidity (70 ± 5%) till adults started emerging. Data were collected on the number of days to adult emergence of *E. cautella* on the beans and the total number of adult *E. cautella* that emerged per treatment. The experiment terminated at 90 days after the beans were infested with first instar larvae.

The proximate, some physicochemical properties of the fat extract and some mineral compositions of the variedly fermented beans were determined using standard procedures (AOAC, 1990). Data were analyzed using analysis of variance (ANOVA) and the means separated using Tukey's studentized range (HSD). Also, proximate, physicochemical and mineral compositions of the beans were correlated with total adult emergents, days to adult emergence and cocoa beans fermented for four days using SPSS 17 Version.

RESULTS AND DISCUSSION

Effect of fermentation periods on proximate composition of the cocoa beans and development of *E. cautella*

The proximate composition of cocoa beans (Amelonado-

N38) variedly fermented at F_0, F_1 F_2, F_3, F_4, F_5, F_6, and F_7 days is shown in Figure 1. The F_0 cocoa beans had significantly (P>0.05) higher mean value of protein (21.33 ± 0.84) than other cocoa beans of F_1, F_2, F_3, F_4, F_5, F_6 and F_7 that had between 13-17% crude protein (Figure 1). The moisture content of all the variedly fermented cocoa beans ranged between 5.23 and 5.95% which was not significantly different (P < 0.05) in all the fermented cocoa beans. Moreover, significant variations (P = 0.05) were recorded in the percentage ash content of F_0, F_1 and F_3 cocoa beans. There was no significant difference (P < 0.05) in the percentage ash content of cocoa beans fermented at F_2, F_4, F_5, and F_7, that had 3.92, 3.86, 3.87 and 3.89%, respectively. The percentage fat content ranged between 26.15% in F_7 and 43.31% in F_1 cocoa beans. The fat content of the cocoa beans decreased significantly (P>0.05) with increase in fermentation period which may be due to the bioactivities of the microorganisms present in the cocoa pulp (Lagunes-Galvez, 2007; Guehi et al., 2008). The F_4 beans had significantly (P<0.05) lower fibre content of 8.14% while F_3 had the highest (18.89%) fibre content. Carbohydrate was significantly higher (P>0.05) in F_5 beans (Figure 1), while F_3 beans had the lowest percentage carbohydrate composition. Metabolizable energy was lowest in F_7 and highest value was recorded in F_0 cocoa beans.

This study shows that development of *E. cautella* from first instar larva to adult stage was influenced by varied fermentation periods of the cocoa beans prior to drying and storage. This might be as a result of differences in

Table 1. Fermentation levels and some mineral elements of dried cocoa beans.

Fermentation level (days)	Lead (mg/L)	Iron (mg/L)	Calcium (mg/L)	Magnesium (mg/L)	Potassium (mg/L)
F_0	$0.15^c \pm 0.00$	$2.41^c \pm 0.02$	$17.54^b \pm 0.02$	$76.51^d \pm 0.18$	$163.13^f \pm 0.93$
F_1	$0.11^b \pm 0.00$	$1.82^a \pm 0.01$	$18.01^b \pm 0.06$	$83.42^e \pm 0.24$	$175.75^h \pm 0.47$
F_2	$0.11^b \pm 0.01$	$2.60^d \pm 0.04$	$19.30^c \pm 0.17$	$67.45^b \pm 0.63$	$166.38^g \pm 1.04$
F_3	$0.10^b \pm 0.004$	$1.89^a \pm 0.01$	$19.99^d \pm 0.04$	$68.67^b \pm 0.26$	$136.08^d \pm 0.12$
F_4	$0.10^b \pm 0.005$	$2.09^b \pm 0.031$	$25.55^g \pm 0.27$	$71.61^c \pm 0.24$	$131.11^c \pm 0.29$
F_5	$0.07^a \pm 0.001$	$1.81^a \pm 0.016$	$24.13^f \pm 0.07$	$71.34^c \pm 0.15$	$158.05^e \pm 0.09$
F_6	$0.03^a \pm 0.001$	$2.45^c \pm 0.016$	$15.96^a \pm 0.04$	$54.56^a \pm 0.45$	$98.13^a \pm 0.27$
F_7	$0.07^a \pm 0.00$	$2.02^b \pm 0.01$	$22.25^e \pm 0.09$	$75.52^d \pm 0.26$	$126.48^b \pm 0.21$

Means following the same letter in the same column are not significantly different (P≤0.05) following Tukey's Studentized Range HSD Test. N=3 replicates/treatment. F0 to F7, fermentations at 0 to 7 days respectively.

the biochemical composition (proximate, physicochemical and mineral) of the cocoa beans being influenced by duration of fermentation vis-à-vis microbial activities of microorganisms which produces metabolites that diffuse from the pulp into the beans. Variation in the proximate and mineral composition of the cocoa beans was influenced by the fermentation levels of the bean prior to storage and this influenced the development of *E. cautella* on the cocoa beans that was stored over a period of 90 days. This finding corroborates earlier studies (Jonfia-Essien, 2006; Guehi et al., 2008) that biochemical variations occur in stored cocoa beans which eventually enhance insect pest population build-up. This study also showed that unfermented cocoa beans had higher protein content than other variedly fermented beans which might have predisposed the F_0 cocoa beans to higher infestation rate and earlier developmental period of *E. cautella* on the beans. This is in agreement with the results of Chapman (2003), (House,1969), Schoonhoven et al. (2005) that optimum-high amount of protein is required and/or essential in insect diet for growth and development even as the larvae have the ability to sense the presence of protein in the food substrates through chemoreception. The lowest fibre content and other biochemical contents found in F_4 cocoa beans in which there was no adult emergents of *E. cautella* at 90 days in store suggests a possibility of nutritional imbalance that occurred in the F_4 cocoa beans, which might be due to incomplete microbial actions and/or reactions during fermentation process. This corroborates (Dadd, 1985) that insects may respond to imbalance diet by moving away from the food or abstain from feeding on such food.

Since no alternate or alternative food host was available to the developing larvae of *E. cautella* in this study, attempt by the developing larvae to abstain from such unsuitable diet like F_4 will result into death of the larvae. Hence, as no adult emerged on F_4 beans. The low fibre content and some few other nutritional factors that

are bio-indicative suggest why F_4 cocoa beans did not support the development of *E. cautella* from first instar larva to adult stage in this study. It also showed that the ash content varied with duration of fermentation which might be due to the effects of varied microbial activities involved during fermentation process as influenced by the fermentation periods. This indication suggests why cocoa beans that were not properly fermented (0-3 days) were more susceptible to insect attack within a shorter period of storage when compared with the beans fermented between four to seven days. The implication is that inadequately fermented cocoa beans support the development of major insect pests of stored cocoa beans like *E. cautella* with shorter developmental period while adequately fermented cocoa beans (5-7 days) delayed *E. cautella*'s development on such beans except four-day fermented beans that did not support *E. cautella* development within 90 days of storage.

Mineral compositions of the variedly fermented cocoa beans and the effect on *E. cautella* larvae

Table 1 shows some mineral elements composition in the cocoa beans fermented at different days. The lead (Pb) content of the cocoa beans was significantly lower (P<0.05) in F_5 to F_7 (0.03 - 0.07 mg/L) cocoa beans while F_0 to F_4 cocoa beans had significantly higher (P>0.05) lead content (between 0.10 and 0.15 mg/L) suggesting the role of adequate fermentation as bio-remediation process of reducing the levels of heavy metals in cocoa beans through the physiological and metabolic activities of fermentation microbes.

The iron content of the variedly fermented beans did not follow a regular trend but was significantly different (P=0.05) with F_0, F_1, F_3, F_4, F_5, F_6, and F_7 having 2.41, 1.82, 2.60, 1.89, 2.09, 1.81, 2.45 and 2.02 mg/L, respectively. The F_4 fermented cocoa beans had a significantly (P>0.05) higher level of calcium content

Table 2. Physicochemical composition of fat extract from cocoa beans fermented at different days.

Fermentation levels (days)	Acid value (mg/g) ($\overline{X}$ ± SE)	Peroxide value (mEq/Kg) ($\overline{X}$ ± SE)	Iodine value (g/100 g) ($\overline{X}$ ± SE)	FFA (g/100 g) ($\overline{X}$ ± SE)	pH ($\overline{X}$ ± SE)
F_0	2.24^f ± 0.02	1.58^e ± 0.01	42.10^g ± 0.12	4.36^f ± 0.05	6.40^e ± 0.00
F_1	1.88^e ± 0.01	1.21^d ± 0.01	29.86^f ± 0.04	3.76^e ± 0.22	6.60^f ± 0.00
F_2	1.78^d ± 0.01	1.18^d ± 0.00	25.28^d ± 0.01	3.59^d ± 0.00	5.70^b ± 0.00
F_3	1.42^b ± 0.01	1.05^c ± 0.02	24.40^c ± 0.00	2.85^b ± 0.02	5.80^c ± 0.00
F_4	1.12^a ± 0.00	2.36^f ± 0.01	18.70^a ± 0.18	2.26^a ± 0.02	6.00^d ± 0.00
F_5	1.69^c ± 0.01	0.79^b ± 0.016	22.54^b ± 0.24	3.32^c ± 0.02	5.80^c ± 0.00
F_6	2.23^f ± 0.00	0.59^a ± 0.01	25.63^d ± 0.06	4.47^g ± 0.00	5.80^c ± 0.00
F_7	1.68^c ± 0.00	1.56^e ± 0.00	27.29^e ± 0.24	3.38^c ± 0.01	5.60^a ± 0.00

Means following the same letter in the same column are not significantly different (P≤0.05) following Tukey's Studentized Range HSD Test. N= 3 replicates/treatment. F0 to F7, fermentations at 0 to 7 days respectively.

(25.55 mg/L) while cocoa beans fermented for 5 days (F_5) recorded the lowest calcium content of 15.96 mg/L. The calcium contents increased with fermentation periods till 4 days fermentation and decreased after 4 day fermentation. This might be as a result of synthesis of these minerals by the microorganisms involved in the fermentation process within the earlier days (0-4 days) before the moisture content is relatively low in latter days (5-7 days) which would also affect the biochemical activities of the microorganisms. The magnesium (Mg) content of the variedly fermented cocoa beans varied significantly (P>0.05) from 54.56 mg/L in 6-days fermented beans to 83.42 mg/L in 1-day fermented cocoa beans. The lowest potassium level was recorded in 6-days fermented cocoa beans with 98.13 mg/L which was significantly lower (P>0.05) when compared with F_1 cocoa beans which had 175.75 mg/L. The decreasing trend of Mg and K as fermentation period increases might be as a result of not storing the cocoa pods before processing. This supports earlier study by Afoakwa et al. (2013) that pod storage before processing enhances the concentration of calcium, magnesium, zinc and potassium as fermentation days increases. The higher level of calcium content in F_4 might be among the main factors limiting adults of *E. cautella* from emerging on the F_4 cocoa beans due to nutritional deficiencies and/or unsuitable diet. This corroborates the findings of Nation (2001) that insects require trace amount of calcium and iron for development. The F_0 and F_1 cocoa beans with the higher mean value of magnesium as well as F_0 to F_2 fermented cocoa beans with higher value of potassium had the highest range of *E. cautella* adult emergents with an average of 48 days to emerge. The magnesium and potassium factors might be significant factors in the development of *E. cautella* to adult stage on such cocoa beans.

This supports was earlier studied by Silva et al. (2005)

that insects need considerable amount of mineral elements like magnesium, phosphorous and potassium but little calcium, sodium and chlorides are required for insect development. It also explains further that a perfect blend of biochemical composition of food host (fermented cocoa beans) determines the population build-up effect of insect pests on such food host which had been greatly influenced by microbial activities during the various fermentation periods. From the mineral analysis, lead content in all the fermented beans seem to be within the acceptable intake level for *E. cautella* larva development except in Four-day fermented beans in which no adult emerged which may be due to other factors like low fibre and calcium content. This is contrary to the expectation that presence of trace amount of lead will reduce the number of adult emergents because lead is a heavy metal that can reduce pathenogenetic activities of the cells during different stages of larval development.

Some physicochemical properties of the fat content extracted from cocoa beans fermented at different days are shown in Table 2. The mean acid values of the fermented beans was significant (P>0.05) with lowest value of 1.12 mg/g in beans fermented at 4 days while the cocoa beans fermented at zero days had the highest acid value of 2.24 mg/g. Peroxide value in cocoa beans fermented at 4 days was significantly higher (P > 0.05) having 2.36 mEq/kg and significantly lowers in cocoa beans fermented at 6 days with 0.59 mEq/kg. Iodine values ranged between 18.70 g/100 g in 4-days fermented beans and 42.10 g/100 g in cocoa bean with zero fermented days. Free fatty acid (FFA) was lowest in 4-days fermented beans (2.26 g/100 g) and highest in 6-days fermented beans (4.47 g/100 g). The physicochemical values of the butter extract from each fermented beans also revealed that the lower the free fatty acid content of cocoa beans, the lower the development of insects on cocoa beans.

Table 3. Effect of fermentation periods of cocoa beans on days to adult emergence and number of adult emergents of *E. cautella* at 90 days after first instar larvae introduction.

Fermentation period (days)	Days to adult emergence (Mean ± SE)	Total number of adult emergents (Mean ± SE)
0	47.50^c ± 3.08	8.25^d ± 0.75
1	49.00^c ± 3.19	8.00^d ± 1.00
2	48.00^c ± 2.89	6.50^{cd} ± 0.87
3	34.25^b ± 13.13	1.50^{ab} ± 0.87
4	0.00^a ± 0.00	0.00^a ±0.00
5	68.75^d ± 2.81	5.00^{bc} ± 0.71
6	75.75^e ± 2.17	3.75^{abc} ± 0.85
7	76.50^e ± 2.95	3.50^{abc} ± 0.96

N= 10/treatment in four replicates; mean with the same letter is not significantly different at P≤0.05 following Tukey's Studentized Range (HSD). F0 to F7, fermentations at 0 to 7 days respectively.

From this study, it was evident that cocoa beans fermented at four days had the lowest FFA, on which *E. cautella* larvae could not develop to adult stage at 90 days in store and this might be as a result of gamut of factors including low FFA that was present in four-day fermented cocoa beans. This is in consonance with Jonfia-Essien and Navarro (2010) whose report directly correlated relationship between FFA and insect infestation that the higher the FFA, the higher the insect infestation and vice-versa. Meanwhile, the proximate analysis of the fermented beans showed that moisture content in the variedly fermented cocoa beans were at lower levels below 6.5% which should have affected the FFA to be on the increase but this was contrary to the report of Jonfia-Essien and Navarro (2010) that cocoa beans with low moisture content of lower than 6.5% were found to contain high FFA levels. Rather than FFA and moisture, gamut of other compositional factors like percentage fibre content, percentage fat content and mineral composition of the cocoa beans could be factors that would enhance or hinder insect pests' development on cocoa beans in store. For instance from this study, cocoa beans fermented for four days were found to have the highest calcium content, lowest FFA, lowest fibre content, highest peroxide value, lowest acid value and lowest iodine value which suggested why four-day fermented beans did not support the development of *E. cautella* till adult emergence. This agrees with the report of Adeniyi et al. (2011), who stated that fermentation periods of cocoa beans has significant effects on the colour, microbial load as well as the quality and nutrition content of the beans. Variations in the physicochemical composition of cocoa beans fermented at different days as shown in this study accounted for the dynamics in the nutrient base that was made available to the developmental stages of *E. cautella* as this affected the biological parameters like number of adult emergents and days to adult emergence. Imbalanced diets as presented in four days fermented cocoa beans with lowest fibre, lowest acid value, lowest free fatty acid, lowest iodine value, highest peroxide value and highest calcium content suggests the reason for non-emergence of *E. cautella* adults on the beans.

Influence of fermentation periods on days to adult emergence and total adult emergents of *E. cautella*

The effects of fermentation periods on total adult emergents and days to adult emergence at 90 days after introduction of day-old first instar larvae of *Ephestia cautella* is shown in Table 3. There were significant differences (P = 0.05) in the number of days to adult emergence of *E. cautella* on the cocoa beans fermented at different days, ranging from 0.00 days on four days fermented cocoa beans and 58.75 days on five days fermented beans. Adults emerged on differently fermented cocoa beans at the average of 47.50, 49.00, 48.00, 34.24, 0.00, 68.75, 75.75 and 76.7 days on F_0, F_1, F_2, F_3, F_4, F_5, F_6 and F_7, cocoa beans, respectively. Similarly, the total *E. cautella* adult emergents on cocoa beans variedly fermented differed significantly (P = 0.05), with the average of 8.25, 8.00, 6.00, 1.50, 0.00, 5.00, 3.75 and 3.50 adults emerging on, F_0, F_1, F_2, F_3, F_4, F_5, F_6 and F_7 fermented cocoa beans, respectively.

The interactions of fermentation periods, the proximate composition of cocoa beans fermented at different days, adult emergence and total adult emergents is shown in a 2-tailed correlation matrix (Table 4). The increase in fermentation period of the cocoa beans significantly (P > 0.01) reduced the percentage composition of protein, fat, metabolizable energy and also reduced the total number

Table 4. Correlation between total adult emergents, days to emergence of *E. cautella* and proximate composition of cocoa beans fermented at different days.

	Fermt.	TAE	DAE	%CP	%Moist	%Ash	% Fat	%Fibre	%CHO	ME
Fermt.	1.00									
TAE	-0.52**	1.00								
DAE	-0.39	0.35	1.00							
%CP	-0.73**	0.27	-0.32	1.00						
%Moist	0.37*	0.21	-0.07	0.20	1.00					
%Ash	0.21	-0.25	-0.11	-0.04	0.06	1.00				
%Fat	-0.94**	0.37	-0.47**	0.78**	0.32	-0.23	1.00			
%Fibre	-0.01	0.05	0.05	-0.16	-0.04	-0.01	-0.05	1.00		
%CHO	0.68**	-0.11	0.56**	-0.42	-0.37*	0.03	-0.69**	-0.52**	1.00	1.00
ME	-0.93**	0.37**	-0.47**	0.82**	0.29	-0.18	0.95**	-0.08	-0.67**	

**Correlation is significant at ($p > 0.01$), (2 tailed). * Correlation is significant at ($p > 0.05$), (2 tailed). TAE, total adult emergents; DAE, days to adult emergence; ME, metabolizable energy, CP, crude protein; CHO, carbohydrate; Fermt., fermentation.

Table 5. Correlation between total adult emergents, days to emergence of *E. cautella* and some mineral composition of cocoa beans fermented at different days.

	Fermt.	TAE	DAE	Fe	Ca	Mg	Pb	K
Fermt.	1.00							
TAE	-0.52**	1.00						
DAE	-0.39	0.35	1.00					
Fe	-0.08	0.07	-0.02	1.00				
Ca	0.36*	-0.55**	-0.23	-0.40*	1.00			
Mg	-0.39*	0.27	-0.05	-0.45**	0.09	1.00		
Pb	-0.83**	0.34	-0.40*	-0.07	-0.12	0.52**	1.00	
K	-0.83**	0.61**	-0.13	-0.17	-0.14	0.45**	0.77**	1.00

** Correlation is significant at $p > 0.01$) (2 tailed). * Correlation is significant at $p > 0.05$ (2 tailed). TAE, Total adult emergents; DAE, days to adult Emergence; Fermt., fermentation.

of *E. cautella* adults that emerged on the cocoa bean samples at 90 days after infestation with day-old larvae. Whereas, percentage composition of carbohydrate significantly ($P > 0.01$) increased with fermentation period of the cocoa beans. Fermentation period of the cocoa beans significantly ($P > 0.05$) lengthened the day to adult emergence of *E. cautella* on the beans, even as the moisture content of the beans significantly decreased. At 99% confidence interval, increase in percentage fat significantly reduced the days to adult emergence of *E. cautella,* but increase in percentage carbohydrate content of the beans significantly lengthened the days to adult emergence. However, increase in percentage fat and metabolizable energy content of the beans significantly ($P > 0.01$) increased the total adult emergents of *E. cautella* on the cocoa beans at 90 days after infestation.

The correlation matrix between total adult emergents (TAE), days to adult emergence (DAE) of *E. cautella* and the mineral values is shown in Table 5. At 99% confidence level, there was a significantly negative correlation between calcium content levels and total adult emergents of *E. cautella* Walker. Also, significantly positive correlation was recorded between the total adult emergents of *E. cautella*, lead content and potassium content of the fermented beans. There was a negative correlation values between the days to adult emergence of *E. cautella* Walker and the mineral values of Fe, Ca, Mg and K which were not significant ($P < 0.01$; $P < 0.05$) except in Pb that was significant ($P > 0.01$). There was a positive correlation between Fe content and the total adult emergents and this is contrary to the findings of Ahmed et al. (2010) that iron fortified wheat resulted in decrease in larval weight of *Tribolium castaneum* as compared to low iron content diet. There was significantly negative correlation ($P > 0.01$) between Ca and Fe but positive correlation between Ca and Mg. There were negative correlations between Ca, Pb and K which were not significant ($P > 0.01$; 0.05). Negative correlation existed between Pb, Fe and Ca while significantly positive correlation existed between Pb and Mg ($P > 0.01$;

Table 6. Correlation between total adult emergents, days to emergence of *E. cautella* and some physicochemical composition of cocoa beans fermented at different days.

	Fermt.	TAE	DAE	Acid value	Peroxide value	Iodine value	Free fatty acid (FFA)	pH
Fermt.	1.00							
TAE	-0.52**	1.00						
DAE	-0.39	0.35	1.00					
Acid Val.	-0.29	0.61**	0.47	1.00				
Perox. Val.	0.30	-0.09	-0.45*	-0.28	1.00			
Iodine Val.	-0.40*	0.50**	0.14	0.68**	0.30	1.00		
FFA	-0.21	0.61**	0.46**	0.96**	-0.27	0.73**	1.00	
pH	-0.66**	0.28	-0.32	0.24	0.29	0.29	0.17	1.00

**Correlation is significant at (p > 0.01), (2 tailed). * Correlation is significant at (p > 0.05), (2 tailed). TAE, Total adult emergents; DAE, days to adult emergence; Fermt., fermentation.

and Pb and K (P>0.01). This study shows that Fe in combination with other mineral elements supported the larval development of *E. cautella* to adult stage at the required proportion in other variedly fermented cocoa beans while Ca at the concentration found in F_4 cocoa beans did not support larval development to adult stage, may be due to ionic imbalance in the larva system and consequent physiological disturbance as reported by Ahmed et al. (2010).

Similarly, the correlation matrix showing the interactions between fermented beans and the physico-chemical properties of fat extracts from the cocoa beans, total adult emergents and days to adult emergence is shown in Table 6. There was no significant differences (P = 0.01; P = 0.05) even as longer fermentation periods, lower the acid value, peroxide value, and free fatty acid values of the fat extract of the cocoa beans fermented for different days. Iodine value (P > 0.05) and pH value (P > 0.01) significantly reduced the longer the fermentation period. Acid value and free fatty acid value significantly (P > 0.01) influenced the total *E. cautella* adult emergents and also causes the days to adult emergence to increase. Similarly, iodine value significantly (P > 0.01) influenced positively the total number of adult emergent.

In conclusion, four-day fermentation of cocoa beans has been found to contain some nutritional and bio-chemical compounds responsible for inhibiting develop-ment of larvae and eventual adult emergence of *E. cautella* on such cocoa beans, but this study need be furthered to assess whether such cocoa beans fit the usage in the chocolate industry or any other medicinal usage, be evaluated for its biocidal and medicinal value at different durations in store.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Selection of *Gossypium hirsutum* genotypes for interspecific introgression from *G. arboreum* using ovule culture

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Ovule culture is one of the techniques currently used to introgress desirable traits from *Gossypium arboreum* germplasm into *G. hirsutum* cultivars. Twenty-six (26) *G. hirsutum* breeding lines were used as female parents in crosses with five *G. arboreum* accessions to determine if the *G. hirsutum* parent influenced the germination and recovery of plants from ovule culture. Variation in boll weight and the number of ovules per boll was observed for crosses with the *G. hirsutum* lines, but heavier bolls and a greater number of ovules per boll were not associated with a higher germination rate. Ovules derived from crosses with 16 *G. hirsutum* lines showed germination. Plants were recovered for seven of these lines (Acala GLS, DES 56, DES 119, Deltapine 50, Stoneville 132, Stoneville 506 and Stoneville 825) with vigorous growing plants derived from four crosses (DES 119 x PI 408763, Stoneville 506 x PI 408763, Acala GLS x PI 529779, and DES 119 x PI 615699). The breeding line DES 119 showed a better success rate and typically produced smaller bolls with fewer ovules. However, results would suggest the *G. arboreum* accessions had a greater influence on the success rate compared to the *G. hirsutum* lines.

Key words: Cotton, germplasm, immature embryo, tissue culture, wide-hybridization.

INTRODUCTION

Tetraploid upland cotton, *Gossypium hirsutum* L., is comprised of over 90% of global cotton production (Zhao et al., 2015). Cultivated *G. hirsutum* genotypes are considered to have a narrow genetic base, due in part to a monophyletic origin and the use of few genotypes in the breeding of new cultivars (Grover et al., 2012; Zhao et al., 2015). In contrast, the diploid species *G. arboreum* L. comprises less than 1% of global cotton production, but is considered an important source of genetic diversity for

several traits (Liu et al., 2006). *Gossypium arboreum* is commonly grown on marginal lands with low inputs and is a source of drought tolerance (Basu, 1996; Maqbool et al., 2007) and pest resistance (Yik and Birchfield, 1984; Thengane et al., 1986; Miyazaki et al., 2012). The ability to transfer genes from *G. arboreum* to *G. hirsutum* is hindered by incompatibility barriers resulting in abortion of developing bolls (Mehetre and Aher, 2004). Techniques such as exogenous hormone application

(Altman, 1988), hexaploid bridging lines (Sacks and Robinson, 2009), *in vitro* interspecific fertilization (Liu et al., 1992), protoplast fusion (Sun et al., 2006), and ovule culture/embryo rescue (Stewart and Hsu, 1978; Gill and Bajaj, 1987) have been used for interspecific hybrid development in cotton. Ovule culture has frequently been used for hybrid development between incompatible diploid species and for crosses between diploid and tetraploid cotton species (Mehetre and Aher, 2004). Several factors can influence the success of ovule culture such as the species being crossed, age of the excised ovules, cultural media, and environmental conditions for culturing and plant growth (Stewart and Hsu, 1978; Mehetre and Aher, 2004). Research has mainly focused on improving the culture media to increase the success rate of germination and plant recovery (Stewart and Hsu, 1978; Thengane et al., 1986; Gill and Bajaj, 1987; Sacks, 2008). Information is lacking to determine if the *G. hirsutum* parent used for interspecific introgression has an influence on the success rate. Thus, the focus of this study was to evaluate 26 *G. hirsutum* breeding lines to determine if the tetraploid parent used in crosses with *G. arboreum* genotypes influenced the growth and germination of immature ovules and the recovery of plants from tissue culture.

MATERIALS AND METHODS

Twenty-six (26) diverse *G. hirsutum* breeding lines (Table 1) and five *G. arboreum* accessions (PI 152088, PI 408763, PI 529724, PI 529779, and PI 615699) were selected from the germplasm collection maintained at the United States Department of Agriculture (USDA) Agricultural Research Service (ARS), Crop Genetics Research Unit in Stoneville, Mississippi. These germplasm lines were planted in the field at the USDA ARS in Stoneville during the 2013 growing season. Each line was planted in a single 3 m row and rows were spaced 0.9 m apart. Standard management practices were conducted for cotton production in Mississippi. The *G. hirsutum* lines were used as the female parent with the *G. arboreum* accessions used as the pollen parent. Flowers of the *G. hirsutum* lines were emasculated by splitting the staminal column with the fingernail and removing the corolla and androecium (Doak, 1934) between 06:30 to 07:30 a.m. Following emasculation, flowers were misted with a single application of a 100 mg L^{-1} solution of gibberellic acid to prolong boll retention (Miravalle, 1964). Pollinations were conducted the same morning between 10:00 to 11:00 a.m. Each emasculated flower was pollinated using a single *G. arboreum* flower. Seven days after pollination, the developing bolls were removed for ovule culture. Bolls were weighed, surface sterilized in an aqueous 0.2 M sodium hypochlorite solution for 15 min., transferred to a 95% ethanol solution for another 15 min., and then air-dried in a biological safety cabinet prior to excision of ovules. The number of immature ovules from each boll was counted. The ovules were cultured on MS basal media (Murashige and Skoog, 1962) with B5 vitamins (Gamborg et al., 1968) and supplemented with 20 g L^{-1} sucrose, 1.9 g L^{-1} potassium nitrate, and 5 g L^{-1} gelrite as a solidifying agent (Sacks, 2008). All ovules from a single boll were cultured in the same 100 × 15 mm sterile plastic Petri plate. Ovules were cultured at 30°C in a growth chamber with a 16 h photoperiod. Ovules were transferred to fresh media every 21 days. Seedlings that developed from the ovules were transferred to magenta plant culture boxes containing

the same media used for ovule culture and grown under the same conditions. Seedlings that developed roots and shoots were planted in pots containing potting media (Metro-Mix 360, Sun Gro Horticulture, Agawam, MA) and covered with a beaker for seven days to prevent desiccation of the developing seedlings. Pots were placed in a growth room at 27°C with a 16 h photoperiod. At flowering, plants were transferred to a greenhouse for further evaluation.

RESULTS AND DISCUSSION

Boll weight and the number of ovules per boll are presented in Table 1. More variation in mean boll weight and mean number of ovules per boll was observed across the *G. hirsutum* breeding lines as compared to the *G. arboreum* accessions. For the *G. hirsutum* breeding lines, the mean number of immature ovules cultured across the five *G. arboreum* accessions ranged from 30 to 42. The breeding line Sure-Grow 747 produced the most ovules across the *G. arboreum* accessions. Mean boll weight across the *G. arboreum* accessions was also greater for Sure-Grow 747 with the highest mean boll weight recorded for Acala GLS. However, the greater number of ovules per boll and higher boll weights did not result in the production of more plants from culture. The breeding line DES 119 showed the lowest mean number of ovules per boll and low mean boll weight, but produced more plants than breeding lines with a greater mean number of ovules per boll and higher boll weights. Across the *G. arboreum* accessions no noticeable trend was apparent. A mean of 37 immature ovules were cultured from the crosses with the *G. arboreum* accessions. Crosses involving accession PI 408763 produced a greater mean number of ovules and heavy bolls across the 26 *G. hirsutum* breeding lines with ovules derived from crosses with PI 408763 and PI 615699 showing a higher frequency of germination (Table 2). However, accession PI 529724, which had the lowest mean boll weight across the 26 breeding lines, produced a greater number of plants. Altman (1988) showed that exogenous hormone application increased boll weight and suggested that heavy bolls may correspond to better quality seed being produced; however, production of plants from *G. hirsutum* x *G. arboreum* crosses was unsuccessful. Flowers for the 26 *G. hirsutum* breeding lines pollinated with *G. arboreum* pollen and not sprayed with gibberellic acid aborted bolls within five days after pollination (data not shown). No comparisons were conducted between bolls treated with gibberellic acid and non-treated bolls to determine if the treatment increased boll weight. Rapid abortion of non-treated bolls was observed for crosses with several *G. hirsutum* lines and the ovules from these bolls failed to grown in culture. The number of ovules per boll was within the range observed for the treated bolls (data not shown); however, no additional data were collected due to poor boll development and ovule growth in culture.

All ovules from the crosses treated with gibberellic acid

Table 1. Boll weight (g) at 7 days after pollination and number of ovules per boll from crosses between 26 *Gossypium hirsutum* breeding lines used as the female parent and five *G. arboreum* accessions (PI 152088, PI 408763, PI 529724, PI 529779, and PI 615699) used as the pollen parent to evaluate the germination and recovery of plants from ovule culture.

Female	PI 152088		PI 408763		PI 529724		PI 529779		PI 615699		Boll weight		Ovules	
	Weight	Ovule	Weight	Ovule	Weight	Ovule	Weight	Ovule	Weight	Ovule	Mean	SD	Mean	SD
Acala GLS	no boll		5.21	40	5.12	40	4.74	37	5.49	39	5.14	0.31	39	1.4
Auburn 82 RNR	4.37	40	4.78	37	3.76	35	4.64	39	3.19	35	4.15	0.66	37	2.3
Auburn 56	4.09	34	2.07	33	1.72	34	1.90	34	4.23	41	2.80	1.24	35	3.3
Bayou 7769	3.52	36	3.39	42	3.05	35	2.61	28	3.92	35	3.30	0.49	35	5.0
Coker 100	3.56	35	0.90	42	1.52	33	2.58	39	2.18	35	2.15	1.02	37	3.6
Coker 312	3.81	38	3.07	46	3.31	36	2.95	35	2.22	45	3.07	0.58	40	5.1
Delcott 277	3.07	36	3.30	35	2.08	31	2.85	39	2.75	39	2.81	0.46	36	3.3
DES 24	3.04	41	3.91	44	3.56	36	3.20	41	4.09	36	3.56	0.45	40	3.5
DES 56	2.93	37	3.32	42	2.18	33	2.49	34	2.78	38	2.74	0.43	37	3.6
DES 119	1.13	27	3.74	34	2.83	35	1.12	20	3.17	35	2.40	1.20	30	6.6
Deltapine 15	2.97	34	4.80	45	3.84	39	3.93	43	4.42	45	3.99	0.69	41	4.7
Deltapine 16	3.57	45	4.24	44	3.06	39	2.62	40	3.94	36	3.48	0.66	41	3.7
Deltapine 50	2.98	32	2.81	37	1.91	36	2.89	34	2.04	31	2.52	0.51	34	2.5
Deltapine 90	2.79	43	3.04	35	2.05	35	2.44	36	3.08	46	2.68	0.43	39	5.1
Empire	5.54	39	6.29	45	3.36	41	3.85	44	4.22	37	4.65	1.22	41	3.3
LA 887	2.54	37	4.26	42	3.67	34	2.85	35	3.27	44	3.32	0.68	38	4.4
MD51ne	3.69	43	3.19	35	3.60	43	2.88	35	2.41	35	3.15	0.53	38	4.4
Sealand 1	3.85	43	2.65	41	2.45	36	5.05	36	4.76	35	3.75	1.19	38	3.6
Stoneville 2	2.40	35	2.06	37	2.40	36	1.50	37	2.14	32	2.10	0.37	35	2.1
Stoneville 132	1.12	25	3.61	38	1.74	30	2.24	39	1.78	38	2.10	0.93	34	6.2
Stoneville 506	2.10	27	3.27	36	3.23	37	3.27	33	3.31	32	3.04	0.52	33	3.9
Stoneville 825	3.21	35	2.84	35	3.46	42	2.76	32	3.57	39	3.17	0.36	37	3.9
Sure-Grow 125	6.17	39	4.53	43	3.82	33	3.70	36	4.21	44	4.48	1.00	39	4.6
Sure-Grow 501	2.48	31	3.85	37	2.62	34	2.91	32	3.13	35	3.00	0.54	34	2.4
Sure-Grow 747	5.04	37	6.03	45	4.58	41	4.89	44	5.02	44	5.11	0.55	42	3.3
STV7A	2.52	32	3.73	45	1.99	40	3.00	34	1.61	14	2.57	0.84	33	11.8
Mean	3.30	36	3.65	40	2.96	36	3.07	36	3.34	37				
SD[1]	1.18	5.2	1.19	4.2	0.92	3.4	0.99	5.0	1.04	6.4				

[1]SD = standard deviation.

showed growth in culture. Variation in ovule growth within and between crosses was observed.

Some ovules showed more than a 10-fold increase in size. These ovules that showed greater growth in culture typically did not germinate and produce seedlings. Germination

Table 2. Number of ovules showing germination in tissue culture and the number of plants recovered from crosses between 26 *Gossypium hirsutum* breeding lines used as the female parent and five *G. arboreum* accessions (PI 152088, PI 408763, PI 529724, PI 529779, and PI 615699) used as the pollen parent. All plants derived from crosses with *G. arboreum* accession PI 529724 died at the flowering stage.

Female	PI 152088		PI 408763		PI 529724		PI 529779		PI 615699	
	Germination	Plant	Germination	Plant	Germination	Plant	Germination	Plant	Germination	Plant
Acala GLS	no boll	no boll	0	0	0	0	1	1	0	0
Auburn 82 RNR	0	0	0	0	0	0	1	0	2	0
Auburn 56	0	0	0	0	0	0	1	0	4	0
Bayou 7769	0	0	0	0	0	0	0	0	0	0
Coker 100	0	0	0	0	0	0	0	0	0	0
Coker 312	0	0	0	0	0	0	0	0	0	0
Delcott 277	0	0	0	0	0	0	0	0	1	0
DES 24	0	0	1	0	0	0	0	0	1	0
DES 56	0	0	0	0	1	1	0	0	0	0
DES 119	0	0	3	1	0	0	0	0	1	1
Deltapine 15	0	0	5	0	0	0	0	0	1	0
Deltapine 16	0	0	0	0	0	0	0	0	0	0
Deltapine 50	0	0	0	0	1	1	0	0	0	0
Deltapine 90	0	0	0	0	0	0	0	0	0	0
Empire	0	0	0	0	0	0	0	0	0	0
LA 887	0	0	1	0	0	0	0	0	1	0
MD51ne	0	0	0	0	0	0	0	0	1	0
Sealand 1	0	0	0	0	0	0	0	0	0	0
Stoneville 2	0	0	0	0	0	0	0	0	0	0
Stoneville 132	0	0	0	0	1	1	0	0	0	0
Stoneville 506	0	0	2	1	0	0	0	0	0	0
Stoneville 825	0	0	0	0	1	1	0	0	1	0
Sure-Grow 125	0	0	2	0	0	0	0	0	0	0
Sure-Grow 501	0	0	0	0	2	0	0	0	1	0
Sure-Grow 747	0	0	0	0	0	0	0	0	0	0
STV7A	0	0	0	0	0	0	0	0	0	0

varied among the ovules derived from the crosses (Table 2). Germination was low compared to results reported by Stewart and Hsu (1978), Gill and Bajaj (1987), and Sacks (2008), but similar to the results reported by Altman et al. (1987) and Altman (1988). Germination was not observed for ovules derived from 10 (Bayou 7769, Coker 100, Coker 312, Deltapine 16, Deltapine 90, Empire, Sealand 1, Stoneville 2, STV7A, and Sure-Grow 747) of the 26 breeding lines. Ovule germination was not observed from *G. hirsutum* crosses with more than two *G. arboretum* accessions. Variation

in ovule germination was also observed across the *G. arboreum* accessions. Ovules derived from *G. hirsutum* crosses with accessions PI 408763 and PI 615699 showed better germination and among a greater number of *G. hirsutum* lines. Germination was observed from crosses with *G. arboreum* accession PI 615699 and 10 of the breeding lines (Auburn 82 RNR, Auburn 56, Deltapine 15, Delcott 277, DES 24, DES 119, MD51ne, LA 887, Stoneville 825, and Sure-Grow 501). In comparison, germination was not observed for ovules derived from any of the crosses with PI 152088.

Many of the developing seedlings showed little or no additional growth when transferred to media in magenta boxes. Other seedlings showed abnormal growth and failed to survive when transplanted into potting media. Seedlings that germinated from ovules derived from crosses between accession PI 529724 and the breeding lines Deltapine 50, DES 56, Stoneville 132, and Stoneville 825, successfully developed into plants; however, when these plants produced flowers they started to wither and die. These plants failed to develop a vigorous root system to support the growth of the plants. Other researchers have reported abnormal seedling growth and poor plant survival (Thengane et al., 1986; Gill and Bajaj, 1987). In crosses involving three of five *G. arboreum* accessions (PI 408763, PI 615699, and PI 529779), healthy and vigorous plants have been recovered (Table 2). Plants have been recovered from crosses DES 119 x PI 408763, Stoneville 506 x PI 408763, Acala GLS x PI 529779, and DES 119 x PI 615699.

Germination was observed for cultured ovules derived from crosses with 16 *G. hirsutum* breeding lines and plants were obtained for seven of these lines (Acala GLS, DES 56, DES 119, Deltapine 50, Stoneville 132, Stoneville 506, and Stoneville 825). Ovules derived from crosses with DES 119 produced more plants hence this line would be a desirable selection. Other *G. hirsutum* lines to be considered include Acala GLS, Auburn 56, DES 24, DES 56, Deltapine 15, Deltapine 50, Sure-Grow 501, Stoneville 132, Stoneville 506, and Stoneville 825. No cultured ovules showed germination from crosses with Deltapine 90; however, four additional crosses between Deltapine 90, and accessions PI 408763 and PI 529724, did result in the recovery of a single plant for each accession. The plant recovered from the Deltapine 90 x PI 529724 crosses failed to survive as was observed for the other plants derived from crosses with this accession. Multiple interspecific crosses were also conducted with Sure-Grow 747, but no plants were recovered from these additional crosses. However, cultured ovules derived from crosses with Sure-Grow 747 and other *G. arboreum* accessions have resulted in the production of plants at a low frequency with the recovery of one plant from crosses with 24 accessions. Sure-Grow 747 is a source of superior fiber quality traits, but the unsuccessful recovery of plants for the majority of the interspecific crosses would suggest other *G. hirsutum* lines evaluated in this study would be more desirable for ovule culture. These results would indicate that evaluation of advance breeding lines or recently release *G. hirsutum* cultivars would be useful for the identification of superior lines for ovule culture. The use of improved cultivars would reduce the time required to recover desirable yield and fiber quality traits. However, the *G. arboreum* accession used in the cross may have a greater influence on the recovery rate and survival of plants compare to the *G. hirsutum* parent. Multiple factors such as tissue culture media, parental genotypes, age of immature ovules cultured, and environment conditions (Mehetre and Aher, 2004) can influence the germination for cultured ovules and the recovery of plants. In this study, germination rates were low and modification to the media could increase the recovery of plants. Several media formulations have been published, but similar results across laboratories have not been achieved (Altman, 1988; Sacks, 2008). Selection of a subset of *G. hirsutum* breeding lines and multiples crosses to the lines would increase the likelihood of generating plants from ovule culture. For some *G. arboreum* accessions, such as PI 529724, other approaches may be required to recover plants. Crosses with this accession and several breeding lines resulted in plants, but all plants failed to survive. Whereas, the success rate for other *G. arboreum* accessions, such as PI 615699, was greater with ovule germination observed from crosses with numerous *G. hirsutum* breeding lines. The successful recovery of plants from three *G. arboreum* accessions used in interspecific crosses provides breeding lines for further evaluation in the introgression of desirable traits for cotton improvement.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Regulation of major cultural components for designing a cost effective medium to increase δ-endotoxin synthesis by *Bacillus thuringiensis*

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The present study was aimed at designing a cost effective medium for increasing the δ-endotoxin (Cry protein) synthesis by *Bacillus thuringiensis (Bt) kurstaki* HD-73 and indigenous *Bt* JSc1 harboring potential *cry* genes active against Lepidoptera insect orders. In this regard, locally available cheap molasses as carbon source, soybean extract as nitrogen source, sea water as trace elements source, cystine as sporulation/growth factor were considered to design a cost effective medium. Molasses and soybean extract in place of glucose and peptone in glucose-peptone (GP) medium supported 78.85% increase in δ-endotoxin synthesis in shake flask culture. The effect of cystine on endotoxin synthesis was highly pronounced in two media with a range of 80.32 to 110% higher δ-endotoxin under comparable fermentation conditions. While, substituting basal salts with sea water, *Btk* HD-73 yielded satisfactory and comparable endotoxin (74.3% of yield with basal salts). It was detected that the rapid decrease of endotoxin synthesis in the culture after 24 h was due to the degradation by the endogenous protease, synthesized with the progress of fermentation. This degradation of the endotoxin was much better protected (1.23 mg/ml endotoxin versus 0.312 mg/ml) by adding 4% ammonium sulfate in the optimized medium. The medium thus formulated with molasses, soybean extract, ammonium sulfate, cystine and sea water was then used in 3.0 L bioreactor cultivation for endotoxin synthesis by both *Btk* HD-73 and *Bt* JSc1 under 30% saturation of dO$_2$ through cascade control of agitation and aeration producing a higher yield of δ-endotoxin (2.1 and 2.63 mg/ml, respectively). The present results may successfully be used for large scale production of *Bt* biopesticide in Bangladesh.

Key words: *Bacillus thuringiensis* biopesticide, cheap substrates, cystine, protease activity, ammonium sulfate.

INTRODUCTION

The control of pest populations by using biological pesticides has been an attractive alternative to the application of chemical pesticides (Glazer and Nikaido, 1995; Ciche and Ensign, 2003). As hazardous and recalcitrant chemical agents damage the environment by causing soil, water and air pollutions and also triggers development of insect resistance, biological pest management is more preferable for the specific toxicity of

its component (Massaoud et al., 2010). An entomopathogenic bacterium, *Bacillus thuringiensis (Bt)*, has been used for more than half a century as important biopesticide of which during sporulation produces crystal (Cry) proteins, toxic against various pests (Özkan et al., 2003). Although, *Bt* based microbial insecticides are available in world market, the high cost makes its application impracticable in large-scale in developing countries. The use of *Bt* as commercial insecticides would remain prohibitively and relatively expensive if it is not produced with high titer of insecticidal proteins by large-scale fermentation. More attention has been given to the regulation mechanisms that ensure the efficient production of the insecticidal proteins which could be achieved by application of an adequate fermentation technology (Zouari et al., 2002), essentially with use of appropriate media (Zouari and Jaoua, 1999), overcoming metabolic limitations (Zouari et al., 2002) etc. It was reported that the commercial application depends on the cost of raw materials, strain efficiency, fermentation cycle, maintenance of process parameters, bioprocessing of fermentation fluid, and formulation of the final product. The cost of raw materials is one of the principal costs involved in overall *Bt* production. In the conventional *Bt* production process, the cost of raw materials varied between 30 and 40% of the total cost depending on the plant production capacity (Ejiofore, 1991). Therefore, local production of this insecticide in developing countries should depend on the use of production media made of cheap, locally available sources including agro-industrial by-products (Ampofo, 1995).

For large scale production of *Bt* biopesticide, different approaches were investigated to develop suitable media that could support good production of spores and toxins at reasonable costs. Various agricultural and industrial by-products used as raw material in *Bt* production were citrus peels, wheat bran, corn meal, seeds of dates, beef blood, silkworm pupal skin, ground nut cake, cane molasses, fish meal, cotton seed meal, soybean meal, residues from chicken slaughter house, fodder yeast, cheese whey and corn steep liquor etc (El-Bendary, 2006). Agro-industrial residues and by-products available in southeastern Brazil were used as ingredients for low-cost culture media for liquid fermentation of *B. thuringiensis* var. *kurstaki*. Highest spore yield was obtained with a medium containing cheese whey, soya bean milk and molasses (Alves et al., 1997). Other wastes, such as, sludge and broiler poultry litters were also utilized for biopesticides production (Adams et al., 2002; Vidyarthi et al., 2002). In general, two methods of fermentations are used for production of microbial products, submerged fermentation and solid state fermentation. *Bt* biopesticides are usually composed of spores and crystals protein mixtures, harvested from the production media, readily produced by aerated liquid fermentation. They are easily harvested and have a long shelf life when formulated properly (Ghribi et al., 2007).

Optimizing different culture conditions and regulating some critical factors, it is possible to obtain higher yield in terms of cell mass, Cry protein concentration and toxicity to develop efficient *Bt* formulations (Dulmage et al., 1990). Critical factors e.g. sugars have significant impact on cell growth but when used at high concentrations, they can cause adverse effects on sporulation due to the acids produced by *Bt* δ-endotoxin from carbohydrates (Dulmage et al., 1990) and moreover, the balance of the ratio between carbon and nitrogen, itself is directly important for the crystal protein production (Farrera et al., 1998).

Other important components for the production of crystal proteins are the trace minerals (Rose, 1979). Again, amino acids are important in the formation of spores and crystal proteins (Sachidanandham et al., 1997). Moreover, it was reported that decreasing the proteolytic activity in the fermentation medium increased the accumulation of δ-endotoxin in the insecticidal crystal proteins (Ennouri et al., 2013). Indiscriminate use of chemical pesticide is a common practice in Bangladesh, an agriculture dependant developing country, where agro-industrial wastes like defatted soybean meal, defatted mustard seed meal, molasses, rice husk, rice bran, citrus peels etc are generated in huge amount every year. The present study was, therefore, carried out with a view to design a cost effective medium comparing the effects of carbon and nitrogen sources, amino acid such as cystine and basal salts on growth, sporulation and δ- endotoxin synthesis for large scale production of *Bt* biopesticide with locally available cheap raw materials such as defatted soybean extract and molasses as nitrogen and carbon source respectively and sea water as the substituent of basal salts. The present study also reports the regulation of certain critical factors that affect the growth, sporulation and δ-endotoxin synthesis by reference *Btk* HD-73 and indigenous *Bt* strain JSc1.

MATERIALS AND METHODS

B. thuringiensis strains and inoculum

Reference strain *B. thuringiensis kurstaki (Btk)* HD-73 and the indigenous *Bt* strain JSc1 were used. Reference strain *B. thuringiensis kurstaki (Btk)* HD-73 was kindly provided by Okayama University, Japan from their *Bt* stock collection and the strain *Bt* JSc1 was isolated from Bangladesh (Shishir et al., 2014). Inoculum was prepared by inoculating a single *Bt* colony into a 250 ml Erlenmeyer flask containing 50 ml of Luria Bertani (LB) broth (per litre: tryptone 10.0 g, yeast extract 5.0 g, NaCl 10.0 g) and incubated 12 h at 30°C and 180 rpm. Each time, inoculum was added into the medium aseptically with sterile micropipette in a manner so that the process starts with an $OD_{600nm} = 0.1$.

Culture medium for bio-insecticide production

Glucose-peptone medium (GP) [10% (w/v) glucose and 5% (w/v) peptone] (Vora and Shethna, 1999) was used either as the control or for substituting the carbon and nitrogen sources, respectively, by

0.5% (w/v) molasses and 10% (w/v) soybean extract (Soybean extract molasses- SeM medium). Optimum cystine concentrations was determined by supplementing it in GP medium. Cystine supplement (10% cystine stock was prepared by suspending 6.0 g cystine in 60.0 ml phosphate buffer) was maintained from 200 to 600 mg/l. The minerals of the soybean extract-molasses (SeM) medium: [(g/l): KH_2PO_4 - 1.0, $MgSO_4.7H_2O$- 0.5, $MnSO_4.H_2O$- 0.1, $FeSO_4.7H_2O$- 0.02, $ZnSO_4.H_2O$- 0.02, $CaCl_2$- 0.01] was substituted with 20% (v/v) sea water (Filtered with Whattman filter paper 1) to formulate Soybean extract molasses sea water medium (SSeM). Ammonium sulfate [4% (w/v)] was added into the medium to inhibit the protease synthesis by *Bt* strains (Liu and Hsieh, 1969). The pH of the medium was adjusted to 6.8 by adding 50 mM phosphate buffer before autoclaving at 121°C for 20 min. Thus, considering different factors and comparing them statistically by paired sample t- test analysis, the final formulation of the medium was 10% soybean extract, 0.5% molasses, cystine 300 mg/l and 20 ml sea water supplemented with ammonium sulphate at a concentration of 4%.

Bioinsectide production

Production in shake flasks

The fermentation method performed in this study was submerged and Batch type. Each flask containing 100 ml of different media were inoculated aseptically with the bacterial inocula of *Btk* HD-73 and *Bt* JSc1 as described in section 2.1 and incubated in an orbital shaker at 30°C with 180 rpm for 72 h. The experiments were performed in triplicate and at least three samples were collected in each case at 24 h interval both from shake flasks and bioreactor for spore count, estimation of δ-endotoxin concentration.

Production in 3.0 L bioreactor

Production experiments were carried out at 30°C in a 3.0 L fully controlled bioreactor (New Brunswick Scientific, USA) containing 2.0 L of finally optimized medium (10% soybean extract, 0.5% molasses, 20 ml sea water, 300 mg/l cystine and 4% ammonium sulfate) as in shake flask. Dissolved oxygen (dO_2) level in the medium was automatically controlled and maintained at 30% by providing aeration, set at 1.0 standard liter per minute (SLPM) and agitation at 250 rpm. Dissolved oxygen was continuously monitored by an oxygen sensor but the pH was not controlled.

Estimation of spore count

The spore counting was performed each time in triplicate with 1.0 ml of sample collected during the culture both from shake flask and bioreactor. It was then heat treated at 80°C for 15 min, serially diluted in sterile distilled water and inoculated 0.1 ml sample using glass rod spreader on the LB agar medium by spread plate method. The plates were then incubated at 30°C for 24 h. The colonies were then counted and multiplied by the dilution factor to estimate their number.

Partial purification and determination of δ-endotoxin concentration

The purification of crystal protein was done by the modified method of Liu et al. (1994) and Öztürk et al. (2009) where 1 ml culture was washed twice with sterile distilled water by centrifugation at 10000 rpm for 10 min. The pellet was treated with 1.0 M NaCl and 5.0 mM EDTA and later with 5.0 mM EDTA alone. Finally, the pellet was re-suspended in 1.0 ml 0.1 N NaOH solution for 1 h at room temperature. Thus, the partially purified crystal protein concentration in the supernatant was estimated by Bradford method (Bradford, 1976).

Proteolytic activity assay

Protease activity was determined by a modified method described by Kreger and Lockwood (1981) using azo-casein (Sigma, USA) as the substrate. In this enzyme assay, 400 µl of the culture supernatant and 400 µl of 1% azo-casein solution (suspended in 0.05 M Tris- HCl; pH 8.5) was taken in microfuge tube. The mixture was then incubated in a water bath at 37°C for 60 minutes. The reaction was stopped by adding 135 µl of 35% TCA and kept on ice for 15 min. The solution was then centrifuged at 13000 rpm for 10 min and 750 µl of the supernatant was collected in which equal volume of freshly prepared 1.0 N NaOH was added by gentle mixing. Absorbance (OD_{440nm}) of the solution was then measured using the solution from a parallel reaction as blank where TCA was added before the enzyme. Enzyme activity was then estimated from the absorbance [OD_{440nm} = 1.0 is equivalent to 100 U Enzyme activity].

SDS-PAGE analysis of delta- endotoxin

SDS-PAGE analysis was performed with the partially purified δ-endotoxin in a 10% separating gel recovered from $(NH_4)_2SO_4$ supplemented and non-supplemented medium to see its influence on inhibiting protease activity (Sambrook et al., 1989).

RESULTS

Efficiency of molasses and soybean extract as C and N source

The δ-endotoxin production was found to be higher in soybean extract-molasses (SeM) medium in comparison to the glucose-peptone (GP) medium by *Btk* HD-73 (Table 1) which can be correlated to the replacement of glucose and peptone with 0.5% molasses and 10% soybean extract as these two formulations differed in carbon and nitrogen sources only. At 24 h, the δ-endotoxin concentration was found to be 0.2 mg/ml in SeM medium which was 78.85% higher than that (0.042 mg/ml) in GP medium. In paired sample t- test analysis, significant difference in endotoxin yield between GP (M = 0.0445, SD = 0.0139) and SeM (M = 0.20933, SD = 0.0139) conditions; t (4) = 14.48 and p = 0.05; was found.

Role of cystine on growth, sporulation and δ-endotoxin synthesis

The maximum sporulation (11.31 $Log_{10}CFU/ml$) and δ-endotoxin yield (0.215 mg/ml) were obtained at 300 mg/l of cystine at 24 h (Figure 1). The role of cystine (300 mg/l) was also tested in SeM medium and an increase of 1 log in spore concentration and 2 fold in δ- endotoxin yield were observed (Figure 2). In paired sample t-test analysis, there was significant difference in the endotoxin

Table 1. Effect of carbon and nitrogen sources on sporulation and δ-endotoxin synthesis by *Bacillus thuringiensis kurstaki (Btk)* HD-73.

Media	Time (h)	Spore count (Log10 CFU/ml)	δ- endotoxin concentration (mg/ml)
GP medium	24	7.32±0.1	0.0423±0.0478
	48	8.13±0.02	0.13±0.034
SeM medium	24	6.024±0.149	0.2±0.076
	48	6.854±0.24	0.187±0.028

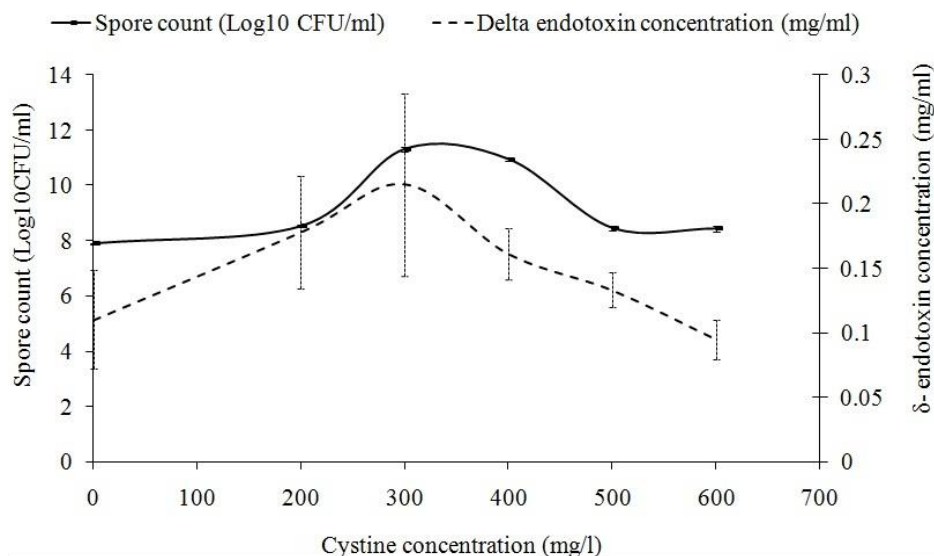GP = Glucose peptone medium; SeM = Soybean extract molasses medium.

Figure 1. Determination of optimum concentration of cystine (300 mg/l) from the curves of spore concentration (11.31 Log10 CFU/ml) and δ-endotoxin concentration (0.215 mg/ml) by *Btk* HD-73 in Glucose-Peptone (GP) medium. (Culture Period: 36 h).
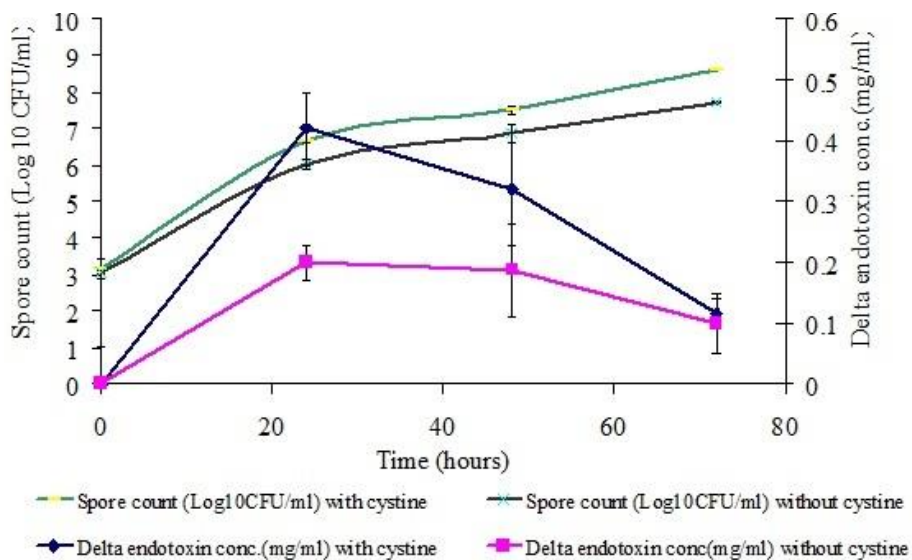

Figure 2. Cystine enhanced both endotoxin yield and sporulation by *Btk* HD-73 similarly in Soybean extract Molasses (SeM) medium.

Table 2. Efficacy comparison between Sea water and basal salts based on sporulation and δ- endotoxin synthesis.

Time (h)	Mineral source	Spore count (Log_{10}CFU/ml)	δ- endotoxin concentration (mg/ml)
0	Basal salts	3.162±0.271	0.001±0.008
	Sea water	3.073±0.0466	0.0014±0.03
24	Basal salts	6.352±0.032	0.420±0.06
	Sea water	6.428±0.072	0.311667±0.0185
48	Basal salts	7.512±0.12	0.320±0.092
	Sea water	6.88±0.047	0.1023 ±0.025
72	Basal salts	8.596±0.0064	0.116±0.024
	Sea water	7.812±0.068	0.075±0.0126

yield at 24 h with cystine (M = 0.42666, SD = 0.061) and without cystine (M = 0.212, SD = 0.061) condition; t (4) = 4.29, p = 0.05. Thus, significant increase in δ- endotoxin yield occurred in the presence of cystine and 80.32 and 110% increase in δ- endotoxin yields were resulted in both GP and SeM media, respectively by *Btk* HD-73 under comparable conditions.

Efficacy of sea water as basal salts substituent

The efficacy of sea water (20%) in sporulation and δ-endotoxin synthesis was comparable with that of basal salts. With both ingredients, maximum sporulation and δ-endotoxin synthesis were obtained at 72 and 24 h, respectively (Table 2). No significant difference was found between endotoxin yield with basal salt (M = 0.4166, SD = 0.384) and sea water (M = 0.313, SD = 0.384) condition; t (4) = 0.33, p = 0.05; and sporulation with basal salt (M = 6.344, SD = 0.058) and sea water (M = 6.439, SD = 0.058) condition; t (4) = 2.00, p = 0.05 at 24 h by paired sample t-test analysis. Maximum yields of sea water in spore count and δ-endotoxin concentration were respectively 90.88 and 74.29% of Basal salt.

Protection of δ-endotoxin degradation by endogenous protease

Sharp decrease in δ- endotoxin concentration was observed to be simultaneous with the rise of endogenous protease activity after 24 h determined by protease assay (Figure 3). When the protease activity reached its maximum, that is, 105.9 U/ml gradually, the δ-endotoxin concentration was also reduced to its minimum (0.075 mg/ml) from the peak (0.312 mg/ml). Protease inhibitor PMSF was added into the culture medium at 0.1 and 0.3 mM concentration to control the proteolytic degradation of δ-endotoxin which inhibited the protease activity partially (Data not shown). As an alternative of PMSF, ammonium

sulfate was used and the protease activity was monitored up to 72 h at 24 h interval. Ammonium sulfate (4%) resulted in maximum endotoxin yield (1.2 mg/ml) as well as restricted the protease activity within 10.1 U/ml (Table 3). This result corresponded to a 295% increase in δ-endotoxin productivity ($51.43×10^{-3}$ g/L/h) in presence of 4% ammonium sulfate. Significant differences in δ-endotoxin yield and enzyme activity in the presence and absence of ammonium sulfate were observed while performing the statistical paired sample t-test analysis. The t (4) =62.08, p=0.05 was obtained under condition with endotoxin yield without ammonium sulfate (M=0.32, SD= 0.018) and with ammonium sulfate (M=1.2276, SD= 0.018) at 24 hours. Similarly, the enzyme activity [At 24 hours, enzyme activity without ammonium sulfate (M=44.24, SD= 1.863) and with ammonium sulfate (M= 4.30, SD=1.863) condition; t(4) = 26.2661, p=0.05)]was found to decrease significantly in the presence of ammonium sulfate in paired sample t-test analysis.

Production of reference and indigenous strains in 3.0 L bioreactor

Finally, optimized medium containing soybean extract, molasses, sea water, cystine and 4% ammonium sulfate was used for the production of *Btk* HD-73 and the indigenous *Bt* JSc1 under controlled conditions in 3.0 L bioreactor with an working volume of 2.0 L. Maximum δ-endotoxin yields were 2100 and 2630 mg/l by *Btk* HD-73 and *Bt* JSc1, respectively, at 24 h (Figure 4). Protease activity was negligible for both strains. The indigenous *Bt* JSc1 resulted in 25% higher δ-endotoxin yield than the reference strain.

Qualitative analysis of effect of ammonium sulfate by SDS-PAGE

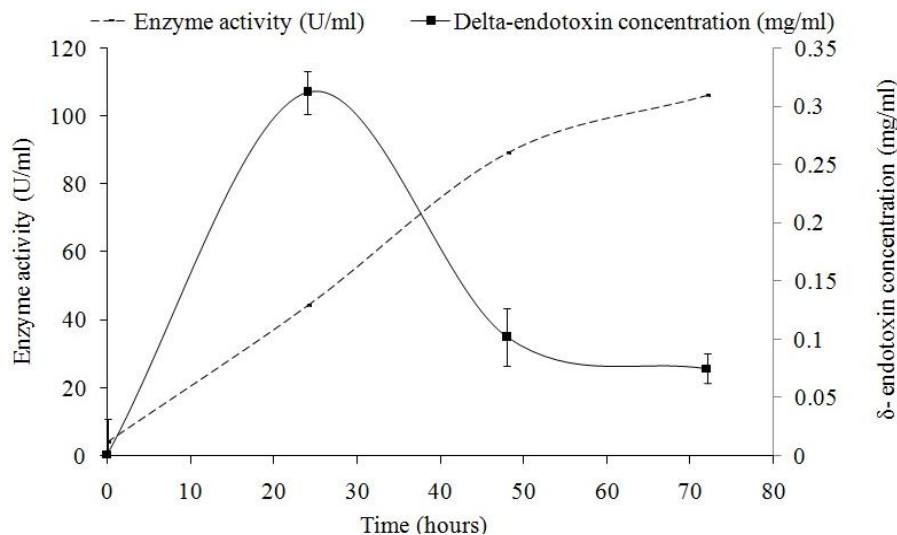Prevention of δ- endotoxin degradation due to

Figure 3. Comparison between increase protease activity and δ-endotoxin degradation with time occurred with *Btk* HD-73 in cystine supplemented SeM sea water medium.

Table 3. Effect of Ammonium sulfate on protease synthesis and δ- endotoxin breakdown in cystine supplemented SeM sea water medium.

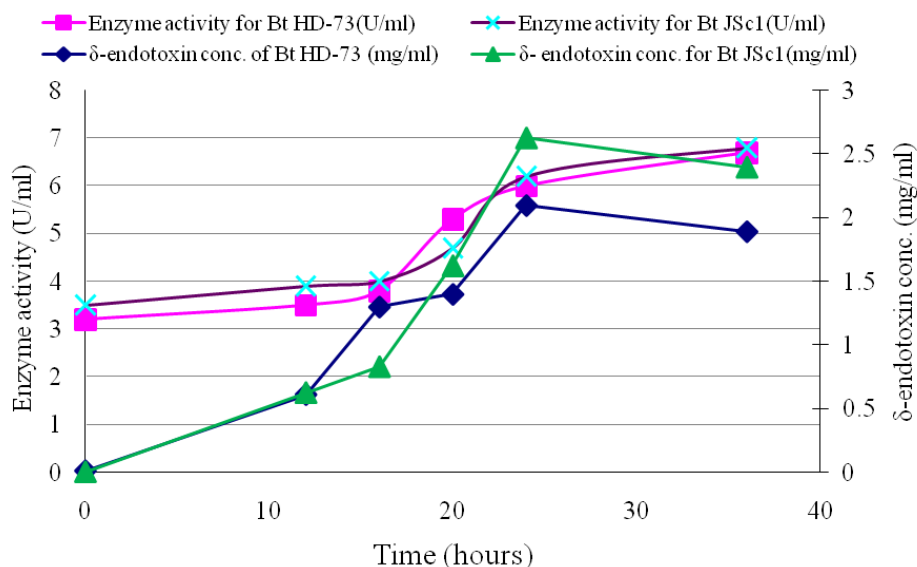| Time (h)	δ- endotoxin concentration (mg/ml)		Enzyme activity (U/ml)	
	Without as	With as	Without as	With as
0	0.0016±0.03	0.0011±0.034	4.21±0.020	3.93±0.132
24	0.312±0.0185	1.2343±0.0153	44.2±0.098	4±0.157
48	0.102±0.0025	0.9027±0.0379	89.2±0.056	9.3±0.047
72	0.075±0.0126	0.839±0.0529	105.9±0.038	10.1±0.116

as = Ammonium sulfate.

Figure 4. Production kinetics of *Bt* JSc1 and *Btk* HD-73 in cystine and ammonium sulfate supplemented SeM sea water medium in a 3.0 L bioreactor.
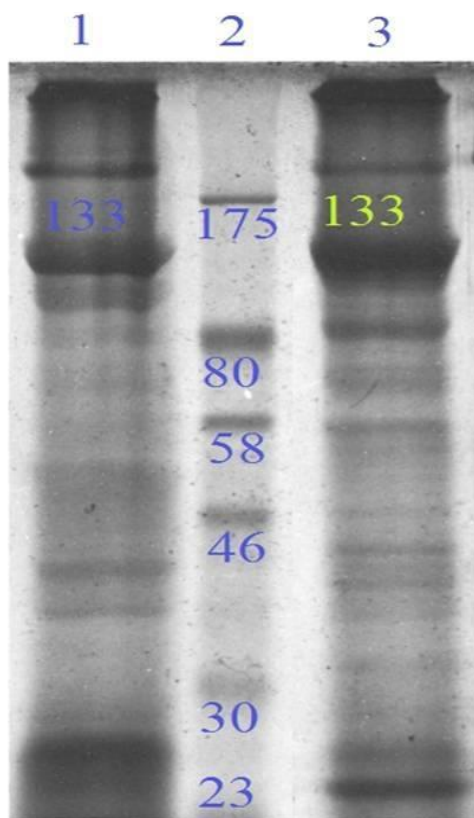
Figure 5. SDS-PAGE analysis of δ-endotoxin partially purified from the cystine supplemented SeM sea water agar medium with and without 4% ammonium sulfate. Lane 1, Without $(NH_4)_2SO_4$; lane 2, marker (ColorPlus Prestained protein marker, Broad range, NEB); lane 3, $(NH_4)_2SO_4$ supplemented.

endogenous protease by ammonium sulfate was visualized by SDS-PAGE analysis (Figure 5). Comparatively thicker band of partially purified 133 kD Cry1Ac protein was observed in the presence of ammonium sulfate than in its absence.

DISCUSSION

One of the main reasons of absence of *Bt* biopesticide in the Integrated Pest Management in Bangladesh agriculture is that it is not yet produced locally. So, industrial production of *Bt* biopesticide can facilitate its application in eco-friendly pest management for which higher yield in spore and δ- endotoxin production with low cost medium is one of the prerequisites to keep the product within farmers' buying capacity. In this connection, present study was performed to formulate a low cost medium regulating different cultural components for higher yield. It was reported that high yield of endotoxin

could be achieved by using inexpensive defatted soybean, ground nut seed meal extract for large scale production of *Bt* biopesticide (Vora and Shethna, 1999). In present study, inexpensive substrate such as molasses and soybean was used as carbon and nitrogen sources for biopesticide production instead of relatively expensive glucose and peptone present in commercial media. Soybean extract and Molasses acted as excellent substituent of peptone and glucose respectively as medium containing soybean extract and molasses resulted in 78.85% higher endotoxin yield than that of GP medium. Molasses is cheap, available throughout the year and easy to store. The defatted soybean meal is also a low cost, readily available item and easy to handle. So, a major cost for the production of *Bt* biopesticide might receive a 10-fold reduction. On the other hand, the increase in δ-endotoxin yield may be due to complex carbon and nitrogen source that permits high sporulation and biomass production.

The optimum concentration of cystine was determined to be 300 mg/l which enhanced the sporulation and δ-endotoxin synthesis by *Bt* strains whereas higher concentration inhibited this phenomena. The cystine was also found to keep statistically significant positive impact on yield. This increase could be explained as the fact that cystine might have interfered with some of the macromolecular changes during sporulation and parasporal crystal formation (Rajalakshmi and Shethna, 1980). On the other hand, when basal salts were replaced with sea water containing most of the minerals, comparable effects on δ-endotoxin synthesis and sporulation were observed as ca. 75 and 90% yield respectively of basal salts was obtained and the differences were not significant at 95% confidence level. So, another cost incurring ingredient, that is, minerals could successfully be replaced with sea water without much negative influence on yield. It was reported in a study that 20% sea water (source: Mediterranean sea) improved the yield in δ-endotoxin concentration and spore count by 2 and 4%, respectively (Ghribi et al., 2007) whereas this study reports slightly lower yield. It indicates that the yield might be variable based on the source of sea water too. Endogenous protease activity was responsible for the decrease in δ-endotoxin concentration that occurred after 24 h (Figure 3). So, initially PMSF and later ammonium sulfate were used to control this protease synthesis and to protect δ-endotoxin degradation. PMSF inhibited the protease synthesis partially and it might be due to the fact that *Btk* HD-73 secreted not only serine protease but also some other classes of proteases which could not be inhibited by this serine protease inhibitor (PMSF). But 4% ammonium sulfate inhibited protease synthesis (10.1 U/ml) to great extent and also resulted in higher endotoxin yield (1.23 mg/ml).

Finally, the medium containing 10% soybean extract, 0.5% molasses, 20% sea water, 300 mg/l cystine and 4%

ammonium sulfate thus found suitable for optimum production of both spores and δ-endotoxin in shake flask culture, was selected for bioprocess development in a 3.0 L bioreactor. From this study, it was found that the fermentation broth should be harvested at 24 h for the strains both reference, *Btk* HD-73 and indigenous, *Bt* JSc1 to recover δ-endotoxin at its maximum yield which also reduces the power consumption, hence cost. So, a low cost medium was designed thus which will facilitate large scale industrial production of *Bt* biopesticide in Bangladesh in a cost effective manner.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Evaluation of the antibacterial activity of *Syzygium cordatum* fruit-pulp and seed extracts against bacterial strains implicated in gastrointestinal tract infections

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Gastrointestinal tract (GIT) infections are the major cause of high morbidity and mortality rates, especially in the developing countries. Fruit and seed extracts possess phytochemicals that are active against bacterial strains implicated in GIT infections. Different parts of *Syzygium cordatum* trees have been investigated pharmacologically against GIT infections previously with the exception of the fruits and seeds. This study aimed at evaluating the antibacterial activity of *S. cordatum* fruits and seeds against bacteria causing GIT infections. The harvested fruits were separated into fruit-pulp and seeds, dried and extracted with methanol using Soxhlet extraction. The extracts were phytochemically screened and micro dilution assay was used to evaluate antibacterial activity of the fruit-pulp and seed extracts against the selected GIT infecting bacteria. The crude extracts of fruit-pulp and seed exhibited the percentage yield of 10 and 6, respectively. The extracts showed the presence of phytochemicals with the total phenolic content of 21.4±1.4 μg/ml for seed extract and 16.4±1.8 μg/ml for fruit-pulp extract. Antimicrobial activity of the pulp extract exhibited the lowest minimum inhibitory concentration (MIC) of 3.13 mg/ml against *Bacillus cereus* (ATCC 10102), *Staphylococcus aureus* (ATCC 25925), *Klebsiella pneumoniae* (ATCC 4352), *Pseudomonas aeruginosa* (ATCC 7700), *Enterococcus hirae* (ATCC 8043) while the seed extract had an equal MIC value against *Klebsiella pneumoniae* (ATCC 4352). The antimicrobial activity was due to the detected phytochemicals and thus promoting *S. cordatum* fruits and seeds as potential new and cost effective sources for prevention and treatment of GIT infections.

Key words: Gastrointestinal, fruits, seeds, phytochemicals.

INTRODUCTION

Gastrointestinal tract (GIT) infections are the major cause of high morbidity and mortality rates, especially in the developing countries. Approximately more than 1.5 billion episodes of GIT infections that result in more than 3 million deaths are reported per year in the developing countries (WHO, 2009). The microbial resistance to most of the available drugs, the prohibitive costs of treatments consequent upon this resistance, the negative side

effects of allopathic medicine and the newly emerging GIT infections have necessitated more research for novel, efficient, safe, and cost effective therapeutic compounds for the prevention and treatment of GIT infectious (El-Mahmood and Doughari, 2008; WHO, 2014). Plants extracts have served as good sources of antimicrobial agents against various pathogenic microorganisms implicated in GIT infections (Pradeep et al., 2008). The pharmacological activity of plant extracts against microbial strains causing GIT infections depends on the presence and concentrations of phytochemicals (Arup et al., 2012). According to Neethirajan et al. (2012), phytochemicals have strong antimicrobial and gastro-protective properties. Apart from the proven antimicrobial efficacy attributed to phytochemicals, plant based antimicrobial compounds possess the margin of safety without toxic side effects and also little chance of development of resistance to microorganisms (Fennell et al., 2004; El-Mahmood and Doughari, 2008; Ahmed et al., 2013; Gakunga et al., 2013). Although, fruits and seeds are excellent sources of therapeutic phytochemicals, fruits and seeds have been rarely used as medicine (van Wyk et al., 2009; Kossah et al., 2011; Srividhya et al., 2013). However, the increasing interest in novel sources for medicine against GIT infections has elevated more research for new therapeutic compounds from fruits and seeds especially from the wild edible plant species.

Syzygium cordatum are myrtaceous, edible trees native to the Republic of South Africa (RSA). *S. cordatum* trees are widely distributed in the Eastern Cape, KwaZulu-Natal and across northern part of RSA (Musabayane et al., 2005; Orwa et al., 2009). *S. cordatum* trees also grow in areas with moist soil and high rainfall. The fruiting season of *S. cordatum* trees is usually from winter to summer in the RSA (Orwa et al., 2009). The fruits are purple, ovoid and fleshy up to 2 cm with 2.8 cm thick seeds. *S. cordatum* fruits rank fourth (after *Sclerocarya birrea*, *Englerophytum magalismontanum* and *Strychnos pungens*) as a preferred delicious fruit among indigenous South Africans (De Lange et al., 2005). The fruits are often fermented to produce potent intoxicating beverages. Jam and jelly are also manufactured from *S. cordatum* fruits (Young and Fox, 1982). The bark and leaves extracts of *S. cordatum* are used for treatment of GIT infections (Sibandze et al., 2010; Amabeoku and Deliwe, 2013). However, the therapeutic value of the fruits and seeds of *S. cordatum* has not been reported. This study was aimed at the evaluation of the antibacterial activity of *S. cordatum* fruit-pulp and seed extracts against bacterial strains implicated in GIT infections.

MATERIALS AND METHODS

Selected bacterial strains

The bacterial strains known to cause GIT infections used in this study included; *Bacillus cereus* (ATCC 10102), *Staphylococcus aureus* (ATCC 25925), *Enterococcus hirae* (ATCC 8043), *Escherichia coli* (ATCC 25922), *Salmonella* Typhimurium (ATCC 700030), *Pseudomonas aeruginosa* (ATCC 7700), *Klebsiella pneumonia* (ATCC 4352), *Vibrio fluvialis* (AL 019) and *Vibrio vulnificus* (AL 042).

Plant materials

Fruits from *S. cordatum* were randomly harvested in summer from the trees at the main campus of the University of Zululand, KwaZulu-Natal, RSA. The seeds from *S. cordatum* were manually removed from the fruit-pulps (fleshy part). The fruit-pulps and seeds were air-dried at room temperature. The dried *S. cordatum* fruit-pulps and seeds were separately ground to a coarse powder using an electric grinder and filtered with a filter of mesh size 1.0 mm to increase the surface area for solvents during the extraction process. The grounded samples were stored at 4°C until required for use.

Extraction

The ground *S. cordatum* fruit-pulp and seed samples (100 g each) were separately subjected to Soxhlet extraction using 400 ml of methanol (Univ.AR). The samples were put on a mechanical shaker at a speed of 200 rpm at 37°C for 12 h. Extractions were repeated three times for each sample. The third extractions were left for 24 h. The extracts obtained were filtered through Whatman filter paper and concentrated using a Bȕchi rotary evaporator at 45°C. The yields of each extract were weighed and re-dissolved in 100 ml of 10% dimethyl sulfoxide (DMSO) to the volume concentration of 100 mg/ml. The extracts were stored at 4°C until they were to be used. The percentage yields from *S. cordatum* fruit-pulp and seed extracts were calculated using the formula below that was used by Shahid (2012).

Phytochemical screening

The extracted crude *S. cordatum* fruit-pulp and seed extracts were phytochemically screened. Phytochemical screening was done for all the extracts using the methods of Harborne (1973).

Betulinic acid -Thin-layer chromatography

An original line of 2 cm from the edge, across the plate was drawn. Betulinic acid was loaded on thin-layer chromatography plate as standard indicator followed by loading of methanol extracts of *S. cordatum* fruit-pulp and seed, respectively. The thin-layer chromatography plate was placed in a chromatography tank containing mixture of hexane and ethyl acetate in the ratio of 7:3, respectively, covering about 1 cm of the plate. The chromatography was allowed to proceed until the hexane-ethyl acetate reaches the top of the plate. At that point, the chromatogram was removed from the tank and dried using hot air dryer. The plate was viewed under ultra violet light at 354 nm. It was then sprayed with 5% sulphuric acid-methanol solution. The appearance of a pink colour indicated the presence of betulinic acid (Walker, 1984).

Quantification analysis of total phenolic content

The total phenolic contents were determined by the Folin-Ciocalteau method according to Makkar et al. (1993). An aliquot (0.2 ml) of 500 μg/ml methanolic fruit-pulp and seed extracts were

Table 1. Minimum inhibitory concentration, minimum bactericidal concentration in mg/ml and total activity in ml of the *S. cordatum* PE and SE extracts on the selected bacterial strains.

Bacterial strain	Pulp extract			Seed extract			Ciprofloxacin	
	MIC	MBC	TA	MIC	MBC	TA	MIC	MBC
S. aureus (ATCC 25925)	3.13	6.25	31	6.25	12.5	16	3.13	6.25
E. coli (ATCC 25922)	6.25	6.25	16	12.5	12.5	8	3.13	6.25
V. vulnificus (AL 042)	6.25	12.5	16	25	50	4	1.56	12.5
B. cereus (ATCC 10102)	3.13	6.25	31	12.5	12.5	8	3.13	3.13
S. typhimurium (ATCC 700030)	6.25	6.25	16	12.5	25	4	1.56	6.25
E. hirae (ATCC 8043)	3.13	3.13	31	6.25	12.5	8	1.56	3.13
P. aeruginosa (ATCC 7700)	3.13	6.25	31	12.5	12.5	4	3.13	12.5
K. pneumonia (ATCC 4352)	3.13	12.5	31	3.13	12.5	31	3.13	12.5
V. fluvialis (AL 019)	6.25	12.5	16	25	50	4	1.56	12.5
Average total activity (ml)			24			10		

MIC, Minimum inhibitory concentration (mg/ml); MBC, minimum bactericidal concentration (mg/ml); TA, total activity (ml); PE, fruit-pulp extract; SE, seed extract.

made up to 1.0 ml with distilled water, respectively. 0.5 ml of Folin-Ciocalteau reagent (1N) was added, followed by 2.5 ml of sodium carbonate solution (20%). The mixtures were mixed properly, and then incubated at room temperature for 40 min. The absorbance of the blue-colored complex formed was measured at 725 nm against the appropriate blank. The total phenollic content was determined from the standard curve of tannic acid and expressed as equivalents of tannic acid (µg/ml).

Antimicrobial activity

The selected bacteria were inoculated into nutrient broth and incubated at 37°C for overnight. Afterwards, 1 ml from each of the bacteria species was pipetted into 9 ml of fresh prepared nutrient broth in separate test tubes labelled with corresponding microorganism. The test tubes were then incubated at 37°C for overnight. After overnight incubation, absorbance of the selected bacterial strains was read in the spectrophotometer (600 nm) for determination of their turbidity. The turbidity of the resulting suspensions was diluted with nutrient broth to obtain an absorbance of 0.132. This absorbance was taken as comparable to 0.5 McFarland turbidity standard. The turbidity was estimated to be equivalent to 1.5×10^8 CFU/ml (Qaralleh et al., 2012).

Minimum inhibitory concentration (MIC)

A serial microdilution method was adapted as described by Eloff (1998) and Qaralleh et al. (2012) to measure the minimal inhibitory concentration (MIC) of the fruit-pulp and seed extracts. The MIC is the lowest concentration of the extract required to inhibit microorganisms. 96-well plates were used to quantitatively determine the MIC of both extracts. The sterile nutrient broth (50 µl) was added to all the wells of the 96-well plate, and 50 µl of the extracts (50 mg/ml, in 10% DMSO) was poured in the wells in the first rows and mixed well on separate plates. The extracts mixtures (50 µl) were removed from all the wells in the row A to perform a 3-fold serial dilution down the columns, respectively. The last 50 µl, in the last column was discarded so that the total volume solution of each well was 50 µl. The selected bacterial strains (50 µl) were transferred into the corresponding wells. 10% DMSO was used as negative control while ciprofloxacin (20 µg/ml) was used as a positive control. The plates were covered and incubated at 37°C for overnight. 0.2 mg/ml of *P*-iodonitrotetrazodium violet (INT) solution was used after the incubation period. 40 µL of 0.2 mg/ml INT solution were added to each well and incubated at 37°C for 30 min. A reddish colour which was the result of INT being reduced by the metabolic activity of microorganism to formazan indicated microbial activity. The clear colour was the indication of the absence of bacterial activity since the INT was not broken-down to form formazan. The tests were replicated three times and the mean values were reported (Table 1).

Minimum bactericidal concentration (MBC)

For the determination of MBC, the agar dilution method was used. The MBC of the extracts was determined by removing a loop full of each culture medium from the wells that no bacterial growths were streaked on different sterile nutrient agar plates. The agar plates were incubated at 37°C for 12 h. The lowest concentrations of the *S. cordatum* fruit pulp and seed extracts that exhibited the complete killing of test microorganisms were considered as the MBC (Qaralleh et al., 2012).

Determination of total activity

The total activity was calculated using the formula:

$$\text{Total activity} = \frac{\text{extracted 1g (mg)}}{\text{MIC (mg/ml)}}$$

The unit was converted to ml and represented the degree to which the bioactive extracts in a gram of powdered sample could be dilute and still inhibited the bacterial growth of the selected strains (Bag et al., 2013).

RESULTS AND DISCUSSION

Bioactive compounds

The percentage yields obtained from *S. cordatum* fruit-

Table 2. Preliminary phytochemical screening of *S. cordatum* PE and SE extracts.

Phytochemical	Test	Sample	Result
Alkaloid	Dragendorff's test:	PE	+
		SE	++
	Mayer's test:	PE	+
		SE	++
Flavonoids	Alkaline reagent:	PE	+
		SE	-
Saponins	Frothing:	PE	++
		SE	+
Cardiac glycosides	Sodium nitroprusside	PE	+
		SE	+
Tannins	Ferric chloride	PE	+
		SE	+
Phenols	Ferric chloride	PE	+
		SE	++
Terpenoids	Salkwosk	PE	+
		SE	+
Betulinic acid (BA)	Tlc:	PE	+
		SE	+

-, Absence; +, low concentration; ++, moderate concentration; +++, high concentration. TLC, Thin layer chromatography; PE, fruit-pulp sample; SE, denotes seed sample.

Table 3. Total phenolic content in 500µg/ml of crude *S. cordatum* PE and SE extracts.

Assay	Expression of results	Concentration (mg/g original sample) ±SER
Total phenolic	TAE:PE	16.4±1.8
	SE	21.4±1.4

TAE, Tannic acid equivalents; PE, fruit-pulp sample; SE, denotes seed sample.

pulp and seed extracts were 10 and 6, respectively, after extracted with methanol. The good percentage yield as showed by both extracts is important when the extracts are needed for their biological activities. The ability of methanol solvent to give better percentage yields is due to its polarity. The results of the phytochemical screening are presented in Table 2. The total phenolic contents of the fruit and seed samples were also measured (Table 3). Bioactive metabolites have been reported to possess strong antimicrobial, anticancer, anti-allergic and gastroprotective properties (Neethirajan et al., 2012). The phytochemicals detected in varying proportions in both extracts were phenolic compounds, alkaloids, cardiac glycosides, phytosterols, flavonoids, saponins, terpenoids and betulinic acid. Flavonoids were not qualitatively detected in the seed extract however other detected bioactive compounds could have been responsible for the antimicrobial activity observed in this study. Moreover, quantitative analysis showed that the *S. cordatum* seed crude extract contained a significantly higher content of total phenolic compounds (21.4±1.4 µg/ml) as compared to *S. cordatum* fruit-pulp extract (16.4±1 µg/ml). This means that the seed extract may possess antibacterial and antidiarrheal activity even though flavonoids were not detected qualitatively. The detected phytoconstituent support the scientific idea that indigenous, traditional

fruits like *S. cordatum* fruits show potential sources of novel lead substances with potential therapeutic and preventive application against GIT infections.

Antibacterial activity of *S. cordatum* fruit-pulp and seed extracts

Ciprofloxacin is a broad-spectrum antibiotic which is effective against Gram-negative and Gram-positive bacteria (Volans and Wiseman, 2010). Ciprofloxacin has bactericidal effect against *E. coli*, *Salmonella* spp., *Pseudomonas aeruginosa*, *Staphylococcus* spp. and *Klebsiella* spp. strains (Paw and Shulman, 2010). It is also active against *Streptococci spp*. Ciprofloxacin is widely used to treat urinary and respiratory infections as well as gastroenterities.

Ciprofloxacin (20 µg/ml) was used as a positive control on the tested bacteria in this study. Ciprofloxacin had inhibitory effects on all the test bacteria with the lowest MIC values of (1.56 mg/ml) on *V. vulnificus* (AL 042), *V. fluvialis* (AL 019) *and S.* Typhimurium (ATCC 700030). The highest MIC value (3.13 mg/ml) of ciprofloxacin was recoded on all other selected bacterial strains. Many naturally occurring compounds found in fruit-pulp and seed extracts have been reported to possess antimicrobial activities. *S. cordatum* fruit-pulp extract showed broad-spectrum antibacterial action with the lowest MIC value of 3.13 mg/ml on *S. aureus* (ATCC 25925), *B. cereus* (ATCC 10102), *E. hirae* (ATCC 8043), *P. aeruginosa* (ATCC 7700) and *K. pneumonia* (ATCC 4352) while the seed extract had similar MIC value on *K. pneumoniae* (ATCC 4352).

Even though the antibacterial action of *S. cordatum* fruit-pulp extract was more pronounced on all Gram-positive bacterial strains, the extract did also show remarkable antimicrobial activities against some Gram-negative bacteria (*P. aeruginosa* (ATCC 7700) and *K. pneumonia* (ATCC 4352)) as well with the same MIC value of 3.13 mg/ml. The seed extract did also show the lowest MIC value of 3.13 mg/ml on the Gram-negative *K. pneumonia* (ATCC 4352).

Gram-negative bacteria, in addition to a thin peptidoglycan layer (2 to 7 nm), possess about 7 to 8 nm of the outer membrane. This outer membrane composes of additional protective lipopolyssachride layer that exhibits toxicity and antigenicity against antimicrobials or chemotherapeutic agents (Martinko and Madigan, 2006). It was concluded that the high resistance shown by some Gram-negative bacteria as compared to Gram-positive bacteria to both *S. cordatum* fruit-pulp and seed extracts was due to the mechanism of action of this layer. Gram-positive bacteria do not possess this layer and therefore they were highly sensitive to the action of antibacterial bioactive compounds found in both extracts. Gram-positive bacteria allow the direct contact of the extract constituents with the phospholipid bilayer of the cell membrane, enabling the antimicrobials to inhibit microorganism`s growth easily. The MIC values shown by *S. cordatum* fruit-pulp were found to be lower than those of *S. cordatum* seed extract generally. The low MIC values displayed by the fruit-pulp extract indicated a higher efficacy against bacteria causing GIT infections than the seed extract. The difference in efficacy of *S. cordatum* fruit-pulp and seed extracts may be due to the unique mechanism of action displayed by various phytochemicals (flavonoids) that were detected in the *S. cordatum* fruit-pulp extract but not in the *S. cordatum* seed extract, although the total phenolic content of seed extract (21.4±1.4 µg/ml) was higher than that of the *S. cordatum* fruit-pulp extract (16.4±1.8 µg/ml).

According to Jayashree et al. (2014), the good potency of methanolic fruit extract has MIC value ranging between 3.125 to 12.5 mg/ml. This implied that *S. cordatum* fruit-pulp and seed extracts have a potential as a source of novel antibacterial agents since they both possessed the MIC values ranging between 3.13 to 12.5 mg/ml against many bacterial strains used in this study. Antimicrobial substances can be considered as bactericidal agents when the ratio is MBC/MIC ≤ 4 and bacteriostatic agents when the ratio is MBC/MIC > 4 (Erhabor et al., 2013). Both *S. cordatum* fruit-pulp and seed extracts exhibited bactericidal effect on all selected bacterial species. However, the standard drug-ciprofloxacin showed bactericidal effect on all selected bacterial species except on *V. fluvialis* (AL 019) and *V. vulnificus* (AL 042) where it showed a bacteriostatic effect. The average total activity of fruit-pulp extract (24 ml) was much higher than that of a seed extract (10 ml). The obtained average total activities implied that, if a gram of extractable bioactive compounds present in one gram of ground *S. cordatum* fruit-pulp and seed was dissolved in 1 ml, it would still inhibit the bacterial growth of the selected strains (Bag et al., 2013). However, *S. cordatum* fruit-pulp would inhibit more bacteria than the seed extract.

Conclusions

S. cordatum fruits and seeds possess antibacterial compounds that can be developed as new and cost-effective phytomedicine for therapy against GIT infections. Further studies will focus on the purification and identification of some of the bioactive compounds that are responsible for antibacterial activity.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Removal of phosphate and nitrate from aqueous solution using seagrass *Cymodocea rotundata* beads

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The aim of the present study was the removal of phosphate and nitrate by sodium alginate seagrass (*Cymodocea rotundata*) beads from aqueous solutions. The adsorption characteristics of phosphate and nitrate on the seagrass beads were optimized under different operational parameters like adsorbent dosage, initial concentration of phosphate and nitrate, retention time and pH. The results showed that 71% of phosphate and 62% of nitrate removal was obtained using this seagrass beads. It is concluded that seagrass beads is a relatively efficient, low cost and easily available adsorbent for the treatment of nutrients rich wastewater.

Key words: Biosorption, seagrass, *Cymodocea rotundata*, nutrients, beads.

INTRODUCTION

Large-scale production of wastewater is an inevitable consequence of all contemporary societies. Most waste-waters are usually hazardous to human health and the environment and must be treated prior to disposal into rivers, lakes, seas, and land surfaces. Secondary treatments of domestic and agro-industrial wastewater still release large amounts of phosphorus and nitrogen. These nutrients are directly responsible for eutrophication of rivers, lakes and seas (Lau et al., 1997; Trepanier et al., 2002) and disposal of partially treated wastewaters produce a constant threat to dwindling freshwater resources on a global scale (Montaigne and Essick, 2002). Prior to discharging wastewater into water bodies,

removing excessive nutrients is usually obligatory, even though it is not performed in many cases, especially in developing countries. The wastewater treatment industry presently uses several methods to remove phosphorus and nitrogen (Duenas et al., 2003) and other pollutants. Some are used in large-scale treatment facilities and a few are experimental projects and used on a small-scale basis (from a process-engineering viewpoint) (Stratful et al., 1999; Van Loosdrecht et al., 1997). In this context, the current study examined the biosorption capacity of seagrass beads on nutrients (phosphate and nitrate) removal from the aqueous solution. Various methods have been carried out on nutrient removal (Azhar et al.,

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2009, 2010a, 2010b, 2011). Several adsorbents like clay and zeolite (Azhar et al., 2011, 2004), limestone (Celik et al., 2001; Aziz et al., 2004), carbon-zeolite composite (Ehret et al., 2001), silica-carbon-calcium carbonate (Farrah and Preston, 1985), microbes (Zeng et al., 2011), marine microalgae (Dinesh Kumar et al., 2013), seaweed (Mithra et al., 2012) and seagrass (Vasanthi, 2012) have been used for biosorption of nutrients. The use of algae in biotechnology has been increased in recent years, these organisms being implicated in food, cosmetic, aquaculture and pharmaceutical industries (Borowitzka and Borowitzka, 1988). Cell immobilization techniques have been developed in order to solve the pollution related problems. The use of immobilized algal cells in water purification processes has been reported long ago (Robinson et al., 1988), as algae form part of the organisms fixed in percolating filters of wastewater treatment plants. But at the end of the sixties of the past century, novel techniques for immobilizing biocatalysts in general (from enzymes to whole cells) began to spread in the literature (Papageorgiou, 1987), and the use of immobilization techniques diversifies. Immobilized algae have been used for biomass obtaining and macronutrient removal. The extremely high accumulation capacity of some of these organisms for potentially dangerous substances (Maeda and Sakaguchi, 1990) has been also exploited for bioremediation techniques applied on polluted waters (especially involving metals; Greene and Bedell, 1990). Therefore, the present attempt has been made on the use of seagrass *Cymodocea rotundata* beads for the removal of nutrients from the aqueous solution. Further, the attempt was also made in the optimization of conditions like pH, incubation time, biomass concentration and initial nutrient concentration on the removal of nitrogen and phosphorus from aqueous solution.

MATERIALS AND METHODS

Collection of seagrass

The healthy and fresh seagrass *C. rotundata* was collected from Palk Bay, Muthukuda (9o 51' 48'' N; 79o 7' 15'' E) Southeast coast of India. The biosorbent powder was prepared according to Bishoni et al. (2004). The seagrass was washed twice with running tap water and then thoroughly rinsed with distilled water to remove particulate matter and adhering salts from the surface. Then the biomass was shade dried and later oven dried at 60°C for 24 h. Dried biomass was subsequently ground with mortar and pestle and sieved to a particle size of 500-850 µm and then stored in desiccators for nutrient biosorption experiments.

Preparation of seagrass beads

The seagrass beads were prepared according to Santos et al. (2002). To prepare 100 ml of alginate solution with the required alginate concentration, the alginate was first carefully dissolved by stirring in 70 ml of distilled water. In the remaining 30 ml of distilled water, 3.5 g of NaCl were dissolved to obtain 3.5 g/l salinity final

solution. When the alginate was completely dissolved, the two parts were mixed in magnetic stirrer. Cation solutions were prepared in nanopure water. Beads were prepared by adding alginate solution drop wise by means of 20 ml of syringe (0.8 mm × 40 mm needle, Braun, Melsunger, Germany) into the cation solution, from a height approximately one drop per second. Beads were kept stirring in the cation solution for 45 min to allow complete hardening of the alginate and washed several times with filtered (0.45 µm) natural seawater to eliminate the remaining cation.

Preparation of nutrient stock solutions

The nutrient stock solutions were prepared by diluting the corresponding salts namely, sodium nitrite and di potassium hydrogen phosphate in distilled water. The stock solutions were diluted with distilled water for the preparation of working solutions. Different initial concentration (10, 1 and 0.1 µmol/l) of working solutions of nitrate and phosphate was prepared. All the nutrient stock solutions (100 ml) were taken in 250 ml conical flasks. The pH of each solution was adjusted from 5 to 9 using diluted sodium hydroxide (NaOH) and hydrochloric acid (HCL) and pH were estimated by using pH meter (ELICO, INDIA).

Adsorption of nutrients by seagrass beads

To the nutrient stock solutions, different densities of seagrass beads were added to the conical flasks. Then the flasks were maintained at 30°C in shaker for various time intervals namely, 30, 60, 90 and 120 min at 200 rpm. At the end of the experiment, the flasks were removed from the shaker and the solution was separated from the biomass by filtration through Whatman filter paper (47 µm) using Millipore filtering apparatus. Then the final concentration of the nutrients was estimated. The amounts of nutrients in the sample were measured spectrophotometrically using Shimadzu Model-2450 as per the standard protocol described by Strickland and Parsons (1972). Nitrate was estimated according to Jenkins and Medsken (1964).

Effect of initial concentration

Three different concentrations 0.1, 1 and 10 µmol/l were used to study the effect of different initial concentration for their nutrient removal capability.

Effect of pH and time interval

The different pH viz.,, 5, 6, 7, 8 and 9 were used for the nutrients removal experiment using seagrass beads. The pH of each solution was adjusted using diluted NaOH and HCL (ELICO, INDIA). Different time interval such as 30, 60, 90 and 120 min were maintained to standardizing the incubation time for nutrients removal. The 250 ml conical flasks filled 200 ml of aqueous solution and beads were kept on the rotary shaker at 30ºC for 200 rpm. At the end of the experiment, the flasks were removed from the rotary shaker and the solution was separated from the biomass by filtration through Whatman filter paper (47 µm). Then the concentrations of the nutrients were estimated.

Effect of biomass dosage

Different seagrass biomass dosages such as 0.1, 0.5 and 1 g were used for the beads preparation and subsequent adsorption processes to optimize the biomass dosage for nutrients removal.
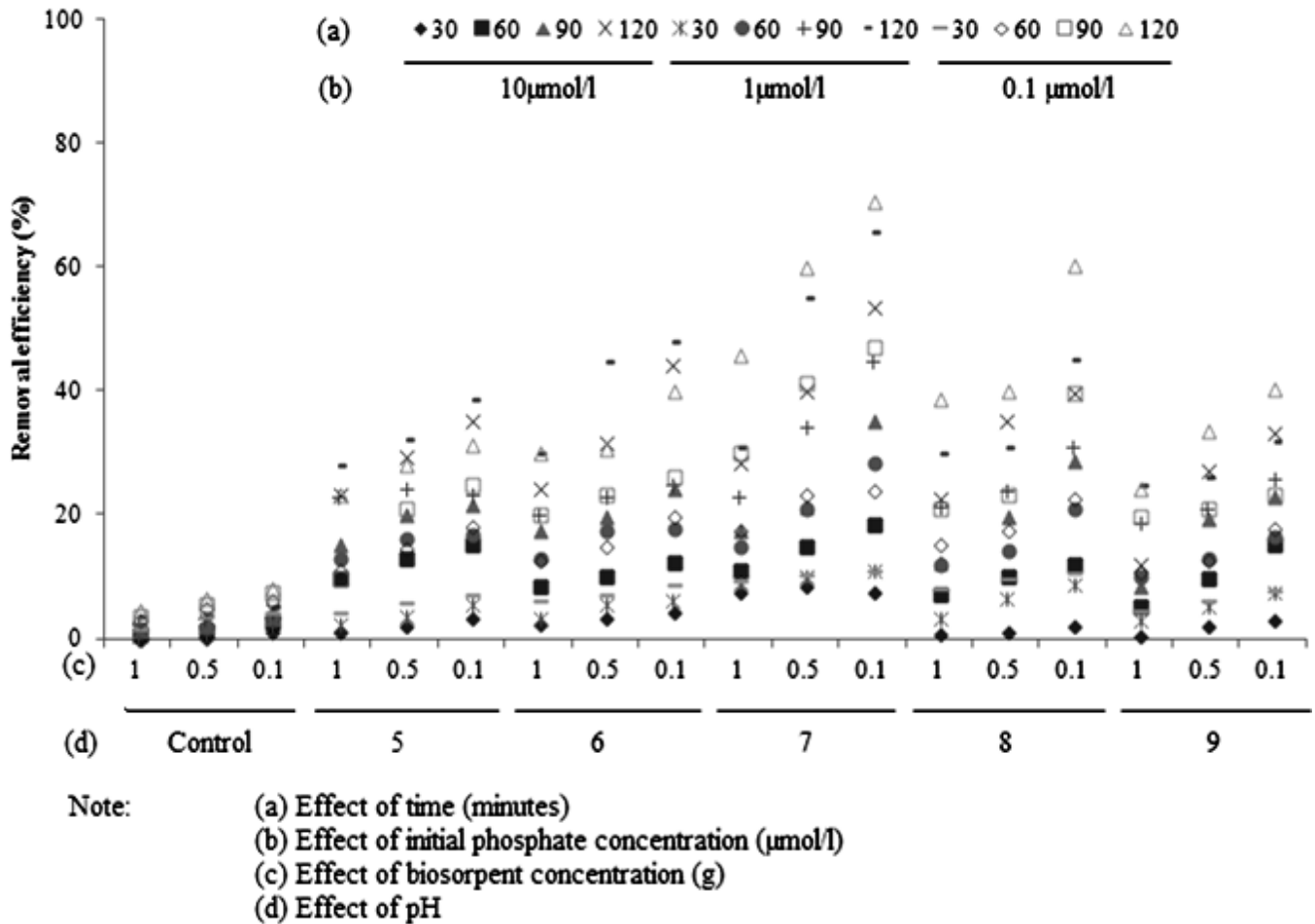
Figure 1. Effect of pH, initial concentration, retention time and biomass dosage on phosphate adsorption.

Calculation

The removal (adsorption) efficiency was calculated using the following equation:

Removal efficiency $=100 (C_0-C_e)/ C_0$

Where, C_0 = concentration of the sample solution before treatment; C_e = concentration of the sample solution after treatment.

RESULTS AND DISCUSSION

Optimization of the pH

The hydrogen ion concentration (pH) is one of the most critical controlling parameters in adsorption processes. This is due to the competitive effect of the H^+ and also due to the fact that the pH affects the ionisation of the functional groups on the surface of the sorbent material (Wahab et al., 2010). Phosphate removal by *C. rotundata* beads was observed at pH values ranging between 5 and 9. Figure 1 shows that as the solution pH increases in the range of 5–9, the removal efficiency of phosphate also

increased gradually and attains a maximum value (70.9%) when the pH value was 7. However, when the pH increases to 8 and 9, the removal efficiency drops dramatically (60.3 and 40.3%). Similarly, for nitrate removal, the effect of pH was investigated by maintaining values between 5 and 9 and the result is presented in Figure 2. In case of nitrate the equilibrium sorption capacity was found to be minimum at pH 5 (1.1%) and maximum at pH 8 (61.8%).

The nitrate adsorption was highly pH dependent, which affects the surface charge of the beads; the overall results were confirmed by those of previous workers (Chatterjee et al., 2009). The adsorption of phosphate onto the biosorbent depends on initial solution pH (Figure 1). The phosphate adsorption was low in pH 5; when the pH increased from 5 to 7 the phosphate adsorption also proportionally increased. For further increases in pH from 7 to 9 however, adsorption of phosphate onto the biosorbent was found to decreased (Figure 1), suggesting the existence of an optimum pH for the maximum phosphate adsorption. This result was consistent with the proposed predominant adsorption mechanism that the optimum pH for phosphate removal by the seagrass beads surface should be around
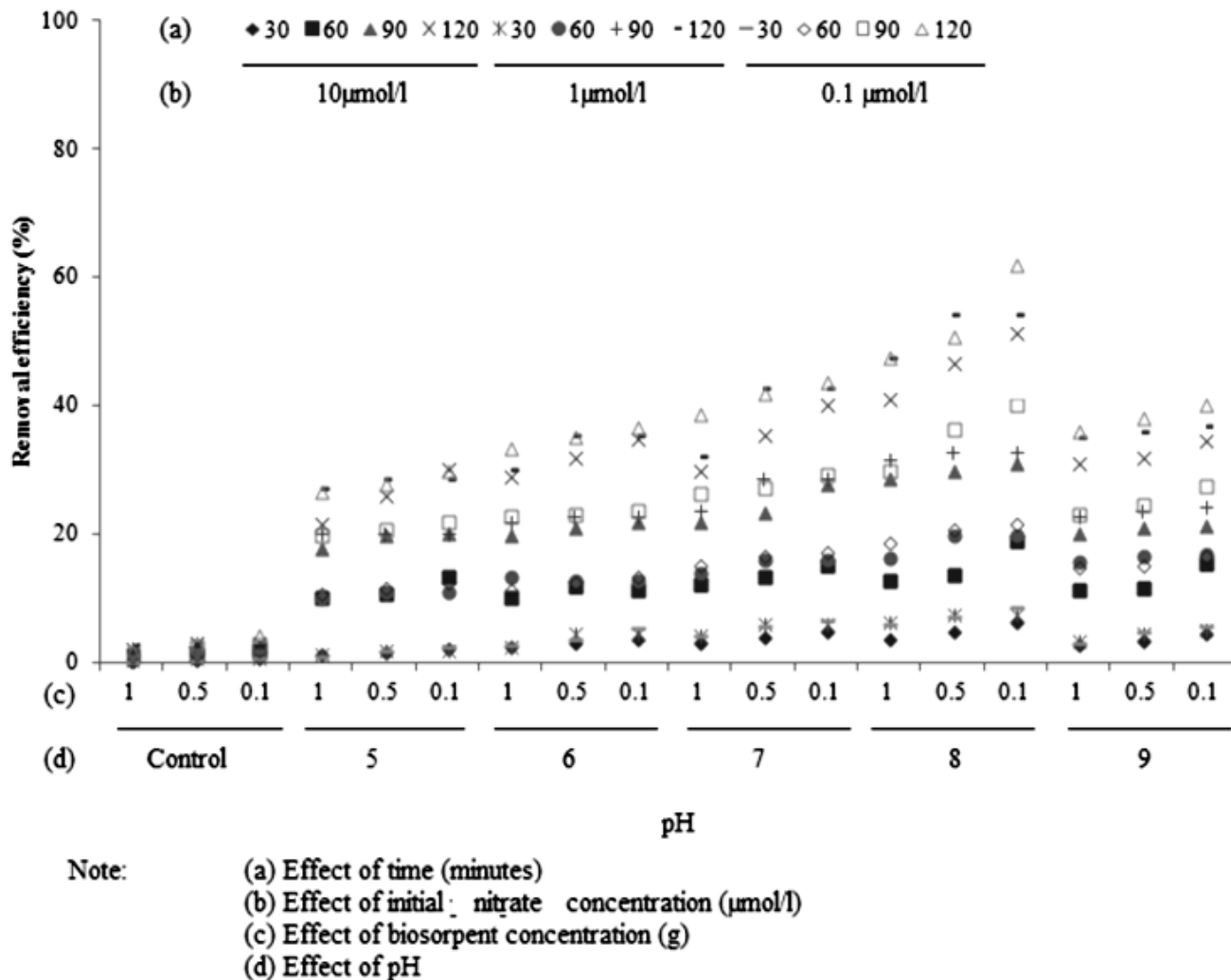
Figure 2. Effect of pH, initial concentration, retention time and biomass dosage on nitrate adsorption.

7 at which almost all phosphate exists in the form of $H_2PO_4^-$ (that is, mononuclear adsorption). If solution pH is higher than the optimum value, polynuclear interactions may be triggered to consume more adsorption sites. Similar results were also reported by some earlier workers (Kumar et al., 2010; Yao et al.., 2011) who studied the pH effect on phosphate removal from aqueous solution by using other carbon-based adsorbents.

Optimization of the time

The equilibrium time required for the biosorption of phosphate and nitrate using *C. rotundata* beads were studied at various initial concentrations and various biosorbent concentrations at different time intervals. Figures 1 and 2 show that removal efficiency sharply increases with increase in time and attains maximum removal in phosphate and nitrate at 120 min incubation

period (Mithra et al., 2012; Dinesh Kumar et al., 2013). The nutrients adsorption increased rapidly during the first 30 min and remained up to 120 min. Such rapid biosorption process has been correlated with the characteristics of the biomass, and its other parameters interact with the aqueous solutions (Ingleton and Simmons, 1996). The contact time is an important consideration for the treatment of wastewater and accordingly, the contact time is fixed as little higher than the respective equilibrium times for each biosorbent (Popuri, et al., 2007; Yan-kui, et al., 2006). Our experiment showed that the 120 min duration was found suitable for maximum adsorption of phosphate and nitrate from the aqueous solution using *C. rotundta* beads.

Optimization of the initial concentration

When determining the sorption capacity of a filtered material

with an agitation experiment the initial concentration of the solution greatly impacts on the sorption results. In general, the sorption increases with increased initial concentration of the species as agreed by some earlier workers (Taneva, 2012; Rahman et al., 2012). Initial concentrations of both nutrients were varied namely, 10, 1 and 0.1 µmol. In our experiment, we observed that removal capacity was increased with decreased nutrients concentration. The maximum removal of 70.9% phosphate through optimized pH (8), time (120 min) and biomass dosage (0.1 g) from the aqueous solution were observed at 0.1 µmol/l and minimum removal of 53.7% was observed at 1.0 µmol/l.

In the case of nitrate removal, the optimized time and biomass dosage was found as 120 min and 0.1 g, respectively, but pH 7 was varying from phosphate. The maximum (61.8%) and minimum (51.2%) removal of nitrate was noticed at 0.1 µmol/l. The removal efficiency was found increased when the initial nutrient concentration decreased. This may be attributed to the saturation of binding sites on the biomass surface leading to increased number of unabsorbed ions from the aqueous solution, because of the lack of binding sites on the biomass at higher concentrations than 0.1 µmol/l (Akçelik et al., 2008).

Optimization of the amount of biosorbent

The effect of the amount of adsorbents on the removal of phosphate is given in Figure 1. The increasing biomass dosage in beads reduced their efficiency in nutrients removal and caused cell leakage. Initially the removal efficiency was rapidly increased due to the rate of adsorption site. With the decrease in adsorbent amount, adsorption sites become profuse compared to phosphate current in the solution. It shows that maximum removal efficiency was 70.9% when the amount was 0.1 g. The phosphate removal at 0.5 g and 1.0 g biomass dosage in beads were recorded to be 60.1 and 45.9%, respectively. In the case of nitrate, minimum removal (47.5%) was recorded at 1.0 g and maximum removal (61.8) was observed at 0.1 g. The removal efficiency at 0.1 g biomass dosage in beads was increased nearly 14.3% than 1.0 g biomass dosage in beads. The 0.1 g biomass dosage revealed higher removal efficiency. But further increases in biosor-bent concentration causes a decrease in percentage of removal. This trend could be due to the formation of biosorbent aggregates at higher biomass concentrations, which in turn could reduce the effective surface area available for the biosorption. With the increasing dose of biosorbent, the value of removal was decreased. The explanation for this behavior can be that adsorption sites remain unsaturated during the adsorption reaction since the number of sites available for adsorption increase by increasing adsorbent dose (Nuhoglu and Oguz, 2003; Zou et al., 2006).

Increasing seagrass powder entrapped within the beads

did not cause any significant improvement in nutrient removal in the present study as agreed by earlier workers (Chevalier and Noue, 1985; Lau et al., 1997). On the contrary, super concentrated cell stockings in the beads posed serious leakage problem (Robinson et al., 1986; Lau et al., 1997). Lukavsky et al. (1986) reported that nutrients such as ammonia and phosphate could first be adsorbed on the surface of the bead, then penetrate slowly into alginate and be continually sorbed into cells. Super concentrated cell stockings may restrict to some extent the nutrient diffusion through the alginate pores (Jimenez-Perez et al., 2004).

Adsorption isotherms

Adsorption isotherms, Langmuir and Freundlich were used to describe the adsorption data for a range of adsorbate concentrations. These isotherms relate adsorption density, q_e (uptake of adsorbate per unit weight of adsorbent) to equilibrium adsorbate concentration in the bulk fluid phase, C_e. The Langmuir isotherm treats surface sites analogous to dissolved complexing ligands. It is derived by combining sorption equilibrium constant with a mass balance on the total number of adsorption sites. The Langmuir isotherm is valid for monolayer adsorption onto a surface containing a finite number of identical sites. The model assumes uniform energies of adsorption onto the surface and no transmigration of adsorbate in the plane of surface. The Langmuir isotherm is represented by the following equation:

$$C_e/q_e = 1/Q_o b + C/Q_o \qquad (1)$$

Where: C_e, is the equilibrium concentration (mg/L); q_e, is the amount adsorbed at equilibrium time (mg/g); Q_o, and $Q_o b$ are Langmuir constants related to adsorption capacity; and energy of adsorption, respectively.

In Langmuir plots for adsorption, the linear plots of C_e/q_e versus C_e (Figure 3) confirm that the adsorption follows the Langmuir isotherm model. Langmuir constants, Q_o and $Q_o b$ were determined from the slope and intercept of the respective plots.

The essential characteristics of a Langmuir isotherm can be expressed in terms of a dimensionless constant separation factor or equilibrium parameter, R_L, which is given by the equation

$$R_L = 1/1 + bC_o \qquad (2)$$

Where, C_o, is the initial dye concentration (mg/L); b, is the Langmuir constant (L/mg); R_L indicates the shape of the isotherm as shown in Table 1.

R_L values of live and other pretreated biomass at different adsorbate concentrations were always less than one and more than zero thereby indicating favourable adsorption of adsorbetes onto the adsorbent. In the
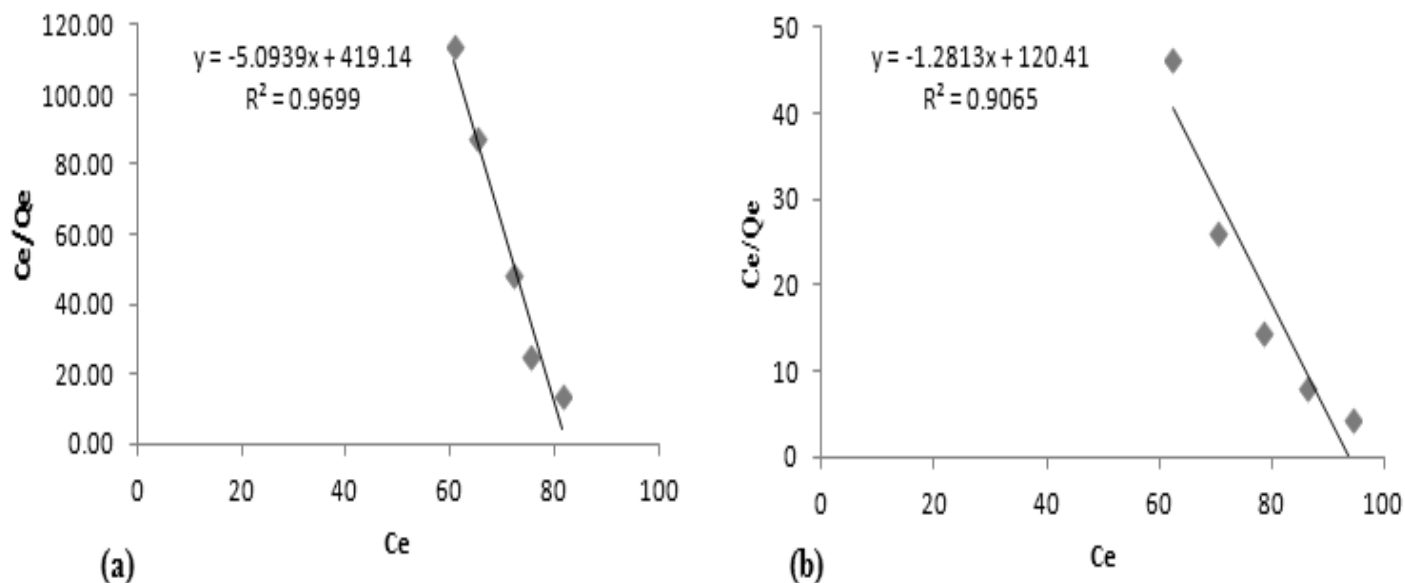
Figure 3. Langmuir adsorption isotherms of (a) phosphate and (b) nitrate on *Cymodocea rotundata* beads.

Table 1. The shape of isotherm.

R_L	Type of isotherms
$R_L > 1$	Unfavorable
$R_L = 1$	Linear
$0 < R_L < 1$	Favourable
$R_L = 0$	Irreversible

present study it was observed that R_L values of phosphate removal was 0.99 whereas for nitrate it was reported as 0.998.

Freundlich equation can be derived by assuming that the free energy of adsorption decreases logarithmically as adsorption density increases. The Freundlich equation is used for heterogenous surface energies in which the energy term, Q_o, in the Langmuir equation varies as a function of the surface coverage, q_e, strictly due to variation in the heat of adsorption.

$$q_e = K_f C_e^{1/n} \tag{3}$$

Rearranging the equation (3) gives

$$Log_{10} X/m = log K_f + 1/n \ log_{10} C_e \tag{4}$$

Where, C_e, is the equilibrium concentration (mg/L); X/m, is the amount adsorbed at equilibrium time (mg/g); K_f, and n are Freundlich constants.

N gives an indication of favourablility and K_f [mg/g (L/mg)^n], the capacity of the adsorbent. This Freundlich adsorption isotherm is applied for the adsorption of phosphate and nitrate removal. Linear plots of log C_e versus log X/m,

Freundlich plots (Figure 4) showed that the adsorption of adsorbates followed Freundlich isotherm model, suggesting that the average energy of adsorption decreases with increasing adsorption density. From the slope and intercept of the plots, the values of n and K_f were calculated. The values of n in the range of 1 to 10 denote good adsorption of the adsorbate onto the adsorbent. In the present study, the n values of phosphate were 0.05 and nitrate was about 0.08 whereas K_f values of phosphate were 1.51 and nitrate in the range of 7.94 for adsorption of nutrients studied onto *C. rotundata* beads. In the present work we have found that 0.1 g biomass containing beads efficiently removed nitrate and phosphate from the aqueous solution compared to other biomass dosage (0.5 g and 1.0 g).

Conclusions

The amount of phosphate adsorption on the *C. rotundata* beads increases with decreased biomass content in the aqueous solution, which indicates that the adsorption depends upon the availability of binding sites for phosphate. Also, the equilibrium of phosphate and nitrate adsorption was attained at about 120 min retention time, while the highest percentage of nutrients adsorption occurred at the pH range 7-8 (8 for phosphate, 7 for nitrate). And lastly, the nutrient's adsorption was found to increase with the decreased initial concentration.

Conflict of interests

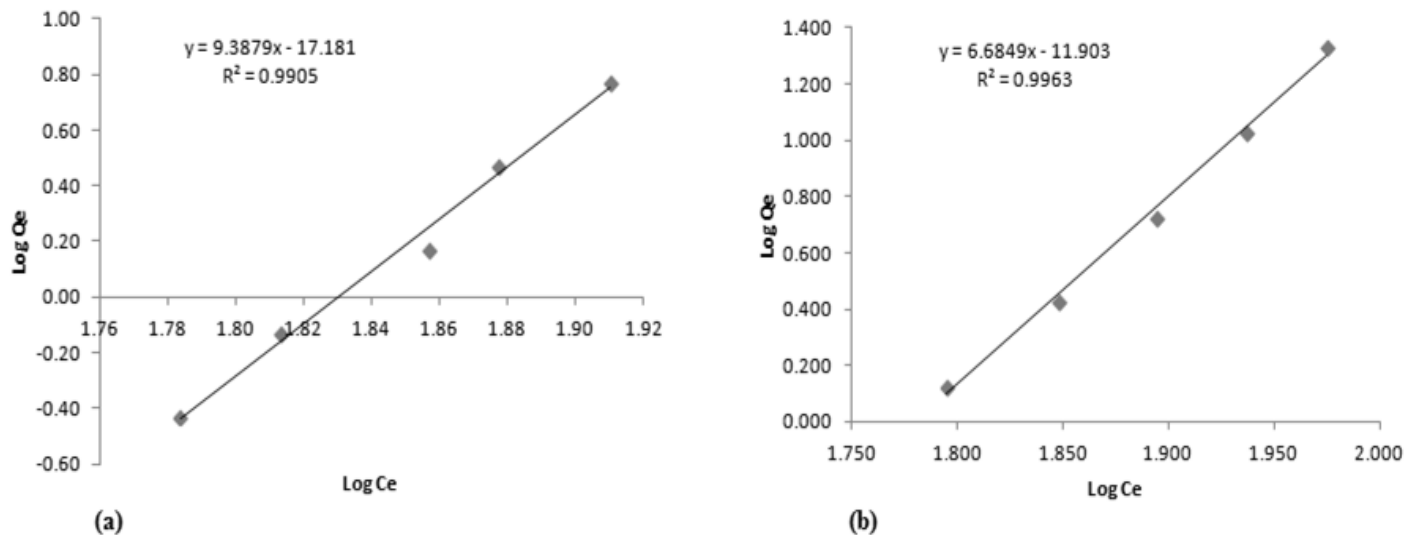The authors did not declare any conflict of interest.

Figure 4. Freundlich adsorption isotherms of (a) phosphate and (b) nitrate on *Cymodocea rotundata* beads.

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Full Length Research Paper

Antioxidant properties of cultivated edible mushroom (*Agaricus bisporus*) in Kenya

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Antioxidant activities and phytochemical compounds of ethanol and hot water extracts of *Agaricus bisporus* species fruiting body and mycelia cultivated in Kenya were spectophotometrically determined and evaluated. The total antioxidant activity was analysed using 1,1-diphenyl-2-picrylhydrazil, hydroxyl, superoxide radical scavenging and reducing power assays, while phytochemicals were assayed through calorimetric assays. Total phenolic, β-carotene, lycopene, flavanoid and ascorbic acid composition of *A. bisporus* extracts was analysed by calometric assays and found to contain 40.26 to 4.61 mg/g, 48.99 to 2.86 mg/g, 67.82 to 11.87 mg/g, 93.8 to17.2 mg/g and 11.62 to 10.22 mg/g) respectively. The mineral elemental analysis done using energy dispenser x-ray fluorescence (EDXRF) analytical method revealed that the samples contain zinc (42.9 mg/l), iron (33 to 48.5 mg/l), copper (18 to 24 mg/l) and manganese (7.5 to 9 mg/l). Generally, the mycelium extracts were more effective radical scavengers than the fruiting bodies. Due to the above characteristics, *A. bisporus* mushroom could be considered a food complement with antioxidative activity in the diet for the health benefits they present. Their effectiveness was also evaluated by their EC_{50} values through interpolation from linear regression analysis of their respective data.

Key words: Button mushroom (*Agaricus bisporus*), edible mushroom, antioxidant, reducing power, scavenging ability, phytochemicals.

INTRODUCTION

The button mushroom, *Agaricus bisporus* (J.E. Lange) Imbach, is the premier cultivated edible mushroom and is consumed throughout the world. The original wild form bears a brownish cap and dark brown gills but more familiar is the current variant with a white form, having white cap, stalk and flesh and brown gills (Loganathan et

al., 2009). In cultivation of *A. bisporus*, fruit bodies are usually produced in polythene bags containing sterilized sawdust. After the completion of vegetative mycelia growth, the bags are opened and the colonized substrate subjected to environmental conditions known to stimulate fruiting. Environmental stresses depending on where/how the mushrooms are cultured may lead to variations in terms of nutritional value including antioxidants. Culturing conditions/feed/substrate type) may also influence the antioxiodants levels in mushrooms (Román et al., 2006). The mushroom is packed by placing the entire cluster or several clusters in each overwrapped package. *A. bisporus* is usually considered to be of lesser value nutritionally and medicinally compared with other cultivated mushrooms that are predominantly grown in Asia (Aisya et al., 2010). Recent evidence suggests that *A. bisporus* also contains high levels of substances of possible medicinal importance, such as tyrosinase, aromatase inhibitors and immunomodulating and antitumour poly-saccharides (Aisya et al., 2010). Cold water extracts of *A. bisporus* have the ability to neutralise genotoxic effects of reactive oxygen species. This genoprotective effect was associated with tyrosinase.

Recent studies illustrate the anticancer activity *in vitro* and *in vivo* of *A. bisporus* extract, and its major fatty acid constituents that suppress aromatase activity and oestrogen biosynthesis are responsible for the potential breast cancer chemopreventive effect (Savoie et al., 2008). Ergosterol, vitamin D_2 content and antioxidant activity are also proposed as interesting components for the development of *A. bisporus* as a nutraceutical. Owing to its nutritional value, with a low calorie, purine, carbohydrate and sodium content as well as a high content of several vitamins, potassium, phosphorus and some trace elements, *A. bisporus*, like other mushrooms, is considered to be a valuable component of the human diet, especially by health-conscious people (Savoie et al., 2008). This suggests that the value of *A. bisporus* as a functional food warrants more detailed study (Savoie et al., 2008). Our objective was to evaluate the antioxidant properties of ethanolic and hot water extracts from *A. bisporus* fruit bodies and mycelia including reducing power, scavenging abilities on hydroxyl, DPPH and superoxide anion radicals. The contents of potential antioxidant components were also determined as well as the trace element contents.

MATERIALS AND METHODS

Mushroom fruit bodies and mycelia

Fresh fruit bodies and mycelia of *A. bisporus* were obtained from 6 bags of substrates for growing the mushrooms. For each *A. bisporus* growing bag, 4 kg wheat straw were used as growing substrate. The wheat straws were watered for 4 days after which a heap was raised and watered overnight. After a day's rest, 2 kg chicken manure, 0.5 kg urea, and 0.5 kg molasses were added to the wheat straw and watered for a day. After a days' rest, the pile

was mixed while sprinkling with a little water. This was repeated six times. The substrate was then filled in a tunnel for pasteurization by allowing the temperature to increase upto about 60°C and allowing to stay for 8 to10 h then cooled to 48°C in a long stack step for a four day conditioning. It was finally allowed 68 to 72% moisture content at 25°C temperature. *A. bisporus* spawns (0.35 kg) was seeded followed by packing into polythene bags 36 x 24 ft with 7 kg substrate each. They were then left to incubate for 14 days until colonization was complete, the polythene bags were then folded and casing done using sterile loam soil, slight watering was done to maintain moisture after which pinning was observed then harvested after three days by gently holding the mushroom body and twisting. The mycelia and fruiting bodies of the harvested mature mushrooms were separated, placed in freezer bag containers and stored in deep freezer at 4°C.

Extraction of the phytochemicals

Ethanolic extraction was done according to Bo et al. (2010). A sub sample (20 g) was extracted by stirring with ethanol (200 mL, 95% pure) at 25°C for 24 h and filtering through Whatman No.1 filter paper. The residue was then extracted with two additional 200 mL portions of ethanol as described above. The combined ethanolic extracts were then rotary evaporated at 40°C to dryness. Hot water extractions was done according to Bo et al. (2010), a subsample (20 g) was heated with deionized water (200 mL) at reflux for 1 h, centrifuged at 4,000 rpm for 15 min, followed by filtering through Whatman No.1 filter paper. The residue was then extracted with two additional 200 mL portions of boiling water as described above. The combined hot water extracts were freeze-dried. The dried extracts were used directly for analyses of antioxidant components or re-dissolved in water or ethanol to a concentration of 50 mg/mL and stored at 4°C for further uses.

Determination of antioxidants component

Phenols, polyphenols, ascorbic acid, flavanoid, β-carotene and Lycopene were determined in both ethanolic and aqueous mushrooms extracts by colorimetric assays, based on previously described procedures except for minor modifications (Barros et al., 2008).

Determination of total phenolic content

A sample of the extract (1 ml) was mixed with 1 ml of Folin and Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Gallic acid was used to calculate the standard curve (0.01 to 0.4 mM). Estimation of the phenolic compounds was carried out in triplicate. The results were in mean values ± standard deviations and expressed as mg of gallic acid equivalents (GAEs) per g of each extract.

Determination of total flavonoid concentration

Mushroom extracts solution (1 ml) were diluted with 4.3 ml of 80% aqueous ethanol and to the test tubes 0.1 ml of 10% aluminium nitrate was added followed by 0.1 ml of 1 M aqueous potassium acetate. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm. Total flavonoid concentration was calculated using quercetin as standard (Park et al., 1997). Absorbance = 0.002108 µg quercetin − 0.01089 (R2: 0.9999).

Ascorbic acid determination

The dried extract (100 mg) was re-extracted with metaphosphoric acid (1%, 10 ml) for 45 min at room temperature and filtered through Whatman No. 4 filter paper. The filtrate (1 ml) was mixed with 2, 6-dichloro-phenolindophenol (9 ml) and the absorbance was measured within 30 min at 515 nm against a blank. Content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.020 to 0.12 mg/ml; $Y = 3.4127X - 0.0072$; $R^2 = 0.9905$) and the results were expressed as mg of ascorbic acid/g of extract.

β-Carotene and lycopene determination

The dried extract (100 mg) was vigorously shaken with acetone–hexane mixture (4:6, 10 ml) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at λ = 453, 505 and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations: lycopene (mg/100 ml) = - 0.0458 A663 + 0.372 A505 - 0.0806 A453; β-carotene (mg/100 ml) = 0.216 A663 - 0.304 A505 + 0.452 A453. The result was expressed as µg of carotenoid/g of extract.

Determination of antioxidant activities

Determining of DPPH radical scavenging activity

This was carried out according to the DPPH spectrophotometric method of Mensor et al. (2001). A range of concentrations (1 to 20 mg/ml) of extract and standards (gallic acid and ascorbic acid) were used. 1 ml of a 0.3 mM DPPH methanol solution was added to 2.5 ml solution of the extract or standards and allowed to react at room temperature for 30 min. The absorbance of the resulting mixture was measured at 518 nm and converted to percentage antioxidant activity (AA%) using the formula: AA% = 100 − [(Abs sample/Abs control) × 100]. Methanol (1.0 ml) plus extract solution (2.5 ml) was used as a blank. 1 ml of 0.3 mM DPPH plus methanol (2.5 ml) was used as a negative control while ascorbic acid and gallic acid solutions were used as positive control.

Determination of hydroxyl radical scavenging activity

The effect of hydroxyl radical was assayed by using the 2-deoxyribose oxidation method (Chung et al., 1997). Hydroxyl radicals (OH-) are generated from Fe^{2+}- ascorbate- EDTA- H_2O_2 system (Fenton's reaction) which attack the deoxyribose and set off a series of reactions that eventually result in the formation of malondialdehyde (MDA), measured as a pink MDA-TBA chromogen at 535 nm. Reaction mixture (1 ml) contained deoxyribose (2.8 mM), $KH_2PO_4^-$ KOH (20 mM; pH 7.4), $FeCl_3$ (100 mM), EDTA (104 µM), H_2O_2 (1 mM) and ascorbate (100 µM). Reaction mixture was incubated at 37°C for 1 h and a colour developed as described above. Catechin was used as positive control.

Determination of superoxide anion radical scavenging activity

Superoxide radical was generated from auto oxidation of hematoxilin and was detected by an increase in absorbance at 560 nm, in a spectrophotometer (Martin, 1987). The reaction mixture contained 0.1 M of phosphate buffer (pH 7.4), EDTA (0.1 M), hematoxilin (50 µM) and incubated at 25°C for different time periods. Inhibition of auto oxidation of hematoxilin by water and ethanolic extracts over the control (gallic acid) was measured.

Reducing power assay

The reducing power was determined according to the method of Oyaizu (Oyaizu, 1986), which measure the power of extracts to reduce ferricyanide to ferrocyanide. Each extract (1 to 20 mg/mL) in water or ethanol (2.5 mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 10 mg/mL potassium ferricyanide (2.5 mL), and the mixture was incubated at 50°C for 20 min. After trichloroacetic acid (2.5 mL, 100 mg/mL) was added, the mixture was centrifuged at 3,000 rpm for 10 min. The upper layer (5 mL) was mixed with deionized water (5 mL) and 1 mg/mL ferric chloride (1 mL), and the absorbance was measured at 700 nm against a blank. A higher absorbance will indicate a higher reducing power. EC_{50} value (mg extract/ml) was the effective concentration at which the antioxidant activity was inhibited by 50%; absorbance was obtained by interpolation from linear regression analysis. Ascorbic acid was used for comparison.

Mineral element analysis

The elemental analysis was done using the Energy Dispenser X-ray Fluorescence analytical method (EDXRF) of Shahidi et al. (1992) for the detection of both detrimental heavy and trace metals in the hot water extracts of the mushrooms. Each mushroom sample was air-dried at 105°C overnight, and crushed using a mortar and pestle. Digestion of the samples was performed using a mixture of HNO_3: H_2SO_4: H_2O_2 (10:1:1, 12 mlg-1of sample) and heated at 100°C for about 10 to 15 min. After cooling, 50 mL of distilled water added and the mixture filtered. The solution was then used for elemental analysis using EDXRF and a concentration of each element was calculated on percentage of dry matter. The mineral elements analyzed included potassium, calcium, chromium, manganese, iron, copper, zinc and lead.

Data analysis

For each of the hot water and ethanoic extractions from fruit body and mycelia, three samples were prepared for every assay of every antioxidant attribute and component. Each value was expressed as mean±SD (n=3).The antioxidant activity was expressed as percentages. The experimental data was also subjected to Analysis of variance (ANOVA) to compare the values of the mycelium and fruiting bodies and test the significance levels at p≤ 0.05. Student's t-test was used to compare the values of the mycelium and fruiting bodies. The efficient concentration of antioxidant required to induce a 50% effect. Also, the data was evaluated by using one-way analysis of variance.

RESULTS

Phytonutrients present in fruiting body and mycelium ethanoic and hot water extracts

The various phytochemical present in mycelium ethanoic extract (MEE) and fruiting body ethanoic Extract (FBEE) of the mushrooms extract as detailed in Table 1. From the analysis, all the mushroom extracts had β-carotene with the content decreasing in the order FBEE > FBHWE > MEE > MHWE. There were significant differences (p < 0.05) in β-carotene amount between ethanol mushroom extracts and the water mushroom extracts. Similarly,

Table 1. Phytochemical levels of fruiting body and mycelium hot water and ethanoic extracts of *Agaricus bisporus*.

Phytochemicals	Concentration of phytochemicas (mg/mL)				
	FBHWE	FBEE	MHWE	MEE	Control
Phenols	31.87±3.89[a]	16.77±4.69[c]	40.26±0.55	4.61±0.31[c]	36.20±1.86
β-Carotene	44.57±1.93[a]	48.99±1.51	2.86±1.15[c]	19.88±0.17[b]	50.86±2.25
Lycopene	67.82±0.39	11.87±0.43[c]	16.80±0.62[b]	42.94±1.08[a]	68.14±1.60
Flavonoids	85.36±1.01[a]	21.87±0.09[b]	93.80±0.36	17.20±0.09[c]	85.10±0.20[a]
Ascorbic acid	10.51±0.18	10.22±0.24	10.39±0.11	11.62±0.09	11.10±1.58

Results are expressed as Mean ± Standard Deviation (SD) of the three independent determinations. Means within respective rows followed by similar lower case letters are not significantly different at p ≤0.05 by ANOVA and Tukey B test. FBHWE, fruiting body hot water extract; FBEE , fruiting body ethanolic extract, MHWE, mycelium hot water extact; MEE, mycelium ethanolic extract.
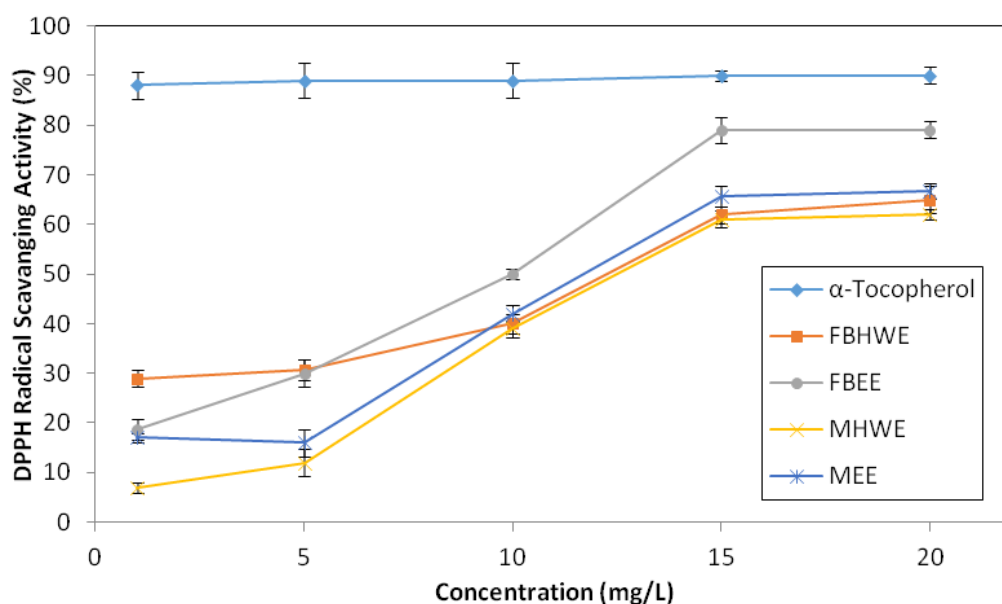

Figure 1. DPPH Radical scavenging activities (%). Results are expressed as Mean ± Standard Deviation (SD) of the three independent determinations. FBHWE, fruiting body hot water extract; FBEE, fruiting body ethanolic extract, MHWE, mycelium hot water extract; MEE, mycelium ethanolic extract.

there were significant differences ($p < 0.05$) in β-carotene amount between the fruiting body extracts and mycelium extracts. However, there was no significant difference ($p < 0.05$) in β-carotene amount among the ethanolic extracts (Table 1). The water extracts of β-carotene amount also differed significantly ($p < 0.05$). All the extracts had high lycopene content. FBHWE had the highest amount of lycopene at 67.82 µg/g. The lycopene amount decreased in the order FBHWE > MEE > MHWE > FBEE. There was a significant difference ($p < 0.05$) in lycopene content between FBHWE and FBEE, MHWE and MEE. The lycopene content differed significantly ($p < 0.05$) between MEE, MHWE and FBEE. Ethanol extracts had a significantly higher ($p < 0.05$) total flavanoid content than the water extracts. Among the ethanol

extracts, MEE had higher total flavanoid content than FBEE. The difference was not significant at $p < 0.05$. Among the water extract, FBWE had a higher, though not significant different ($p < 0.05$) total flavonoid content than MHWE. All the extracts had substantial amount of ascorbic acid content. MEE had the highest amount of ascorbic acid at 11.62±0.13 mg/g. There was no significant difference ($p < 0.05$) in the ascorbic acid content among the mushroom extracts (Table 1).

Determination of antioxidant activities

Figure 1 shows that there was significant DPPH radical scavenging activity in the fruiting body and mycelium hot
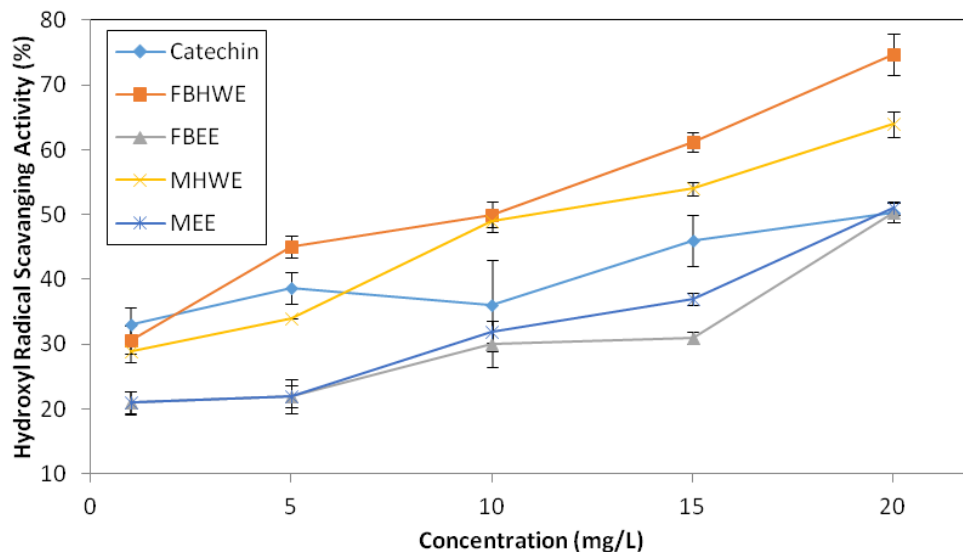
Figure 2. Hydroxyl Radical Scavenging Activity. Results are expressed as Mean ± Standard Deviation (SD) of the three independent determinations. FBHWE, fruiting body hot water extract; FBEE, fruiting body ethanolic extract, MHWE, mycelium hot water extract; MEE, mycelium ethanolic extract.

Figure 3. Superoxide Anion Radical scavenging Activity. Results are expressed as Mean ± Standard Deviation (SD) of the three independent determinations. Gallic acid was used as the control. FBHWE, fruiting body hot water extract; FBEE, fruiting body ethanolic extract, MHWE, mycelium hot water extract; MEE, mycelium ethanolic extract.

water and ethanoic extracts of *A. bisporus*. The ethanoic extracts of both the mycelium and the fruiting body have a greater DPPH scavenging activity than the water extracts. Result also shows the hydroxyl radical scavenging activity of the fruiting body and mycelium hot water and ethanoic extracts of *A. bisporus* (Figure 2). The hydroxyl radical scavenging activity increased with the increasing concentration of the extracts. The fruiting body hot water extracts had a better hydroxyl radical scavenging activity. The order of hydroxyl radical scavenging activity was fruiting body hot water extract followed by mycelium hot water extract followed by fruiting body ethanoic extract. Figure 3 shows the superoxide anion radical scavenging activity of the fruiting

Figure 4. Reducing power of *Agaricus* bisporus. Results are expressed as Mean ± Standard Deviation (SD) of the three independent determinations. FBHWE, fruiting body hot water extract; FBEE, fruiting body ethanolic extract, MHWE, mycelium hot water extract; MEE, mycelium ethanolic extract.
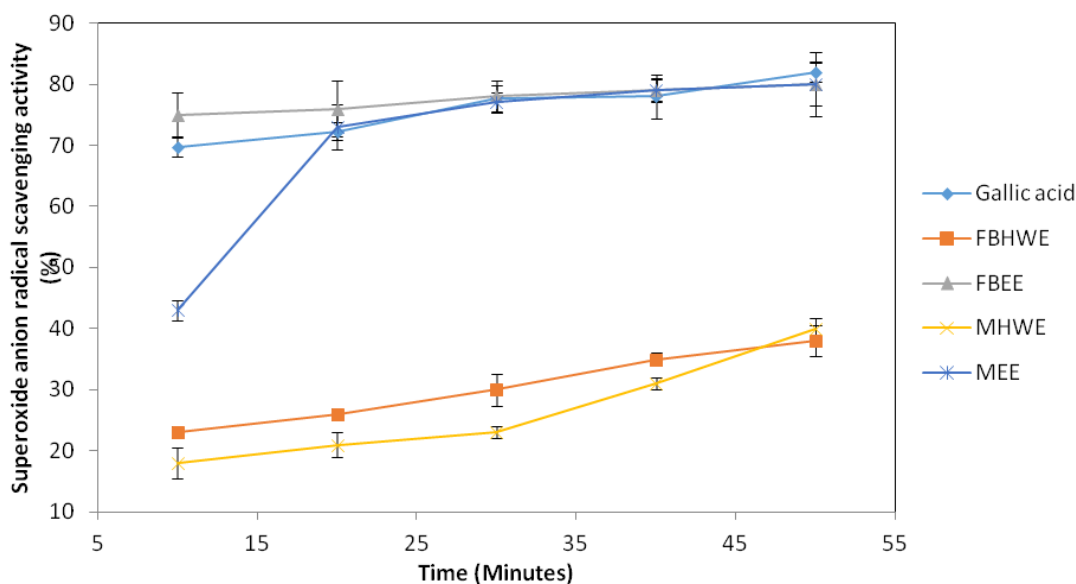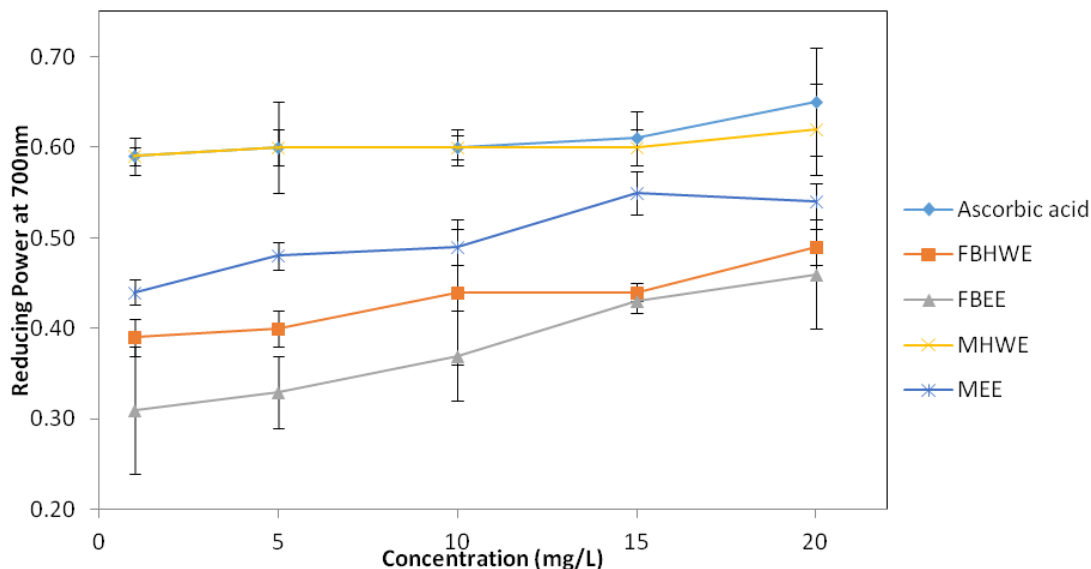
body and mycelium hot water and ethanoic extracts of *A. bisporus.* Results show that generally the superoxide anion radical scavenging activity was similar in fruiting body ethanoic and mycelium ethanoic extracts which were then followed by similar lower superoxide anion scavenging activity of the fruiting body and mycelium hot water extracts. An increasing superoxide anion radical scavenging activity was noted with increasing incubation time for fruiting body and mycelium hot water extracts.

Figure 4 shows the reducing power of the fruiting body and mycelium hot water and ethanoic extracts of *A. bisporus.* Results show that the reducing power of both ascorbic acid and mycelium hot water extracts were similar for concentrations ranging from 1 to 15 mg/L but this was significantly increased when the concentrations of the two was raised to 20 mg/L (Figure 3); for the fruiting body hot water extracts, fruiting body ethanoic extracts, and mycelium ethanoic extracts, increasing the concentrations increased the reducing power. In general, the order of reducing power decreased from ascorbic acid and mycelium hot water extracts followed by mycelium ethanoic extracts followed by fruiting body hot water extracts. EC_{50} values showed that antioxidant activity was inhibited by 50% at 0.5 absorbance for reducing power. DPPH and Hydroxyl radicals were also scavenged by 50%

Mineral element analysis

Some antioxidant enzymes such as cytoplasmic and mitochondrial superoxide dismutases require micro-

Table 2. Mineral element composition of the fruiting body and mycelium of *Agaricus bisporus*.

Mineral	Mineral concentration (mg/kg)	
	Fruiting body	**Mycelium**
Copper	24.0±2.7	18.0±2.7
Zinc	46.7±3.6	42.0±1.0
Manganese	9.0±3.0	7.5±0.1
Iron	33.0±2.7	48.5±1.0

Results are expressed as Mean ± Standard Deviation (SD) of the three independent determinations.

elements for their activities. All the samples from *A. bisporus* analysed recorded substantial amounts of the elements tested (Table 2). The Fruiting body sample contained high amounts of zinc (46.7±3.6 mg/kg) while mycelium recorded 42.0±1.0 mg/kg zinc. The amount of iron was 33.0±2.7 mg/kg in fruiting body and 48.5±1.0 mg/kg in mycelium. The amount of copper recorded was 24.0±2.7mg/kg in fruiting body and 18.0±2.7mg/kg in mycelium. Fruiting body had the highest amount of Zinc while mycelium had the highest amount of iron.

DISCUSSION

A. bisporus extracts were found to be rich in secondary plant metabolites like total phenols, flavonoids, ascorbic acid, β-carotene and lycopene. *A. bisporus* usually grow

in estuarine swamps; have unique adaptations to combat environmental stress conditions for example high salinity, high temperature, low nutrient and excessive radiation. An inevitable consequence of this process results in the production of ROS and accordingly the antioxidant enzymes were upregulated due to altered expression of these antioxidant genes (Jitesh et al., 2006). Phenolics have been considered classic defence compounds for protecting plants from herbivores. Ever since plant secondary metabolites were suggested to have evolved for that reason. In contrast to these concepts, it has been suggested that the main role of many plant phenolics may be to protect leaves from photo damage, not herbivores; they can achieve this by acting as antioxidants; and their levels may vary with environmental conditions in order to counteract this potential photo damage (Banerjee et al., 2008). The phenolics especially flavonoids were shown to protect mushrooms from UV radiation (Agati et al., 2007). The different extracts from mushroom were high in phenolic content and reflected greater synthesis since these were grown and survived in stress condition. The high content of total phenols in the mushroom extracts was partially responsible for their effective antioxidant properties (MHWE-40.26±0.9 and 31.87±0.42 mg/g for FBHWE). In fact, it had been reported that the antioxidant activity of plant materials is well correlated with the content of phenolic compounds (Ferreira et al., 2007). The level of phenolics in mushroom samples analysed (40.26 to 4.61 mg/g) was higher than the content of the total phenolics obtained from other edible wild mushroom like *Lactarius deliciosus* (pine mushroom), which was 17.25 to 0.65 mg/g as reported by Ferreira et al. (2007). As a result *A. bisporus* is a potential superior natural source of phenols which are known to be effective antioxidants.

However, with regard to solvents used, it was obvious that water extracts contained higher TPC than ethanol extracts. This could be due to the availability of more water soluble phenols in the extract. Higher amounts of phenolic compounds were obtained with increasing the concentrations of extracts, which was in agreement with reports (Chirinang and Intarapichet, 2009). The high phenolic contents of *A. bisporus* in this study are comparable to those in commonly consumed vegetables such as lettuce, celery and cucumber (Chu et al., 2002). It has been reported that phenolic groups such as flavonoids, carotenoids, anthocyanins, phenolic acids, tannins, lignans, and phenolic acids were found in *A. bisporus* (Button mushroom) Gursoy et al. (2010). A relationship between the reducing power, scavenging ability on hydroxyl radicals, scavenging ability on super-oxide anion radical and scavenging ability on DPPH and antioxidant components was found, indicating that the mechanisms of action of the extracts for the antioxidant activity may be identical, being related to the content of total phenols. The RSA of ethanolic extracts (FBEE and MEE) were found to be higher ($p < 0.05$) than those of

water extracts (FBHWE and MHWE). Generally, ethanolic extracts were more effective in scavenging abilities than hot water extracts for most mushrooms reported previously (Savoie et al., 2008). Similar observation was found in our experimental data. From the analysis of Figure 1, we can conclude that the scavenging effects of mushrooms ethanolic extracts on DPPH radicals increased with the concentration increase. The higher scavenging abilities in ethanolic extracts might be attributed to the high level of antioxidant components in extracts, which could react rapidly with DPPH radicals and reduce most DPPH radical molecules. These results indicated that extracts were free radical scavengers, acting possibly as primary antioxidants (Savoie et al., 2008).

The results of hydroxyl radical scavenging powers of the mushroom extracts showed that MHWE exhibited the highest hydroxyl radical scavenging activity (70%) at the highest dose of 20 mg/ml concentration. Oyetayo et al. (2009) had earlier reported a concentration dependent increase in the scavenging abilities of all hot water extracts from *Ganoderma tsugae* on hydroxyl radicals while methanolic extract from *G. tsugae* showed a non-concentration dependent hydroxyl radical scavenging ability. Therefore, the sample from *A. bisporus* has an appreciable scavenging power on hydroxyl radicals. The superoxide anion scavenging effect of both ethanolic and water mushroom extract peaked at the 30th min, after which the superoxide anion radical scavenging effects decreased. This is probably due to exhaustion of radical-scavengers (Kim et al., 2009). This supports previous observations by Oyetayo et al. (2009) that hot water extract from natural and cultured mycelium of *Cordyceps sinensis* had a better superoxide scavenging effect than ethanolic extract affirming the role of extraction solvent polarity in superoxide anion scavenging ability. Generally, the samples have some appreciable effect on superoxide free radical scavenging. The result also showed that all the 4 extracts exhibited good antioxidant activity as evidence by their lower EC_{50} values. With regard to the scavenging activity on hydroxyl radicals, various extracts were effective in order of their EC_{50} values: Hot water extracts of mycelium was the most effective with EC_{50} value of 9.8± 0.5 against fruiting body hot water extract (10.2± 0.7). The ethanoic extracts with EC_{50} values of 15.3±0.5 and 15.1±0.5 of fruiting body and mycelium, respectively, were also effective. Therefore, the hydroxyl ion scavenging ability exhibited suggests that extracts have potentials of being used as alternative to synthetic antioxidants in arresting oxidative activity of hydroxyl ions (Oyetayo et al., 2009). There is an excellent correlation between contents of total phenols and EC_{50} values of antioxidant activity (Savoie et al., 2008). The presence of zinc and iron in high levels also explains the effectiveness of the samples in scavenging superoxide anion radicals. Cytoplasmic and mitochondrial superoxide dismutase enzyme requires Cu, Zn and Mn to catalyse

the removal of superoxide radicals. Furthermore H_2O_2 in the cell is removed by catalase (C 1.16.1.16) which require Fe (Duthie, 1993). Iron is also required for transport of oxygen and oxidative metabolism (Bothwell et al., 1979). *A. bisporus* could therefore be considered as a complement in the human diet for the health benefits they present.

In conclusion, the ethanolic and hot water extracts from fruit bodies and mycelia were effective in antioxidant properties which represent antioxidant agents to provide prophylaxis against various diseases related to oxidative stress. Phytochemical screening of samples from *A. bisporus* indicated presence of compounds responsible for antioxidant and antibacterial activity. The major anti-oxidant components found in hot water extracts were total phenols and in ethanolic extracts were total toco-pherols. *A. bisporus* is therefore a cheaper and more natural source of antioxidants useful for the wellbeing of a living body system. The obtained results could form a good basis for selection of mushroom species for further investigation in the potential discovery of new valuable bioactive compounds.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

Lipid profile and levels of omega-3 polyunsaturated fatty acids present in jackfruit (*Artocarpus heterophyllus*) Lam. (Moraceae) seeds and variation in different treatments

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The intake of polyunsaturated fatty acids especially omega-3 is projected to be way below the recommended intake in Kenya. Thus, there is need to find other sources of polyunsaturated fatty acids (PUFAs). This study screened for the lipid profile and levels of omega-3 PUFAs in jackfruit and explored the variation in lipid profile of jackfruit seeds in different areas and treatments. The extracted lipids were characterized and analysis done using gas chromatography. The lipid content was found to be 0.45 ± 0.24%, iodine number was 60.76 ± 3.25, saponification number was 353.65 ± 14.21, and levels of omega-3 and of omega-6 PUFAs were also found to be 9.94 ± 0.99% and 31.19 ± 0.82%, respectively. Boiling and drying of seeds were found to greatly decrease the levels of polyunsaturated fatty acids such omega-3 and omega-6 and thus, not suitable methods for processing or preservation of jackfruit seeds.

Key words: Jackfruit, omega-3 polyunsaturated fatty acids, lipid profile.

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus*) is one of the most significant trees in tropical home gardens and perhaps the most widespread and useful tree in the important genus *Artocarpus*. Jackfruit tree belongs to the family Moraceae and is believed to have originated from the rainforests of the Western Ghats. It is mostly found in Asia, mainly in India, China and Philippines. In Africa, it is found in Kenya, Uganda and Zanzibar (Morton, 1987). The tree is easily recognized by its fruit, which is considered to be among the largest in the cultivated plants and the fruit's weight ranges from 2 to 30 kgs. The succulent, aromatic, and flavorful fruit is eaten fresh or preserved in numerous ways (Elevitch and Manner, 2006). Jackfruit has been reported to contain high levels

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of protein, starch, calcium and thiamine (Mukprasirt and Sajjaanantakul, 2004) as well appreciable levels of lipids (Ajayi, 2008). One fruit contains about a 100 to 500 seeds, which are processed in different ways before consumption.

The seeds can be boiled, roasted or preserved in syrup like chestnuts. Roasted, dried seeds are ground to make flour, which is blended with wheat flour for baking (Morton, 1987). This fruit is always termed as a 'neglected fruit' as little research has been done on it and is also considered to be for the lower socioeconomic class in Asia, where the fruit is most dominant (Ocloo et al., 2010). Studies on jackfruit seeds have shown that it has appreciable levels of lipids and knowledge on lipid profile, will help in determining the nutritive content of the fruit (Ajayi, 2008).

Fatty acids exist in two main categories namely: saturated and unsaturated fatty acids. Most saturated fatty acids are synthesized by the body, while the unsaturated fatty acids need to be provided in the diet. The most important polyunsaturated fatty acids are the omega-3 and omega- 6 PUFAs. Clinical studies have established that the omega-6 fatty acid and omega-3 PUFAs collectively protect against coronary heart disease (Vasuki and Hayes, 2004).

Omega-3 PUFAs also improve vascular endothelial function and help lower blood pressure, platelet sensitivity and the serum triglycerides (Vasuki and Hayes, 2004). Studies show that fatty acids are among the most crucial molecules that determine one's brain integrity and ability to function well (Chang et al., 2009). Epidemiological studies suggest that diets rich in omega-3 polyunsaturated fatty acids reduce the risk of cancer (Berquin et al., 2007).

It is projected that the intake of omega-3 PUFA falls below the recommended amount of 250 to 500 mg daily for individuals without cardiovascular heart diseases (Lee et al., 2009). The main source of omega-3 fatty acid in the diet is fish (Christian et al., 2007). However, due to over fishing, there is need to find an alternative dietary source of omega-3 PUFAs. The vegetarians on the other hand, solely rely on plant sources to obtain all the important fatty acids. Knowledge of plants lipid profile will enable them to make informed choices, so that they can also meet the daily recommended intake of PUFAs (Lee et al., 2009).

A few plant oils have been found to contain omega-3 fatty acids. The plant oils include: flaxseed, carola oil, soybean and pumpkin (Coupland, 2008). Despite the fact that the presence of lipids in jackfruits has been documented, no extensive studies have been carried out on the nature of the lipids present (Ocloo et al., 2010). This study seeks to screen the lipid profile and levels of omega-3 PUFAs in Jackfruit seeds and determine if the levels vary in different areas, the effect of boiling and drying, which are both common processing and storage methods used in Kenya, on the total lipid profile.

MATERIALS AND METHODS

Research design

Jackfruits were obtained from six regions namely: Nairobi, Mombasa, Malindi, Kakamega, Uganda and Kisumu. The study was done at the Department of Biochemistry, University of Nairobi. Three fruits were obtained per region and seeds extracted from these fruits. This was done by slicing the fruits and removing the seeds, which were approximately 100 to 500 per fruit. The seeds from each fruit were then divided into three groups, where the seeds from the first group were extracted while fresh. The seeds from the second group were dried for one week in sunlight, after which lipid was extracted and the third group was boiled in water for 2 h then lipid extracted. The seeds were crushed using a blender to break it to smaller pieces and further ground using mortar and pestle and then weighed. Thirty grams of the crushed seeds were used in each case for lipid extraction using Folch method. The lipid extracted was then characterized using saponification and iodine number.

The lipid profile of Jackfruit was determined using gas chromatogram (GC) analysis. Prior to G.C analysis, the unsaturated fatty acids were methylated through the derivatization process using methanolic sulfuric acid. The methylation was done to ensure accurate separations during the G.C analysis. The methylated samples were then injected into the GC and the peaks noted. The standard, which consisted of a mixture of fatty acid methylesters were also run in the GC. The retention time of the fatty acid in the analytes were compared to those of the standards to identify the fatty acids present. The levels of fatty acids in all regions was then determined to obtain the Jackfruit lipid profile, comparison of how the levels varied in the different region was also determined using analysis of variance (ANOVA) at $p = 0.05$ and the variation of the fatty acid profile both in fresh, boiled and dried seeds was also done using ANOVA to determine the effect of boiling and drying on fatty acid profile and levels of omega-3 and omega-6 fatty acids.

Fruit variety sampling and preparation

Jackfruits were obtained from six different geographical locations namely: Nairobi, Mombasa, Malindi, Kakamega, Uganda and Kisumu. Three fruits were picked from each of the six regions and the seeds extracted from each fruit. Each fruit had seeds ranging from one 100 to 500 seeds. The seeds of each fruit were then subdivided into three groups. The first group was analyzed while fresh, the second group dried in the sun for one week and the last one boiled in water for 2 h prior to analysis. The seeds were then crushed using a blender for 5 to 10 min until they were broken into smaller pieces and further ground using mortar and pestle prior to lipid extraction.

Lipid extraction and quantification

Thirty grams of crushed seeds from each group were homogenized with chloroform/methanol (2/1) of volume 600 ml. The whole mixture was agitated for 1 h in a magnetic stirrer at room temperature. The homogenate was then centrifuged to recover the liquid phase (Folch et al., 1957). The solvent was washed with 120 ml volumes of 0.9% NaCl solution. The mixture was then centrifuged at low speed (2000 rpm) to separate the two phases. Upper phase was removed by siphoning after centrifugation and the lower chloroform phase containing lipids was evaporated under vacuum in a rotary evaporator. The lipid extract was then weighed after it had dried completely and stored at -20°C in a freezer (Folch et al., 1957).

Table 1. Percentage weight of lipid content in g per 100g, Saponification (mg of KOH/ g of lipid and Iodine number (g of I/ 100 g of lipid) of fresh samples from all regions.

Region	Lipid content (% weight)	Saponification number (mg of KOH/ g of fat)	Iodine number (g of I_2/ 100g of fat)
NBI FRESH	0.45 ± 0.25	346.8 ± 4.95	62.91 ± 2.39
UG FRESH	0.46 ± 0.22	346.17 ± 21.92	63.55 ± 2.32
MSA FRESH	0.45 ± 0.21	347.43 ± 9.00	60.54 ± 4.71
MLD FRESH	0.44 ± 0.15	362.28 ± 17.48	61.81 ± 4.05
KSM FRESH	0.44 ± 0.34	350.60 ± 12.09	61.29 ± 4.62
KKMG FRESH	0.47 ± 0.26	368.59 ±19.79	53.88 ± 1.41
MEAN	0.45 ± 0.24	353.65 ± 14.21	60.76 ± 3.25

Characterization of the lipid extracts

Saponification number is defined as the amount in milligram of KOH required to saponify one gram of fat. In order to determine the saponification number of the lipid extracts, 200 mg of lipid extract was mixed with 2 ml of chloroform. 0.2 ml of the mixture was boiled with 2.5 ml of 0.3 M potassium hydroxide under a reflex condenser in a 250 ml flux. The mixture was then removed and allowed to cool. Two drops of phenolphthalein indicator was added to the mixture, after which it was titrated with 0.3 M hydrochloric acid until endpoint. Titration of blank KOH was also done and the value was noted. To obtain the volume that was saponified, the volume of HCl used to titrate the mixture was subtracted from volume of HCl used to titrate the blank. The number of moles of KOH in this volume was then determined and the results expressed in mg of KOH per 1 g of fat (Masukawa et al., 2010). Iodine number is the mass of iodine in grams that is consumed by 100 g of lipid. Iodine number was determined by a drop wise addition of iodine and mercuric chloride solution to 200 mg of lipid dissolved in 2 ml chloroform, until the lipid solution turned brown. The solution consisted of 26 g iodine and 30 g of mercuric chloride in 250 ml ethanol, which were mixed and made up to 1 L by addition of ethanol. The solution was made in such a way that, 1 ml of the solution, contained 26 mg of iodine. The iodine number was calculated by multiplying the volume in (ml), of iodine and mercuric chloride solution used to titrate the lipid solution by 26 and results were expressed in grams of iodine per 100 g of lipid (Gupta and Kanwar, 1994).

Gas chromatogram analysis of the lipid extracts

Fatty acid methyl ester (FAME) was prepared following the method described by (Mbatia et al., 2010). 20 mg of lipid sample was mixed with 2 ml of toluene and 2 ml of 1.5% sulphuric acid in dry methanol. The mixture was then vortexed to mix and incubated at 55°C for the whole night. 4 ml of saturated NaCl solution was then added and the mixture vortexed to mix. 2% of NaHCO$_3$ was also added and mixed well until the lower phase had a pH of 7. The mixture was centrifuged for 10 min at 2000 r.p.m and the upper phase collected through siphoning and stored for gas chromatography (Mbatia at al., 2010). The fatty acid analysis was carried out using Varian CP 3800 GC system equipped with a flame ionization detector (FID). Supelco wax 10 capillary column (60metres by 0.32 mm by 25 µm film thickness; Supelco, Bellefonte, PA, USA) was used to separate the FAME. The carrier gas was Helium at 1.79 * 10^{-1} MPa. The temperature programmed for separation was as follows: initial temperature of 50°C was held for 5 min, increased to 220 at 25°C/min and held for 21 min. The temperature was then increased to 240°C at 15°C/min and held for 10 min. The injector temperature was maintained at 60°C/min and held for 2 min. The detector temperature was kept constant at 250°C.

The instrument was calibrated with one-point calibration method, using a standard mixture of fatty acid methyl esters of known proportions and an internal standard used to spike the samples to monitor the detector response. 1 µ of both the standards, which were made of different concentrations and the samples, which were injected into the GC. Individual methyl esters were identified by comparison of retention times to those of known standards. The peak areas of the standards obtained from the chromatograms were used to calculate the detector response. This was done by division of all standard peak areas, by their corresponding internal standard peak areas. The detector response of the standards, which were of different concentrations, were then used to plot seventeen calibration graphs of the fatty acid methyl esters present in the standards. The calibration graphs were then used to determine the levels of each fatty acid present in the samples (Ackman, 1980). A ratio of the peak areas of the standards to the internal standards were calculated and a calibration graph of the ratio of the detector response against concentration, plotted for each of the seventeen fatty acids (Ekeberg et al., 2006). The concentration of each fatty acid was determined by multiplying the concentrations, which were in p.p.m (mg/L) present, by the percentage weight of the fatty acids in the standard.

Data analysis

The mean and standard deviation of the lipid content and fatty acid values were determined, and one-way analysis of variance was used to detect variation in means at significance level of p = 0.05.

RESULTS

Lipid content and characterization of Jackfruit seeds

The samples from each region were done in triplicates, and the mean and standard deviation per region determined were as shown in Table 1, the lipid content of samples from all regions showed no variation at significance p = 0.05. The average lipid content for the fresh seeds was found to be 0.45 ± 0.24 g per 100 g of crushed seeds. The mean saponification and iodine values were also found to be 353.65 ± 12.51 and 60.76 ± 3.25, respectively, as shown in Table 1.

Lipid profile of jackfruits seeds

The lipid profile was found to contain sixteen fatty acids. The fatty acids were namely tetradecanoic acid (C 14:0),
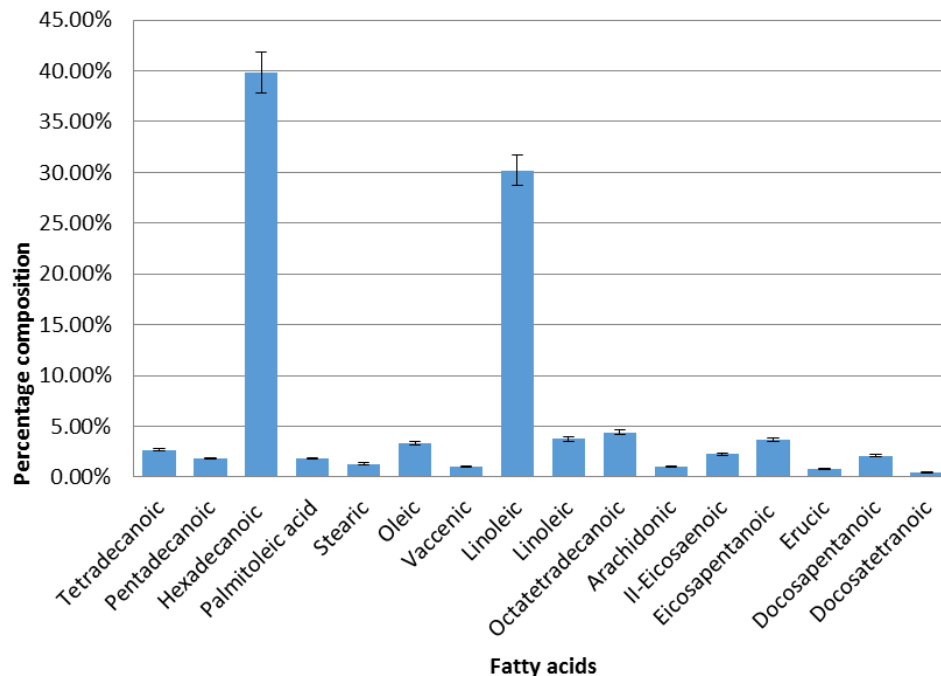
Figure 1. Percentage composition of Jackfruit seeds fatty acid profile of fresh samples from all the six regions.

pentadecanoic acid (C15:0), Hexadecanoic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C 18: 1), vaccenic acid (C18:1), linoleic acid (C 18:2), linolenic acid (C18:3), octadecatetranoic acid (C18:4), 11-eicosanoic acid (C20:1), arachidonic acid (C20:4), eicosapentanoic acid (C22:5), erucic acid (22:1), docosatetranoic acid (C22:4) and docosapentanoic acid (C22:4) as shown in Figure 1. Hexadecanoic acid recorded the highest relative percentage composition of 39.85 ± 2.68%. Linoleic acid, which is an omega-6 fatty acid, equally recorded in high values and was also among the most dominant fatty acids, with the relative percentage of 30.18 ± 0.59%. The other saturated fatty acids that were consistently present were tetradecanoic acid (C14:0), pentadecanoic acid (C15:0) and stearic acid (C18:0), whose percentages ranged from 1 to 3%, which implies that they were present in low levels. The total levels of saturated fatty acids were found to be 45.64±4.90%. The monounsaturated fatty acids present were palmitoleic acid, oleic acid, vaccenic acid and 11-eicosanoic. The total percentage of monounsaturated fatty acids is 6.55±2.32. The polyunsaturated fatty acids present are linoleic acid, linolenic acid, octadecanoic acid, arachidonic acid, eicosapentanoic acid, erucic acid, docosatetranoic and docosahexanoic acid. They constituted the greatest percentage composition of 46.37%. The total monounsaturated and polyunsaturated fatty acids is 52.92%, which implies that Jackfruit mostly consists of polyunsaturated fatty acids. The omega-3 fatty acids were found to be linolenic acid,

eicosapentanoic acid, docosapentanoic acid and docosatetranoic acid. Linoleic and eicosapentanoic acid were found to be the most abundant omega-3 fatty acids, with the least being docosatetranoic acid and docosapentanoic acid as shown in Table 2. The total percentage levels of omega-3 polyunsaturated fatty acids were found to be 9.94 ± 0.99%. The levels of omega-6 fatty acids were found to be 31.26 ± 0.82%, this could be attributed to the fact that linoleic acid was among the most dominant fatty acids.

Lipid content, profile and levels of omega-3 fatty acids in Jackfruit seeds from different areas

The levels of fatty acids from all the six regions showed no significant variation at significance p = 0.05. The percentage composition of the fatty acids of the saturated fatty acids such as tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, and stearic acid showed a lower variation compared to the values of unsaturated fatty acids such as oleic acid, vaccenic acid (11 – Octadecenoic acid), linoleic acid, linolenic acid, octadecatetraenoic acid, 11-eicosenoic acid, arachidonic acid, eicosapentanoic, (erucic acid 22:1), docosatetranoic and docosapentanoic acid as shown in Figure 2. There was also a variation in percentage composition of omega-3 PUFAs at significance p = 0.05, which included eicosapentanoic acid, docosapentanoic acid, linolenic acid and docosatetranoic acid. The seeds were also

Table 2. Overall percentage fatty acid composition of saturated, monounsaturated and polyunsaturated fatty acids from all regions.

Fatty acid	Percentage composition	Type of fatty acid
Tetradecanoic acid (C14:0)	2.66±0.51	Saturated
Pentadecanoic acid (C15:0)	1.83±1.21	Saturated
Hexadecanoic acid (C16:0)	39.85±2.68	Saturated
Stearic acid (C18:0)	1.30±0.50	Saturated
Oleic acid (C 18: 1)	3.30±1.38	Monounsaturated
Vaccenic acid (C18:1)	1.01±0.44	Monounsaturated
11-Eicosanoic acid (C20:1)	2.24±0.02	Monounsaturated
Linoleic acid (C 18:2)	30.19±0.59	Polyunsaturated
Linolenic acid (C18:3)	3.73±0.25	Polyunsaturated
Octadecatetranoic acid (C18:4)	4.38±0.53	Polyunsaturated
Arachidonic acid (C20:4)	1.07±0.23	Polyunsaturated
Eicosapentanoic acid (C22:5)	3.66±0.65	Polyunsaturated
Erucic Acid (22:1)	0.79±0.02	Polyunsaturated
Docosatetranoic acid (C22:4)	0.47±0.04	Polyunsaturated
Docosapentanoic acid (C22:4)	2.08±0.06	Polyunsaturated
Total saturated fatty acids	45.64±4.90	
Total monounsaturated fatty acids	6.55±2.32	
Total polyunsaturated fatty acids	46.37±2.12	

found to contain a high levels of omega-6 fatty acids, namely the linoleic acid and arachidonic acid. Linolenic acid was the most abundant Omega-3 fatty acid followed closely by eicosapentanoic acid as shown in Table 3.

The effect of boiling and drying on the lipid content and lipid profile

The boiled seeds had the lowest lipid content in most regions compared to the fresh and dried seeds. There was a significant variation in iodine values in the fresh, dried and boiled seeds at significance of p = 0.05. There was no significant variation in saponification values for fresh seeds, boiled and dried seeds in all regions at p = 0.05. Hexadecanoic acid showed a small variation in fresh, dried and boiled seeds though the values of the boiled seeds were slightly lower. Tetradecanoic acid, hexadenoic acid, palmitolenic acid and stearic acid were also found to show slight variations in the levels of both fresh, boiled and dried seeds and they are all saturated fatty acids as shown in Figure 3. The difference in fresh and boiled seeds was however higher in oleic, vaccenic acid, linolenic, linoleic acid and octadecatetranoic acid, which are all unsaturated fatty acids. Linoleic acid was also found to be in high levels in fresh fruits and lower in boiled and dried seeds. Octadecatetraenoic acid was found to be present only in fresh fruits and the levels in both boiled and dried seeds were found to be below the GC detection limit, which is the lowest concentration of

an analyte that can be detected by the GC. The levels of eicosapentanoic acid were relatively high in fresh seeds but below the G.C detection limit in both dried and boiled seeds. arachidonic, erucic and docosapentanoic acid were also found to be present in fresh seeds but below G.C detection limit in both dried and boiled seeds except for docosapentanoic acid, which was present in small quantities in dried seeds as shown in Figure 4.

DISCUSSION

The average mass of the oil content per 100 g was 0.45±0.24 g. This implies that the oil content of Jackfruit seeds constitutes 0.45 ± 0.24%. This value is different from those found in other studies, as the oil content was found to be 11.39% (Ajayi, 2008). The value was also different from the findings of Sign et al. (1991) whose value was 3.2, 0.91% by Mukprasirt and Sajjaanantakul (2004) and Tulyathan et al. (2002), who recorded 0.99% lipid content in Jackfruit. The value was however, close to (Madrigal-Aldana et al., 2011), which was 0.71%. The saponification number was 353.65 ± 14.21 mg of KOH per 1 g of fat, which is above 250 upper limit for lipids with long chained fatty acids, implying that Jackfruit fatty acids mainly consists of short chained fatty acids (Dosumu and Ochu, 1995). The iodine number was 60.76 ±3.25, which is relatively high, implying that the oil contains an appreciable level of unsaturated bonds (Akubugwo and Ugbogu, 2007). The polyunsaturated
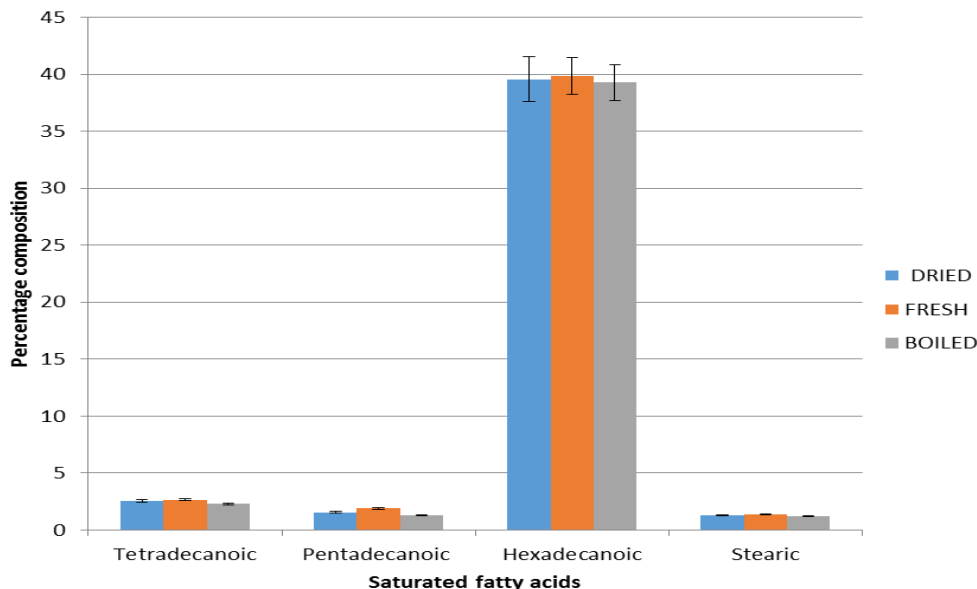
Figure 2. Fatty acids composition of fresh samples of Jackfruit seeds from all the six regions.

Table 3. Overall percentage composition of omega-3 and omega-6 PUFAS in Jackfruit.

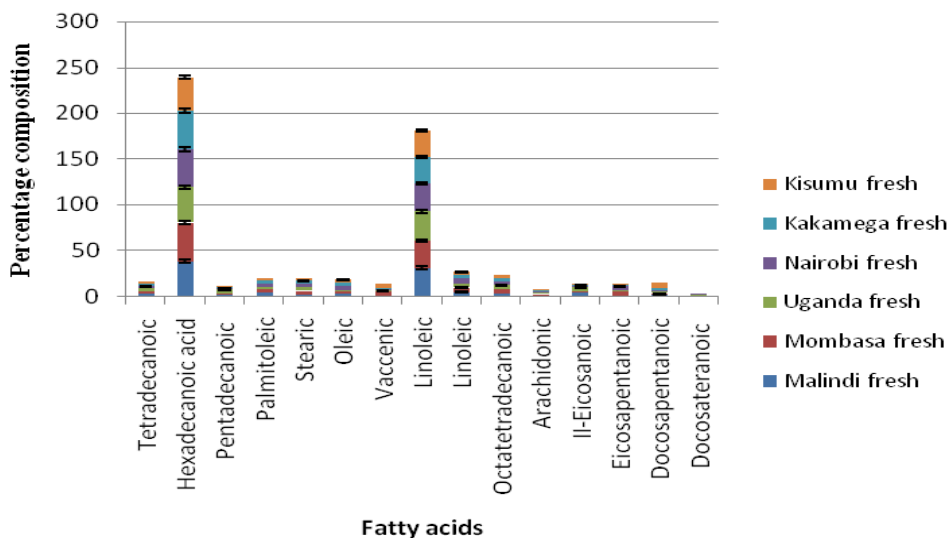| Fatty acid	Percentage composition	Type of fatty acid
Linolenic acid (C18:3)	3.73±0.25	omega-3
Eicosapentanoic acid (C22:5)	3.66±0.65	omega-3
Docosatetranoic acid (C22:4)	0.47±0.04	omega-3
Docosapentanoic acid (C22:4)	2.08±0.05	omega-3
Linoleic acid (C 18:2)	30.19±0.59	omega-6
Arachidonic acid (C20:4)	1.07±0.23	omega-6
Total omega-3 PUFAs	9.94±0.99	
Total omega 6 PUFAs	31.26±0.82	

Figure 3. Percentage compositions of saturated fatty acids in fresh, boiled and dried samples from all regions.
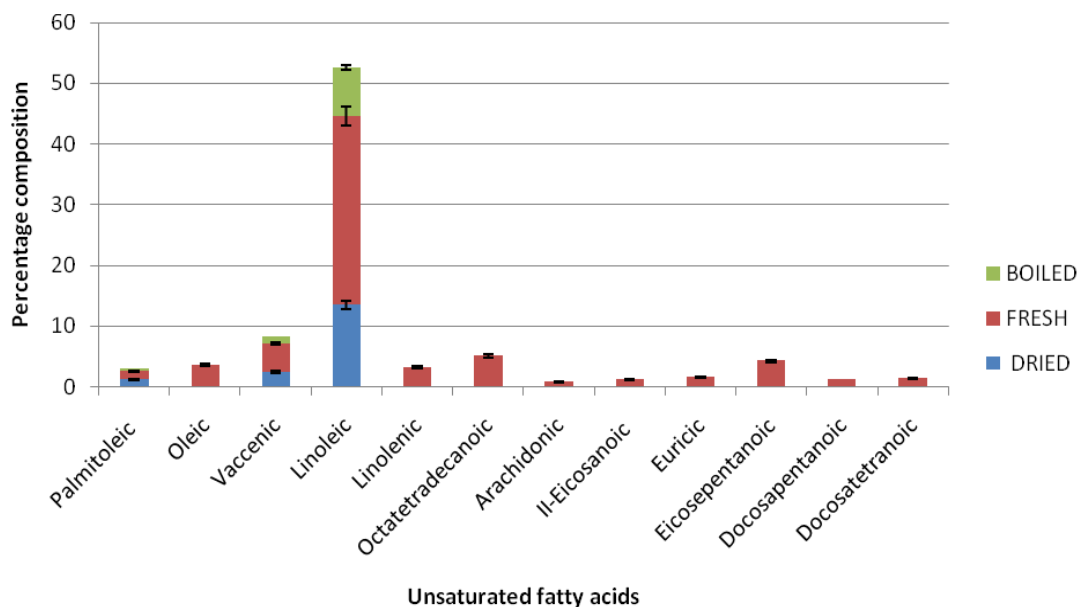
Figure 4. Percentage composition of unsaturated fatty acids in fresh, boiled and dried samples from all regions.

fatty acids found were linoleic acid, linolenic acid, octadecanoic acid, arachidonic acid, eicosapentanoic acid, erucic acid, docosatetranoic and docosahexanoic acid. They constituted the greatest percentage composition of 46%. This implies that Jackfruit lipid is very beneficial to our health. The total monounsaturated and polyunsaturated fatty acids is 52.92, which shows that Jackfruit mostly consists of polyunsaturated fatty acids and thus healthy as most polyunsaturated fatty acids have been associated with numerous health benefits.

The omega-3/omega-6 ratio was 1:3 which is within the margin of the recommended intake ratio of 1:4. This study was consistent with Kikuta and Erickson (1968) and Mazliak (1965), whose findings indicated, that one of the most dominant saturated fatty acids in Avocado is hexadecanoic acid, which was also found to be the most dominant saturated fatty acid in jackfruit seeds. The monounsaturated fatty acids oleic acid (18:1) and palmitoleic acid (16:1) and the polyunsaturated fatty acids linoleic acid (18:2) and linolenic acid (18:3) were also found to be present, which is consistent with this study as these fatty acids were also found to be present in the fatty acid profile of Jackfruit seeds (Kikuta and Erickson, 1968).

Studies also indicate that the fatty acids profile of different plant oils vary from one plant oil to another. Coconut oil has been found to contain 90% saturated fats with lauric acid constituting a greater percentage and it is therefore likely to cause heart conditions if consumed in high quantities (Vasudevan, 2010). This is contrary to the popular belief that plant oils consist of a greater percentage of unsaturated fatty acids. Peanut on the

other hand, has been found to have a higher percentage of unsaturated fatty acids with oleic acid being the most dominant fatty acid and other dorminant fatty acids present were linoleic acid, linolenic acid and palmitic acid (Misuna et al., 2010). The linoleic acid and hexadecanoic acid had the highest percentage, with each having 39 and 30%, respectively. The levels of palmitic acid were lower in Jackfruit unlike in other plant oils.

Jackfruit seeds were found to contain omega-3 fatty acids and the average level of Omega-3 percentages in lipid is 9.94% with the average in 100 g of crushed seeds being 54.18 mg. This falls below the recommended intake of 350 mg (Lee et al., 2009). On the other hand, the fruit has a high level of Omega-6 fatty acids, which are equally important and good for the body and works hand in hand with Omega-3 in controlling heart disease. The fruit also has other unsaturated fatty acids that are healthy for the body apart from the hexadecanoic acids, which are equally in high levels. The World Health Organization stated that intake of hexadecanoic acid puts one at more risk of developing cardiovascular diseases (World Health Organization, 2003), however other studies indicate that hexadecanoic acid has no hyper-cholesterolaemic effect, if the intake of linoleic acid is 4.5% of the energy (French et al., 2002). From this study the levels of linoleic acid were 30%, which were way above the 4.5% and thus the seed oil may not have any negative cardiovascular effects.

The lipid profile of jackfruit of fresh fruits from all the six regions showed no variation at significance p = 0.05. A comparison of the means of fatty acid in all the six regions, showed variation in levels of omega-3 polyunsa-

turated fatty acids at significance p = 0.05. This could be attributed to factors such as slight difference in weather patterns, which may in turn lead to difference in temperature. Studies show that temperature affects the lipid profile of organisms, with organisms in lower temperature regions having a higher level of polyunsaturated fatty acids especially the omega-3 fatty acid as opposed to regions of higher temperatures (Yaniy et al., 1988). The difference could also be attributed to difference in varieties, which in turn implies difference in genetic makeup may cause variation in composition of polyunsaturated fatty acid. A study done by Green et al. (1984) revealed that difference in fatty composition is also under genetic control. The lipid content was found to show variation in fresh, boiled and dried seeds at p = 0.05, this could be attributed to the fact that some of the oil may have been lost in the process of boiling, as some of the lipids may have been lost in water (Simopoulos, 2002). There was a significant variation in iodine values in the fresh, dried and boiled seeds at significance of p = 0.05. This implies that the boiling and drying affects the degree of unsaturation of fatty acids. The double bonds in the unsaturated fatty acids undergo hydrolytic and oxidative reactions in high temperatures leading to a decrease in the iodine number (Ramezanzadeh et al., 2000). The saponification numbers showed no variation in p=0.05 for fresh, dried and boiled samples, which implies that boiling and drying may not have an effect in the chain lengths (Dosumu and Ochu, 1995).

The levels of saturated fatty acids were found to show no variation in fresh, boiled and dried seeds at p = 0.05, this may be attributed to the fact that saturated fatty acids can withstand high temperatures (Antunes and Sfakiotakis, 2008). This implies that boiling and drying of seeds, does not affect the composition of saturated fatty acids. This is because, the four bonds attached to carbon are all attached to other atoms thus they are not susceptive to oxidative and hydrolytic reactions that cause rancidity (Dosumu and Ochu, 1995). There was however variation in levels of unsaturated fatty acids in fresh, boiled and dried seeds at p = 0.05. This implies that, drying and boiling of seeds affects the levels of unsaturated fatty acids. The dried and boiled samples of unsaturated fatty acids were in low levels, with most levels of polyunsaturated fatty acids in boiled seeds being below the GC detection limit. This is an indication that most of the polyunsaturated fatty acids are very sensitive to heat and exposure to sunlight and is readily destroyed by both of them (Ramezanzadeh et al., 2000). This means that, the processing and storage conditions that involve high temperatures often lead to enormous loss of the essential fatty acids especially the omega-3 and Omega-6 fatty acids, which have numerous health benefits (Simopoulos, 2002) and both temperature and sunlight has a negative effect on the levels of polyunsaturated fatty acids (Yaniv et al., 1988). This may be attributed to the fact that the unsaturated fatty acids readily

undergo hydrolytic and oxidative reactions, leading to decrease in levels of polyunsaturated fatty acids. The oxidative reactions usually take place at the position of the double bonds (Antunes and Sfakiotakis, 2008). The storage procedures need to ensure protection from Hydrolytic and oxidative deterioration to minimize the ability of the oil to go rancid (Williams et al., 1988).

In most samples the levels of fatty acids in the dried seeds were lower than those in the fresh seeds especially the monounsaturated and polyunsaturated fatty acids. This is consistent with the studies done by Su and Babb (2007), whose evaluation on cooking methods recorded a sharp decrease in levels of omega-3 PUFAs in cooking methods, which involved more exposure to higher temperature (Su and Babb, 2007). The omega-3 PUFA levels are greatly affected by heat and therefore, jackfruit lipid would not be a suitable for cooking oil but instead, it can be added to the margarine or used in spices such as creams or in cake icing as it is high in unsaturated fatty acids and hence provides a healthy diet. The levels of omega-3 were also significantly affected at p = 0.05 in both boiled and dried seeds. This implies that, both drying and boiling the seeds greatly decreases the levels of omega-3 polyunsaturated fatty acids. Future research should focus on coming up with better processing and storage methods that will help in preserving the levels of Omega-3 fatty acids in Jackfruit seeds.

Conflict of interests

The authors did not declare any conflict of interest.

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Full Length Research Paper

High proportion of mosquito vectors in Zika forest, Uganda, feeding on humans has implications for the spread of new arbovirus pathogens

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There is a steady increase in the contact between humans and wildlife, brought about by encroachment, destruction of natural forests, climatic and environmental changes. Mosquitoes get exposed to hosts and pathogens; creating possibilities for new disease patterns. Therefore, the identification of blood-meal sources is important to determine the linkages between hosts and vectors. Engorged mosquitoes were collected in Zika forest (Uganda) for a period of 12 months using carbon dioxide (CO_2)-baited light traps. Total genomic DNA was extracted from the abdominal contents of the mosquitoes and the diagnostic regions of the mitochondrial genes, *cytochrome oxidase subunit 1* (COI) and *cytochrome b* (cytb) sequenced. The sequences were subsequently blasted in the GenBank. Sequence analyses revealed that feeds were derived from mammalian and avian hosts. Blood-meal sources from *Aedes africanus* (Theobold) examined were entirely human. There were significant differences between host species from which the mosquito species fed (Krusakal Wallis test, χ^2 = 19.118, df = 5, p = 0.018). Several mosquitoes were considered as potential bridge vectors for a number of arboviruses and other diseases have been collected from the forest. Taking of mammalian origin blood-meals, including humans, may facilitate exposure to new pathogens and disease patterns.

Key words: Blood-meal, bridge vectors, cytochrome oxidase sub unit I, cytochrome b, Entebbe.

INTRODUCTION

Female mosquitoes take blood-meals from various verte-brates, in preparation for oviposition (Lefèvre et al., 2009).

Consequently, humans are at risk of infections with pathogens circulating within the blood system of other

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animals especially mammals (Taylor et al., 2001; Lambin et al., 2010). Mosquitoes are bridge vectors of emerging and re-emerging parasitic (Apperson et al., 2002; Kent and Norris, 2005; Diallo et al., 2012) and arboviral pathogens causing infections including West Nile virus (Apperson et al., 2004; Turell et al., 2005; Hamer et al., 2008) and Chikungunya (Diallo et al., 1999; 2012) responsible for millions of infections and deaths of humans and animals globally. Knowledge of host choice and preferences is critically required for effective vector-based disease control. Host choice depends on a number of factors including; host availability, abundance, flight behaviour, feeding periodicity and region which vary according to innate, seasonal, and environmental conditions (Hamer et al., 2008; Molaei et al., 2008). The basis for blood-meal host's choice, a consequence of a mosquito feeding and pathogen transmission in the process, currently remains unclear. Despite their relevance to public and veterinary health, knowledge of mosquito feeding patterns in Uganda is still poor.

In Uganda, earlier mosquito studies from forests including Zika focused on vertical feeding patterns of especially the genera *Aedes*, *Coquillettidia* and *Mansonia*. These studies reported many anopheline and culicine species preferring avian, amphibian, reptilian, or mammalian blood-meals (Williams, 1964; Haddow and Ssenkubuge, 1965; Mukwaya, 1972). Blood-meal sources could be directly estimated by analyzing the abdomen contents of field-derived females. All estimations during earlier studies were drawn using the precipitin test, visual observations and attraction to animal bait traps (Gilles and Wilkes, 1974; Savage et al., 2008). The precipitin test has a disadvantage of forming a cloudy antibody - antigen complex at the confluence of sera and antisera and only a small number of hosts can be identified due to limitations of commercially available reagents. Thus, serological tests would be limited in identifying hosts to species level. Visual observations and animal attractions may not be true to source.

Over the years, a new method of identifying blood-meals using polymerase chain reaction (PCR) was developed. During this process sized DNA fragments have been used to identify blood-meals (Kent and Norris, 2005), establish host choice (Garcia-Rejon et al., 2010; Lee et al., 2002) and feeding patterns of mosquitoes (Apperson et al., 2004). PCR has an advantage over older serological methods by being relatively simple to perform and more sensitive. With many DNA-size fragments, animal hosts of mosquitoes can be reliably identified to species level and if a complete match to the unknown DNA sequence is not present in the databases, the sequence data may be subjected to phylogenetic analyses. Once a given blood-meal is identified, the PCR product can be used to generate a new reference sample. Simple (Molaei et al., 2008) multiplex (Kent and Norris, 2005) and heteroduplex PCR assays (Lee et al., 2002) have been used with cytb determining hosts that

play a significant part in vector borne diseases to species level through blood-meal analysis. On the other hand, (Alcaide et al., 2009) and (Roiz et al., 2012) have used the HOTSHOT protocol with *cytochrome oxidase subunit I* (COI) to identify blood-meal hosts. Along with these identifications, a number of arboviruses including Chikungunya (Weinbren et al., 1958), Zika (Weinbren and Williams, 1958; Haddow et al., 1964; Henderson et al., 1968), Rift Valley Fever (Woodall, 1964), O'nyong-Nyong (Rwaguma et al., 1997; Lanciotti et al., 1998; Kiwanuka et al., 1999), Sindbis (Smithburn et al., 1946), Bunyamwera (Smithburn et al., 1946), Ntaya (Smithburn and Haddow, 1951), Semliki Forest (Finter, 1964), West Nile and Usutu (Smithburn et al., 1940; Williams et al., 1964), Witwatersrand and Germiston (Monath et al., 1972), Uganda S virus (Dick and Haddow, 1952) and yellow fever (Kirya et al., 1977) were isolated from mosquitoes.

However, mosquito blood-meal studies from forests in Uganda including Zika, were discontinued after the 1970's due to instabilities in the country. From the 1980's, very little was documented about mosquito feeding. Presently, anthropogenic activity has greatly modified the environment around the forest. New homes and/or crop fields and plantations are adjacent to the forest and many are still coming up with increasing urbanization. Changes around the forest might have great implications on the blood-meal host sources, and the emergence or re-emergence of pathogens and infections. It is therefore necessary to closely examine the interaction between animals and man. The aim of this study was to examine mosquito blood meal sources from Zika forest and provide information on the potential interactions that could lead to transmission of zoonoses.

MATERIALS AND METHODS

Study area

Zika is a small isolated tropical forest found at 32° 30' E and 0° 7' N and approximately 11 km (6.2 mi) from Entebbe. It is located at Kisubi on Entebbe/Kampala road, Wakiso district, Uganda. The forest covers approximately 25 hectares (61.8 acres) and forms part of a narrow sinuous strip skirting the extensive grass and papyrus swamps of Waiya Bay, a sheltered inlet of Lake Victoria near Entebbe. Using GIS, Arcview and ArcInfo software, base maps of the collection points were produced to give a pictorial representation of the study area (Figure 1). Sixteen representative sites were selected along the vertical and horizontal gradients. Six sites were located along the tower platforms, seven along the horizontal gradient within the forest and three along the forest edge (Figure 1). The sites were selected based on distance from the open swamp and tower.

Mosquito sampling

Sampling was conducted weekly for 12 months along a vertical (steel tower) and horizontal (wet, raised and marginal forest, outliers, and grassland) gradient. Adult mosquitoes were collected using carbon dioxide (CO_2)-baited CDC light trap collections
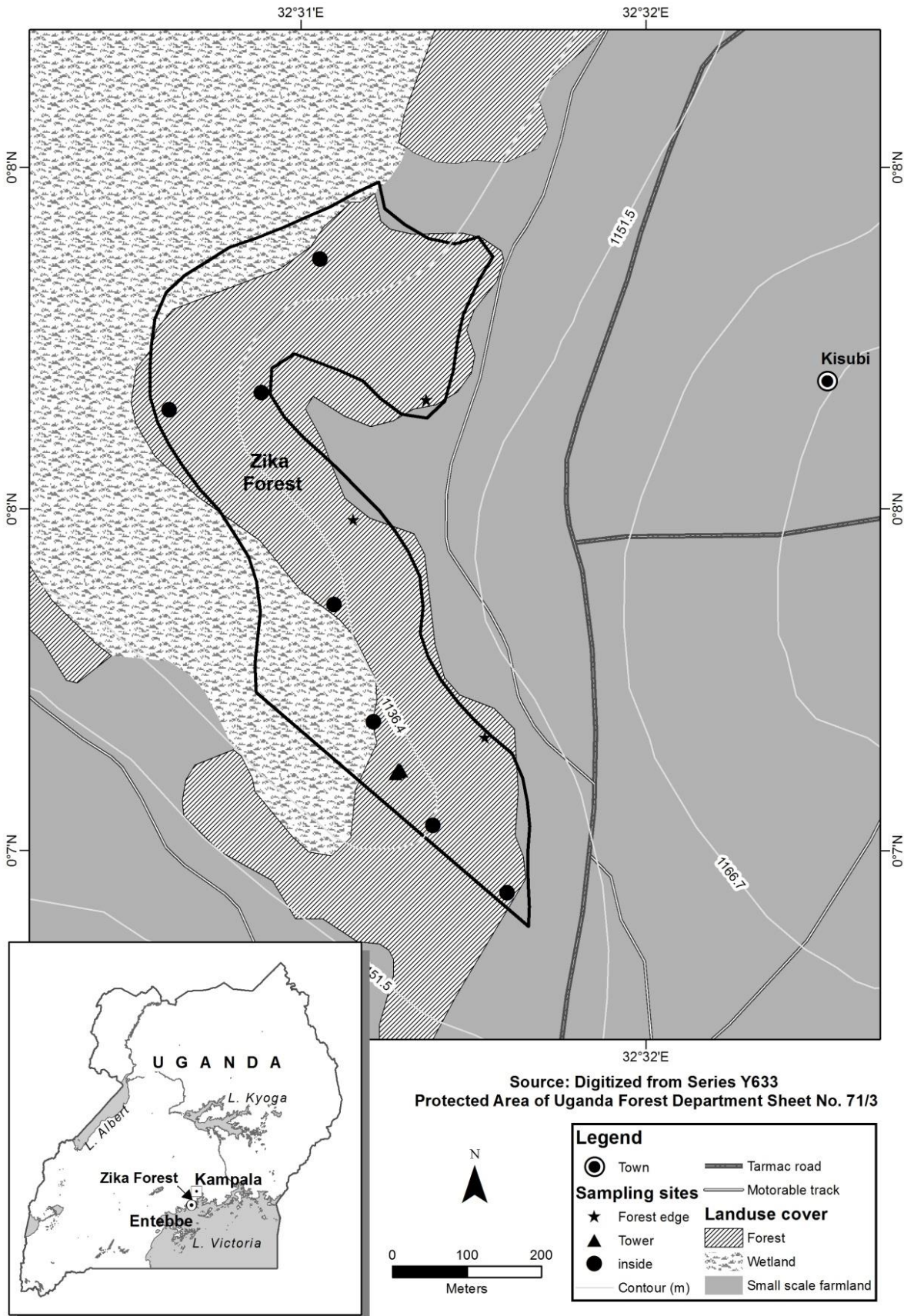
Figure 1. Sampling sites in Zika forest.

(Service, 1993). The light/CO$_2$ trap collections were done from the steel tower platforms (vertical) at 20 ft (6 m) intervals from 20 ft upwards to 120 ft and along the horizontal transect at 10 m intervals. Light/CO$_2$ trap were set weekly over 12 h period (6.00 pm to 6.00 am) just before sunset and collected early in the morning. After collection mosquitoes were quickly counted, put in vials and placed on dry ice and transported to the laboratory for identification. Mosquito species identifications were done with available keys (Edwards, 1941; de Meillon, 1947; Gillett, 1972; Gillies and Coetzee, 1987; Jupp, 1996). We used the nomenclature of (Knight and Stone, 1977) supplemented with notes and updates from the Walter Reed Biosystematics Unit website (http://www.wrbu.org/docs/mq ClassificationTraditional201307.pdf). After mosquito sorting, we selected engorged mosquitoes in the laboratory at the Uganda Virus Research Institute (UVRI). Mosquitoes were inspected for a visible blood-meal and positive samples stored at -80°C in individual cryo-vials with silica gel. Voucher specimens for each species are currently stored at the Uganda Virus Research Institute, Entebbe, Uganda.

Blood meal identification assay

The abdomens of engorged mosquitoes were carefully detached from the thorax using sterile fine forceps and scalpel blades. Each abdomen was macerated in 200 µl 0.01 M Phosphate Buffer Saline (PBS), pH 7.4 in 1.5 ml Eppendorf tube using a sterile micro-pestle for 20 min.

DNA extraction

DNA was isolated from the abdominal contents using the QIAGEN DNeasy blood and tissue kit (Qiagen Inc., Maryland, CA, USA), following manufacturer's instructions. DNA extracts from the mosquito blood-meals served as the DNA template in a standard polymerase chain reaction (PCR) assay (Kumar et al., 2007; Cywinska et al., 2006). The protocol run was revised by eluting in 32 µl AE buffer. The mixture was incubated for 1 min at room temperature followed by centrifuging at 8000 rpm and the process repeated thrice for maximum yield. Success of the DNA extracts was tested by electropheresing 5 µl of total genomic DNA on 1% NuSeive agarose gel, stained with ethidium bromide (EtBr) and visualized under ultraviolet light. Extracted DNA was stored at -20°C until needed.

DNA Amplification

Blood meal sources were assessed using standard PCR on a 9800 Fast thermal cycler (Applied Biosystems). Amplification of the fragment of a vertebrate COI mitochondrial gene using previously described primers (VFd1_t1 and VRd1_t1, Ivanova et al., 2007) (Barcode of Life Database (BOLD) and cytb gene (L14181 and H15149) was done. This assay was a modification of previously published protocols (Kocher et al., 1989; Ngo and Kramer, 2003). Universal mammalian-specific primers that amplified the 648-bp region of the COI gene VF1d_t1 5'-TAAAACGACGGCCAGTTCTCAACCAACCACAAAGACATTGG-3' and VR1d_t1 5'-AGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA-3' (Kocher et al., 1989); and the 358-bp region from the cytb gene L14181 5'- CCATCCAACATCTCAGCATGATGAAA-3' and H15149 5'- GCCCCTCAGAATGATATTTGTCCTCA-3' (Ngo and Kramer, 2003) were used. A 30 µl reaction volume was prepared with 1 µl template DNA, 1 µl each primer (0.1 to 0.5 pmol/L), 6 µl 5X HF buffer (comprising of 1.5 mM MgCl$_2$), 0.6 µl dNTP mix (10 mM/L

each), 0.25 µl Phusion High Fidelity DNA polymerase (Thermal Scientific, Finland) and 19.15 µl double distilled water. The thermocycling conditions consisted of initial denaturation at 98°C for 30 s, 39 cycles at 98°C for 10 s, annealing at 57°C for 30 s, primer extension of 72°C for 1 min and a final extension of 72°C for 7 min for COI. Conditions for cytb were similar, apart from annealing temperature at 61°C for 20 s and the cycles repeated 35 times.

Amplicon purification and sequencing

To detect the presence of amplified DNA fragments, eight micro litres of PCR products were electrophoresed on separate 1% agarose gels (NuSieve, FMC) in 40 mM Tris acetate (pH 8.0) and stained with 0.5 µg/ml EtBr. Products were run at 120 V for 30 to 60 min and viewed under Ultra Violet light with a 100-bp Gene Ruler (Fermentas Life Science, Hanover, MD, U.S.A.). Expected amplicon products from the reactions were purified using the Gen Script kit (GenScript USA Inc) following manufacturer's instructions. Purified products were commercially sequenced for both forward and reverse strands at the World Meridian Venture Centre Macrogen, Seoul, Korea. The same PCR amplification primer pairs were re-synthesized for sequencing.

Sequence analysis

Peak identification and fragment sizing of the chromatograms for the blood meal sequences was done using the programme Peak Scanner[TM] v1.0 (Applied Biosystems). The resulting sequences were edited and stored in Bioedit software (Hall, 1999). Sequences were then aligned using the programme Codon code aligner. Within the same programme, sequences were used to query the Genbank database using the BLAST algorithm (Altschul et al., 1990) and the barcode of life database (BOLD) (Ratnasingham and Herbert, 2007); BOLD-ID platform (http://www.barcodinglife.org/views/idrequest-php) for specific identification of hosts. All the host species identified from mosquitoes were the most similar species at more than 99% sequence identity. Sequences which did not meet the level were left out of the analysis. Collection sites were categorized according to 3 groups; within forest, forest edge and tower and host species according to 4 groups (Human, Bovid, Suid, and Aves). Differences between the mosquito blood meal host species were examined using the Mann-Whitney U test at $p < 0.05$.

RESULTS

Species composition and relative abundance of mosquitoes collected from this study are shown by Kaddumukasa et al. (2014). Sequencing of COI and cytb from 56 mosquito blood meals yielded a number of hosts. From the 56 wild-caught mosquitoes, 47 amplified with cytb and 9 from COI. Sequences obtained from the mosquito blood meal sources from Zika forest showed two main groups; human sources and the other group containing animal sequences (Figure 2). The sequences show that blood was obtained by mosquitoes from all sites in the forest and mosquitoes were able to access the different sites for their blood meal sources. After identification, the obtained mosquito blood meal sequences showed a distribution of blood meal hosts from all points of collection within the forest. Table 1 shows the vertebrate blood meal sources from engorged mosquito species detected by PCR. Blood meal sources were most identified from sites within the forest,
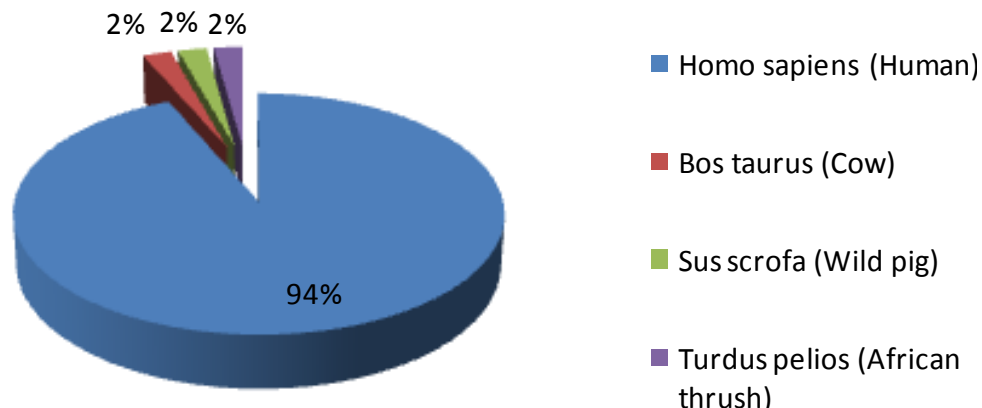
Figure 2. Blood meal sources identified with cytochrome b from Zika forest mosquitoes.

Table 1. Mosquito species and their blood meal sources identified by PCR and sequencing.

Primer	Habitat	Species	Human	Pig	Cow	Bird	No.
Cytochrome b	Forest edge	*A. coustani*	1				1
		A. implexus	2				2
		C. fuscopennata	15				15
		M. africana nigerrima	1				1
	Within Forest	*C. fraseri*	1				1
		C. fuscopennata	10		1		11
		C. pseudoconopas	1				1
		C.annulioris	1	1			2
		C. antennatus	2				2
		C. insignis	5				5
		C. univittatus	1				1
		M. africana africana	1				1
	Tower	*A. africanus*	2				2
		C. aurites				1	1
Total			43	1	1	1	46
Cytochrome oxidase I	Forest edge	*A. implexus*			3		3
		C. fuscopennata			1		1
		C. annulioris			1		1
	Within Forest	*C. pseudoconopas*			1		1
		C. annulioris			1		1
	Tower	*C. fuscopennata*			2		2
Total					9		9

followed by the edge of the forest and least at the tower platforms (Table 1). All species of *Aedes, Anopheles, Coquillettidia,* and *Mansonia* were found to feed on mammalian hosts. *Coquillettidia fuscopennata* mosquitoes showed the highest composition of blood meals from within the forest and at the forest edge. The number of blood meal hosts was higher in the forest by this species than at the edge of the forest. In addition, *C. fuscopennata*'s blood source was identified from *Bos taurus*, *Cq. aurites*

from *Turdus pelios* and *Culex annulioris* from *Sus scrofa.* For both COI and Cytb, majority of hosts identified were mammals and found to represent the most common host class (Table 1). Cytochrome b sequences represented 47 samples which were identified as 44 human, one (cow, pig and bird) respectively. All the nine COI sequences were identified as cow blood sources from four mosquito species. From means of hosts identified man, bovids and birds were major hosts. However, hosts from which

mosquitoes derived their blood-meals differed significantly (χ^2 = 19.118, df = 5, p = 0.0018). Sites inside the forest had the greatest variety of hosts followed by sites along the forest edge and least was tower sites. Non-parametric Kruskal Wallis test was used to examine variation of hosts because data did not conform to a normal distribution. There were no significant differences amongst sites within, at the forest edge and along the tower, in the number of mosquito blood-meal hosts identified (χ^2 = 0.301, df = 2, p = 0.861). To explore the differences between host species, Mann-Whitney U test was used and significant differences were observed between man and other hosts (p < 0.05).

DISCUSSION

This study has shown the first molecular analysis of mosquito blood-meal sources from Zika Forest with two important findings. First, from the host variety revealed, the majority of the blood meals were from human sources. The big number of positive blood-meals from humans than other hosts is of particular interest as humans may pick infections from animals. The close association of humans with forest habitats may easily promote emergence of new pathogens. Intra-population variation within the human sequences was revealed, showing the diversity of people interacting with the forest. The cytb assay detected more sources from the sequences from each blood meal taxon, representing different individuals; human, cow, pig and bird, which were different from each other. In contrast, for COI all the cow sequences were identical. Our study was able to identify a larger number of blood meals with cytb than COI probably because smaller products are detectable over a longer time of degradation than longer ones (Fornadel et al., 2008). Negative results may have included blood-meals that were too small or probably completely too digested for identification. Blood meals have been noted to degrade quickly (Fornadel et al., 2008). A small delay of hours may be the difference between an identifiable blood meal and one that will not be identified.

A variety of host feeding preferences namely human and a number of animals even for those species previously assumed to feed exclusively on birds or mammals was presented in this study (Table 1). For example, *C. fuscopennata* was reported to mainly have preference for man (Mukwaya, 1972). However, this species presented more than one host in this study. *C. fuscopennata* had more than one host choice from its amplifications (Table 1). This may open the possibility for transferring pathogens across distantly related vertebrate hosts, including humans. For the *Anopheles* spp., in particular, *An. implexus* bovine–derived blood sources were revealed from this study. Other studies showed *Anopheles* species host sources were mainly human

sources showing their anthropophilic nature (Haddow and Ssenkubuge, 1973; Fornadel et al., 2010). Other authors however, reported *Anopheles* host sources as zoophilic, taking blood from cows and a few other wild mammalian sources (Muriu et al., 2008). This may suggest that the blood-seeking activities of this species from Zika extend beyond the forest's confines. In other studies, increased interaction between man, domestic animals and the forest was reported by Hamer et al. (2008) while examining *Culex pipiens* (Linnaeus). Humans served as a bigger blood source and more readily available target than the normal wild animal sources. No non-human primates were found as sources of blood for the collections examined. Other members of genus *Coquillettidia* (*Cq. aurites and Cq. pseudoconopas*) fed almost exclusively on birds. Cows were an additional host for *Cq. pseudoconopas* from this study. The results of this study agree with a previous study that reported this species to be primarily ornithophilic (Mukwaya, 1972). Other mosquito species in the genus *Coquillettidia* have been reported to take blood from avian and primate hosts (Mukwaya, 1972). Blood-meals from birds may be taken at any available opportunity. In other parts of the world, *Coquillettidia* species have been reported to feed predomi- nately upon mammalian hosts (Molaei et al., 2008).

Secondly, the *Aedes africanus* mosquitoes examined had human-derived blood-meals. Interestingly, *A. africanus* mosquitoes have been known to take primarily primate blood (Mukwaya, 1972). Opportunistic and easy human blood feeding may be provided by the available people living close to the edge of the forest than the primates. However, since only two mosquitoes were examined we may not draw conclusions. Blood-meal sources from other *A. africanus* have revealed host varieties including entirely bovine (Muriu et al., 2008; Linthicum et al., 1985), avian and mammalian sources. Blood-meal sources from *Mansonia* mosquitoes were mainly from humans. This may have serious implications because this is one of the main vectors of Rift Valley fever in Kenya (Lutomiah et al., 2011). Previously, *Mansonia* species records have reported reptilian blood-meal sources. *Culex* blood-meals were recorded from human and other animal sources (Table 1). *Culex annulioris* had human and pig sources from cytb and cow hosts from the COI amplifications but had not been reported in any previous study. *Culex* species from West Africa have been reported to feed on a variety of mammal and bird sources (Snow and Boreham, 1973). *Culex quinquefasciatus* was reported to take mainly animal sources (Lee et al., 2002), while *C. pipiens* fed exclusively on bird sources (Hamer et al., 2008). Along with identifications of blood meals, arboviruses were isolated from mosquitoes. From earlier studies, some of these arboviruses include; Chikungunya (Weinbren et al., 1958), Zika (Haddow et al., 1964), Rift Valley Fever (Woodall, 1964), O'nyong-Nyong (Rwaguma et al. 1997; Lanciotti et al., 1998), and yellow Fever viruses (Kirya et al., 1977).

The mosquitoes therefore pose a serious threat to communities living around the forest and need to be constantly monitored. Variations of the host species selected for feeding may be brought about by several factors namely; the availability of host-species at the time of feeding, innate behaviour of each species selecting for particular blood types and location of host. Species in one particular part of the world specialize in certain hosts while others feed on a variety of animals. Types of hosts may be chosen as a result of environmental conditions, such as environmental temperature which later determines disease outbreak (Molaei et al., 2008). The emergence of a disease in a particular region is associated with changes that influence people's livelihood strategies, their rapid conversion of natural habitats, and urbanization (Smith, 1975). Host specificity may be influenced by factors such as; host type present, mosquito behaviour and environment (Patz et al., 2008; Norris, 2004), namely visual, thermal, and olfactory stimuli (Costantini et al., 1996). These direct a mosquito to which species to feed on and in so doing pass on its pathogens.

Based on our results, more than half of the blood meals were derived from humans (Figure 2). In addition to a better understanding of mosquito blood feeding habits for ecological and disease transmission purposes, cytb and COI should be used to explore more interactions between man and the mosquito. Further investigations using a range of collection methods, targeting ecologically diverse sites should be done to determine mosquito contribution as reservoirs or amplifying hosts in the transmission of arboviruses. Special precaution should be taken when interpreting sequence data for mammals where a limited number of specimens were obtained. Some mammals had not been examined before and most bird species in Uganda are not recorded in the BOLD or GenBank database. There is a possibility that the blood-meals were derived from vertebrates for which *cytochrome b* and *cytochrome oxidase subunit I* sequences are not yet available because some failed to yield a host and were left out of the analysis.

Conflict of interests

The authors did not declare any conflict of interest.

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Abbreviations: UVRI, Uganda virus research institute; **PCR,** polymerase chain reaction; **COI,** cytochrome oxidase subunit 1; **cytb,** *cytochrome b*; **PBS,** phosphate buffer saline; **EtBr,** ethidium bromide; **BOLD,** barcode of life database.

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Review

An overview of the role of rumen methanogens in methane emission and its reduction strategies

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Methane is the most effective global warming greenhouse gas and methanogens are the key microbiota in methane emission. Emerging research focuses on ruminant methanogens due to their emission of methane globally; of which around 20% is from livestock. Enhanced techniques revealed the methangens diversity, adaptation in rumen, methanogenesis and their reduction strategies. Based on diet, geographical location, type of ruminant species, methanogen population shows vast diversity. Many strategies also interfere to reduce the methane emission worldwide such as dietary composition, vaccines, plant secondary metabolites, analogs and fungal secondary metabolites. This review gives a concise knowledge of methanogens' interference in methane emission and research and development techniques used for reducing methane emission.

Key words: Methane, plant secondary metabolites, ruminants, ionophores, lovastatin.

INTRODUCTION

Methane is a more potent greenhouse gas, having 21 folds greater global warming potential than carbon dioxide (Sirohi et al., 2013). Livestock are major source of methane emission contributing about 81 to 92 MT methane per annum globally (IPCC, 2007; Patra, 2012a). India has livestock wealth of 272.1 million cattle, 159.8 million buffaloes, 71.6 million sheep, 140.6 million goats and 13.1 million (GOI, 2012, Sridhar et al., 2014) other ruminants, which produce large amounts of CH_4 as a part of their normal digestive process. This constitutes about 20% of the world's ruminant population. The rumen of the dairy cow contains a rich and diverse population of microbes that produce significant quantities of methane during feed digestion; it contributes to greenhouse gas emissions (GHG). Methane emissions represent between 30 and 50% of the total GHG emitted from the livestock sector; with enteric methane from ruminant production systems representing by far the most numerically important source. It is responsible for approximately 80% of the methane emissions from the sector (Gill et al., 2010). Strategies for reducing methane provide opportunities to improve livestock productivity and reduce greenhouse gas emission. In order to develop the strategies, vast knowledge on methanogens' diversity and genomic capability is required. Enhanced research and technology on rumen metabolism revealed the rumen methanogen

diversity, methane emission and mitigation. Rumen contains a microbial population of 10^{11} bacterial cells, 10^3 fungal cells and 10^6 protozoa cells. Methanogen cells are roughly present in 1 ml of rumen fluid (Sunil et al., 2012), but only 10% of the microbial population was identified (Pers-Kamczyc et al., 2011). Methanogen population varies based on the geological locations. Like in India, *Methanomicrobium* phylotype is the most dominant methanogens in buffaloes, whereas *Methanobrevibacter* phylotype is the predominant in Australia (Chaudhary and Sirohi, 2009).

RUMEN MICROBIOTA

Ruminants are mainly fed by lignocellulosic based bi-products which are rich in complex carbohydrates; hence the active microbial populations present are derivatives of this feed. The rumen epithelial or epimural bacterial community performs a vast diversity of functions necessary for host health including the hydrolysis of urea, scavenging of oxygen and the recycling of epithelial tissues (Cheng et al., 1979; Dinsdale et al., 1980; McCowan et al., 1978; Petri et al., 2013). *Fibrobacter succinogenes* (Hungate et al., 1950; Flint et al., 1990), *Ruminococcus flavefaciens* (Dehority et al., 1986), *Ruminococcus albus* (Dehority, 1967; Stewart, 1979; Bryant, 1986), *Clostridium cellobioparum* (Hungate, 1944), *Clostridium longisporum, Clostridium lochheadii* (Hungate, 1957)*, Eubacterium cellulosolvens* (*Cillobacterium cellulosolvens*) (Bryant, 1958; Van Gylswyk, 1970) were the most active cellulose degrading microbes; *Butyrivibrio fibrisolvens* (Bryant, 1953; Bryant, 1956; Cotta, 1992), *Prevotella ruminicola* (Cotta, 1992), *Eubacterium xylanophilum,* and *Eubacterium uniformis* (Van Gylswyk, 1985) greatly participated in hemicelluloses degradation, while *Streptococcus bovis* (Latham et al., 1986), *Ruminobacter amylophilus* (*Bacteroides amylophilu*) (Hamlin and Hungate, 1956) and *Prevetella ruminicola* (*Bacteroides ruminicola*) (Cotta, 1992) were dominating group of starch degrading microbes.

METHANOGEN POPULATION IN RUMEN

Maximum rumen has anaerobic microbiota; hence it is very difficult to maintain them. Methanogens are very important for the functioning of rumen and to control hydrogen pressure maintenance. Archea can be found in the limb rumen 30 h after birth (Morvan et al., 1994). So far 113 species of methanogens are recognized in the ecosystem but only few species of methanogens are found in the rumen (Janssen and Kirs, 2008). *Methanobrevibacter spp.* were initially colonized methanogens in the limb rumen and less population of *Methanobacterium spp.* while seven weeks after birth, lambs contained only *Methanobrevibacter* spp. (Skillman

et al., 2004); but, *Methanobrevibacter* disappeared 12th to 19th day after birth (Zhu et al., 2007). *Methanobacterium formicicum, Methanobrevibacter ruminantium, Methanosaricina barkeri, Methanosaricina mazei* and *Methanomicrobium mobile* are the predominant methanogens (Stewart et al., 1997; St-Pierre and Wright, 2012); hence *M. ruminantium* (Leahy et al., 2010), of the order Methanobacterials is predominant in the rumen (Jarvis et al., 2000).

METHANOGENESIS IN RUMEN

Feed components like complex carbohydrates, proteins and other organic substances are degraded to monomer components by the fibrolytic or primary anaerobes. These monomers are further converted into volatile fatty acids, carbon dioxide and hydrogen. Methanogens utilize H_2 and CO_2 as a substrate produced from the fermentation of feeds; these are the main electron acceptor and donor and produce methane. However, along with methanogens, other microbes also participate in methane emission either by involving in hydrogen metabolism or by affecting the methanogen population. The synthesis of methane contributes to the efficiency of the system in that it maintains the partial pressure of H_2 to levels that might inhibit the normal functioning of microbial enzymes involved in electron transfer reactions, particularly NADH dehydrogenase. This results in NADH accumulation, and ultimately reduces rumen fermentation (Morgavi., 2010) (Figure 1). The capturing of the H_2 produced by fermentative species to hydrogen utilizing species is referred to as interspecies H_2 transfer (Wolin et al., 1997). Attachment of methanogens to the external pellicle of protozoa has been reported by Krumholz et al. (Krumholz, 1983; Stumm et al., 1982). Some *in vitro* and *in vivo* studies demonstrated that the lack of the protozoal population in the rumen ecosystem has a significant effect on both the population of methanogens and the level of methane production (Cieslak et al., 2009a; Morgavi et al., 2012). The research also showed that sheep maintained without protozoa for more than 2 years have reduced methanogenesis in comparison with sheep kept without protozoa for only 2 months (Morgavi et al., 2012). Formate, which is formed in the production of acetate, can also be used as a substrate for methanogenesis, although it is often converted quickly to hydrogen and carbon dioxide instead (Hungate, 1970; Archer and Harris, 1986). By removing hydrogen from the ruminal environment as a terminal step of carbohydrate fermentation, methanogens allow the microorganisms involved in fermentation to function properly and support the complete oxidation of substrates (Sharp, 1998). The fermentation of carbohydrates results in the production of hydrogen and if this end product is not removed, it can inhibit metabolism of rumen microorganisms (Sharp, 1998).
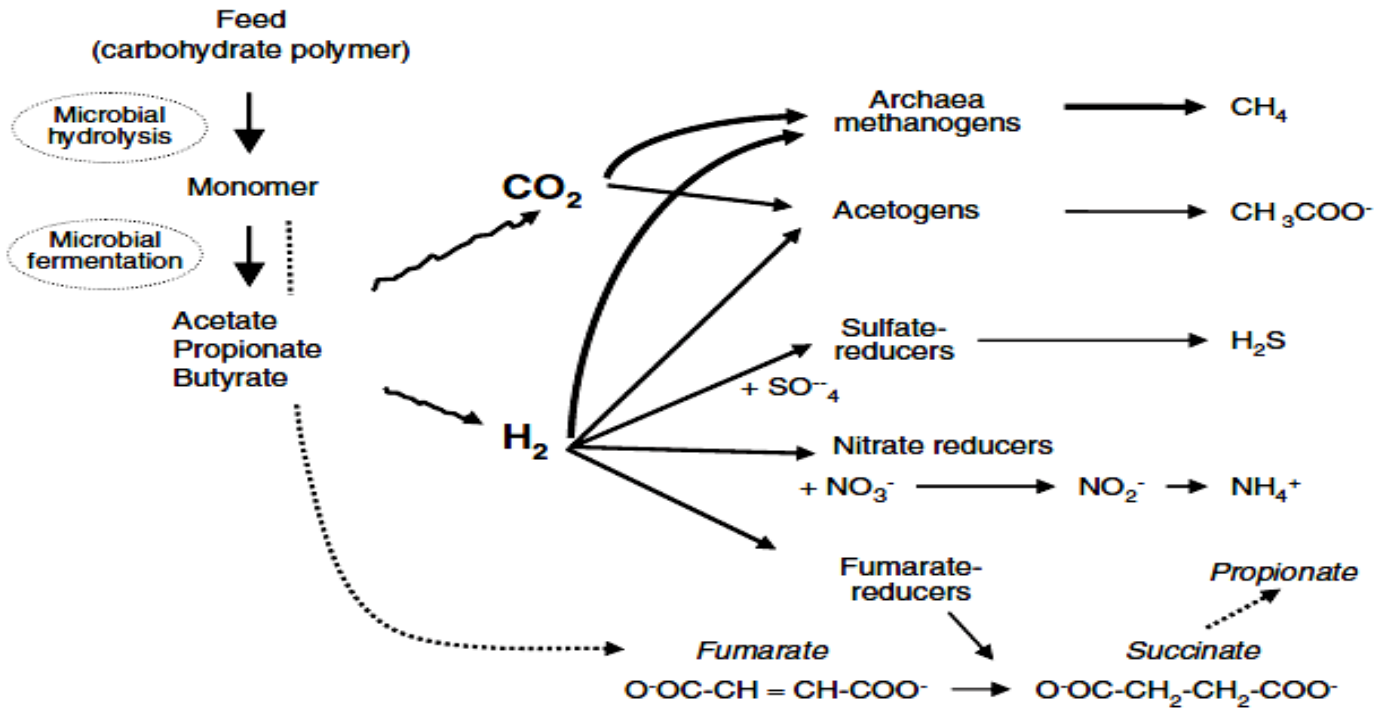
Figure 1. Methane production/ synthesis in the ruminants (Adapted from Morgavi et al., 2010).

STRATEGIES INVOLVED IN METHANE REDUCTION

Methane mitigation depends on the relationship methanogens have with other organisms in the rumen. Mitigation is caused either by attacking the methanogens directly or indirectly by the substrate available for methanogenesis (Hook et al., 2010). Some of the strategies to reduce methane production are given in Table 1.

Dietary composition impact on methane emission

The type of diet composition and the carbohydrate rate in diet are very important in methane synthesis. Diet can alter the pH of the rumen by rumen microbial composition (Johnson and. Johnson, 1995). Corn silage based diet increased the propionate concentration but decreased ruminal pH, CH_4, L/kg of dry matter intake, and concentrations of acetate and butyrate (Benchaar, 2013). The compositional basis of a cow's diet has been known to have effects on methane expulsion, with corn and soybean meal concentrate diets generally resulting in less gas production than forage diets. Concentrate and forage diets also affect ruminal pH differently, which may contribute to the activity of the enteric methanogens. The levels of methane expulsion from forage-fed and concentrate-fed cows in relation to ruminal pH showed that cows fed with all-forage diet maintain pH of more or less constant around 6.7 to 6.9; meanwhile concentrate-fed cows' ruminal pH decreased dramatically to as low as 5.45 immediately after feeding. Mixed ruminal bacteria from the forage-fed cow converted carbon dioxide and hydrogen to methane, while no methane was produced by the concentrate-fed cow (Kessel and Russell., 1996). Yan et al. (2010) studied the relationship between methane emission, animal production and energy utilization in lactating dairy cows fed with diet containing grass silage. They concluded that dairy cows capable of high milk yielding and energy utilization efficiency are effective for reducing methane emission from lactating cows.

Ionophores as methane mitigators

Ionophores are highly lipophilic ion carriers. They pass through the permeable peptidoglycan layer of gram-positive bacteria and penetrate into the lipid membrane. Therein, they destroy ion gradients at the expense of ATP, ultimately resulting in the depletion of energy reserves, impaired cell division, and the likely death of the microorganism (Tedeschi et al., 2003). Microbiota which produces hydrogen and formate is gram negative and sensitive to ionophore, thereby preventing the formation of necessary substrates for methanogens. This leads to an effective dramatic reduction in methanogen population in the rumen. Many ionophores will not inhibit the propionate-producing bacteria, resulting in an increased proportion of this volatile fatty acid (Callaway et al., 2003). Propionate is efficiently utilized by ruminants,

Table 1. Different types of Nutritional substrates used for reduction strategies of methane

Substrate	Quantity	Method applied	Incubation period	Digestibility	Methane	Reference
Nitrate supplement	3% in diet	Open-circuit respiration chambers	6 weeks	NA	35.4%	Hegarty et al., 2012
50 : 50 forage : concentrate ratio diet *ad libitum*	375 g/day	Sulphur hexafluoride tracer gas technique	93 days	No significant change	39%	Jordan et al., 2006
50 : 50 forage : concentrate ratio	250 g/day coconut oil	Sulphur hexafluoride tracer gas technique	105 days	No significant change	18%	Jordan et al., 2006b
10 : 90 forage : concentrate ratio diet	10% soya oil / 12% whole soya bean	SF_6 tracer technique	103 days	Reduced	40%/ and 25%	Jordan et al., 2006
60 : 40 forage : concentrate ratio diet	3% Soya oil on DM bases	Open-circuit respiratory chambers	60 days	Reduced	14%	Mao et al., 2010
45 : 55 forage : concentrate ratio diet	sunflower seeds (SFS), linseed oil (LO) or rapeseed (RS) oilseeds (3.3% of DM)	Respiration chambers	112 days	Increased	18%	Beauchemin et al., 2008
maize silage, grass hay and concentrate,	linseed oil (6.6% of DM	Respiration chambers	63 days	Increased	10% reduction	MacHmüller et al., 2000
maize silage, grass hay and concentrate	sunflower seed (6.0% of DM)	Respiration chambers	63 days	Reduced	27%	MacHmüller et al., 2000
Alfalfahay (4.2 kg/DM/cow) and rye grass silage (6.6 kg/DM/cow)	48% cottonseed (CS)	SF_6 tracer technique	84days	NA	23%	Grainger et al., 2010
grass silage, grass silage plus concentrate (GS+C), maize silage (MS) with monensin	120 mg feed DM/syringe.	Hohenheim Gas Test	24 h	NA	30%, 17%, and 18%	Gerald Wischer et al., 2012

Table 1. Contd.

Substrate	Quantity	Method applied	Incubation period	Digestibility	Methane	Reference
defaunation with fumaric acid defaunation with fumaric acid, Barely, grain39%, Berseem hay 40%, Wheat straw 20.14%, Vitamin and mineral premix 0.30%	200 mg	*In vitro*	24 h	NA	43.07%	Abdl-Rahman, 2010
Hay: concentrate (1:1)	10.2 and 20.4 g/kg of *Knautia arvensis* extract	*In vitro*	24 h	No significant effect on TVFA, A/P and methanogens	5.8 and 7.1%	Makkar and Becker, 2008b
Barley silage: concentrate (51:49)	15, 30, 45 g/kg DM of *Quillaja saponaria*	Serum bottle	24 h	IVDMD and A/P decreased; TVFA unaffected	5.33% 4.43%	Holtshausen et al., 2009
Hay: concentrate (1:1)	14.8 and 30.4 g/kg DM of *Trigonella foenum-graecum*	*In vitro*	24 h	No significant effect on TVFA, A/P and methanogens	2.21 and 2.21%	Makkar and Becker, 2008b
Lucerne hay: concentrate (1:1)	0.5 g/L of *Yucca schidigera*	RUSITEC	22 days	No significant effect	12.8%	Wang et al., 1998
Grass silage and hay: barley (77:23)	0.001 and 0.02, and 0.1 g/kg DM of effective sarsaponin of *Medicago sativa*	RUSITEC	10 days	IVDMD, TVFA, A/P, total bacteria unaffected	-5.16, 3.87 and 1.29%	Sliwinski and Machmuller, 2002
Hay: concentrate (32:68)	1.65 g/l or 174 g/kg Substrate of *Sesbania sesban*	*In vitro*	24 h	50.5% reduction in protozoa	11.9%	Makkar and Becker, 2008b
Wheat straw: Concentrate (1:1)	0.2 g/kg DM *of Acacia concinna*	*In vitro*	24 h	TVFA & IVDMD unaffected, A/P and protozoa numbers decreased	3.8 and 18.6%	Patr, and Agarwal, 2006
Wheat flour: wheat straw (4:1)	0.2 g/kg DM of *Sapindus mukorossi*	*In vitro*	24 h	IVDMD, A/P and protozoa decreased (70-90%), TVFA unaffected	22- 96%	Agarwal and Patra, 2006

Table 1. Contd.

Substrate	Quantity	Method applied	Incubation period	Digestibility	Methane	Reference
Corn starch	1.2–3.2 g/l or 180–480 g/kg substrate of *Medicago sativa*	Serum bottle	24 h	TVFA increased, A/P decreased, protozoal numbers decreased	36.0–64.1%	Lila et al., 2003
Corn grain/Chinese wild rye (50:50)	0.30, 60, 80 g/l cultural media of *Tribulus terrestris*	*In vitro*	24 h	TVFA, acetate and Ammonia decreased, propionate and A/P increased, protozoa decreased	23.43%	Feng et al., 2012
Lucerne hay: concentrate (60:40)	5 g/kg DM of *Camellia sinensis*	*In vivo*	21 days	No significant effect	8.71%	Yuan et al., 2007
Wild rye: concentrate (60:40)	4.1 g/kg DM	*In vivo*	60 days	TVFA increased; A/P unaffected; protozoal and methanogen decreased	27.2%	Mao and Liu, 2010
Hay: concentrate	0, 400, 600, 800 mg/kg DM of *Ilex kudingcha*	*In vivo*	10 days	No significant effect	ND	Zhou et al., 2012
Hay : concentrate (1:1)	10.2, 20.4 g/kg DM of *Medicago sativa*	*In vivo*	14 days	TVFA,A/P,methanogens unaffected	5.8- 7.1%	Makkar and Becker, 2008a
Corn: corn silage	0.25-1.5% DM of *Quillaja saponaria,*	*In vivo*	22 days	ND	No effect	Li and Powers, 2012
Ryegrass hay: concentrate (3:2)	13.5 g/kg of diet or 16.1 g/day of *Q. saponaria*	*In vivo*	18 days	TVFA decreased, digestibility, A/P, protozoa not affected	21.7%	Pen et al., 2007
Barley silage: concentrate (51:49)	10 g/kg of DM	*In vivo*	28 days	No significant effect	7%	Holtshausen et al., 2009
Forage: concentrate (49.2–56:21)	5 g/kg body wt of *Sapindus saponaria*	*In vivo*	21 day	Digestibility, A/P and protozoa decreased; TVFAandmethanogens increased	7.8%	Hess et al., 2004
3 kg concentrate mixture and chopped maize fodder (*Zea mays*)	fumaric acid @ 2% of DMI	*In vivo*	21 days	No change in digestibility	20.7%	Mohini et al., 2008
Wheat straw based diet.	2 ml of neem leaf extract in 30ml of medium	*In vitro*	24 h		40%	Malaiyappan et al., 2012

Table 1. Contd.

Substrate	Quantity	Method applied	Incubation period	Digestibility	Methane	Reference
Wheat straw containing diets	40R:60C	*In vitro*	24 h	Propionic acid levels increased, no significant changes in digestibility	22.60%	Sirohi et al., 2011
Myristica fragrans fruit powder	roughage 50% and concentrate 50%	*In vitro*	24 h	decreased	48%	Sirohi et al., 2012

and thus may enable increased derivation of energy from feed. The efficacy of ionophores in ruminant diets is examined (Guan et al., 2006).

Methane analogs as inhibitors

Methanogens can be inhibited by the addition of methane analogues such as commonly 2-bromoethanesulphonate (BES), a structural analog to coenzyme M, 3-bromopropanesulfonate (BPS). It mimics methyl-coenzyme M lumazine, and ethyl 2-butynoate. Some inhibitors, however, are more effective against certain species of methanogens than others, and some only offer short-term protection (Ungerfeld et al., 2004). *M. ruminantium* was the most sensitive to the effects of BES, *M. ruminantium* was most sensitive to ethyl 2-butynoate, *Mm. mobile* was somewhat sensitive, and *M. mazei* was unaffected. Lumazine is a structural analogue of some important co-factors in methanogenesis, but slight methanogen recovery was observed six days post-feeding, jeopardizing the chance of significant long-term benefits. Cell envelope differences may be related to the differences observed in toxicity of the methanogens to ethyl 2-butynoate. The presence of an S-layer in *M. mazei* and *M. mobile* (absent in *M. ruminantium*) may have conferred some resistance, which is a problem for the practical

use of this inhibitor *in vivo* (Ungerfeld et al., 2003). Like BES, selective resistance to ethyl 2-butynoate among different species may favor these species over long-term, rendering obsolete any initial decreases in enteric methane production. Dihydrogen (H_2) is the key element that maintains methane production in the rumen. Among H_2 producers, protozoa also play prominent role. This is strengthened by their close physical association with methanogens, which favors H_2 transfer from one to the other. H_2, formate and ethanol can accumulate during the process of ruminal methanogen inhibition. By the addition of precursors the formation of these products would be avoided and the electrons would be relocated. A case in point is the butyrate precursor that can relocate the electrons into butyrate. But, the butyrate precursors were ineffective as electron acceptors because they were not completely converted to butyrate and were also metabolized through other pathways (Ungerfeld et al., 2006).

Effect of lipids on methane emission

Lipids such as fatty acids and oils also show some effect on the rumen methanogens. Fatty acids inhibit methanogens by binding to their cell membrane and disturbing their membrane

transport (Dohme, 2001). In the meta-analysis of methane, lipid supplemented in the diet of lactating dairy cows showed a 2.2% decrease in methane per 1% of supplemented lipid in the diet (Eugene, 2008). 5.6% methane reduction per percentage unit of lipid added to the diet was observed in cattle and sheep (Beauchemin et al., 2008). Methane was reduced by 22% in sheep fed with myristic acid in a 58% concentrate based diet (Machmuller et al., 2003). Plant extracted oils naturally contain a medium to long chain fatty acids (Soliva et al., 2004). Refined soy oil based diet fed to beef bulls reduced methane by 39% (Jordan, 2006). Sunflower oil also had good impact on methane production; it resulted in 11.5 to 22.0% reduction in methanogenesis (McGinn, 2004). Linseed oil supplemented at a level of 5% of DM to lactating dairy cows resulted in a 55.8% reduction in grams of methane per day (Martin, 2008). Garlic (*Allium sativum*), Eucalyptus (*Eucalyptus globules*) and Neem (*Azadirachta indica*) oils were tested *in vitro* for methane emission, but garlic oil with low fiber diet reduced methane by 55.8% (Sirohi et al., 2012). Fatty acids, with medium chain length such as coconut oil, canola oil, kernel oil, sunflower oil reduce the methane emission in ruminants (Machmuller and Kreuzer, 1999; Dohme et al., 2000). Supple-mentation of coconut oil (7%) with 100 g/day of garlic powder increased the end products

and improved rumen microbial population; and 9% methane gas was reduced (Kongmun et al., 2011). According to Kumar et al. (2009), in vitro inclusion of eucalyptus (E. globules) oil (EO) at 1.66 µl/ml showed positive effect by reducing 56% methane mitigation, but has negative effect on fatty acid; 0.33 µl/ml of EO reduced 10% methane but had no effect on fatty acid synthesis. Szumacher-Strabel et al. (2011)'s experiment proved methane mitigation was reported only in wild dog rose seeds oil treatment, but had no negative impact on the rumen. Also, there was no change in rose seed residue.

Plant extracts as effective methane mitigators

Plants secondary metabolites such as, saponins, tannins and oils have anti-microbial activity, which can be used as alternative additives to reduce methanogen population in the rumen (Kamra, 2008). Herbal plant extracted products have a prominent effect on rumen microbiota either directly changing the methanogens or indirectly affecting protozoa. It has the ability to change the methane emission (Navneet et al., 2012). Saponins mitigate methane by reducing the protozoa population; tannins and essential oils have toxic effect on methanogens (Cieslak et al., 2013). Methanol extract of Terminalia chebula reduced 95% methane and double level of the extract was inhibited completely. Phenolic acids such as p-coumaric acids, ferulic acids, cinnamic acids and phloretic acids and some monomeric phenolics have been found to decrease methane, acetate and propionate production (Ushida et al., 1989; Asiegbu et al., 1995). The ethanol extract of Emblica officinalis fruit and methanol extracts of the fruits inhibited methanogenesis significantly ($P < 0.05$). The anti-methanogenic and anti-protozoal activity of the saponins has to be further investigated by long term in vivo trials on different feeds; as earlier reports indicated that the rumen microbes get adapted to saponins by prolonged feeding of such feeds (Wallace et al., 2002). Supplementation of coconut oil with garlic powder improves the ruminal fluid fermentation of volatile fatty acids and reduces the methane emission along with protozoal population (Kongmun et al., 2010). Zmora et al. (2012)'s 24 h study on *in vitro* dry matter digestibility (IVDMD) showed that Xanthohumol inhibited the rumen methanogens directly. Cieslak et al. (2012) showed that *Vaccinium vitis idaea* tannin had antimicrobial activity potential to indirectly mitigate methane and thereby ammonia.

Vaccines and antibiotics

Vaccines are used to prevent or control disease for a particular period, but the utilization of vaccines reduces methanogens population and increase productivity is a current topic. The anti- methanogen vaccine triggers the immune system of ruminants and produces antibodies against methanogens in the ruminants. A vaccine against three selected methanogens has been developed in Australia. Immunization in sheep lowered CH_4 production by 8%, while further testing failed to confirm its efficacy in other geographical regions (Wright et al., 2004). *Streptomyces cinnamonensis* secondary metabolite known as monensin inhibits the gram positive bacteria, which is responsible for supplying substrate to methanogens. Monensin acts on the cell wall of the gram positive bacteria; it interferes with ion flux and decreases the acetate-to-propionate ratio in the rumen, effectively decreasing CH_4 production. The effect of monensin on lowering CH_4 emission is dose-dependent: at lower doses (10 to 15 ppm), it results in the production of profitable milk, but has no effect on CH_4 (Grainger et al., 2008; Waghorn et al., 2008); but at higher doses (24 to 35 ppm) (McGinn et al., 2004; Sauer et al., 1998; Van Vugt et al., 2005), it reduces CH_4 production by up to 10% (g/kg DMI). However, there have been unanswered questions over the perseverance of CH_4 suppression (Johnson and Johnson, 1995).

Role of a fungal secondary metabolite, lovastatin in methane mitigation

Lovastatin ($C_{24}H_{36}O_5$) is a secondary metabolite of idiophase of the fungi with a molecular weight of 404.55 (Lai et al., 2003). It inhibits the key enzyme of cholesterol biosynthesis such as enzyme 3-hydroxy-3-ethyl glutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) (Alberts, 1988). Isoprenoid is a central component in Archeal cell wall and it is an intermediate step in cholesterol synthesis (Konrad and Eichler, 2002). As an inhibitor HMG-CoA reductase, lovastatin can suppress isoprenoid synthesis, thereby cell wall synthesis in archeal cell membrane and methanogen population (Smit and Mushegian, 2002). The Fermented Rice Straw Extract of lovastatin significantly reduced total CH_4 production by rumen methanogenic Archaea after 48 h of incubation by 19.47% (Juan et al., 2012). Biological control strategies such as bacteriophages or bacteriocins could prove effective for directly inhibiting methanogens and redirecting H_2 to other reductive rumen bacteria such as propionate-producers or acetogens (McAllister and Newbold, 2008). However, most of these options are in the early stages of investigation and still require significant research over an extended period to deliver commercially viable vaccines and biological control options that will be effective over a range of production systems and regions.

Potential of *genetics* to reduce *methane* emissions in ruminants

The key microbiota Archea is a very small population and it emits large portion of methane in rumen. Molecular

analysis provided that methyl coenzyme-M reductase gene (Martino et al., 2013) is a genetic marker common for the Methanogenic population. De Haas et al., (2011) analyzed the association between cumulate enteric methane emission and Genome wide Single Nucleotide Polymorphism. Though SNP effect could be identified, no large regions were significantly associated. The cows with lower residual feed intake have lower predicted methane emission grams/day. Hence, it is possible to reduce methane emission. Genetic variation suggests that 11 to 26% methane mitigation in 10 years could be more in a genetic selection program.

CONCLUSION

For more than 20 years, research has been done on rumen methanogens. Along with key enzymes methane emission, which causes global warming, made an important task to reduce methanogen population. Various strategies have been implemented to mitigate methane such as by changing diet, especially by providing diet rich in oil seed or proteins rather than carbohydrates. Ionophores, antibiotics and vaccine also have positive effect on methane mitigation, but chance of developing resistance to vaccines is also there. Fungal secondary metabolites such as lovastatin and plant extracts had significant effect on methane emission and a vast deal of information have revealed mitigation strategies. Genomic analysis showed that methyl coenzyme-M reductase is a marker gene for methane production and correlation between food intake. SNP in the genome and breed selection has significant results against methane emission. Now, more work has to be done on the direct effect on rumen methanogens to mitigate methane.

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Conflict of interest

The authors did not declare any conflict of interest.

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