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A list of non-standard Abbreviations should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer’s name and address. Subheadings should be used. Methods in general use need not be described in detail.
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Examples:

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Genetic variability of indigenous cowpea genotypes as determined using inter-simple sequence repeats markers

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Cowpea [Vigna unguiculata (L.) Walp.] is cultivated widely by small farmers in the semiarid region of Northeastern Brazil for subsistence purposes, especially to complement the family income. However, owing to the limited availability of water in this region, there is an urgent need for novel highly productive drought-tolerant cultivars. The aim of the present study was to establish the genetic variability of 14 cowpea populations (60 indigenous genotypes from 13 microregions of Rio Grande do Norte and 4 domesticated cultivars produced by Embrapa) using inter-simple sequence repeats (ISSR) markers. The set of 13 selected primers generated a total of 257 loci, 247 (96.11%) of which were polymorphic, with sizes ranging between 200 and 2000 bp. Genetic similarities between accessions were estimated from Jaccard coefficients and genetic relationships were determined from the dendrogram constructed using the unweighted pair group method with arithmetic average (UPGMA) technique. Bayesian statistics coupled with the Markov chain Monte Carlo technique was applied to determine population structure, while the genetic variability was established by analysis of molecular variance. UPGMA analysis allowed the separation of the genotypes into three groups, but no relationship between the genetic and geographical distances was observed. The fixation index was considered intermediary (FST = 0.0818), the average heterozygosity was low (Hs = 0.39) and the coefficient of endogamy was high (f = 92.6%). The results show the presence of genetic diversity among the studied populations and revealed that such variability could be attributed mainly to intra-population variability (91.82%).

Key words: Vigna unguiculata, genetic diversity, plant improvement, drought stress.

INTRODUCTION

The climatic factors that most influence the production of beans are temperature and degree of precipitation...
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Figure 1. Origin of the cowpea genotypes analyzed in the study. The map of Brazil shows the location of the State of Rio Grande do Norte and its microregions; the number of genotypes collected within each of the microregions is shown in brackets.

(Barteko et al., 2010). In the semiarid region of the State of Rio Grande do Norte in Northeastern Brazil, water availability is very limited owing to scarce and irregular rainfall and high prevailing temperatures. Hence, there is available to family farmers in this area.

In this context, *Vigna unguiculata* (L.) Walp. (Fabaceae), commonly known as cowpea, represents an excellent target because of its high genetic variability and its versatility in terms of cropping possibilities and application of the final product (Freire Filho et al., 2005). Furthermore, the species constitutes one of the main sources of protein for the populations of Northern and Northeastern Brazil, and provides an alternative source of income for small family farmers who have little or no access to advanced production technologies.

An understanding of the genetic variability within a species, as assessed by the proximity and diversity between genotypes, is an essential prerequisite in plant improvement. Such knowledge not only permits the identification of divergent and complementary genotypes that can be used as progenitors in a breeding program, but also increases the chance of selecting elite genotypes in segregating generations (Cruz and Regazzi, 2006).

Molecular markers are valuable tools in the investigation of genetic variability within a plant collection or among wild species. Amongst available markers, inter-simple sequence repeats (ISSR; Zietkiewicz et al., 1994) have been widely used by virtue of the repeatability and reproducibility of the banding patterns obtained and the simplicity of the techniques involved. A number of reports have been published recently on the application of ISSR in the determination of genetic diversity in cowpea (Silva et al., 2009; Ghalmi et al., 2010; Santos et al., 2013).

Since small farmers in the Northeast of Brazil mainly grow indigenous cultivars of cowpea, it is expected that the genetic variability within this species would have been preserved. This valuable resource could be exploited in the development of novel cultivars with enhanced traits by transferring desirable characters from their indigenous counterparts. On this basis, the present study aimed to establish, with the aid of ISSR markers, the genetic variability of indigenous genotypes collected in Rio Grande do Norte and of commercial genotypes produced by the Empresa Brasileira de Pesquisa Agropecuária (Embrapa).

MATERIALS AND METHODS

Plant material

A total of 64 cowpea genotypes were analyzed, 60 of which were indigenous genotypes collected in 13 of the 19 microregions of Rio Grande do Norte (Figure 1) and four were the domesticated
phases of the plants and transferred to labeled plastic bags that were cooled over ice during transportation to the Laboratory of Biotechnology and Molecular Biology of Embrapa Meio-Norte (Teresina, PI, Brazil). Plant material was subsequently stored at -20°C until required for analysis.

### ISSR fingerprinting

DNA was extracted from leaf material using Invisorb® Spin Plant Mini Kits (Stratec Molecular, Berlin, Germany) following the recommendations of the manufacturer. Quantification of the extracted DNA was carried out by electrophoresis on 0.8% agarose gel in Tris-borate-EDTA (0.5 x TBE) buffer, staining with GelRed™ (40 x; Biotium, Hayward, CA, USA), and comparison with co-analyzed diluted DNA standards (50 and 100 ng). The quality and quantity of genomic DNA samples were verified spectrophotometrically using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). DNA samples were diluted to 7.0 ng/μL and stored at -20°C until required for ISSR analysis.

In order to select appropriate primers for ISSR reactions, genomic DNA from five cowpea genotypes was initially amplified using 40 primers obtained from the University of British Columbia, Vancouver, Canada. The 13 primers that generated the largest numbers of amplified loci with the best band resolution and the highest levels of polymorphism (Table 1) were chosen for the PCR amplifications of DNA from the 64 cowpea accessions. Assays were performed according to the method described by Silva et al. (2009). The reaction mixture employed in PCR amplifications contained 1.0 × buffer (20 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 50% glycerol [v/v]), 2.0 mM MgCl₂, 0.8 mM dNTPs, 0.8 μM primer, 1U Invitrogen Taq DNA polymerase (Life Technologies Corporation, São Paulo, Brazil), 0.5 μL DNA template (7.0 ng/μL) and ultrapure distilled water to a final volume of 10 μL. Amplifications were carried out in a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 94°C for 4 min, 40 cycles each comprising denaturation at 94°C for 1 min, annealing at a temperature that varied according to the melting temperature of the primer (Table 1) for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The resulting amplicons were separated by electrophoresis on 1.5% agarose gel in 0.5 x TBE buffer for 4 h at 110 V, stained with GelRed (40 x), visualized under a UV transilluminator and subsequently photographed. The sizes of the amplicons were estimated by comparison with Invitrogen 100 bp and 1 kb DNA ladder.

### Cluster analysis

Analysis of the band pattern generated by each of the 13 primers allowed the construction of a binary matrix in which “1” indicated the presence of a band and “0” the absence. Genetic similarities between cowpea genotypes were estimated from the Jaccard similarity coefficients (sgj), calculated according to Rohlf (1992), and a dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA) clustering technique. Cophenetic correlation coefficients (r) were determined from the similarity matrix and the dendrogram, and the bootstrap confidence index was calculated based on 1000 permutations. Cluster analysis was performed with the aid of the software PAST version 1.34 (Hammer et al., 2001).

### Analysis of population structure

The presence of population structure in the samples was identified using the program STRUCTURE version 2.3.4 (Pritchard et al., 2000), which generates a posterior distribution based on a Bayesian model and Markov chain Monte Carlo (MCMC) simulation. Application of this approach with an admixture model enabled the proportion of the genome deriving from another population to be assessed for each genotype in the absence of a priori information. Each analysis considered a fixed number of 300,000 MCMC iterations with a “burn-in” of 50,000 iterations, and three runs were performed for each K value. The most probable number for K in relation to the proposed population structure was determined from the values ΔK (Evanno et al., 2005).

Analysis of molecular variance (AMOVA), performed using ARLEQUIN software version 3.1 (Excoffier et al., 1992), was employed to estimate the components of variance attributed to
differences between: (i) the 14 populations (13 relating to indigenous genotypes and one comprising the four commercial genotypes), (ii) sites within the microregions, and (iii) individuals within the sites. The significances of the variance components were obtained using 1000 permutations. The magnitude of genetic differentiation between populations was expressed by the fixation index \((F_{ST})\). The average heterozygosity \((H_S)\) and the coefficient of inbreeding \((f; \text{an analogue of the coefficient } F_{IS} \text{ for dominant markers})\) were estimated using the Bayesian approach implemented in HICKORY software version 1.1 (Holsinger et al., 2002).

**RESULTS**

**Genetic diversity**

The set of 13 primers selected for ISSR analysis of the 64 genotypes amplified 257 loci of which 247 (96.11%) were polymorphic. The average number of loci per primer was 19.77 and the sizes of the fragments ranged between 200 and 2000 bp. The loci generated by primers UBC 810, UBC 822, UBC 827, UBC 828 and UBC834 were all polymorphic (100% polymorphism), while the remaining primers (except for UBC 826) presented loci with \(\geq 90\%\) polymorphism (Table 1). The efficiency of the selected ISSR primers is exemplified by the electrophoretic profile generated by primer UBC 834 (Figure 2). The average \(H_S\) value, which is considered to be a measure of genetic diversity, was 0.39 (95% confidence interval 0.37 to 0.40) with minimal variation (standard deviation of 0.0001).

**Genetic similarity**

Genetic relationships between the 64 cowpea genotypes were established from the Jaccard coefficients, the values of which ranged between 0.203 and 0.796. The dendrogram shown in Figure 3 was constructed from the 257 amplified loci using the UPGMA method. A cut-off

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**Figure 2.** Electrophoretic profiles of ISSR amplicons generated by primer UBC 834 with DNA derived from 13 indigenous genotypes (lanes 2 - 14) and four commercial genotypes (lanes 15 - 18; T1 - T4) of cowpea \([\text{Vigna unguiculata} \text{ (L.) Walp.}]\). Lane B represents the negative control (complete reaction mixture minus DNA).
Figure 3. UPGMA dendrogram based on the 13 selected ISSR polymorphic markers showing similarity relationships between 60 indigenous genotypes and four commercial genotypes of cowpea [Vigna unguiculata (L.) Walp.]. The numbers in brackets indicate the microregions in Rio Grande do Norte where the genotypes originated (see Figure 1).
point of 0.40 was established from the average coefficient of similarity between the genotypes, and this enabled the 14 cowpea populations to be discriminated into three genotypic groups. The excellent performance of cluster analysis based on ISSR markers was verified by the high value of the cophenetic correlation coefficient ($r = 0.92$).

Group I comprised 42 genotypes representing populations from all 13 microregions of Rio Grande do Norte, together with the four commercial genotypes from Embrapa. Group II was composed of eight genotypes from the microregions Pau dos Ferros, Chapada do Apodi, Mossoró, Borborema Potiguar and Agreste Potiguar located at the extremes of the State. Group III included 10 genotypes from the geographically close microregions of Angicos Borborema Potiguar, Agreste Potiguar and Litoral, Sul. Consequently, the strongest association between genetic similarity and geographic distance was observed in this group.

Pair wise comparison of populations revealed that genotypes from Serra de São Miguel (39-1) and Baixa Verde (42-1) presented the highest coefficient of genetic similarity (0.796) even though the distance between sampling sites was 380 km. The lowest coefficient of genetic similarity (0.203) was observed between the genotypes from Agreste Potiguar (1-1) and Pau dos Ferros (50-1) and, in this case, the distance between sampling sites was 441 km.

**Population structure**

Application of the Bayesian MCMC strategy using the program STRUCTURE confirmed the existence of admixture between the studied genotypes, thus corroborating the results from cluster analysis. However, the Bayesian MCMC approach indicated that the genotypes could be organized into two main groups ($K = 2$) (Figures 4 and 5) rather than three as suggested by cluster analysis. It should be noted, however, that in all of the analyses, $K = 3$ was identified as the second best value for this parameter (Figure 5), and that the cut-off point in the dendrogram represents a somewhat tenuous limit since a small dislocation to the left would divide the samples into just two groups (Figure 3).

Bayesian MCMC analysis revealed that five of the 64 studied genotypes had ancestors from the other group, as indicated by individuals presenting different coloured bars in Figure 4. Furthermore,
results of the analysis allowed relationships between individual genotypes to be identified. Thus, all genotypes from Serra de São Miguel, Vale do Açú, Macau, Baixa Verde and Macaíba were grouped together with the commercial genotypes as in the cluster analysis.

The results of AMOVA indicated that the total variability among the genotypes studied was explained mainly by intra-population differences (91.82%), while inter-population differences were very low ($F_{ST} = 0.0818$) (Table 2). The coefficient of endogamy determined using the Bayesian model was high ($f = 92.6\%$), while the average heterozygosity value was low ($H_S = 0.39$).

**DISCUSSION**

According to Grativol et al. (2011), the efficiency of a molecular marker can be assessed by the amount of polymorphism detected. The efficiencies of all of the ISSR primers employed in the present study, with the exception of UBC 822, had been previously established by Muthusamy et al. (2008) in an investigation of the genetic diversity of *Vigna umbellate* (rice bean). In this study, four of the primers generated 100% of polymorphic bands and the overall polymorphism was 61.79%.

Additionally, Silva et al. (2009) assessed the genetic variability of 46 short-cycle erect cowpea genotypes using eight of these ISSR primers and reported that, of the 62 loci generated, 49 (79.04%) were polymorphic with an average of 6.12 polymorphic bands per primer. In the present study, an average of 19 polymorphic bands were generated per primer, implying that the data obtained using the ISSR primers could be employed with a high degree of confidence in explaining the genetic diversity of the cowpea genotypes.

The high degree of polymorphism (96.11%) detected in the present study can be attributed mainly to the 60 indigenous genotypes, since they had been subjected only to natural selection. During the domestication of a plant species, the frequencies of alleles relating to desirable characteristics are augmented until they are fixed in the offspring. The implementation of such strong selective pressure generally results in a considerable loss of genetic diversity (Wang et al., 1999). Selection is one of the main tools employed by plant breeders irrespective of the breeding method, but this strategy depends on the availability of populations presenting the genetic variability that is normally present in indigenous varieties (Bespalhok et al., 2007a). In our study, the 60 indigenous cowpea genotypes analyzed showed an average
heterozygosity ($H_s$) of 0.39, a value that would justify the development of a breeding program. The genetic richness of the indigenous genotypes may be exploited not only for genetic studies but also for the purpose of crossing divergent progenitors in order to maximize heterosis and augment the possibility of creating commercial cultivars with novel traits.

Most of the genotypes (46/64), including the domesticated cultivars, could be combined into a single group (group I) after cluster analysis, and this result may raise doubts regarding the genetic distance between individuals. However, it should be emphasized that these genotypes originated from small subsistence farms where seed interchange and, consequently, crossbreeding within a crop is common. Nonetheless, it was possible to observe the existence of two well-defined subgroups (Figure 3), one of which included 16 genotypes from 23-1 to 46-1, while the other included 30 genotypes from 48-1 to the commercial genotype Marataoaã. This division was confirmed through analysis of resampling data (Bootstrap index $= 91\%$).

The results presented herein reveal that the indigenous genotypes from Rio Grande do Norte and the domesticated cultivars produced by Embrapa are closely related and suggest that crossing between these populations may lead to the transfer of desirable characters. In this context, Soares (2012) has recently identified an indigenous cultivar, AM-63-3-Lizão Carioquinha or Lizão, originating from the Macau microregion that is highly resistant to the seed beetle *Callosobruchus maculatus* (Fabr. 1775) (Coleoptera: Crysomelidae).

Group II contained genotypes sampled in distant microregions, such as 20-1 from the municipality of Itaú (Pau dos Ferros) and 12-1 from the municipality of Passa e Fica (Agreste Potiguar), locations that are 400 km apart. Paradoxically, this group also contained the genotypes 20-1 and 21-1 collected in the municipalities of Itaú and Severino Melo, respectively, which are located 13 km apart in the Pau dos Ferros microregion. These results confirm that the local farmers cultivate seeds from various origins in the same geographical area. Interestingly, genotype 20-1 has been screened by Soares (2012) and shown to exhibit moderate resistance to the seed beetle. Considering that the genetic similarity between the indigenous genotype 20-1 and the domesticated cultivars is less than 30\%, crossing would likely result in a novel cowpea cultivar with enhanced resistance traits.

Previous studies have shown that genetic and geographical distances are not always correlated. For example, Oliveira et al. (2003) evaluated the genetic divergence among 16 cowpea genotypes originating from Brazil and Nigeria, and observed that genotypes from the same geographical origin exhibited wide genetic distances.

Analogous results were reported by Bezerra (1997) and Vidal et al. (2006), but these authors justified their findings by emphasizing that the sampling site is not necessarily the site of origin of the plant. Indeed, the misconception among some researchers that geographical distance between cultivated species is an indicator of genetic divergence has been the focus of some criticism since, in many cases, a relationship between genetic diversity and geographical distance cannot be verified (Cruz, 1990; Cruz and Carneiro, 2003).

In order to understand better the genetic distances between cowpea genotypes, we employed the program STRUCTURE that has been used in various studies to verify the groupings of bean genotypes generated by dendrograms (Blair et al., 2007; Burle et al., 2010; Silva 2011). The results of such analyses may have value in the identification of progenitors that offer the highest likelihood of selecting elite genotypes in segregating generations within future breeding programs (Cruz and Regazzi, 2001).

In contrast to the cluster analysis, the optimum $K$ value generated by the STRUCTURE program was two, suggesting the formation of only two groups. However, the genotypes incorporated into groups II and III in the dendrogram formed a single group (represented by orange bars in Figure 4) according to Bayesian MCMC analysis. In reality, groups II and III are very close genetically and a slight dislocation of the cut-off point on the dendrogram would eliminate the division into two groups. In this sense, the results of cluster and Bayesian MCMC analyses are compatible and consistent one with another.

The high levels of genetic dissimilarity between some of the indigenous genotypes and the domesticated cultivars suggest that crossings would probably produce
improved cowpea varieties with advantageous traits such as superior quality and yield of seed with better resistance to biotic and abiotic stress. Considering the similarity matrix presented herein, it is possible to suggest crossings between 1-1 (Agreste Potiguar) and 50-1 (Pau dos Ferros), 21-1 (Pau dos Ferros) and Guariba, Marataoa and 21-1 (Pau dos Ferros), Nova Era and 4-1 (Agreste Potiguar), 24-1 (Serra de São Miguel) and 10-1 (Angicos), and 20-1 and Marataoa, the Jaccard similarity coefficients of which were 0.203, 0.221, 0.227, 0.239, 0.240 and 0.230, respectively.

Intra-population diversity was responsible for most (91.82%) of the genetic differentiation among the 14 populations of cowpea studied. One explanation of this finding is that small farmers in Northeastern Brazil plant different varieties of seeds in the same location and, consequently, there may have been a mixture of seeds in a single collection. Thus, some individuals considered to be from one population may have been from different populations. Although the assessment of genetic diversity between populations was very low (8.18%), the value of \( F_{ST} \) may have been underestimated because some populations comprised only a small number of individuals. Moreover, since \( V. unguiculata \) is predominantly autogamous and cleistogamous, that is, pollination of the stigma occurs before the opening of the flower bud or anthesis (Bespalkh et al., 2007b), the rate of natural crossing is very low and generally less than 1% (Ehlers and Hall 1997). According to Carvalho (2009), the distribution patterns of genetic variability among populations are correlated with the mode of reproduction.

Nevertheless, greater divergence between the studied populations was expected since inter- and intra-population genetic diversities of 71.50 and 28.50%, respectively, had been reported by Ghalmi et al. (2010) following an ISSR analysis of indigenous cowpea populations in Algeria. However, although the theoretical value of \( F_{ST} \) varies from 0 (indicating no divergence) to 1 (indicating the fixation of alternative alleles in different subpopulations), the maximum value observed is normally much smaller than 1 (Hartl and Clark 2010). According to Wright (1978), an \( F_{ST} \) value ranging between 0.05 and 0.15 indicates the existence of moderate genetic differentiation, as in the case of the cowpea populations presently evaluated. Moreover, statistical analysis showed that the \( F_{ST} \) value was significant and that genetic divergence existed among the study populations.

The high coefficient of endogamy (\( f = 92.6% \)) established in the present study likely reflected the autogamous nature of the species, while the low average heterozygosity value (\( H_s = 0.39 \)) was probably due to self-pollination of cowpea accessions resulting in descendents with identical ancestral alleles in a single locus (autozygous) (Hartl and Clark 2010).

Conclusions

The ISSR markers used in this study were very efficient in revealing the genetic diversity and population structure of the indigenous cowpea genotypes collected in Rio Grande do Norte and of the cultivars produced by Embrapa. Most of the diversity was attributed to intra-population variability and no association between genetic and geographical distances was observed. The best combinations of genotypes for future breeding programs were 1-1 (Agreste Potiguar) and 50-1 (Pau dos Ferros), 21-1 (Pau dos Ferros) and Guariba, Marataoa and 21-1 (Pau dos Ferros), Nova era and 4-1 (Agreste Potiguar), 24-1 (Serra de São Miguel) and 10-1 (Angicos), and 20-1 and Marataoa. The genetic divergence between the 14 cowpea populations, expressed by the \( F_{ST} \) coefficient, was considered moderate although it was lower than expected possibly due to the cultivation of mixed seeds from different sources in the small farms of Rio Grande do Norte where sampling was performed.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES


Carvalho SVA (2009). Parâmetros genéticos populacionais como indicadores de sustentabilidade em populações naturais de pimenta rosa - Schinus terebinthifolius Raddi (Anacardiaceae) no baixo curso do rio São Francisco SE/AL. MSc Dissertation, Universidade Federal de Sergipe.


Essential oil composition of different fractions of *Piper guineense* Schumach. et Thonn from Cameroon using gas chromatography-mass spectrometry and their insecticidal effect on *Sitophilus oryzae* (L.)

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Essential oil fractions from dried seed powder of *Piper guineense* were analyzed by gas chromatography-mass spectrometry (GC-MS) and evaluated for their insecticidal effects on *Sitophilus oryzae* L. The GC-MS analysis showed quantitative and qualitative differences between the oil fractions. Chromatographic results revealed chemical constituents like eugenol, piperanol, pinene, carene, copaene with insecticidal properties. New chemotypes were seen in the different fractions. Instead of β-caryophyllene reported in literature, α-caryophyllene was found in all the different fractions. Caryophyllene oxide, an oxygen-containing sesquiterpene was present in all fractions except n-hexane. In addition to α-phellandrene present in all, β-phellandrene, a monoterpene hydrocarbon was found in the n-Hexane fraction. Contact toxicity on wheat grains showed that all fractions caused significant (P < 0.001) mortality of the weevils. The oil fractions also showed variable contact toxicity on impregnated filter paper. All doses of the n-hexane fraction were very toxic to the test insect than the control, causing 100% mortality after five days of exposure. All the fractions produced a strong repellent activity against the test insect. These results suggest that *P. guineense* has potentials for development as an organic insecticide against *S. oryzae* and other pests of stored grains.

Key words: *Piper guineense* Schum. et Thonn., essential oil fractions, chemotypes, toxicity, repellency, *Sitophilus oryzae* (L.).

INTRODUCTION

Stored maize is infested by several important cosmopolitan pests such as weevils which cause considerable economic losses (Ndemah, 1999). Weevils have been reported to cause up to 30% grain damage in Cameroon, where

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stored grains constitute the most important food crop (Nukenine et al., 2010). Control of these insect pests is primarily dependent upon applications of synthetic insecticides (Bekele et al., 1996). Sometimes, insecticides may seem effective, but repeated application has disrupted biological control by natural enemies, leading to outbreaks of other pest species and also the development of resistance (Park et al., 2003). The problems caused by synthetic pesticides and their residues have increased the need for effective biodegradable pesticides of plant origin with greater selectivity and history of traditional use. Botanicals plants have been successful against a number of pests in Africa (Bekele et al., 1997; Ogendo et al., 2008).

Botanical products are based on powders, extracts or purified substances of plant origin and are promising alternatives to synthetic residual pesticides in Africa, where subsistence farming practice predominates. In many parts of Cameroon, farmers use different kinds of plant products for insect control (Tapandjou et al., 2000; Ngamo et al., 2007). Although, very little use is known by farmers in the South West region of Cameroon on plant products for insect pests’ control. Plant products are known to have negligible effects on beneficial insects and lower environmental impacts. They are easily affordable, available and play useful role in integrated pest management IPM programs in developing countries. Since most developing nations suffer from high cost of synthetics, crop protection products with modest efficacy are preferred if they are readily available and less expensive than the conventional pesticides. Plants powders or extracts could be produced with limited skills and knowledge and their use entails little or no financial expenditure.

Of the 700 species of *Piper*, only three are recognized to be indigenous to Cameroon (Hutchinson and Dalziel, 1963). The most widely distributed, *Piper guineense*, is a native to tropical Africa, ranging from Guinea to Kenya and South Zambia. It is a forest liana with branchlets spiralling up to shrubs to about 10 m. The leaves are elliptical in shape and have a pleasant aroma when crushed. The spherical fruits (berries) are yellow becoming orange, red and finally black (Iwu, 1993). The fruits are commonly called “West African Black pepper” or “Poivrie” in French. The fruits are sold in the market as edible medicinal plant or food additives due to their strong, pungent aroma and flavour. In the South West region of Cameroon, the leaves (fresh or dried) are eaten as vegetables in ‘Soups’, while the ground powder is used as additives in sauce. Powders obtained from ground seeds are used as stimulants (Sofowora, 1982).

*P. guineense* has demonstrated wide potentials against major noxious insect pests of arable crops (Ukeh et al., 2008; Ntonifor, 2011). A laboratory assessment of the repellent and anti feedant properties of both aqueous extracts and powders of *P. guineense* against *Callosobruchus maculatus* and *Putella xylostella* revealed potent repellent and anti feedant activities of this plant (Ajayi and Wintola, 2006; Ntonifor et al., 2010). Insecticidal activities of the essential oil have been tested (Asawalam et al., 2007). Several studies have been done on the essential oil composition of *P. guineense* seeds from different geographic origins (Amvam et al., 1998; Jirovtez et al., 2002). Many investigators have worked on whole fruit powder of *P. guineense* from Cameroon (Jirovtez et al., 2002; Tchoumbougoungang et al., 2009). Their essential oil composition depended on the geographic locations and climatic conditions where the seeds were taken. Essential oil composition of different fractions of this plant by GC-MS was not reported. Therefore, the purpose of the present study was: (1) to identify the aroma compounds of different fractions of this plant responsible for the characteristic odour and taste, (2) to evaluate the insecticidal activity of the different fractions against *Sitophilus oryzae*, a pest of stored grains found in Cameroon.

**MATERIALS AND METHODS**

**Plant material and extract preparation**

Dried fruits (berries) of *P. guineense* were purchased from a local market in Yaounde, Cameroon. The spices were authenticated by comparing with herbarium specimens at the Limbe Botanic Garden (Limbe, Cameroon). Samples were air-dried for 3 days at room temperature and crushed to a fine powder using a mechanical blender. The powder was then packed in air-tight bags and exported to Faculty of Agriculture and Environment (University of Sydney, Australia). Extracts of the spice were prepared in a sequential manner as follows: 100 g of seed powder was dissolved in 200 ml methanol, vortexed for 15 min and sonicated for 1 h under high frequency in an ultrasonic cell disruptor (Microson™). This process was to break and disrupt the plant cells for easy extraction. The process was repeated 3 times. The resulting solution was filtered with Whatman No. 1 filter paper using a vacuum pump. The filtered solution was evaporated in a BUCHI (R-114) rotavapor under reduced pressure at a temperature of 60°C to concentrate the samples. The sample was re-dissolved in 50 ml methanol and transferred into a separating funnel and eluted successively in hexane, chloroform, ethyl acetate and methanol. Each solvent was evaporated in a BUCHI rotavapor under reduced pressure at a temperature of 60°C. The resultant residues were re-dissolved in acetone, to give a stock volume of 10 ml for each fraction. The solutions were refrigerated prior to application and GC-MS analysis.

**Gas chromatography- mass spectra analysis**

The essential oil fractions of *P. guineense* were analyzed by GC-MS and identification of their constituents was achieved based on their retention indices determined with reference to standards and by comparing with those reported in the literature (Jirovtez et al., 2002; Adams, 2007; Tchoumbougoungang et al., 2009). Analytes were tentatively identified by reference to the NIST 2008 mass spectral library. An Agilent 7890A series GC with 5975C inert MSD triple axis detector (Agilent Technologies, USA) was used for GC-MS analysis. A Markes Series 2 ultra-unity system (Markes International Ltd., UK) was used for automated thermal desorption. Extract (3 µL) was injected onto sampling tubes containing Tenax TA (Supelco Inc., Bellefonte, PA, USA) adsorbent resin via a stream
of zero air (100 mL/min). The samples were desorbed by heating the sample tubes for 6 min at 300°C and focused onto a Tenax TA cold trap at -30°C for 6 min. The cold trap was then flash-heated to 300°C for 5 min and the sample injected onto a HBS column (30 m × 250 μm × 0.25 μm ID) via a heated transfer line held at 260°C. The GC oven was initially held at 40°C for 2 min, heated to 160°C at 5°C min⁻¹, 320°C at 10°C min⁻¹, then held for 2 min.

**Insecticidal activity of the oil fractions against S. oryzae**

**Insect culture**

*S. oryzae* adults were obtained from a stock maintained at the Faculty of Agriculture and Environment (University of Sydney) and reared on whole wheat grains in a constant temperature and humidity chamber (26°C, 65% RH) in darkness.

**Bioassays**

Bioassays were conducted using one month old adults of *S. oryzae*. Doses of 0, 0.5, 1 and 2 ml of the stock solution of each oil fraction were serially diluted in acetone to make up 10 ml, giving a series of dilutions 0, 0.05, 0.1 and 0.2 of each solution.

**Grain contact toxicity**

One millimeter from the new preparation was mixed with 40 g of wheat grains and stirred thoroughly for 5 min to allow even distribution over the grains. The control grains were treated with acetone only. Treated grains were kept for 20 min to allow the solvent evaporation. Twenty (20) adults of one month-old from the laboratory culture were introduced to the grains in a 250 ml glass vial with perforated lids. Each treatment and control was replicated four times. Mortality was evaluated after 24 h for up to five days.

**Filter paper contact toxicity**

Contact effect of extract fractions was evaluated on filter paper, 7 cm Whatman no.1. The filter paper was placed in Petri dishes and 0.5 ml of the serially diluted extract fractions was applied on the filter paper disc. The control filter papers were sprayed with acetone only. The acetone was allowed to evaporate for 20 min, after which, 20 unsexed one month-old adult insects were introduced at the center of each disc. This was kept under laboratory conditions (25±1°C, 60% RH). The treatments and control were replicated 4 times. Insect mortality was recorded after 24 h for up to five days. Percent mortality was calculated using Abbott correction formula for natural mortality in untreated controls (Abbott, 1925) (PT), as follows:

\[
PT = (PO-PC)/(100-PC) \times 100
\]

Where, PO = observed mortality of treated adults (%), PC = control mortality.

**Repellency bioassay**

A repellence effect was evaluated using the modified area preference method of McDonald et al. (1970) (Taponjciu et al., 2005). Test areas consisted of 7 cm Whatman no. 1 filter paper cut in halves. Test solutions were prepared by series of dilutions of the extract fractions in acetone as above. Each solution (0.5 ml) was uniformly applied to a half-filter paper disc using a micropipette. The other half disc was treated with acetone alone and served as control. Treated and untreated discs were air-dried for 10 min to evaporate the solvent completely. Full discs were remade by attaching treated halves to untreated halves with clear adhesive tape. Each remade disc was placed in a 7 cm Petri dish and 20 unsexed adult insects of one month-old were released at the center of the filter paper disc and the Petri dishes were covered. Each treatment and control was replicated 4 times. The number of insects present on the control (Nc) and treated (Nt) areas of the discs was recorded after 3 h. Percent repellency (PR) was calculated as follows:

\[
PR = \left(\frac{\text{Nc} - \text{Nt}}{\text{Nc} + \text{Nt}}\right) \times 100
\]

And assigned to repellency classes (0 - V) according to Talukder and Howse (1993) as follows: Class 0 (PR < 0.1%), class I (PR = 0.1 - 20%), class II (20.1 - 40%), class III (PR = 40.1 - 60%), class IV (PR = 60.1 - 80%), class V (PR = 80.1 - 100%).

**Statistical analysis**

The results of the experiments were analysed by one-way analysis of variance (ANOVA) using the General Linear Model Procedure (GLM) of GenStat 13th edition. Data were log-transformed before analysis. Duncan multiple range tests were used to compare the means. Percentage mortalities were calculated from the overall number of dead insects. Significant levels were set at 0.05.

**RESULTS**

**Chemical composition of dried fruit of *P. guineense***

The screening results of the plant revealed the presence of various chemical groups such as terpenes and flavonoid (Table 1). The fruit essential oil of the different fractions had colours ranging from pale yellow (chloroform) to deep yellow (n-hexane) with the characteristic pungent and aromatic odor of *Piper* plants. These essential oils were composed (90%) mainly of terpenes (mono- and sesquiterpenoids) with the most important ones being identified as copaene (99%), caryophyllene (99%), eugenol (98%), α-cubebene (98%), γ-elemene (94%). In the n-hexane fraction the most abundant constituents were Longifolene (91%), copaene (97%), caryophyllene (99%), α-caryophyllene (97%) and α-cubebene (96%). In addition to these, the methanol fraction also had piperanol (97%), camphor (94%), and eugenol (98%). The chloroform fraction contained oleic acid (78%), while all the fractions contained β-myrcene instead of myrcene as seen in literature.

**Insecticidal activities of *P. guineense* essential oil fractions**

**Contact toxicity by grain treatment**

Insect mortality by grain treatment of the different oil fractions differed significantly (P < 0.05) (Table 2). The n-hexane fraction produced 100% mortality of *S. oryzae*
Table 1. Comparative percentage composition of essential oil major fractions from dried seeds of *Piper guineense* bought from Yaounde, Cameroon.

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<th>Quality</th>
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Ethyl acetate fraction

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Chloroform fraction

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<th>Compound</th>
<th>Retention Time</th>
<th>Relative Intensity</th>
<th>Relative Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Mycene</td>
<td>13.19</td>
<td>49</td>
<td>136</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>13.19</td>
<td>48</td>
<td>136</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>13.19</td>
<td>43</td>
<td>136</td>
</tr>
<tr>
<td>1S-α-Pinene</td>
<td>13.19</td>
<td>43</td>
<td>136</td>
</tr>
<tr>
<td>3-Carene</td>
<td>13.19</td>
<td>30</td>
<td>136</td>
</tr>
<tr>
<td>4-Carene (1S,3S,6R)</td>
<td>13.19</td>
<td>30</td>
<td>136</td>
</tr>
<tr>
<td>(+)-4-Carene</td>
<td>20.24</td>
<td>94</td>
<td>136</td>
</tr>
<tr>
<td>Ocimene</td>
<td>20.24</td>
<td>93</td>
<td>136</td>
</tr>
<tr>
<td>α-Cubebeene</td>
<td>20.58</td>
<td>98</td>
<td>204</td>
</tr>
<tr>
<td>Ylanglenc</td>
<td>21.78</td>
<td>83</td>
<td>204</td>
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Table 1. Contd.

<table>
<thead>
<tr>
<th>Compound</th>
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<th>Relative</th>
<th>Area %</th>
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<tbody>
<tr>
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<td>204</td>
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<td>Seychellene</td>
<td>22.26</td>
<td>89</td>
<td>204</td>
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<tr>
<td>Patchouline</td>
<td>22.26</td>
<td>86</td>
<td>204</td>
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<tr>
<td>β-Guaiene</td>
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<td>β-Panasinsene</td>
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<td>204</td>
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<tr>
<td>(Z,Z) α-Farnesene</td>
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<td>204</td>
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<tr>
<td>Trans-α-Bergamotene</td>
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<td>87</td>
<td>204</td>
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<tr>
<td>Caryophyllene</td>
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<td>99</td>
<td>204</td>
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<tr>
<td>(-)-Aristolene</td>
<td>23.02</td>
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<tr>
<td>(+)-Epi-bicyclosesqui-phaellendrene</td>
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<td>204</td>
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<tr>
<td>α-Phallendrene</td>
<td>23.61</td>
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<td>136</td>
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<tr>
<td>Camphene</td>
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<tr>
<td>α-Muurolene</td>
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<tr>
<td>Longifolene -(V)</td>
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<td>94</td>
<td>204</td>
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<tr>
<td>Thujepsene-(12)</td>
<td>24.54</td>
<td>64</td>
<td>204</td>
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<tr>
<td>β-Humulene</td>
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<tr>
<td>γ-Elemene</td>
<td>26.32</td>
<td>55</td>
<td>204</td>
</tr>
<tr>
<td>Aromadendrene</td>
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<td>204</td>
</tr>
<tr>
<td>γ-Himachelene</td>
<td>26.32</td>
<td>70</td>
<td>204</td>
</tr>
<tr>
<td>Caryophyllene oxide</td>
<td>26.84</td>
<td>87</td>
<td>220</td>
</tr>
<tr>
<td>Bergamotol-(Z)-α-trans</td>
<td>26.84</td>
<td>25</td>
<td>220</td>
</tr>
<tr>
<td>Epizonarene</td>
<td>27.13</td>
<td>80</td>
<td>204</td>
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<tr>
<td>δ-Selínene</td>
<td>28.32</td>
<td>90</td>
<td>204</td>
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<tr>
<td>cis-α-Bisabolene</td>
<td>28.89</td>
<td>90</td>
<td>204</td>
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<tr>
<td>Cis-α-Santalol</td>
<td>28.89</td>
<td>38</td>
<td>220</td>
</tr>
<tr>
<td>α-Bisabolol</td>
<td>28.89</td>
<td>38</td>
<td>222</td>
</tr>
<tr>
<td>Spathulenol</td>
<td>31.00</td>
<td>50</td>
<td>220</td>
</tr>
<tr>
<td>Trans-Longipinocarveol</td>
<td>31.63</td>
<td>60</td>
<td>220</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>35.12</td>
<td>78</td>
<td>282</td>
</tr>
</tbody>
</table>

Table 2. Summary of analysis of contact toxicity by grain treatment.

<table>
<thead>
<tr>
<th>Source</th>
<th>d.f.</th>
<th>deviance</th>
<th>Mean deviance</th>
<th>Deviance ratio</th>
<th>Approx. chi pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>3</td>
<td>387.74</td>
<td>129.25</td>
<td>129.25</td>
<td>&lt;0.001</td>
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<tr>
<td>Residual</td>
<td>256</td>
<td>693.81</td>
<td>2.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>259</td>
<td>1081.55</td>
<td>4.18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

after five days of grain treatment, while the aqueous methanol fraction produced less than 50% mortality after five days of treatment. The chloroform and ethyl acetate fractions showed very little mortality and were not significantly (P < 0.05) different from the control (Figure 1). All the different doses of n-Hexane fraction of the essential oil induced 100% mortality after five days of exposure. This mortality could have been due to the high percentages of sesquiterpenes hydrocarbons present in this fraction. Methanol fraction, on the other hand, had percentages of eugenol and piperanol which induced high mortality in the essential oil. The 0.2 ml/cm² of the methanol fraction induced more than 30% mortality different from the control.

**Filter paper contact toxicity**

The percentages of insect mortality recorded after five days of exposure to increasing concentrations of volatile
Figure 1. Percentage mortality of *Sitophilus oryzae* exposed to different fractions of *Piper guineense* essential oil coated on wheat grains (A- Hexane fraction; B- chloroform fraction; C- ethyl acetate fraction and D- methanol fraction).

oil on filter paper discs showed similar results. The n-Hexane fraction had a significantly (P < 0.05) higher mortality on *S. oryzae* and doses of 0.1 and 0.2 ml/cm² were able to induce 85 and 100% mortality, respectively. The Chloroform, Methanol and Ethyl acetate fractions were almost nil within the first three days, and were not significantly (P < 0.05) different from the control, causing less than 50% mortality after five days of exposure on impregnated filter paper disc (Figure 2). There was a significant correlation between treatments, duration and concentrations as seen below (Table 3).

**Repellency testing**

Repellency bioassay using the area preference test showed that *P. guineense* extract fractions significantly repelled *S. oryzae* with an overall repellency of 87.5% (Table 4). The effect of the volatile oil on repellency of *S. oryzae* after 3 h of exposure was not dose-dependent. The n-Hexane fraction produced the highest repellency at different concentrations, while chloroform, methanol and ethyl acetate had 50% repellency at the highest concentrations.

**DISCUSSION**

The composition of different fractions of *P. guineense* essential fruit oil in this study was remarkably different from those reported earlier in Nigeria (Oyedeji et al., 2005; Owolabi et al., 2013) and Cameroon (Jirovetz et al., 2002; Tchoumgoungang et al., 2009). In all the different fractions, α-caryophyllene was present in high amounts instead of β-caryophyllene reported in literature. Such high content has not been found in *P. guineense* essential oil from Cameroon until now. Eugenol was abundant only in the Methanol fraction. These and others represented a number of new chemotypes revealed from the GC-MS analysis. The presence of constituents such as aromadendrene, piperazine and piperidine, though in low amount in the n-Hexane may have accounted for its
Figure 2. Percentage mortality of *Sitophilus oryzae* exposed to different fractions of *Piper guineense* essential oil impregnated on filter paper discs (A- Hexane fraction; B- Chloroform fraction; C- Ethyl acetate fraction and D- Methanol fraction).

Table 3. Correlation parameters for contact toxicity by grain treatment and impregnated filter paper assay.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact toxicity by grain treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Constant</td>
<td>1.000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Treatment</td>
<td>-0.592</td>
<td>1.000</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Concentrations</td>
<td>-0.558</td>
<td>0.030</td>
<td>1.000</td>
</tr>
<tr>
<td>4</td>
<td>Durations</td>
<td>-0.554</td>
<td>0.064</td>
<td>-0.006</td>
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<tr>
<td>Impregnated filter paper assay</td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>Constant</td>
<td>1.000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Treatment</td>
<td>-0.518</td>
<td>1.000</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Concentrations</td>
<td>-0.547</td>
<td>0.043</td>
<td>1.000</td>
</tr>
<tr>
<td>4</td>
<td>Durations</td>
<td>-0.612</td>
<td>-0.012</td>
<td>0.003</td>
</tr>
</tbody>
</table>
Table 4. Repellent effects of different fractions of essential oil from *Piper guineense* on *Sitophylus oryzae*.

<table>
<thead>
<tr>
<th>Extract fraction</th>
<th>Concentration (ml/cm²)</th>
<th>% Mean Repellency</th>
<th>Repellency class</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane</td>
<td>0.05</td>
<td>80±7.1</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>87.5±2.5</td>
<td>V</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>72.5±13.8</td>
<td>IV</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.05</td>
<td>20±14.7</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>10±10.8</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>50±16.8</td>
<td>III</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>0.05</td>
<td>45±15.6</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>28.7±15.6</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>50±23.8</td>
<td>III</td>
</tr>
<tr>
<td>Methanol</td>
<td>0.05</td>
<td>22.5±24.3</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>42.5±9.3</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>50±18.7</td>
<td>III</td>
</tr>
</tbody>
</table>

‘heat’ and aroma. *P. guineense* have been demonstrated to have 5 to 8% Piperine which gives them their ‘heat’ (Oparaeke, 2006). It also contains about 31-mono-sesquiterpenoids that have high insecticidal properties due to their pungent nature (Taponjou et al., 2005). The variability of the different constituents of *P. guineense* is important in light of its use as flavouring and medicinal agent. Therefore the efficacy varies depending on the chemotypes used. In the present study, the essential oil fractions of *P. guineense* exhibited different levels of toxic and repellent effects on *S. oryzae* at various doses. These effects may be due to factors such as the chemical composition of the plant as well as the insect susceptibility. The different doses of the n-Hexane fraction caused 100% mortality of *S. oryzae* on filter paper fumigation toxicity and grain contact after five days of exposure. The insects appeared to avoid the treated areas. The mortality of weevils on treated grains and filter paper varied with dosage of essential oil fractions. High mortality rates were recorded with the n-Hexane fraction at all doses compared to the other fractions. The toxicity of volatile oil from *P. guineense* is generally attributed to the presence of the alkaloids, piperine, chavicine and piperidine which are reported to be the major active components in *P. guineense* seeds (Lale and Alaga, 2000). This suggests that the toxicity of *P. guineense* was not due to ingestion of treated grains, but due to suffocation. The oil fractions evoked a very high repellency against the insect, suggesting that the oils could have contained a very pungent substance that caused high repellency of the insects. n-Hexane again demonstrated the highest level of repellency probably due to presence of constituents like Piperazine and Piperidine, though in very low quantity. Many other researchers have demonstrated the toxic and repellent effects of constituents of this oil such as 1S-α-Pinene, Copaene, β-caryophyllene, Eugenol, Piperanol and many others (Amvam Zollo et al., 1998; Owolabi et al., 2013). They are similar to those reported by Jirovetz et al. (2002) and Tchoumbougnang et al. (2009), in *P. guineense* obtained from Cameroon and Nigeria.

The results demonstrated a scientific rationale for the traditional incorporation of the dried seeds oils of *P. guineense* into grain protection practices in rural communities of Cameroon. However, there is need for further investigations into such practices to improve their efficacy and reliability in rural communities. With the right dosage and proper formulations this essential oil could be exploited at the small scale farmers’ level against insect infestations in the fields and in storage. Most of the oils are more effective, less cumbersome and are not particularly dangerous to consumers because they are used in pharmaceutical preparations (Bauer et al., 1990). They are also less expensive, safe to the environment and harmless to humans and other mammals. This study concludes that all of the fractions from the seeds of *P. guineense* demonstrated toxic and repellent effect on *S. oryzae*, a storage pest in Cameroon. The study also showed that the n-Hexane fraction of the essential oil was more toxic to *S. oryzae*.

**Conflict of interests**

The authors did not declare any conflict of interest.

**ACKNOWLEDGEMENTS**

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REFERENCES


Nukenen EN, Adler C, Reichmuth C (2010). Efficacy of Clausena anisata and Plectranthus glandulosus leaf powder against Prostephanus truncatus (Coleoptera: Bostrichidae) and two strains of Sitophilus zeamais (Coleoptera: Curculionidae) on maize. J. Pest Sci. 83:181-190.


Ukeh DA, Arong GA, Ogban EI (2008). Toxicity and oviposition deterrence of Piper guineense (Piperaceae) and Monodora myristica (Annonaceae) against Sitophilus zeamais (Motsch) on stored maize. J. Entomol. 5(4):295-299.
Heritability of polyphenols, anthocyanins and antioxidant capacity of Cameroonian cocoa (Theobroma cacao L.) beans

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This study investigates the heritability of polyphenolic, anthocyanin and antioxidant capacity of beans derived from four cocoa clones and their offsprings. These compounds were analyzed at 280 nm (polyphenols) and 520 nm (anthocyanins) by reversed-phase high-performance liquid chromatography (RP-HPLC) method using a photodiode array detector (PDA). The antioxidant capacity of methanolic extracts prepared from cocoa beans was measured by the DPPH and ABTS assays. Samples displayed catechin and epicatechin as the two main polyphenols. Epicatechin, which represents 1 to 5% of defatted cocoa seed powder, was hundred-fold higher than catechin while cyanidin-3-arabinoside was three-fold higher than cyanidin-3-galactoside. The two main anthocyanins found in our samples represent about 0.05% of defatted cocoa seed powder. All these compounds were genotype-dependent. Unidentified substances called A, B and C were also found in cocoa seeds. Substance A is discussed as a derivative of caffeic acid and an ester-bound compound. Substances B and C are oligomers of proanthocyanidins. Antioxidant capacity of cocoa beans obtained by 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay was higher than those obtained by DPPH assay. According to correlation tests, flavanols contributed better in the antioxidant capacity than anthocyanin. No maternal effect was detected in the transmission of polyphenol compounds suggesting a nuclear heritability.

Key words: Theobroma cacao, cocoa beans, polyphenolic compounds, anthocyanins, heritability.

INTRODUCTION

Cocoa beans are the fruit from the plant Theobroma cacao L., a plant tree originated in the rain forests of America whose culture has extended to equatorial areas of Africa and Asia. Cocoa beans are important in the economy of several countries such as Ivory Coast, Ghana, Indonesia, Cameroon and Nigeria. Cameroon is the fifth largest producer of cocoa beans in the world and the fourth in Africa. Cameroonian cocoa germplasm is constituted of two main varieties: Forastero and Trinitario. Criollo, is considered to exhibit one of the best flavor qualities which is scarce in Cameroon. Cacao powders, chocolate, cocoa-related products are phenolic-rich foods derived from the fermented, roasted and milled seeds of T. cacao L. These products, consumed all over the world,
are popular because of the antioxidant and antiradical properties of some of its phenolic constituents (phenolic acids, procyanidins and flavonoids) (Wollgast and Anklam, 2000). Flavonoids in cocoa are mainly flavan-3-ols, either monomeric (catechin and epicatechin) or oligomeric procyanidins (ranging from dimers to decamers), with appreciable amounts of anthocyanins (especially cyanidin glycosides) and flavonols (quercetin glycosides) (Keli et al., 1996; Hertog et al., 1993). Several methods are intensively studied to determine antioxidant capacities of samples. These methods differ in terms of their assay principle and experimental conditions. Most of them are based on the study of a reaction in which a free radical is generated and how this reaction is inhibited by the addition of the sample of interest. Stratil et al. (2006) determined antioxidant activities of several species of commonly consumed vegetables by the Trolox equivalent antioxidant capacity (TEAC), using diphenyl-p-picrylhydrazyl radical (DPPH), and ferric reducing antioxidant power (FRAP) methods. The phenolic content values of test substances and total antioxidant activity of the sets of samples correlate very well for all the methods used (Paixao et al., 2007). The antioxidant properties of simple polyphenols have been largely studied by means of in vitro DPPH scavenging tests (Lee et al., 2003; Othman et al., 2007) and many studies have confirmed the protective action of cocoa procyanidins and flavanols using in vitro cellular models (Kenny et al., 2004; Zhu et al., 2005). Moreover, other benign properties related to the bioactivity of phenolics from cocoa were largely studied. They can increase the antioxidant capacity of solutions and slow the oxidation of LDL. They may also induce endothelium-dependent vessel relaxation (Karim et al., 2000). Cocoa procyanidins can reduce the production of inflammatory cytokines, while increasing the production of anti-inflammatory cytokines (Mao et al., 2000a; Mao et al., 2000b). Schramm et al. (2001) reported that cocoa procyanidins can increase the synthesis of the antithrombotic lipid prostacyclin, while reducing the production of the proinflammatory cysteinyl leukotrienes. Cocoa polyphenol oligomers have been reported to protect against pteroxynitrate-dependent oxidation and nitration reactions (Arteel and Sies, 1999). Finally, cocoa was shown to decrease the expression of the activated conformation of glycoprotein IIb/IIIa and CD62P (Pselectin) on epinephrine-activated platelets (Rein et al., 2000).

Cocoa beans used in the confectionery industry come from a wide range of geographical areas, and may have different chemical and organoleptic properties. The chocolate producer must therefore select and combine these beans in various proportions in order to meet certain quality standards and economic specification. This task can be avoided if a screening of some cocoa clones in terms of their polyphenolic components can be done. So, some clone with high potential of bioactive compounds can be exhibited and exploited at industrial levels. As it is true that there are at least two parental clones in cocoa plantation, pollination will augment the number of unidentified hybrids. It will be very interesting then, to know if the content of polyphenol of cocoa hybrid can be predicted on the basis of those of its parental clones.

The aim of this study was therefore (i) to use high performance liquid chromatography (HPLC) to determine polyphenolic contents in cocoa beans from two Trinitario and two Forastero cocoa clones and their offsprings, (ii) and to determine and compare the antioxidant capacity of these samples applying two commonly used spectrophotometric methods (DPPH and ABTS assay methods). In addition, the correlation between specific polyphenols and the antioxidant activity in samples were investigated. The heritability related to these traits was also undertaken.

MATERIALS AND METHODS

Cocoa plant materials

Four Clones available in gene banks of the Cameroon Cocoa Development Corporation (SODECAO) at Mengang Station (South Cameroon) were used to create ten progenies: One local Trinitario (SNK16), one Trinitario introduced from Trinidad (ICS40), and two Forastero (Sc12 and T79/501). Crossings were realized in Mengang Station of SODECAO in May, June and July 2012 using hand-pollination techniques (Cilas, 1991) (Table 1).

Post-harvest treatment of cocoa

Two thousand ripe cocoa pods from different parental cocoa clones and hybrids were harvested from the experimental plots of the SODECAO at Mengang Station in the South Region of Cameroon. The ripe pods were split and beans obtained were fermented using the traditional heap method. The fermentation was done by heaping the extracted cocoa beans on the fermenting platform covered with banana leaves. The heaped beans were again covered with

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Abbreviations: ABTS, 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); Cya-ara, cyanidin 3-O-α-L-arabinoside; Cya-gal, cyanidin 3-O-β-D-galactoside; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GAE, gallic acid equivalents; HPLC, high performance liquid chromatography; PDA, photodiode array detector; SPSS, statistical package for the social sciences; TEAC, trolox equivalent antioxidant capacity.

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banana leaves and fermented for six days with consecutive openings and turnings after every two days. The fermented cocoa beans were then sun dried on the bamboo mat for twelve days.

Reagents and standards

Epicatechin and quercetin were obtained from Sigma. Protocatechic acid and catechin were obtained from Aldrich and Fluka respectively. 3-α-L-arabinosyl cyanidin and 3-β-D-galactosyl cyanidin were purchased from polyphenols AS. All solvents used were of analytical grade and purchased from Merck (Darmstadt, Germany). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Extraction and analysis of total phenol content (TPC) and total flavonoids (TF)

Two grams of dry cocoa beans were milled in 10 mL of n-hexane for fat removal. Most of the residual seed fat was extracted by flushing the powder with 25 mL n-hexane in a Buchner funnel. The phenolic compounds were extracted by agitation with 0.5 g of the fat-free sample on ice three times with 50 mL 60% aqueous acetone with constant shaking. After centrifugation at room temperature at 5000 rpm for 15 min, the three supernatants were combined in a flask containing 2 mL of glacial acetic acid. The acetone was removed by rotary evaporation under partial vacuum at 40 ±1°C. The aqueous phase obtained was adjusted to 100 mL with Milli-Q Plus water in a volumetric flask. Total contents of polyphenolic compounds were analyzed from this aqueous phase. TPC of cocoa extracts was determined spectrophotometrically according to a modified method of Lachman et al. (1998). Briefly, to a 0.5 mL aliquot of samples, 2.5 mL Folin-Ciocalteu’s reagent, 30 mL distilled water and 7.5 mL of 20% Na2CO3 were added and filled up to 50 mL with distilled water. After 2 h the absorbance of blue coloration was measured at 765 nm against a blank sample. To determine the content of total flavonoids, these compounds were precipitated using formaldehyde, which reacts with C-6 or C-8 on 5, 7-dihydroxy flavonoids to form methyl derivatives that further react with other flavonoid compounds also at positions C-6 and C-8. The condensed products of these reactions were removed by filtration and the remaining non-flavonoid phenols were determined as previously described. Total flavonoid content was calculated as the difference between total phenol and non-flavonoid content. Gallic acid was used as the standard and the results were expressed as mg gallic acid equivalents (GAE) per gram of defatted cocoa product (Kramling and Singleton, 1969). All measurements were performed in triplicate.

Purification of polyphenols

To clean the sample, 30 mL of the previous aqueous phase were mixed five times with 30 mL ethyl acetate. After 1 min of shaking, the aqueous phases were discarded and the organic phases combined, dried by the addition of 20 g anhydrous Na2SO4 and filtered after 5 min in the dark with Whatman paper No. 2. The salt residue was discarded and the clear organic phase was dried at 40°C under vacuum. The dried extract of polyphenolic compounds was dissolved in 5 mL of pure methanol (Lichrosolv, Merck) and filtered with Millipore (0.45 mm). The pure polyphenol extracts were stored at -20°C until HPLC analyzed.

Purification of anthocyanins

Purification of anthocyanins was conducted from the 100 mL aqueous phase using a Sep-Paks Vac C18 6cc column (Waters). The column was first eluted with a mixture of pure methanol (10 mL): 2% acetic acid (10 mL). A 20 mL aliquot of the aqueous phase sample was loaded onto the column and washed with 2.5 mL of 2% acetic acid. Anthocyanins were then eluted twice from the column with 5 mL pure methanol analytical grade (Lichrosolv, Merck). The eluted fractions were combined and dried by rotary evaporation. The residues were re-suspended in 2 mL of a mixture of pure methanol and acetic acid 2%.

Analysis of polyphenolic and anthocyanin compounds by reverse-phase HPLC

Chromatographic analyses were carried out on Waters HPLC system equipped with an A2-200 automatic injector, Knauer HPLC pump 64, Knauer HPLC program 50 solvent controller, Waters 996 Photodiode Array Detector (PDA) and analyzed with Millenium TM 3.2 software (Millipore Corporation, Milford, MA, USA). Separation of polyphenols was performed on a LicroCart 250-4 octadecylsilyl (ODS) C18, 5 mm particle [RP-18 (5 mm)] column (Merck) at 26°C. The guard column consisted of a LicroCart 4-Lichrospher 100 RP-18 (5 mm) (Merck). The binary mobile phase (Table 2) consisted of 2% acetic acid in water (A) and acetoni-trile-water-concentrated acetic acid mixture (4:9:1 v/v/v) (B). Twenty microliters of sample was injected into the column. The separation of polyphenols was monitored using a PDA detector at 280 nm and anthocyanins were recorded at 520 nm. Identification of each peak was confirmed by comparison of retention time and coelution with authentic standards of Protocatechic acid, catechinhidrate, epicatechin, cyanidin-3-galactoside and cyanidin-3-arabinoisde.

Table 1. General description of cocoa crossings evaluated in this study.

<table>
<thead>
<tr>
<th>Family</th>
<th>Crossings</th>
<th>Families</th>
<th>Back-crossings</th>
</tr>
</thead>
<tbody>
<tr>
<td>F40</td>
<td>(♀) ICS40 × (♂) Sca12</td>
<td>F12</td>
<td>(♀) Sca12 × (♂) ICS40</td>
</tr>
<tr>
<td>F45</td>
<td>(♀) ICS40 × (♂) T79/501</td>
<td>F50</td>
<td>(♀) T79/501 × (♂) ICS40</td>
</tr>
<tr>
<td>F20</td>
<td>(♀) ICS40 × (♂) SNK16</td>
<td>F25</td>
<td>(♀) SNK16 × (♂) ICS40</td>
</tr>
<tr>
<td>F61</td>
<td>(♀) Sca12 × (♂) T79/501</td>
<td>F15</td>
<td>(♀) T79/501 × (♂) Sca12</td>
</tr>
<tr>
<td>F79</td>
<td>(♀) SNK16 × (♂) T79/501</td>
<td>F16</td>
<td>(♀) T79/501 × (♂) SNK16</td>
</tr>
</tbody>
</table>

ABTS radical scavenging assay

The Trolox equivalent antioxidant capacity (TEAC) of cocoa extracts was also estimated by the ABTS radical cation decolorization assay (Re et al., 1999). Stock solutions of ABTS (7 mM) and potassium peroxodisulfate (140 mM) in water were prepared, and mixed together to a final concentration of 2.45 mM

Table 2. Binary gradient used for the separation of polyphenolic and anthocyanin compounds in cocoa beans.

<table>
<thead>
<tr>
<th>Times (min)</th>
<th>Flow rate (ml/min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>8</td>
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<td>10</td>
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<td>38</td>
<td>1.1</td>
<td>77</td>
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<td>90</td>
</tr>
<tr>
<td>78</td>
<td>1.2</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>93</td>
<td>1.2</td>
<td>90</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 3. Rate of success of hand-pollination.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Family</th>
<th>Crossing</th>
<th>Number of test</th>
<th>Number of success</th>
<th>Percentage of success (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>1000</td>
<td>360</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>F12</td>
<td>(♀) SCa12 × (♂) ICS40</td>
<td>1000</td>
<td>460</td>
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<td>2</td>
<td>F45</td>
<td>(♀) ICS40 × (♂) T79/501</td>
<td>1000</td>
<td>110</td>
<td>11</td>
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<tr>
<td></td>
<td>F50</td>
<td>(♀) T79/501 × (♂) ICS40</td>
<td>1000</td>
<td>220</td>
<td>22</td>
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<td>3</td>
<td>F20</td>
<td>(♀) ICS40 × (♂) SNK16</td>
<td>1000</td>
<td>20</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>F25</td>
<td>(♀) SNK16 × (♂) ICS40</td>
<td>1000</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>F61</td>
<td>(♀) SCa12 × (♂) T79/501</td>
<td>1000</td>
<td>360</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>F15</td>
<td>(♀) T79/501 × (♂) SCa12</td>
<td>1000</td>
<td>410</td>
<td>41</td>
</tr>
<tr>
<td>5</td>
<td>F79</td>
<td>(♀) SNK16 × (♂) T79/501</td>
<td>1000</td>
<td>320</td>
<td>32</td>
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<tr>
<td></td>
<td>F16</td>
<td>(♀) T79/501 × (♂) SNK16</td>
<td>1000</td>
<td>410</td>
<td>41</td>
</tr>
</tbody>
</table>

Potassium peroxodisulfate. The mixture was left to react overnight (12 to 16 h) in the dark, at room temperature. On the day of analysis, the ABTS radical solution was diluted with methanol to an absorbance of 0.70 (± 0.02) at 734 nm. All measurements were performed as follows: 100 µL of cocoa extract were added to 2.0 mL of the ABTS radical solution, and the absorbance readings were taken after exactly 6 min against the appropriate reagent blank of 100 µL of methanol instead of the sample. The results, obtained from triplicate analyses, were expressed as Trolox equivalents, and derived from a calibration curve determined for this standard (100 to 1000 µM).

DPPH radical scavenging assay

Antioxidant capacity of the cocoa extracts was determined using the DPPH radical scavenging assay described by Brand-Williams et al. (1995), with some modifications. Briefly, 100 µL of methanol cocoa extract was added to 1.9 mL of 0.094 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. The free radical scavenging capacity using the free DPPH radical was evaluated by measuring the decrease of absorbance at 517 nm every 2 min until the reaction reached its "plateau" state. Antioxidant capacity was expressed as mmol/L Trolox equivalents, using the calibration curve of Trolox (0 to 1000 µM), a water soluble vitamin E analogue.

Estimation of the heritability

For the different parameters measured, heritability was estimated according to Falconer (1974). This estimation considers the regression slope between means of parents and progeny.

Statistical analyses

Values are given as means of three measurements. Where appropriate, the data were tested by one-way ANOVA using the software SPSS 18.0 for windows, followed by Tukey post hoc test. Correlations between antioxidant capacities using DPPH and ABTS essays and content of polyphenolic compounds were also performed using the same software package.

RESULTS AND DISCUSSION

Hand-pollination

Hand-pollination test was less successful in F5 and F9 families with 11 and 18%, respectively. These results were better in F15 (41%) and F61 (36%) families (Table 3).
Total phenol content and total flavonoid content of cocoa extracts

Total polyphenols, total flavonoids, catechin, epicatechin, cyanidin-3-galactoside and cyanidin-3-arabioside contents in defatted cocoa powder (DCP) determined by HPLC analysis.

<table>
<thead>
<tr>
<th>Group</th>
<th>Clone/family</th>
<th>PPT (mg/g)</th>
<th>Flavonoid (mg/g)</th>
<th>Flavanol (µg/g)</th>
<th>Cyanidin (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Catechin</td>
<td>Epicatechin</td>
</tr>
<tr>
<td>1</td>
<td>ICS40</td>
<td>112.85^a</td>
<td>60.15^a</td>
<td>245.58^ef</td>
<td>30001.8^d</td>
</tr>
<tr>
<td></td>
<td>Sca12</td>
<td>138.4^cd</td>
<td>66.41^b</td>
<td>234.3^de</td>
<td>28940.4^c</td>
</tr>
<tr>
<td></td>
<td>F40</td>
<td>147.14^cd</td>
<td>121.81^d</td>
<td>260.1^g</td>
<td>31210.1^de</td>
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<tr>
<td></td>
<td>F12</td>
<td>141.68^d</td>
<td>105.89^c</td>
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<td>2</td>
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</tr>
<tr>
<td></td>
<td>T79/501</td>
<td>114.95^a</td>
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<td>159.7b</td>
<td>36513.8^ef</td>
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<td></td>
<td>F45</td>
<td>168.53^d</td>
<td>129.53^e</td>
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<td>30722.1^d</td>
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<tr>
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<td>117.65^ab</td>
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<td>30001.8^d</td>
</tr>
<tr>
<td></td>
<td>SNK16</td>
<td>130.18^c</td>
<td>84.51^b</td>
<td>754.5^k</td>
<td>45513.7^g</td>
</tr>
<tr>
<td></td>
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<td>147.51^cd</td>
<td>115.21^cd</td>
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<td>31203.2^de</td>
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<tr>
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<td>136.14^cd</td>
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<td>195.7^c</td>
<td>28613.9^c</td>
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<tr>
<td></td>
<td>T79/501</td>
<td>114.95^a</td>
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<tr>
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<tr>
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<td>110.16^cd</td>
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<td>84.51^b</td>
<td>754.5^k</td>
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</tr>
<tr>
<td></td>
<td>T79/501</td>
<td>114.95^a</td>
<td>70.16^a</td>
<td>159.7^b</td>
<td>36515.8^ef</td>
</tr>
<tr>
<td></td>
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<td>150.82^d</td>
<td>105.71^cd</td>
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<td>88.77^c</td>
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<td>41690.4^g</td>
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<tr>
<td></td>
<td>Means</td>
<td>142.51^d</td>
<td>98.85^c</td>
<td>305.59^g</td>
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<tr>
<td></td>
<td>Sums</td>
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<td>1198.23</td>
<td>4278.28</td>
<td>417232.7</td>
</tr>
</tbody>
</table>

*Values with the same letter in the same column are not significant different (P < 0.05).

Depending on the cocoa bean variety, geographical origin, ripeness degree (harvest season) and post-harvest conditions, such as fermentation, drying, roasting, processing and storage (Wolgast and Anklem, 2000). In the evaluation of major chemical components in beans of some selected international cocoa clones in Ghana, Dadzie et al. (2014) confirmed the existence of significant correlations among the major chemical components in cocoa beans and the influence of genotype on the chemical component concentration. In 2-day-old fermented-like cocoa beans, the average total content ranged from 101.3 to 143.6 mg g⁻¹ DM (Niemark et al., 2006). Those values are close to our results and this may be because, the authors values were obtained in 6-day-old fermented-like beans. TFC in our samples represented about 70% of TPC and the assertion that flavonoids are the main polyphenol compounds found in cocoa is confirmed. Each sample of defatted cocoa seed polyphenols exhibits seven significant peaks at λ = 280 nm (Figure 1). Two of these peaks
Figure 1. Representative chromatogram of a sample of defatted cocoa at $\lambda = 280$ nm. (AU: Absorption Units).

Figure 2. Representative chromatogram of a sample of defatted cocoa at $\lambda = 530$ nm. Cya-galcyanidin 3-$\beta$-D-galactoside, Cya-ara: cyanidin 3-$\alpha$-L-arabinoside.

correspond to the two purine alkaloids; caffeine and theobromine, which are commonly found in cocoa seeds. HPLC chromatograms revealed three unidentified compounds, namely substances A (RT = 12.41 min), B (RT = 20.99 min), C (RT = 29.56 min). Protocatechic acid and quercetin were not detected. Two peaks were present at $\lambda = 520$ nm in defatted cocoa seed samples that contained anthocyanins (Figure 2). These two peaks were identified as cyanidin 3-galactoside and cyanidin 3-arabinoside.

Epicatechin content by the sum of means (29802.7 $\mu$g/g DCP on average) was hundred-fold higher than catechin content (305.6 $\mu$g/g DCP on average). Likewise the quantity of cyanidin-3-arabinose by the sum of means (349.2 $\mu$g/g DCP on average), was about three-fold higher than Cyanidin-3-galactoside (132.02 $\mu$g/g).
DCP on average). Among the four cocoa clones, SNK16 was found to be highest in catechin and epicatechin content, while T79/501 possessed the greatest quantity of cyanidin-3-galactoside and cyanidin-3-arabinoside.

Among hybrids, individuals of F16 family displayed the highest content in flavanols (catechin and epicatechin) and cyanidins. Except F40, F12 and F16 hybrids which displayed flavanol values similar to those of at least one of their parent, all other hybrids presented identical values to those of their parent in term of flavanols and cyanidins (Table 4). For Niemenak et al. (2006), it was well established that the predominant polyphenols identified in dried defatted cocoa bean was epicatechin followed by catechin.

In the same way, Wu et al. (2004) and Gu et al. (2004) also stated that cocoa beans are a concentrated source of antioxidants and flavonoids, with the flavan-3-ols and their derivatives being present in high concentrations. Among unidentified compounds, substance A, due to the PDA results will be a derivative of caffeic acid and an ester-bound compound. Substance B and C will be oligomers of proanthocyanidins that have been detected in cocoa seed (Adamson et al., 1999). As for the cyanidin 3-O-β-D-galactoside, a variation between 0 and 319.19 µg.g⁻¹ defatted cocoa was found, whereas the content of cyanidin 3-O-α-L-arabinoside ranged from 42.46 to 789.13 µg.g⁻¹ in defatted cocoa. These values were a little lower than those obtained by Elwers et al. (2009) in cocoa seeds and this can be due to variation in the methods used by the authors for the extraction of anthocyanins.

Antioxidant capacity of cocoa extracts
A standardized method for the determination of antioxidant properties of certain foods and beverages has not yet been established, so using more than one method for evaluating antioxidant capacity is highly recommended. In this study, two different antioxidant assays (ABTS and DPPH) for the evaluation of antioxidant capacity of various cocoa beans were applied and the obtained results are shown in Figure 3. Comparing the results of the applied radical scavenging assays on cocoa extracts, it can be noticed that methanol cocoa extracts exhibited high antioxidant potential, but the efficiency of radical scavenging differs markedly with regard to each cocoa clone.

Methanol extracts of SNK16 and T79/501 cocoa clones were the most efficient DPPH radical scavengers (30.2 and 30.1 mmol/L Trolox, respectively). F12 and F25 cocoa hybrid exhibited the least DPPH radical scavenging capacity in methanol extracts with 17.4 and 15.4 mmol/L Trolox, respectively. In the case of ABTS radical scavenging assay, methanol cocoa extract of F25 hybrid showed the best ABTS radical scavenging properties (114.42 mmol/L Trolox), better than F79 (86.5 mmol/L Trolox). F61 hybrids showed the lowest ABTS scavenging efficiency (44.1 mmol/L Trolox).

The antioxidant capacity of cocoa extracts obtained by ABTS assay was higher than that obtained by DPPH assay (Figure 3). Considering the fact that DPPH radical reacts only with lipophilic antioxidants, while ABTS radical reacts with both hydrophilic and lipophilic antioxidants, the difference between the results of these two radical scavenging assays becomes more obvious (Prior et al., 2005). Similar methodological differences were previously observed by Kim et al. (2002) and Arnao (2000), who also claim that these differences may be due to absorbance interruption at 517 nm by other compounds in the DPPH assay.
Several studies showed a correlation between antioxidant activity and phenolic content (Nagai et al., 2003; Velioglu et al., 1998). Our findings report that cocoa beans exhibited the highest antioxidant activity. However, low correlation between ABTS, DPPH essays and TPC of cocoa beans were noticed (data not shown).

Our result is in agreement with those of Belščak et al. (2009) who stated that lower correlation coefficients between TPC and DPPH in both methanol and water extracts indicate that only a small content of the phenolic antioxidants in cocoa products account for the activity by scavenging free DPPH radicals. Arlorio et al. (2005) and Othman et al. (2007) suggested that high scavenging ability of cocoa extract compounds on DPPH and ABTS radicals could be attributed to other methanol-soluble compounds like methylxanthines, minor flavonoids and pigments.

Quite big correlation coefficients between flavanol and DPPH on one hand, flavanol and ABTS on the other hand in methanol extracts were found. These coefficients were about $r = 0.64$ for catechin and $r = 0.57$ for epicatechin (Table 5). For cyanidins, these coefficients were weaker than those of flavanol with $r = 0.40$ for cyanidin-3-galactoside and $r = 0.39$ for cyanidin-3-arabinoside (Table 5). These results indicated that flavanol contribute better than cyanidins in cocoa antioxidant capacity. These results also assumed that high scavenging ability on DPPH and ABTS radicals could not be exclusively due to flavanols and cyanidins in cocoa extracts. Arlorio et al. (2008), studying the impact of cocoa polyphenols in the antioxidant capacities, found a poor correlation between the contents of a specific polyphenolic fraction of cocoa (clovamide) and the antioxidant properties of cocoa. In Contrast, Othman et al. (2010) found that both ethanolic ($r = 0.92$) and water ($r = 0.90$) extracts of cocoa beans showed a significant positive and high correlation between epicatechin and ABTS value. These authors stated that epicatechin content in cocoa beans could be responsible for the antioxidant capacity.

### Heritability

Heritability of biochemical compound contents was estimated using the regression between the average contents in flavanols and cyanidins of parents and those of their progenies (Figures 4 and 5). The calculated heritability ($h^2$) for catechin was 0.51 for the first crossings and 0.61 for reciprocal crossings. Concerning epicatechin, the heritability values obtained were 0.45 and 0.50, respectively, for the first crossings and their reciprocals. Cyanidin contents obtained in different crossings permitted estimation of the heritability of these characters. Heritability of the accumulated cyanidine-3-galactoside was weak for the two different crossings ($h^2 = 0.42$ for the first crossing and $h^2 = 0.55$ for reciprocal crossing).

For cyanidine-3-arabinoside, the estimation of the heritability was very close with 0.57 and 0.44. There was a weak relationship between flavanol contents of parents and those of progenies in the first crosses than reciprocal crossings (data not shown). These results were also observed for cyaniding-3-galactoside. Regardless of the studied character, the absence of a significant difference between the heritability values from reciprocal crossing portrays the absence of maternal heritability. This observation suggests that the heritability of biochemical compounds studied is nuclear rather than cytoplasmic. The relationship between phenolic compounds, amino acids, carbohydrates and resistance to Phytophthora megakarya in T. cacao detected no maternal effect in the transmission of these characters (Djicogoue et al., 2011; Ondobo et al., 2013).

### Conclusion

Our results indicate that cocoa beans displayed high contents of polyphenolic compounds. Epicatechin content was hundred-fold higher than catechin content, likewise the quantity of cyanidin-3-arabinoside was about three-fold higher than Cyanidin-3-galactoside. However, those values were clone-dependant. According to multiple comparison tests, none of the offsprings displayed polyphenolic compounds values similar to those of their parent. Cocoa bean extracts gathered antioxidant capacity and those obtained by ABTS assay was higher than those obtained by DPPH assay. Flavanols contributed better in the antioxidant capacity than cyanidin and no maternal effect was detected in the transmission of polyphenol compounds.

### Conflict of interests

The authors did not declare any conflict of interest.

### ACKNOWLEDGEMENT

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Figure 4. Evaluation of heritability ($h^2$) using the regression slope between parental clones and their progenies for catechin contents [crossing (a) and back-crossing (b)] and for epicatechin contents [crossing (c) and back-crossing (d)].

Figure 5. Evaluation of heritability ($h^2$) using the regression slope between parental clones and their progenies for Cyanidine-3-Galactoside contents [crossing (a) and back-crossing(b)] and for Cyanidine-3-Arabinoside contents [crossing (c) and back-crossing(d)].
Figure 5. Contd.

REFERENCES


Ondobo ML, Efua OP, Djougoe PF, Boudjeko T, Manga NJ, Djoko KJC, Omokolo ND (2013). Influence of Phytophthora megakarya inoculation on necrosis length, phenolic content, peroxidase and polyphenoloxidase activity in cocoa (Theobroma cacao L.) plants.


Full Length Research Paper

Preliminary characterization of residual biomass from *Hibiscus sabdariffa* Calyces

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*Hibiscus sabdariffa* calyces are mainly used for different agro-food and beverages applications. The residual biomass generated contains various useful substances that were extracted and characterized. It contained 23% (w/w) soluble pectic material, a food additive, extracted with hot acidified water (80°C, pH = 1.5) and precipitated with ethanol. The molecular weight (28.5 and 109.7 kDa), the degree of methylation (70.6 and 44.3%) and the degree of acetylation (19.0 and 4.9%) were determined for two Senegalese cultivars (koor and vimto, respectively). The effect of the extraction method on these parameters was highlighted. The residual lignocellulosic material (LCM) was chemically degraded to monosaccharides and the amount of glucose and xylose (39% of dry LCM) determined to estimate its potential as feedstock for biofuels production. However, an enzymatic degradation test revealed a recalcitrant LCM, as only 50 to 55% of its polymeric glucose content was degraded to monosaccharides without pretreatment. Xylo-oligosaccharides (XOS) are functional foods with a real market potential as prebiotics, characterized by their degree of polymerization (DP). The production of XOS synthetized by the enzymatic degradation of LCM was monitored. The results of analyses performed showed that XOS produced had mainly DP3 and DP4 values.

**Key words:** Pectin, lignocellulosic material, enzymatic degradation, xylo-oligosaccharides.

INTRODUCTION

*Hibiscus sabdariffa* var. sabdariffa is a member of the malvaceae family. This plant is an erect, annual shrub characterized by large and succulent sepals particularly rich in anthocyanins (Pouget et al., 1990; Tsai et al.,...
2002). Once considered a secondary crop, it is gaining increasing interest for the food industry as a source of anthocyanins for nutraceuticals (Da-Costa-Rocha et al., 2014; Patel, 2014) or food color production (Giusti and Wrolstad, 2003). In Senegal it was traditionally used to manufacture drinks and only a small proportion of calyces were processed locally. The national production of red H. sabdariffa was of 2885 tons in 2012, 200 tons of which were consumed locally (Cissé et al., 2008). However newly developed industrial products (spray dried instant drinks, energy drinks, bottled drinks etc.) made from dry calyces are manufactured by local small and medium facilities. The biggest amounts processed by a single company are estimated at 100 tons for the current year. The residual biomass produced is sometimes used as animal feed after a thorough washing designed to eliminate residual acidity, otherwise it is discarded as waste material. Due to environmental and economic considerations agro-industrial byproducts are processed to recover and/or produce valuable products. To date H. sabdariffa’s residual biomass has not been considered with the objective of increasing its value.

H. sabdariffa calyces contain pectic polysaccharides whose chemical structure and biological activity have been studied (Muller and Franz, 1992; Brunold et al., 2004; Badreldin et al., 2005). Generally, industrial juice extraction processes are designed to avoid the extraction of pectins (Taylor, 2007); therefore, considerable amounts are still present in the residues. Apple pomace and citrus peels are the industrial byproducts traditionally used for extraction of commercial pectins, additives commonly used in food, cosmetic and pharmaceutical industries as gelling agent, thickener or emulsion stabilizer (Sila et al., 2009; Smith and Hong-Shum, 2011). However, alternative sources with new interesting characteristics are constantly sought (Happi Emaga et al., 2008; Fissore et al., 2013). Lignocellulosic material (LCM), primarily made of cellulose, hemicellulose and lignin, have the potential to be used as feedstock for the production of bioenergy, nutraceuticals, chemicals, biodegradable composites (Moure et al., 2006; Satyanarayana et al., 2009; Faruk et al., 2012; Menon and Rao, 2012). The products considered depend on the characteristics and composition of the biomass and on the profitability of the process selected (Uçkun Kiran et al., 2014).

The objective of the present study was to characterize the waste material from the processing of H. sabdariffa calyces in order to determine the potential of this by-product as a source of new derived products. Pectic polysaccharides from the two main Senegalese cultivars (Koor and Vimto) were extracted and characterized. The determination of cellulose, hemicellulose and lignin content together with an enzymatic degradation assay were used to estimate the potential as feedstock for biofuels production. Finally, the enzymatic release of xylooligosaccharides (XOS), nutraceuticals with a real market potential, was studied (Aachary and Prapulla, 2011; Finegold et al., 2014; Singh et al., 2015).

MATERIALS AND METHODS

Equipment, solvents and reagents

The centrifuge used throughout this work was a Jouan B4i centrifuge. Unless specified, centrifugation was performed for 15 min at 4000 rpm. All solvents and chemicals (acetone, formic acid, dibasic sodium phosphate, chloroform, and acetonitrile) were analytical grade and purchased from VWR International BVBA (Leuven, Belgium).

Residual biomass samples preparation

The experiments were made on calyces of Vimto and Koor cultivars from the Louga region (Northwest of Senegal). Dry whole calyces were extracted at 70°C in water with a 1/20 (v/v,ol) ratio for 90 min under constant agitation. After filtration the calyces residues (CR) were dried at 55°C. CR was used in the following experiments.

Characterization of soluble polysaccharides

Extraction and purification of soluble polysaccharides

10 g of CR sample was submitted to extraction with distilled water (150 ml) at room temperature for 24 h. The supernatant, called crude water-soluble extract (CWE) hereafter, was collected by centrifugation and stored for further analysis. A second extraction was performed on the sediment with water acidified to pH 1.5 with hydrochloric acid (HCl). Temperature was kept, at 80°C for 2 h under constant stirring. The slurry was centrifuged and the supernatant, called crude acid-soluble extract (CAE) hereafter, was collected by centrifugation and stored for further characterization. Crude extracts (CWE and CAE) were purified as follows (Lama-Muñoz et al., 2012): 4 volumes of 96% ethanol were added to an aliquot of the extract. After 24 h at 4°C, the ethanol-insoluble fraction was recovered by centrifugation, dispersed in ethanol, allowed to settle for 60 min. The liquid fraction was removed; the ethanol insoluble fraction was dissolved in pH 2 water (0.01 M HCl), precipitated again with one volume of ethanol (96%), allowed to settle for 60 min then centrifuged (15 min at 4000 rpm) to recover the precipitates. The sediment was recovered and dissolved in acidified water (0.01 M HCl), to give purified water extract and purified acid extracts (PWE and PAE) stored at 4°C before analysis.

Molecular distribution of H. sabdariffa soluble polysaccharides

Molecular mass (MM) distribution of polysaccharides contained in samples of CWE, CAE, PWE and PAE was determined by High-Performance Size Exclusion Chromatography (HPSEC). The apparatus used was a Waters 2690 - High Performance Liquid Chromatography (HPLC) system (Waters Inc., Milford, MA, USA) equipped with a TSKgel GPWXL column (300 mm × 7.8 mm) (Tosoh Co. Ltd., Tokyo, Japan) and coupled on-line with a Waters 2410 differential refractometer that measures the refractive index (RI). Elution was performed at room temperature with 50 mM sodium nitrate solution containing 0.05% sodium azide at a flow rate of 1 ml/min. Elution was performed at room temperature with 50 mM sodium nitrate solution containing 0.05% sodium azide at a flow rate of 1 ml/min.
rate of 0.7 ml/min. The system was calibrated using dextran standards (Sigma-Aldrich NV / SA, Bornem, Belgium).

**Galacturonic acid content**

Enzymatic hydrolysis was performed with the commercial enzyme Frimapec W70 (Beldem, Groot Bijgaarden, Belgium), generally used to extract anthocyanins from red grape mashes. A 10 mg sample was mixed with 20 ml of enzyme dissolved in 20 mM of pH 5 sodium acetate buffer. The mixture was incubated at 40°C for 24 h and then heated at 100°C for 5 min to inactivate the enzymes. The final hydrolysate was filtered through a 0.2 µm filter membrane before analysis. Galacturonic acid (GalA) and glucuronic acid contents were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as previously described (Combo et al., 2013).

**Degree of methylation and acetylation**

The samples were submitted to de-esterification by alkaline treatment, and the amount of methanol and acetic acid released were determined by HPLC as described by Happi Emaga et al. (2008).

**Characterization of H. sabdariffa LCM**

**Composition in xylose and glucose**

Total monosaccharides concentration of H. sabdariffa LCM was determined as follows: 3 ml of 72% sulfuric acid was added in a 100 ml flask to a 300 mg sample. After 60 min of incubation at 30°C, 84 ml of distilled water were added to the flask that was put in an autoclave at 121°C for 60 min. Subsequently the supernatant was collected, neutralized with Ba(OH)$_2$, then centrifuged. An aliquot of the supernatant was taken for HPLC analysis. Total lignin content was determined by weighing accurately the solid residue previously dried to constant weight at 105°C.

**Enzymatic hydrolysis**

LCM samples were submitted to enzymatic degradation with a previously optimized (Rodriguez et al., 2005) mixture of commercial cellulases and hemicellulases in order to estimate the amount of LCM that could be degraded to monosaccharides (called enzymatic glucose or enzymatic xylose hereafter). This value was reported to the total concentration of monosaccharides previously determined. Biodegradability was defined here as the ratio (w/w) of enzymatic glucose or xylose to total LCM dry matter. Recovery was defined as the ratio (w/w) of enzymatic glucose or xylose to their total respective content. The enzymes used were: Viscozyme L (an endo-β-glucanase that hydrolyzes (1,3)- and (1,4)-linkages in β-D-glucans, and had enzymatic side-activities such as cellulase, hemicellulase and xylanase), Celluclast 1.5 L (a cellulase with an endoglucanase activity) and Celluloseclean Classic 700T (a cellulase). The samples were added (0.05% w/v) to prevent enzymatic hydrolysis and (1,4)~D~ glucans, and had enzymatic side-activities such as cellulase, hemicellulase, and xylanase). Celluclast 1.5 L (a cellulase with an endoglucanase activity) and Celluloseclean Classic 700T (a cellulase). All the enzymes used were from Novozymes A/S ( Bagsvaerd, Denmark). Celluloseclean Classic 700T was dissolved in phosphate buffer (0.1 N, pH 5.5). NaN$_3$ was added (0.05% w/v) to prevent microbial growth. The solutions were then filtered on a GF/C membrane (Whatman, Maidstone, England). Celluloseclean Classic 1.5 L and Viscozyme L were dialyzed overnight in phosphate buffer (0.1 N, pH 5.5 with 0.05% NaN$_3$) using nitrocellulose membranes with a cutoff of 10 kDa (Sigma-Aldrich, Bornem, Belgium). One liter of enzymatic preparation was obtained by mixing 500 ml of 20 g/l Celluloseclean Classic 700T, 100 ml of dialyzed Viscozyme L and 50 ml of dialyzed Celluclast 1.5 L. H. sabdariffa LCM hydrolysis was performed as follows: 30 ml of enzyme preparation was added to 500 mg sample in a 100 ml flask incubated in a water bath at 40°C. After 48 h an aliquot was centrifuged, the supernatant was analyzed by HPLC as described hereafter. Enzymatic activity was tested as follows: 1.5 ml of enzyme preparation was incubated in test tubes with 50 mg of cellulose (Whatman filter paper grade 1) for 60 min at 40°C under constant agitation. The test tubes were then put for 5 min in a 100°C water bath to deactivate the enzymes. The supernatant’s monosaccharides content was determined by HPLC analysis as described hereafter.

**HPLC analysis**

HPLC analysis was performed on an Agilent 1100 series apparatus (Agilent Technologies, Diegem, Belgium) equipped with a RI detector. Separation was made with a C-610-H ion exchange column (300, 7.8 mm, Supelco, Bellefonte, PA, USA) and quantified using standards. All samples were filtered through 0.2 µm Minisart Syringe filter (Vivascience, Hannover, Germany).

**Enzymatic production of xylo-oligosaccharides (XOS)**

XOS production rate by enzymatic hydrolysis of H. sabdariffa LCM was monitored. The enzyme preparation and the enzymatic hydrolysis procedure were identical to those previously used. A sample was removed at increasing time periods and after deactivation of enzymes at 100°C for 5 min, the samples were analyzed by HPAEC-PAD as described above to determine the amount of XOS produced.

**RESULTS AND DISCUSSIONS**

**Main characteristics of residual biomass from H. sabdariffa calyces**

Residual biomass (CR) accounted for more than 50% of calyces’ dry mass (Table 1). It presumably contained residual amounts of anthocyanins, soluble and insoluble polysaccharides, and other compounds whose extraction was minimized with the aqueous extraction method previously used. In the present study focus was put on the polysaccharide composition. CR were submitted to a sequential extraction procedure. A first extraction was performed at room temperature with distilled water (CWE) followed by a second extraction with acidified water (CAE). CWE accounted for 10.9 and 16.5% weight of soluble dry matter from koor and vimto cultivars total CR. Combined crude extracts accounted for around 40% of CR dry mass (Table 1). This was in accordance with the study of Chan and Choo (2013) who found no significant effect of water temperature (50 to 95°C) on the extraction yield of cocoa husk pectin, whereas hot acidified water helped increase pectin solubility leading to an improved extraction yield. Similarly, Yapo et al. (2007) showed that pectin extraction yield was increased significantly with increasing acid strength but also with increasing extraction time. Extraction temperature on the other hand did not have any significant effect on the
Table 1. Composition of residues from H. sabdariffa calyces.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>CR (% of dry calyces (w/w))</th>
<th>Crude pectic material extracts(% of dry CR (w/w))</th>
<th>LCM (% of dry CR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CWE</td>
<td>CAE</td>
</tr>
<tr>
<td>Vimto</td>
<td>54.8±1.3</td>
<td>16.5±1.7</td>
<td>29.4±4.9</td>
</tr>
<tr>
<td>Koor</td>
<td>55.6±2.1</td>
<td>10.9±0.3</td>
<td>28.6±2.0</td>
</tr>
</tbody>
</table>

Results are expressed as average of 3 determinations ± standard deviation (sd); Vimto and koor are the local names of two H. sabdariffa cultivars.

Table 2. Composition of precipitated water soluble (PWE) and acid soluble (PAE) polysaccharides of H. sabdariffa calyces.

<table>
<thead>
<tr>
<th></th>
<th>PWE (% of dry CR)</th>
<th>PAE (% of dry CR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimto</td>
<td>2.7±0.8</td>
<td>21.0±3.3</td>
</tr>
<tr>
<td>Koor</td>
<td>6.2±1.6</td>
<td>17.0±1.2</td>
</tr>
</tbody>
</table>

Results are expressed as average of 3 determinations ± sd; Vimto and koor are the local names of two H. sabdariffa cultivars.

Extraction yield. Both crude extracts, CWE and CAE, were submitted to precipitation aiming at removing low molecular mass (MM) and ethanol-soluble compounds from the extracts. Results in Table 2 show that the amount of ethanol-precipitated extracts (PWE and PAE) represent 23.7 and 23.2% of CR (dry mass basis). CR can thus, be favorably compared to citrus peel and apple pomace, the main raw materials used for industrial extraction of commercial pectin, that contain 25 and 12%, respectively, pectin (Kalapathy and Proctor, 2001; Sila et al., 2009). In order to further characterize H. sabdariffa polysaccharides, some characteristics influencing their functional properties such as molecular mass (MM), degree of methylation (DM), degree of acetylation (DA) and galacturonic acid content (GalA) were determined.

**Characterization of soluble polysaccharides**

**Biochemical characterization**

Results of characterizations performed on PWE and PAE samples are summarized in Table 5. PWE samples had DM values of 70.6 and 44.3% for vimto and koor cultivars respectively. These values decreased in the samples extracted with hot acidified water (Table 5). Yapo (2009) observed similar effects on yellow passion fruit pectins and pointed out the least deesterifying action of citric acid compared to mineral acids. The DA was 19.0 and 4.9% for the vimto and koor cultivars pectic polysaccharides respectively. These values dropped to 3.2% and null for the pectic polysaccharides extracted with hot acidified water. Such a decrease in DA was also reported by Garna et al. (2007) and it was probably due to the hydrolysis of acetyl groups from the GalA during the extraction step with acidified water. Levgine et al. (2002) evidenced the more marked effect of pH on DA compared to extraction time and temperature. Furthermore, the use of water as an extractant did not have a significant effect on DA values of extracted pectins even at 95°C (Chan and Choo, 2013).
Figure 1. Overlay of HPSEC chromatograms of crude (A and C) and precipitated (B and D) extracts of residual biomass from of *H. sabdariffa* calyces. Cultivars studied were Vimto (top) and Koor (bottom). Precipitated water extracts (PWE) and crude water extracts (CWE) in grey, precipitated acid extracts (PAE) and crude acid extracts (CAE) in white.
Table 3. HPSEC analysis of crude extracts of *H. sabdariffa* calyces residues: apparent MW and relative abundance of polysaccharides.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>MW (Da)</th>
<th>Percentage of total area* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CWE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimto</td>
<td>12.429</td>
<td>34.1</td>
</tr>
<tr>
<td>Koor</td>
<td>62.226</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>219.377</td>
<td>41.8</td>
</tr>
<tr>
<td>CAE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimto</td>
<td>766</td>
<td>17.3</td>
</tr>
<tr>
<td>Koor</td>
<td>157</td>
<td>45.6</td>
</tr>
</tbody>
</table>

*calculation based on the total HPSEC determined surface. Results are expressed as average of two determinations; Vimto and koor are the local names of two *H. sabdariffa* cultivars.

Table 4. Molecular Weight distribution and main characteristics of polysaccharides from extracts of two cultivars of *H. sabdariffa* calyces.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Fraction</th>
<th>Major polysaccharide</th>
<th>MW (Da)</th>
<th>Percentage of total area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimto</td>
<td>PWE</td>
<td>28.500</td>
<td>86.3</td>
<td></td>
</tr>
<tr>
<td>Koor</td>
<td></td>
<td>109.700</td>
<td>72.1</td>
<td></td>
</tr>
<tr>
<td>Vimto</td>
<td>PAE</td>
<td>13.300</td>
<td>66.2</td>
<td></td>
</tr>
<tr>
<td>Koor</td>
<td></td>
<td>50.300</td>
<td>49.3</td>
<td></td>
</tr>
</tbody>
</table>

Vimto and koor are the local names of two *H. sabdariffa* cultivars – PWE and PAE are pectins obtained by ethanol precipitation of water extracts (PWE) and acidified water extracts (PAE). Results are expressed as average of two determinations.

Table 5. Biochemical properties of pectin extracts precipitated with ethanol after aqueous extraction (PWE) or after acidified water extraction (PAE).

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Fraction</th>
<th>Biochemical properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DM (%)</td>
</tr>
<tr>
<td>Vimto</td>
<td>PWE</td>
<td>70.6±27.4</td>
</tr>
<tr>
<td>Koor</td>
<td></td>
<td>44.3±28.1</td>
</tr>
<tr>
<td>Vimto</td>
<td>PAE</td>
<td>13.0±8.5</td>
</tr>
<tr>
<td>Koor</td>
<td></td>
<td>13.0±8.5</td>
</tr>
</tbody>
</table>

DM (Degree of methylation): % moles methanol/moles Galacturonic acid (GalA) - DA (Degree of acetylation): % moles acetic acid/moles of galacturonic acid. Vimto and koor are the local names of two *H. sabdariffa* cultivars. Results are expressed as average of 3 determinations ± SD.

GalA content determined by HPAEC-PAD after enzymatic hydrolysis of PWE and PAE extracts ranged from 6.6 to 11.2% (w/w) of soluble extract (Table 5). The pectins extracted with acidified water contained more GalA than pectins extracted with water for both cultivars. This confirms the findings by Yapo et al. (2007) who showed that the extraction pH was the parameter that mostly influenced GalA content. Previous studies by Muller and Franz (1992) on chemical structure of *H. sabdariffa* polysaccharides extracted with water at room temperature, revealed the presence of two neutral polysaccharides and a pectin-like polysaccharide (84% GalA) whose average MM were respectively 20.0, 6.3 and 100.0 kDa. The main characteristics of polysaccharides contained in CR are shown in Table 5. According to American Chemical Society classification, these are considered pectinic acids (Marshall and Joseph, 1986). In the present work, the more soluble polysaccharides were presumably extracted during the aqueous extraction procedure (90 min at 70°C) previously used on *H. sabdariffa* calyces to produce CR samples.
Table 6. Composition of Lignocellulosic material (LCM) from residual biomass of *H. sabdariffa* calyces.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Glucose* (%)</th>
<th>Xylose* (%)</th>
<th>Lignin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimto</td>
<td>29.7±3.7</td>
<td>9.5±0.7</td>
<td>15.8±2.4</td>
</tr>
<tr>
<td>Koor</td>
<td>25.3±1.9</td>
<td>9.3±0.7</td>
<td>16.2±4.8</td>
</tr>
</tbody>
</table>

Results are expressed as average of 4 determinations ± standard deviation and on a dry mass bases as percentage of total LCM. Vimto and koor are the local names of two *H. sabdariffa* cultivars. *Expressed as polymeric form.

Table 7. Biodegradation of Lignocellulosic Material (LCM) from residual biomass of *H. sabdariffa* calyces cultivars.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Glucose LCM Bioavailability* (%)</th>
<th>Glucose Recovery** (%)</th>
<th>Xylose LCM Bioavailability* (%)</th>
<th>Xylose Recovery** (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vimto</td>
<td>15.0±0.8</td>
<td>50.8±4.0</td>
<td>3.0±0.1</td>
<td>31.5±1.5</td>
</tr>
<tr>
<td>Koor</td>
<td>13.8±1.8</td>
<td>54.4±7.5</td>
<td>3.4±0.5</td>
<td>37.5±8.6</td>
</tr>
</tbody>
</table>

Results are expressed as average of 4 determinations ± sd. Vimto and koor are the local names of two *H. sabdariffa* cultivars. *Ratio (w/w) of enzymatic glucose or xylose to total LCM dry matter. **Ratio (w/w) of enzymatic glucose or xylose to total glucose or xylose content.

Lignocellulosic residues from *H. sabdariffa* calyces

**Potential as bioenergy resource**

After the previously described pectin extraction procedure, 40 to 45% of CR mass was extracted as pectic material. The remaining insoluble plant material, representing 55 to 60% of CR dry mass, was characterized by determination of total glucose, xylose and lignin content. Results summarized in Table 6 show that the total amount of glucose and xylose under polymeric form represented 34.6 and 39.2% (w/w) of koor and vimto cultivars dry LCM, respectively. These results were used to estimate the potential of *H. sabdariffa* residual biomass as feedstock for biofuels production. Considering total glucose content, a potential of 25.3 to 29.7% of *H. sabdariffa* LCM dry mass could be enzymatically hydrolyzed then fermented to ethanol by *Saccharomyces cerevisiae*. Based on these results and on the assumption that 100 tons of *H. sabdariffa* calyces are processed annually by the same plant, considering the theoretical ethanol yield of 0.51 kg ethanol/kg glucose (Drapcho et al., 2008b), the amount of ethanol that could be produced is estimated at 4500 L. This amount is in our opinion too small to justify the investment for a bioethanol production plant that are profitable only for production capacities of several millions liters of ethanol (Hamelinck et al., 2005; Drapcho et al. 2008b). To further characterize *H. sabdariffa* LCM a biodegradation test was performed. The amount of monosaccharides released by a containing cellulase and hemicellulase was determined in order to evaluate the biodegradability of the waste material (Table 7). The fraction of material degraded to glucose represented 15.0 and 13.8% (w/w) of total LCM dry mass, whereas the amount of xylose was 3.0 and 3.4% for vimto and koor cultivars, respectively. These results suggest that a further degradation was hindered by intrinsic characteristics of *H. sabdariffa* LCM. Such recalcitrance was described in literature as being directly proportional to lignin content (Fang and Dixon, 2007). This issue is usually overcome with a pretreatment preceding the enzymatic treatment designed to increase the depolymerization yield and to minimize the amount of enzyme used (Öhgren et al., 2007; Mussatto et al., 2008). Lignin content that varies with the plant species was estimated at 16.0% of LCM dry mass for both *H. sabdariffa* cultivars.

Results of the biodegradability test showed that *H. sabdariffa* LCM could also be used as feedstock for biomethane production by anaerobic fermentation. Indeed, biodegradability is correlated to the amount of biomethane produced as documented by various authors (Rodríguez et al. 2005; Fang and Dixon, 2007; Khalid et al., 2011; Monlau et al., 2012). It is generally assumed that vegetable wastes 1 kg of organic wastes could generate 1.0 m³ of biogas that contains 6.5 kWh of energy (Drapcho et al., 2008a). Based on these assumptions we calculated that 195 MWh of energy could be theoretically produced each year by the biomethanation of LCM from 100 t of *H. sabdariffa* calyces. However, the biodegradation test showed that only 50.8% of glucose and 31.5% of xylose under polymeric form was degraded to fermentable monosaccharides (Table 7). The results show a lower
biodegradation of hemicellulose that has been described elsewhere as being caused by its more complex structure. The energy recovered from the biomethanation could be reinjected in the production system and help reduce the environmental impact of the processing plant. Unlike bioethanol production, biomethanation plants that allow the processing of small amounts of wastes in a profitable way are available (Cheng et al., 2014; Surendra et al., 2014). The removal of lignin should be considered in order to exploit the full potential of these residues (Adl et al., 2012; Shafiei et al., 2013).

**Potential as a source of xylooligosaccharides**

Alternatively, *H. sabdariffa* LCM could be used for the production of xylooligosaccharides (XOS). These oligosaccharides mainly made of xylose are characterized by their molecular size, defined by the degree of polymerization (DP) which represents the number of monosaccharide residues (2 to 10). *H. sabdariffa* LCM enzymatic hydrolysis was monitored by determination of the types and amounts of XOS generated at increasing time periods. The analysis revealed that xylose and mainly oligosaccharides with DP3 and DP4 were produced during the enzymatic hydrolysis of *H. sabdariffa* LCM. Figure 2 shows the kinetics of xylose production: *H. sabdariffa* LCM is rapidly degraded into xylose that reached 20 mg/g (dry mass) after 30 min and a maximum of 30 mg/g that remained constant after 4 h. DP3 and DP4 were detected at much lower concentrations but with production kinetics similar to xylose, as illustrated by Figure 3 for Vimto cultivar (shown for illustration). DP3 reached a maximum concentration of 2.0 mg/g (dry mass bases) and DP4 a maximum of 5.2 mg/g after 48 h. The overall XOS production was low throughout the reaction. It was due to the fact that the degradation of LCM into monosaccharides was favored, indicating that the commercial enzyme mixture contained a high exoxylanase activity. The enzymes hydrolyzed (1,3)- and (1,4)-linkages in β-D-glucans and also had enzymatic side-activities such as cellulase, hemicellulase and xylanase. The types and amounts of oligosaccharides generated by LCM enzymatic hydrolysis are dependent on the substrates and enzymes employed (Akpinar et al., 2009), therefore, other available enzymes should be tested to improve the production of monosaccharide-free XOS. An additional step could also be considered, where an extraction of hemicellulose using alkali (sodium or potassium hydroxide) would precede the enzymatic treatment in order to increase hemicellulose recovery (Akpinar et al., 2009; Samanta et al., 2012).

**Conclusion**

The work presented here studied the various fractions extracted from the processing byproducts from calyces of two *H. sabdariffa* cultivars. Besides, pectins, the total
content of monosaccharides and lignin of the pectin-free biomass were evaluated. This residue was then treated by enzymes and the release of mono- and oligosaccharides was followed. The whole constitutes an original approach for a possible integrated valorization of this agro-waste. More precisely, extraction of polysaccharides using a procedure similar to industrial extraction of pectin from apple pomace was performed. The two cultivars appeared to originate different types of pectins in terms of MM and degree of esterification. Considering the amounts extracted (23%, dry mass basis), this byproduct has the potential to be used as a low price feedstock for pectin production. Further studies should be made for a complete characterization of these polysaccharides including a study of their functional properties. LCM represented over 50% of the byproduct studied, 39% of which were made of xylose and glucose. This makes it a potentially highly biodegradable material that could be used to make biogas to inject in the processing plant. However, its enzymatic degradation was limited (50.8 to 54.4% of total glucose) presumably by the high lignin content (16% w/w of LCM dry mass). Otherwise, enzymatic production of xylooligosaccharides with DP3 and DP4 as neutraceuticals would be an alternative to bring added value to the residual biomass from H. sabdariffa calyces.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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REFERENCES


Lebensmittel Wissenscha 1913


Full Length Research Paper

A study of the optimal conditions for glucoamylases obtained from *Aspergillus niger* using amylopectin from cassava starch as carbon source

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A fourteen day pilot study carried out showed that high glucoamylase activities were obtained on the 4th and 11th day of fermentation and the enzymes were harvested on the respective days giving the codes GluAgCSV4 and GluAgCSV11. The optimal pH and optimal temperatures for enzyme activities GluAgCSV4 and GluAgCSV11 were in a range of 6 to 7 and 50 to 55, using cassava, guinea corn and tiger nut starch as substrates, respectively. The enzyme activity (GluAgCSV4) was enhanced by Ca$^{2+}$, Mn$^{2+}$, Fe$^{2+}$ and Zn$^{2+}$. Co$^{2+}$ had inhibitory effect on the enzyme while Pb$^{2+}$ completely inactivated the enzyme. The enzyme activity (GluAgCSV11) was enhanced by Ca$^{2+}$ and Co$^{2+}$. Zn$^{2+}$, Fe$^{2+}$, Mn$^{2+}$ and Pb$^{2+}$ completely inactivated the enzyme. The Michaelis constant $K_M$ and maximum velocity $V_{max}$ obtained form Lineweaver-Burk plot of initial velocity data at different substrate concentrations were found to be 90.06 mg/ml and 188.67 µmol/min (using cassava starch as substrate), 173.70 mg/ml and 434.78 µmol/min (using guinea corn starch as substrate) and 28.57 mg/ml and 227.27 µmol/min (using tiger nut starch as substrate), respectively for GluAgCSV4. Also, 271.30 mg/ml and 1000 µmol/min (using cassava starch as substrate, 3093 mg/ml and 10000 µmol/min (using guinea corn starch as substrate) 2625 mg/ml and 10000 µmol/min (using tiger nut starch as substrate), respectively, were obtained for GluAgCSV4.

**Keywords:** Glucoamylases, *Aspergillus niger*, amylopectin and starch.

INTRODUCTION

Glucoamylases of microbial origin are divided into exo-acting, endo-acting, debranching and cyclodextrin producing enzymes. Glucoamylases hydrolyze α-1, 4 and α -1, 6 linkages and produce glucose as the sole end-product from starch and related polymers (Shenoy et al., 1985; Svensson et al., 2000; Parbat and Singhal, 2011). Beside cellulose, starch is the most abundant carbohydrate in the world (Betiku, 2010; Ozienbge and Onilude, 2011). The primary industrial use of starch is its hydrolysis to sugar syrups that are employed by the food

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**Abbreviations:** PDA, Potato dextrose agar; DNSA, 3, 5-dinitrosalicylic acid.

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industry to make sweets, drinks, juices and for fermentation into products like citric acid, ethanol, bakers' 'yeast, as well as in paper and textile. The original method of starch hydrolysis known as acid hydrolysis (Kolusheva and Marinova, 2007) requires the use of corrosion resistant materials, gives rise to high colour and salt ash content (after neutralisation), needs more energy for heating and is relatively difficult to control. As a result of these problems, enzymatic hydrolysis becomes a better method for starch hydrolysis since the above mentioned constraints are not peculiar. Starch hydrolysis in industry most times requires a combination of enzymes for complete hydrolysis. There is the need therefore to optimize the necessary conditions for glucoamylase activity to achieve complete starch hydrolysis without necessarily involving multiple enzymes. Starch is a major storage product of economically important crops like cassava, guinea corn and tiger nut. These plants have the potentials to become the principle feedstock for fuel, alcohol and glucose syrup market. This research is aimed at production of glucoamylases from Aspergillus niger in a submerged fermentation using amylopectin fractionated from cassava starch as the only carbon source and possibly optimize the conditions for the applications in hydrolysis of cassava, guinea corn and tiger nut starch.

**Materials and Methods**

**Materials**

**Collection of plant samples**

The plant materials (cassava tuber, guinea corn, and tiger nut) were obtained at Orba main market, Udenu Local Government, Enugu State, Nigeria.

**Processing of cassava and cassava starch**

Cassava starch was processed using the method described by Corbishley and Miller (1984) with the following modifications. Freshly harvested cassava tubers were peeled washed clean and grated. The grated cassava (1.2 kg) was soaked in 4 L of distilled water for 1 h after which it was sieved (3 times) with muslin cloth. This was allowed to stand for 4 h and the supernatant decanted. The isolated wet starch was sun dried and packaged in plastic air tight container and stored at room temperature. The starch from cereals was processed using the method described by Agboola et al. (1990) with the following modifications. The seeds were sun dried and ground to fine flour. 300 g of the flours were suspended in 3 L of distilled water for 24 h. The suspended flour was sieved using muslin cloth. The extracted starch was allowed to sediment for 4 h at room temperature. The supernatant was decanted off and the starch washed with 3 L of distilled water twice and finally allowed to stand for 4 h. The supernatant was then decanted and the resulting wet starch was sun dried and then packaged in an air tight container and stored at room temperature.

**Fractionation of starch into amylose and amylopectin**

Fractionation of amylose and amylopectin was carried out by following the general procedure of (Sobukola and Aboderin, 2012). This consists of heating and stirring starch dispersion (0.8%, w/v in water) in water bath at 100°C until starch is gelatinized. Starch solutions were filtered using filter paper to remove insoluble residues, and the pH adjusted to 6.3 using phosphate buffer. The solution was stirred in a boiling water bath for 2 h to disperse the starch molecules. Thereafter, n-Butyl alcohol was added (20%, v/v), and the solution was stirred at 100°C for 1 hr, followed by cooling to room temperature over a period of 24-36 h. Amylose butyl alcohol complex crystals was formed and precipitated during cooling, and was separated by filtration. The amylopectin remaining in the supernatant was recovered by adding excess methyl alcohol.

**Isolation of glucoamylase producing fungi**

Glucoamylase producing fungi were isolated by adopting the method of Martin et al. (2004) as modified by Okoye et al. (2013). Tiger nut starch was fractionated into amylose and amylopectin. The wet amylopectin was left open on shelf to allow microorganisms to grow on it. A loop of each organism was streaked onto potato dextrose agar PDA under the flame of Bunsen burner. Streaks were made from each side of the plate, marking an initial point, with sterilization of the wire loop after each side has been completed. The plates were thereafter incubated at 35°C till visible colonies were observed. All morphological contrasting colonies were purified by repeated streaking and sub-culturing on separate plates. This process was continued till pure fungal culture was obtained. Pure fungal isolates were maintained on potato dextrose agar (PDA) slopes or slants as stock cultures. PDA media were prepared according to the manufacturer’s description. In the description, 3.9 g of PDA powder was weighed and added in small volume of distilled water and made up to 100ml. The medium was autoclaved at 121°C, 15 psi for 15 min. It was allowed to cool to about 45°C and then poured into petri dishes and allowed to gel. The plates were then incubated in a B and T Trimline incubator at 37°C for 24 h to check for sterility. Three day old pure cultures were examined. The colour, texture, nature of mycelia or spores and growth patterns were also observed. The three day old pure cultures were used in preparing microscopic slides. A little bit of the mycelia was dropped on the slide and a drop of lactophenol blue was added to it. A cover slip was placed over it and examination was performed under the light microscope at X400 magnification. Identification was carried out by relating features and the micrographs to “Atlas of mycology” by Barnett and Hunter (1972).

**Glucoamylase production**

Glucoamylase was produced by adopting the method described by Bagheri et al. (2014) with the following modifications. A 250 ml Erlenmeyer flask containing 100 ml of sterile cultivation medium optimized for glucoamylase with 0.3% ammonium sulphate (NH4)2SO₄, potassium dihydrogen phosphate (0.6% KH₂PO₄), magnesium sulphate hepta hydrate (0.1% (MgSO₄.7H₂O), 0.01% ferrous sulphate hepta hydrate (FeSO₄.7H₂O), 0.1% calcium chloride (CaCl₂) and 1%, amylopectin. The flask was stoppered with aluminium foil and autoclaved at 121°C, 15 psi for 15 min. From the PDA slants, fresh plates were prepared and three day old cultures were used to inoculate the flasks. In every sterile flask, two discs of the respective fungal isolates were added using a cork borer of diameter 10 mm and then plugged properly. The culture was incubated for 7 days at room temperature (30°C). At each day of harvest, flasks were selected from the respective groups and mycelia biomass separated by filtration using filter paper. Each day,
the filtrate was analyzed for glucoamylase activity and extracellular protein concentration till the 14th day of fermentation. After the 14 days pilot SmF studies, 4 and 11th day of fermentation were chosen for mass production.

Glucoamylase assay

Glucoamylase activity was assayed by the method of Parbat and Singhal (2011) with the following modifications. 0.5 ml of the enzyme was added into a clean test tube followed by 0.5 ml 1% soluble starch solution in 50 mM acetate buffer (pH 5.5) at 50°C and was allowed to stand for 20 min. 1 ml of 3, 5-dinitrosalicylic acid (DNSA) reagent was added and boiled for 10 min to stop the reaction. 1 ml of sodium potassium tetaurate was added to stabilize the red colour produced. The mixture was then allowed to cool and the glucose released was measured using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Huston Texas) at 540 nm. Absorbance values were converted to glucose concentrations by extrapolation from the glucose standard curve. Glucoamylase activity unit (U) was expressed as the amount of enzyme releasing one μ mole of glucose equivalent per minute per ml.

α-amylase assay

The α-amylase activity was assayed by adopted the method of Bernfield (1955). The reaction mixture contains 0.5 ml of the enzyme preparation and 0.5 ml of (1% w/v) starch solution in 20mM sodium phosphate buffer (pH 7.0). The reaction mixture was incubated at 55°C for 60 min after which the reaction was stopped by addition of 1 ml 3, 5-dinitrosalicylic acid (DNSA) reagent and boiling for 10 min. 1 ml of sodium potassium tetaurate was added to stabilize the red colour produced. The mixture was then allowed to cool and the glucose released was measured using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc., Huston Texas) at 600 nm. α-amylase activity unit (U) was expressed as the amount of enzyme releasing one μ mole of reducing end groups (maltose) per minute under assay conditions.

Protein determination

Protein content of the enzyme was determined by the method of Lowry et al. (1951), using Bovine Serum Albumin as standard.

Partial purification

The crude enzyme preparation was made up to 20 and 70% ammonium sulphate saturation with solid (NH₄)₂SO₄ for both GluAgCSV4 and GluAgCSV11, respectively. This was kept at 4°C for 30 h; thereafter it was centrifuged with Cole-palmer VS-13000 micro centrifuge at 4000 rpm for 30 min. The precipitate was collected and re-dissolved in 20 mM acetate buffer pH 5.5. The glucoamylase activity and protein were determined as described above.

Gel filtration chromatography

A volume (20 ml) of the precipitated enzyme was introduced into a (50 × 2.5 cm) gel chromatographic column and subjected to gel filtration with sephadex G-100 pre-equilibrated with 0.02 M sodium acetate buffer pH 5.5. Fractions were collected at a flow rate of 5 ml/20 min. The protein concentration of each fraction was monitored using a JENWAY 6405 UV/VIS spectrophotometer (Beckman/Instruments, Inc. Huston Texas) at 280 nm. The glucoamylase activity of each fraction was assayed as earlier discussed with the active fractions pooled together and stored at -10°C.

Optimizing glucoamylase activity with respect to pH, temperature, metal ion concentration and substrate concentration

Optimum pH

The optimum pH for enzyme activity was determined using 0.02 M sodium acetate buffer pH 3.5 to 5.5, phosphate buffer pH 6.0 to 7.5 and Tris-HCl buffer pH 8.0 to 10.0 at intervals of 0.5. 0.5 ml of gelatinized starch solution (1%) was equilibrated with 1 ml of the buffers (20 mM) of respective pHs for 5 min at 37°C. 0.5 ml of the enzyme was added and the reaction mixture was mixed properly and allowed to stand for 20 min at 50°C. The glucoamylase activity was assayed as described above using starch as substrate.

Metal ions concentration

The concentrations, 20, 30, 40 and 50 mM of metal salts (ZnCl₂, CoCl₂, MnCl₂, FeCl₂, PbCl₂ and CaCl₂) were prepared in 20 mM sodium acetate. Each of the reaction mixtures contains 0.5 ml of enzyme solution, 0.5 ml of starch solution (1%) and 1 ml of metal ion solutions (Ca²⁺, Mg²⁺, Mn²⁺, Fe²⁺, Co²⁺ and Zn²⁺). The mixtures were incubated for 20 min at the predetermined optimal pHs and temperatures. To study the effect of metal ions on glucoamylase activity/stability, the reaction was carried out with and without metal ions. In all the above experiments, the enzyme activity was calculated as the average of three independent sets of experiments and the standard deviation in all cases was negligible.

Optimum temperature

The optimum temperature for glucoamylase activity was determined by incubating the enzyme with gelatinized starch solution (1%) at 30 to 80°C for 20 min at respective predetermined optimal pHs. Glucoamylase activity was assayed as described above using starch as substrate.

Substrate concentration

The effect of substrate concentration on glucoamylase activity was determined by incubating 0.5 ml of enzyme with 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg/ml of starch for 20 min at respective predetermined optimal pHs and temperatures. The glucoamylase activity was assayed as described above using starch from cassava, guinea corn and tiger nut starch as substrate.

RESULTS AND DISCUSSION

In all the above experiments, the results were calculated as the average of three independent sets of experiments and the standard deviation in all cases was negligible. A fourteen day pilot study was carried out to determine the day of highest protein production, α-amylase and glucoamylase activity in submerged fermentation using amylopectin obtained from cassava starch as carbon source. Enzyme activities were assayed using cassava,
Two major peaks were obtained on day 4 and 11 with activities 299.56 and 298.47 µmol/min, respectively, using cassava starch as substrate. Also, using guinea corn starch as substrate, a major peak was obtained on day 4 with glucoamylase activity 236.15 µmol/min, while day 4 exhibited the highest peak with glucoamylase activity (304.30 µmol/min) using tiger nut starch as substrate. Therefore, day 4 and 11 were chosen for mass production of the enzyme. The high increase in glucoamylase activity observed in the study could be due to de-branching activity exhibited by glucoamylase in addition to its α-1, 4-glucosidic activity. Glucoamylase first hydrolyses the amorphous regions before attacking the crystalline regions and both amylase and amyllopectin are hydrolysed simultaneously to smaller molecular sizes (Wang and Wang, 2001). The decrease in glucoamylase activity could be due to depletion in the level of branch points in the carbon sources, product inhibition as well as depletion in growth supplement in the broth (Nahar et al., 2008). Alva et al. (2007) reported that the productivity of glucoamylase production is generally subjected to catabolite repression by glucose and other readily
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**Figure 3.** Monitoring the day of highest glucoamylase, α-amylase activity and protein concentration in liquid broth using amylpectin from cassava starch as the only carbon source.

**Table 1.** Purification table for GluAgCSV4 and GluAgCSV11.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Total Protein</th>
<th>Activity (U)</th>
<th>Total Activity</th>
<th>Specific Activity (U/mg)</th>
<th>Purification fold</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>1000</td>
<td>0.149</td>
<td>149</td>
<td>193.79</td>
<td>193790</td>
<td>1300.60</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>GluAgCSV4</td>
<td>1000</td>
<td>0.132</td>
<td>132</td>
<td>266.58</td>
<td>266580</td>
<td>2019.55</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>350</td>
<td>0.423</td>
<td>148.05</td>
<td>35.29</td>
<td>12351.5</td>
<td>83.43</td>
<td>0.1</td>
<td>6.37</td>
</tr>
<tr>
<td>GluAgCSV4</td>
<td>350</td>
<td>0.284</td>
<td>85.20</td>
<td>44.54</td>
<td>13362</td>
<td>156.83</td>
<td>0.1</td>
<td>5.01</td>
</tr>
<tr>
<td>GluAgCSV11</td>
<td>300</td>
<td>0.286</td>
<td>8.58</td>
<td>53.79</td>
<td>1613.7</td>
<td>188.08</td>
<td>2.3</td>
<td>13.06</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>30</td>
<td>0.159</td>
<td>4.77</td>
<td>38.99</td>
<td>1169.7</td>
<td>245.22</td>
<td>1.56</td>
<td>8.75</td>
</tr>
</tbody>
</table>

Ammonium sulphate saturation (20 and 70%) were found suitable to precipitate proteins with highest glucoamylase activity in GluAgCSV4 and GluAgCSV4. After ammonium sulphate precipitation and gel filtration, the specific activities were found to increase from 83.43 to 188.08 U/mg protein with purification fold 2.3 and 156.83 to 245.22 U/mg protein with purification fold 1.56 for GluAgCSV4 and GluAgCSV11, respectively (Table 1). The increase in specific activity could be as a result of exclusion of impurities contributed by ammonium sulphate salt that may have inhibited the enzyme activity.

The optimal pH for GluAgCSV4 were 7.5, 7.0 and 6.0 using cassava, guinea corn and tiger nut starch as substrates, respectively while that for GluAgCSV11 were 7.0, 5.0 and 6.0 using cassava, guinea corn and tiger nut starch as substrates (Figures 4 and 5). This suggests that at optimum pH range of 5.0-7.5, cassava, guinea corn and tiger nut starch hydrolysis by GluAgCSV4 and GluAgCSV11 could be very much enhanced. Parbat and Singhal, (2011) reported optimum pH of 5.0 for glucoamylase from *Aspergillus oryzae* using agro industrial products. Similarly, Puri et al. (2013) reported optimum pH of 5.0 for glucoamylase from *A. oryzae*. Rangabhashiyam et al. (2012) reported optimum pH of 5.2 for glucoamylase obtained from *A. niger*. Kumar et al. (2013) reported optimum pH 6.0 for glucoamylase from *A. oryzae* using wheat bran as carbon source. Koc and Metin (2010) also reported optimum pH of 6.0 for glucoamylase produced by *Aspergillus flavus*. Zambare (2010) reported optimum pH of 6.0 for glucoamylase from *A. oryzae*. The result of this study has similarity with that of aforementioned authors. The disparity could be as a result of difference substrates used for the enzyme assay. The optimum pH range suggests that, glucoamylases obtained in this study have

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**Figure 4.** Glucoamylase activity and α-amylase activity in liquid broth using amylpectin from cassava starch as the only carbon source.

**Figure 5.** Protein concentration in liquid broth using amylpectin from cassava starch as the only carbon source.

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**Table 2. Optimization table.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Starch source</th>
<th>optimum pH</th>
<th>Optimum Temperature (°C)</th>
<th>K_M (mg/ml)</th>
<th>Vmax (µmol/min)</th>
<th>Activator</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluAgCSV4</td>
<td>CSV</td>
<td>7.5</td>
<td>55</td>
<td>90.06</td>
<td>188.67</td>
<td>Ca^{2+}, Zn^{2+}, Fe^{2+}, Pb^{2+}, Mn^{2+}, Co^{2+},</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>7.0</td>
<td></td>
<td>173.7</td>
<td>434.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TN</td>
<td>6.0</td>
<td></td>
<td>28.57</td>
<td>227.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GluAgCSV11</td>
<td>CSV</td>
<td>7.0</td>
<td>50</td>
<td>271.30</td>
<td>1000.00</td>
<td>Ca^{2+}, Co^{2+}, Pb^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>5.0</td>
<td></td>
<td>309.3</td>
<td>1000.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TN</td>
<td>6.0</td>
<td></td>
<td>262.50</td>
<td>1000.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.** Effect of pH on glucoamylase activity obtained from *Aspergillus niger* on the 4th day of fermentation using amylopectin from cassava starch as carbon source (GluAgCSV4).

**Figure 5.** Effect of pH on glucoamylase activity obtained from *Aspergillus niger* on the 11th day of fermentation using amylopectin from cassava starch as carbon source (GluAgCSV11).

Wide pH stability which is of industrial importance.

Most of amylases are known to be metal ion-dependent enzymes (Deb et al., 2013). The effect of metal ion on glucoamylase activity showed that, GluAgCSV4 activity was enhanced by Ca^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+} while Co^{2+} had inhibitory effect on the enzyme but Pb^{2+} inactivated the enzyme (Figure 6). Also, GluAgCSV11 activity was enhanced by Ca^{2+} and Co^{2+} however, Zn^{2+}, Fe^{2+}, Mn^{2+}
and Pb$^{2+}$ completely inactivated the enzyme (Figure 7). The optimum pH range obtained suggest that is more likely to readily form nucleophilic hydroxide ions with metals like Ca$^{2+}$, Co$^{2+}$, Fe$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$ by activating a bound water molecule, this stabilizes the negative charges that are formed at the active sites allowing more weak interactions to hold the substrates in proper orientation at the active site of the enzyme thereby enhancing the activity. Also, it has been suggested that metal ions probably force amylases to adopt a compact structure, by salting out the hydrophobic residues of the enzyme, thereby inducing resistance to extreme pH and temperatures (Kareem et al., 2014). Since glucoamylases are used together with α-amylase, which requires Ca$^{2+}$ ions in the liquefaction process, stimulation of glucoamylase activity by Ca$^{2+}$, Co$^{2+}$, Fe$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$ ions can make it more suitable for use in industrial starch bioconversion process. The inhibition on glucoamylase activity inhibition may be due to the complexes formed between metals and the enzyme which prevent it from binding to the substrate and form the product (Aziz and Ali, 2012). Koc and Metin, (2010) reported that Mn$^{2+}$, Ca$^{2+}$ and Co$^{2+}$ activated glucoamylase obtained from A. flavus while Fe$^{3+}$, and Zn$^{2+}$ inhibited the enzyme activity. Jambhulkar (2012) reported that glucoamylase activity from Rhizopus nigricans was enhanced by Mn$^{2+}$ while supplementation of K$^+$, Fe$^{2+}$, Zn$^{2+}$, Cu$^{2+}$ and Ca$^{2+}$ significantly reduced the glucoamylase activity. Kareem et al. (2014) also, reported that Mn$^{2+}$, Ca$^{2+}$, and Fe$^{2+}$ increased activity for glucoamylase obtained from...
**Rhizopus oligosporus** SK5 mutant. Bagheri et al. (2014) reported that glucoamylase activity was strongly inhibited by Fe$^{2+}$ but Ca$^{2+}$, Zn$^{2+}$ and Mg$^{2+}$ showed no significant effect on glucoamylase activity.

The optimum temperature for GluAgCSV4 was found to be 55°C while that for GluAgCSV11 was 50°C (Figure 8). Increase in temperature from 35 to 50°C and for the respective enzymes was accompanied by increase in glucoamylase activity beyond which the glucoamylase activity decreased rapidly making 50°C and 50°C the optimal temperatures for both GluAgCSV4 and GluAgCSV11, respectively. These results suggest that GluAgCSV4 and GluAgCSV11 will perform best at 50°C and 55°C in biotechnological applications. The increase in glucoamylase activity as temperature increases may be as a result of change in the enzyme conformation which brings the essential residues to close proximity for catalysis.

The decrease in the activity could be as a result of thermal denaturation at high temperature. Jebor et al. (2014) reported optimum temperature of 40°C for glucoamylase from *A. niger*. Sarojini et al. (2012) also used *A. niger* and reported optimum temperature of 45°C. Deshmukh et al. (2011) reported optimum incubation temperature of 40°C for glucoamylase obtained from sorghum. Cereia et al. (2000) reported temperature optimum of 60°C for glucoamylase obtained from *Scytalidium thermophilum*. El-Gendy (2012) reported optimum temperature range of 50 to 60°C for glucoamylase obtained from marine endophytic *Aspergillus* sp. JAN-25 under optimized solid-state fermentation conditions on agro residues. Koc and Metin (2010) also reported optimum temperature of 60°C for glucoamylase obtained from *A. flavus*. Norouzian et al. (2006) reported optimum temperature of 70°C for glucoamylase obtained from *A. niger*.

The Michaelis constant (Km) and maximum velocity (Vmax) obtained from the Lineweaver-Burk plot of initial velocity data at different substrate concentrations were 90.06 mg/ml; 188.67 µmol/min, 173.7 mg/ml; 434.78 µmol/min, and 28.57 mg/ml; 227.27 µmol/min for GluAgCSV4 using cassava, guinea corn and tiger nut starch as substrates while the Michaelis constant for GluAgCSV11 were 271.30, 309.30 and 262.50 mg/ml using cassava, guinea corn and tiger nut starch as substrates with the same maximum velocity of 1000 µmol/min (Table 2). This suggests that GluAgCSV11 could be of better industrial use since it has very high affinity for the 3 different substrates than GluAgCSV4 and as such has the chance of becoming the better amylase for industrial application than GluAgCSV4 (Figure 9 and 10). Jebor et al. (2014) worked with glucoamylase from *A. niger* and reported K$_{M}$ and V$_{max}$ of 2.8 mM, 9.8 mM/min, respectively. The results of this study show that glucoamylases obtained from this research have high affinity for their respective substrates, especially starch obtained from underutilized plant (tiger nut).

**Conclusion**

The results of the study suggest that the glucoamylases obtained from *Aspergillus niger* using amylopectin as carbon source possess the properties to solve biotechnological applications. Also, GluAgCSV11 could be of better industrial use since it has very high affinity for the 3 different substrates than GluAgCSV4 and as such has the chance of becoming the best amylase for local starch fermentation industry than GluAgCSV4.

**Conflict of interests**

The authors did not declare any conflict of interest.
Figure 10. LineWeaver-Burk plot for (GluAgCSV11) using Cassava, guinea corn starch and tiger nut starch as substrate.

REFERENCES


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