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Full Length Research Paper

Yield coefficient for the growth of *Pseudomonas* sp. AQ5-04 at various concentrations of phenol

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Phenol is widely used by many industries in Malaysia, and is one of the highly toxic environmental pollutants with levels exceeding the regulatory standard that have been reported in Malaysia. A phenol-degrading bacterium; *Pseudomonas* sp. AQ5-04, a metabolically versatile xenobiotic-degrading bacterium, has been isolated from a local contaminated site. The bacterium was able to withstand a high concentration of phenol. This work aims to determine the effect of concentration and to establish the yield factor on phenol as a carbon source. It was established that the isolate can degrade up to 900 mg/L phenol within 72 h; it was discovered that the yield factor ($Y_{X/S}$) varied between 0.3 to 0.6 mg cells per mg phenol for different initial phenol concentrations with increasing phenol concentrations resulting in a lower yield. This shows that growth on phenol is strongly inhibitory and resulted in low yield at an inhibitory concentration of phenol.

Key words: *Pseudomonas* sp. AQ5-04, yield factor, phenol, inhibitory concentration.

INTRODUCTION

Phenols and phenolic compounds are injurious to organisms even at low concentrations with many of them categorized as dangerous pollutants due to their toxicity towards human health for various reasons Some of the phenolic compounds include chlorophenols, nitrophenols, methyl phenols, alkylphenols, aminophenols, butylhydroxytoluene, nonylphenol and Bisphenols A (Maiti and Mannan, 1999; Hirooka et al., 2003). In Malaysia, the 2014 Environmental Quality Report showed that nearly all groundwater monitoring stations had phenol concentrations exceeding the National Guidelines for Drinking Water Quality Standard (0.002 mg/L). Phenol and phenolic compounds continue to be the top scheduled wastes generated in Malaysia as the demand for phenol by the industries are increasing annually (Gami et al., 2014a).

Phenols and phenolic mixes are damaging to living things even at low amount with huge numbers of them

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classified as poisons because of their poisonous quality towards human wellbeing (Das and Maiti, 2007; Zulkharnain et al., 2013; Ghazali and Johari, 2015). Part of the phenolic substances are nitrophenols, chlorophenols, methyl phenols, aminophenols, alkylphenols, nonylphenol, butylhydroxytoluene and Bisphenols A (Anuar, 2013; Ghazali and Johari, 2015). In Malaysia, the National Guidelines for Drinking Water Quality Standard is 0.002 mg/L and the 2014 Environmental Quality Report demonstrated that nearly all of the groundwater observing stations had exceeded this level for phenol. This is due to phenol and phenolic substances are the number one scheduled waste product in Malaysia as their usage in the industrial sector is one of the highest (Ahmad et al., 2011a, 2012, 2017; Arif et al., 2013; Department of Environmental, 2014; Gami et al., 2014b; AbdEl-Mongy et al., 2015; Norazah et al., 2015; Sabullah et al., 2017).

Physicochemical methods for phenol removal are abounded and include photodecomposition (Akbal and Onar, 2003), chemical polymerization (Kulkarni and Kaware, 2013), ion exchange (Alkaram et al., 2009), electrocoagulation (Abdelwahab et al., 2009) and advanced oxidation (Liotta et al., 2009). Having said that, a natural or biological approach continues to be considered as the preferable means of taking care of phenol pollution. Organic methods are powerful at small concentrations of phenol, is value effective and do not create secondary pollutants compared to some physicochemical methods (Hank et al., 2010; Kulkarni and Kaware, 2013; Agarry et al., 2008; Kulkarni and Kaware, 2013).

The ability of several microorganisms to utilise phenol and other phenolic substances are considered as a tool for the removal of noxious waste material (Agarry et al., 2008). In reality, the endeavour of utilizing microorganisms to eliminate phenol and other organic contaminants has been escalating in recent years. There are a considerable variety of microorganisms that coexist in almost all-natural surroundings which includes water and soil. These microorganisms can break down organic substances by generating intracellular or extracellular xenobiotics-degrading enzymes (Agarry et al., 2008; Luo et al., 2009, 2012; Nordin et al., 2013; Zulkharnain et al., 2013).

Phenol biodegradation is affected by various factors such as temperature, pH, nitrogen source and salinity (Ahmad et al., 2011b). In this work, a phenol-degrading bacterium has been isolated from a waste site in the Langat River Basin where intensive studies for many years have shown the severity of the contamination of this area by industrial activities near and surrounding the basin (Yusof et al., 2002; Taweel et al., 2013). In general, there is limited information on the growth yield of bacterial cells on phenol, of which this paper is attempting to address. This value will be very useful in predicting the growth on phenol during bioremediation works.

MATERIALS AND METHODS

Growth and maintenance of phenol-degrading bacteria

The phenol-degrading bacterium AQ5-04 was isolated in 2014 in Selangor and was identified as *Pseudomonas* sp. AQ5-04 with an accession number KT693288.1. A minimal salt medium (MSM) containing (g/L) of NaCl (0.1), $MgSO_4$ (0.1), Fe_2 (SO4).H_2O (0.01), K_2HPO_4 (0.4), KH_2PO_4 (0.2), $MnSO_4.H_2O$ (0.01), $(NH_4)_2SO_4$ (0.4), $NaMoO_4.2H_2O$ (0.01) was utilized in this work. The MSM was supplemented with 500 mg/L of phenol as a carbon source (Ahmad et al., 2012). The best isolate was utilized in this work, its optimization and characterization works (Aisami, 2017). The phenol-degrading activity was determined using the 4-aminoantipyrine colourimetric assay at 510 nm (Ahmad et al., 2012). Bacterial growth was measured using colony forming-units (CFU/ml) and dry cellular weight. Biomass concentration as a function of optical density was estimated from the liquid medium by centrifuging the bacterial cells culture at 10,000 g for 15 min. After centrifugation, residual phenol was measured in the supernatant. The cellular pellet was resuspended in 1 mL of fresh culture medium and the optical density measured at A 600 nm using a UV/visible spectrophotometer (UV 1240 Mini Shimadzu Japan) with the culture medium as the blank. Biomass of the bacterium as dry weight of washed cells was determined by vacuuming filtered culture filtrates onto a pre-weighed Whatman filter disc (0.2 cm, 4.7 cm). The filter was then dried in a vacuum oven at 80°C until a constant weight was achieved and subtraction of this weight to the initial mass of the filter represents cell dry weight. A calibration curve was constructed for the cellular biomass concentration against this wavelength. Samples exceeding an absorbance value of 1.0 were diluted before measured. A linear relationship between OD_{600} nm and cell dry weight of up to 750 mg/L (R^2 of curve was 0.97) was calculated.

Effect of various phenol concentrations on the bacterial growth and phenol removal

Phenol concentration of between 400 and 1200 mg/L was used to determine the effect on the bacterial growth and phenol degradation.

Growth kinetics

In a batch system, the biomass growth rate is typically defined as follows:

$$\left(\frac{dX}{dt}\right) = \mu_g X - k_d X \quad (1)$$

Where the growth rate of biomass (h^{-1}) is represented by μ_g, X is the cellular biomass concentration (mg/l), and the endogenous decay coefficient (h−1) is represented by k_d. The substrate utilisation rate is defined as follows;

$$\left(\frac{dS}{dt}\right) = -\left[\frac{1}{Y_x}\left(\frac{dX}{dt}\right) + mX\right] = -\mu_s X \quad (2)$$

S is defined as the rate-limiting substrate concentration at time t(mg/l), the specific substrate consumption rate (h^{-1}) is μ_s the observed yield coefficient, which is defined as the ratio of biomass formed to the mass of substrate utilized (mg/mg) is represented as

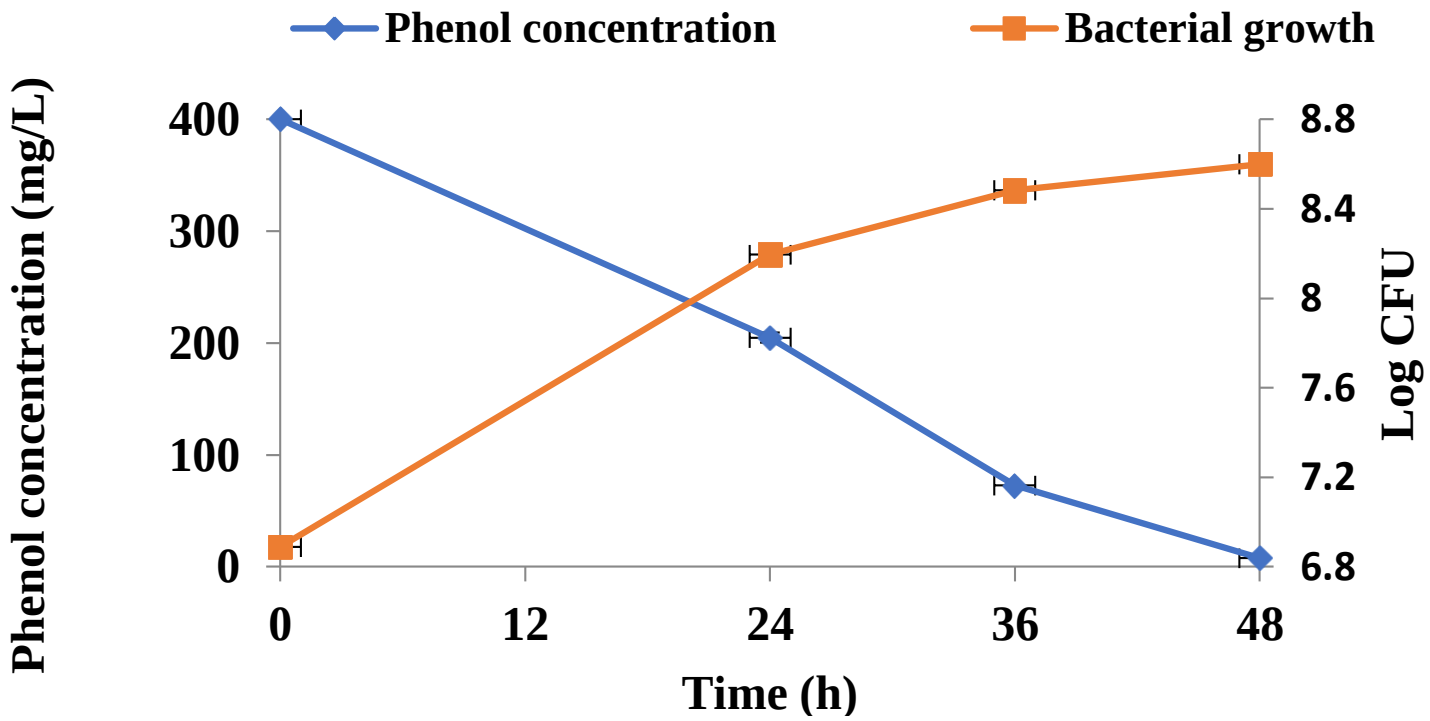

Figure 1. Effect of phenol concentration (400 mg/L) on bacterial growth and phenol degradation.

Y_X, and m is maintenance energy coefficient (h^{-1}) or the percentage of the energy sources utilized by the bacterial cells for needs other than growth. The endogenous decay coefficient at the exponential growth phase can in many cases be neglected.

Hence, Equation 2, upon integrating with the boundary condition ($X = X_0$ at $t = 0$), is reduced to;

$$\ln \frac{X}{X_0} = -\mu_g t \quad (3)$$

Yield coefficient on phenol

The plot of (X–X_0) versus (S_0–S) at the exponential growth region of the bacterial growth curve at any particular value of an initial concentration of substrate gives a straight line. The value of the observed yield coefficient or Y_{xs} can be obtained from the slope of this line. The formula for the observed yield coefficient is as follows

$$(Y_{xs})_{obs} = \frac{(X - X_0)}{(S_{l0} - S_l)} \quad (4)$$

RESULTS AND DISCUSSION

As shown in Figure 1, *Pseudomonas* sp. AQ5-02 can tolerate 400 mg/L phenol and up to 50% (200 mg/L) was removed within the first 24 h and there is an increase in the bacterial growth and almost 100% was removed within 48 h. But at 800 mg/L, it took the isolate 60 h for complete degradation (Figure 2) while for 1000 mg/L, almost 90% was removed after 60 h and no increase in the percentage removal after 72 h (Figure 3). At the concentration of 1200 mg/L, the bacterial growth increases after 24 h but no further significant increase in the growth at 48, 60 and 72 h. This indicates that at concentration, the phenol became very toxic to the bacterial leading to the inhibition of the growth and also phenol degradation as shown in Figure 4. This result is in agreement with some previous studies that reported that there is decrease in phenol degradation at higher phenol concentrations (Mohanty and Jena, 2017). Also, as the concentration of phenol increases there is increased in degradation time and decreased in bacterial growth as reported by other authors (Bakhshi et al., 2011).

Yield coefficient ($Y_{X/S}$)

The apparent bacterial yield on phenol was determined through linear regression of the corresponding slope of the plot of cell dry weight concentration (mg/mL) vs. initial phenol concentration (mg/mL) during the exponential portion of the growth phase (published elsewhere).

As shown in Figure 5, the highest yield coefficient was 0.6 mg cells per mg phenol at 200 mg/L of initial phenol concentration. The yield was down to 0.3 mg cells per mg

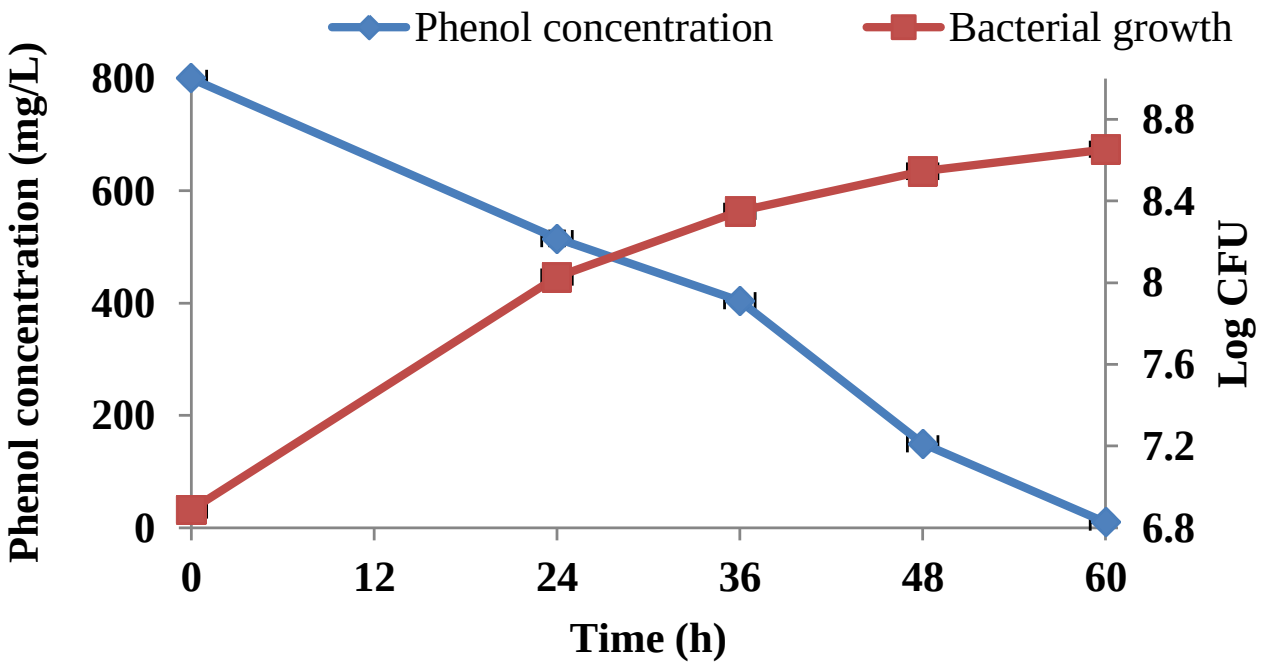

Figure 2. Effect of phenol concentration (800 mg/L) on bacterial growth and phenol degradation.

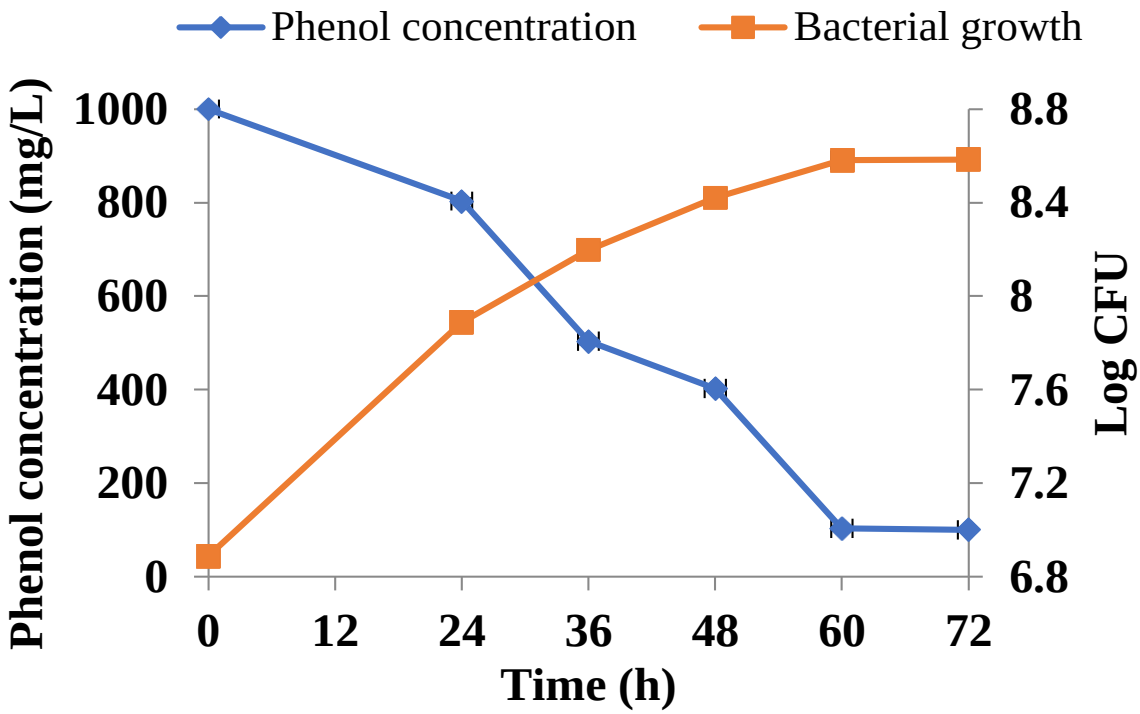

Figure 3. Effect of phenol concentration (1000 mg/L) on bacterial growth and phenol degradation.

phenol at 2000 mg/L of initial phenol concentration, indicating a trend of lower growth yield at inhibiting phenol concentrations. The value obtained in this work is quite far from the highest theoretical value of $Y_{X/S}$ for growth on phenol, which is 0.94 g/g (Reardon et al., 2000). This is not surprising since the conditions of

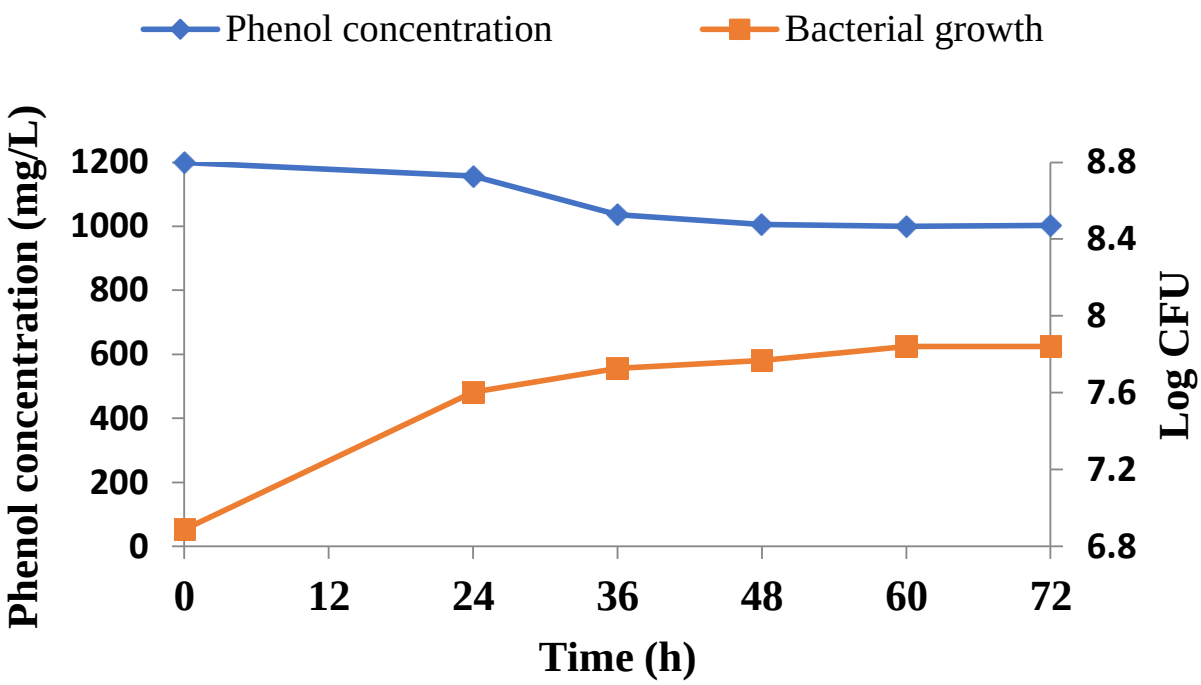

Figure 4. Effect of phenol concentration (1200 mg/L) on bacterial growth and phenol degradation.

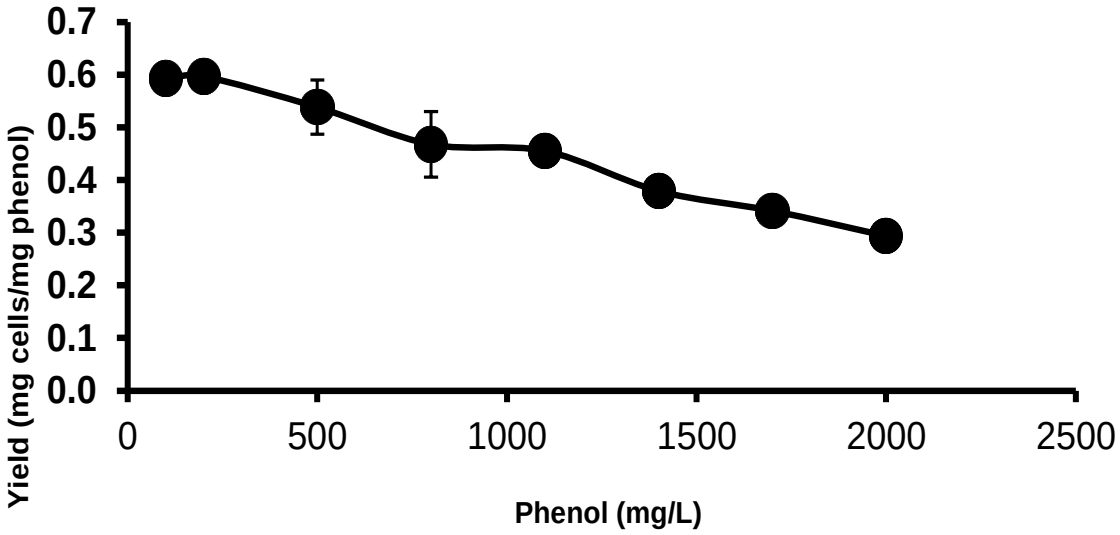

Figure 5. Variation in yield coefficient with initial phenol concentration.

growth are not as optimal as in chemostat conditions. Comparison of various growth yield date on phenol for several phenol-degrading bacteria available from the literature indicates that the yield reported on phenol varies dramatically with the lowest yield exhibited by *Pseudomonas putida* DSM 548 at 0.0017 mg cells/mg phenol while the highest yield is exhibited by *Bacillus cereus* at 0.88 mg cells/mg phenol. However, the yield is reflected by the tolerance of the phenol-degrading bacteria wherein *P. putida* DSM 548, the inhibition constant or K_i value is just 54.1 mg/L, a low value compared to the better phenol-tolerant bacterium *B. cereus* where the K_i value is 238.1 mg/L.

In general, the higher the concentration of phenol, the lower the yield as phenol is toxic and much of the energy from the substrate is needed to maintain cellular function to combat the toxic phenol (Onysko et al., 2000). Phenol can easily cross bacterial cell wall and membrane and

Table 1. Yield factors observed by some previous studies.

Bacterial strain	Phenol concentration range (mg/L)	$Y_{x/s}$ observed (mg cells/mg phenol)	Reference
Bacillus cereus	50-600	0.102-0.880	Zhang et al. (2013)
Pseudomonas putida MTCC 1194	10-1000	0.65	Kumar et al. (2005)
Peudomonas putida DSM 548	1-100	0.0017	Monteiro et al. (2000)
Alcaligenes faecalis	0-1800	-	Jiang et al. (2007)
Pseudomonas putida LY1	20-800	0.765 (for concentration 50 mg/L)	Wang and Loh (1999)
Pseudomanas putida (Tan1) *Staphylococcus aureus* (Tan2)	100-800	0.4258	Li et al. (2010)
Pseudomonas putida F1 strain ATCC 700007	10-200	0.44	Abuhamed et al. (2004)
Mixed cultures	25-1450	0.6	Senthilvelan et al. (2014)
Stenotrophomonas maltophilia strain KB2	25-500	0.65	Szczyrba et al. (2016)
P. putida (PTCC 1694)	300-1000	0.012-0.177,	Bakhshi et al. (2011)
Pseudomonas putida Q5	20-600	0.42-0.89 (estimated)	Onysko et al. (2000)
P. putida ATCC 17484	5-200	0.70	Hutchinson and Robinson (1988)
C. tropicalis PHB5	20-2400	0.185 -0.96	Basak et al. (2014)
Mixed culture	1000-1500	0.16 - 0.27	

can transform the presence of oxygen by various oxygenases to form toxic phenoxy radicals that attack various cellular structures and cause lipid peroxidation. Furthermore, the production of phenol-degrading metabolites including quinine methides and semiquinones can damage protein and DNA (Gami et al. 2014a). The yield coefficient is dependent on initial phenol concentration, with higher concentrations reducing yield significantly as was observed in many studies on phenol-degrading bacteria (Chi and Howell, 1976; Hutchinson and Robinson, 1988; Wang and Loh, 1999; Onysko et al*.,* 2000; Abuhamed et al*.,* 2004; Jiang et al*.,* 2007; Li et al*.,* 2010; Bakhshi et al*.,* 2011; Senthilvelan et al*.,* 2014). Hence, the average value of the yield coefficient is usually not reported. The trend in the yield of cellular biomass on phenol for many phenol-degrading microorganisms is a diminishing yield as the phenol concentrations were increased. Yields in many cases started to dramatically decline at concentrations of phenol of above 100 mg/L (Onysko et al*.,* 2000; Bakhshi et al*.* 2011; Wolski et al*.,* 2012; Zhang et al*.,* 2013; Basak et al*.* 2014) indicating the toxicity of phenol is causing cells to direct their resources for survival. Under toxic concentrations of phenol, it is anticipated that the maintenance energy will be quite high. Table 1 shows some established yield factors, the concentration range and also the name and strain of the bacteria.

Conclusion

Pseudomonas sp. strain AQ5-04, a phenol-degrading bacterium showed tolerace of 1000 mg/L phenol and growth yield inhibition in the presence of phenol. The yield obtained in this work is within the range observed on the growth on phenol by several other bacteria, although the yield obtained in this work is well below the theoretical value of yield on phenol, which is 0.94 g cells per g phenol. The inhibition of the growth yield at high concentration of phenol is anticipated but this bacterium showed some resilience in terms of growth yield data at very high concentration of phenol.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Analysis of genetic diversity in four Sudanese provenances of *Balanites aegyptiaca* (L.) Del. based on random amplified polymorphic DNA (RAPD) marker

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***Balanites aegyptiaca* (L.) Del. (Heglig) is a multipurpose tree with considerable potential. The wide range of environments under which it is growing suggests high pattern of variation among and within locations. The scrutiny was undertaken to assess genetic diversity among and within four geographical genotypes of Heglig growing naturally in four different regions of Sudan based on their seed performance using random amplified polymorphic DNA (RAPD) markers. The DNA was extracted from dried leaf materials and subjected to PCR using ten primers. A total of 57 bands were scored and 42 were polymorphic with polymorphism level ranging from 50 to 87.5% and a mean of 71.7%. Cluster analysis using NTSYS-PC software, showed three main clusters. The dissimilarity values ranged between 77 and 93%. Effective gene flow among the three populations of Obied, Damazin and Gedaref and limited gene flow with Genaina was observed. Populations of *B. aegyptiaca* from different provenances were confirmed to have significant genetic diversity. Results indicate that RAPD could be efficiently used for studying genetic variation of *B. aegyptiaca.* The study recommends that local provenances of *B. aegyptiaca* should be properly conserved and immediate efforts to be made to widen the genetic base through research and collection from other regions.**

Key words: *Balanites aegyptiaca*, random amplified polymorphic DNA (RAPD), marker, genetic variation, DNA, Sudan.

INTRODUCTION

Balanites aegyptiaca (L.) Del. (Zygophyllaceae), commonly known as desert date, is a multipurpose tree or spiny shrub with considerable potential. The plant is mainly distributed in the Middle East and Africa. Natural selection, genetic drift and gene flow collectively affect the genetic diversity of populations and result in promoting or hampering local and range-wide adaptation (Magri et al., 2006; Liepelt et al., 2009). Genetic diversity among and within individuals or populations can be determined using morphological traits and molecular markers. Molecular markers have proven to be valuable tools in the characterization and evaluation of genetic

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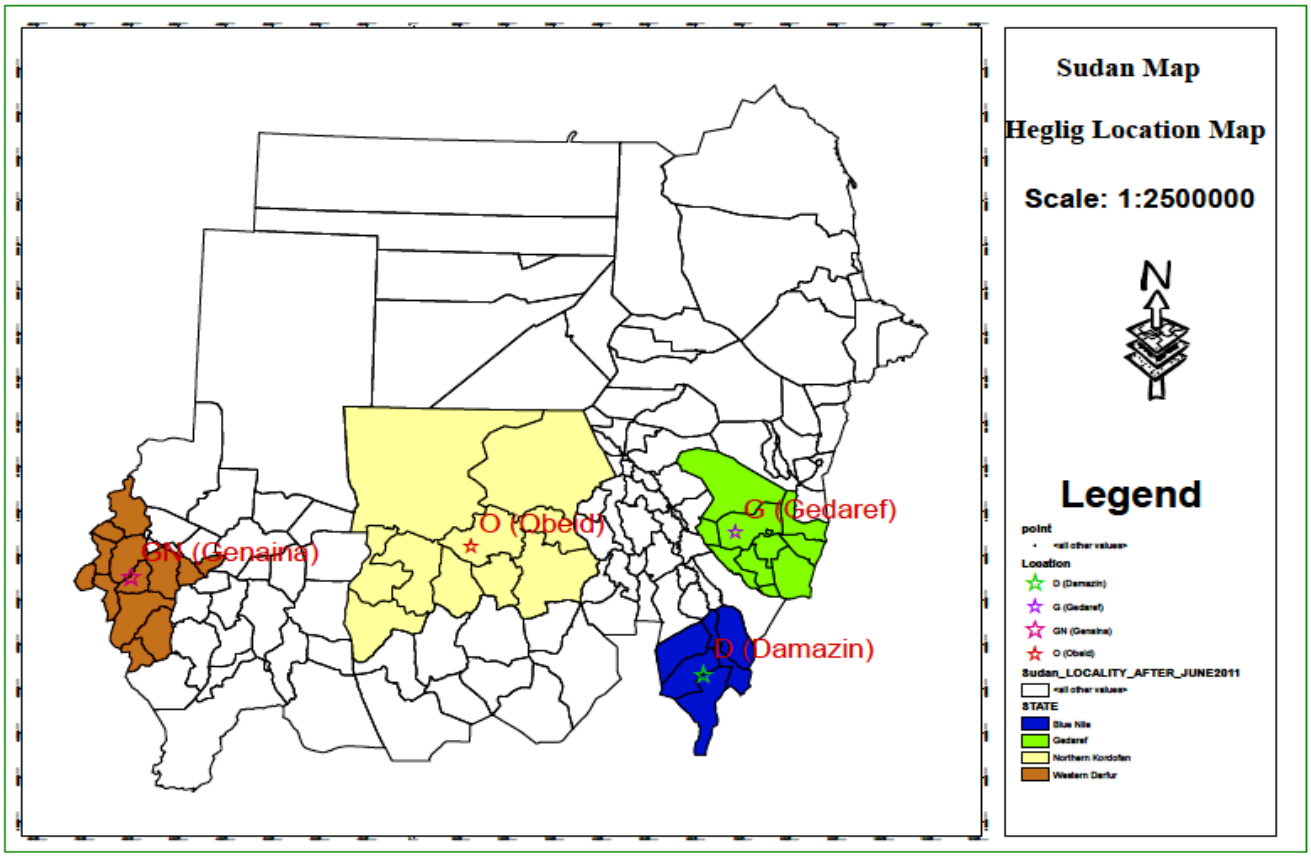

Figure 1. Location of the investigated *Balanites aegyptiaca* provenances collected from different regions of Sudan.

diversity within species (Nejia et al., 2007; Muchigi et al., 2008; Missihoun et al., 2015; Khamis et al., 2017). The preference of a molecular marker system should be conciliated between consistency and simplicity of analysis, low cost, statistical rule, and assurance of exposing polymorphisms.

Various types of molecular markers have been developed to investigate the genetic variation of plants (Idrees and Irshad, 2014) and forest trees in the past few decades (Mulcahy et al., 1993). Random amplified polymorphic DNA (RAPD) markers have been used extensively in analyzing genetic diversity (Garacia et al., 1998; Artyukova et al., 2004; Sureja et al., 2006; Guerra et al., 2010). It is a simple, reliable, speed and relatively low cost, straight forward technique to apply and the number of loci that can be examined is unlimited. RAPD markers have generally been used for the detection of genetic variation within and among populations in several plant species without the need for detailed knowledge of DNA (Holsinger et al., 2002; Wei et al., 2008; Zarek, 2009). Recently RAPD analysis of PCR- amplified DNA regions have been used to detect DNA variations for taxonomic purposes, particularly at the intra- and inter-specific levels, the genotype stability of species and others (Abkenar et al., 2004; Liesebach and Gotz, 2008; Ibrahim et al., 2010; Rahman and Al Munsur, 2009; Agbidinoukoun et al., 2017).

The wide range of environmental conditions under which *B. aegyptiaca* is growing suggests high pattern of variation among and within locations (Elfeel, 2010) and was reflected in a very wide inconsistency in its morphology and products (Abasse et al., 2011). At present, very little is known about genetic variation in this species and base line information are needed to initiate conservation strategies. The aim of this study was to determine the current state of genetic diversity and to assess the level of genetic variation based on molecular variation within and between populations of *B. aegyptiaca* growing naturally in different geographical regions by using RAPD markers.

MATERIALS AND METHODS

Plant materials

Four different provenances within the natural habitats of *B. aegyptiaca* were selected, namely, Genaina (GN), Obied (O), Damazin (D) and Gedarif (G) (Figure 1 and Table 1). Ten trees (families) within (D) and (G) and eight trees within (O) and (GN) provenances were marked at least 150 m a part. Seeds from each

Table 1. Provenance data for *Balanites aegyptiaca* seed sources used in this study.

Location	Lat. N	Long. E	Soil type	Mean annual rainfall (mm)
GN	13° 27'	22° 27'	Sand	500-600
O	13° 16'	30° 27'	Sand	300-400
D	11° 81'	33° 86'	Clay	800-1000
G	14° 03'	35° 40'	Clay	600-700

Table 2. The sequence of the ten RAPD primers that showed amplification.

No.	Name	Sequence
1	A00	5´-ATC AGC GCA CCA-3´
2	A06	5′- ACT GGC CGA GGG-3′
3	OPA-13	5′- CAGCACCCAC-3´
4	OPB-07	5′- GGTGACGCAG-3´
5	OPC-11	5′- AAAGCTGCGG-3´
6	OPO-02	5′- ACACACGCTG-3´
7	OPO-07	5′- CAGCACTGAC-3´
8	OPU-03	5´-CTA TGC CGA C-3´
9	OPU-16	5´-CTG CGC TGG A-3´
10	OPZ-04	5′- AGGCTGTGCT-3´

family were collected separately and planted in the nursery. Fresh leaves from each tree per provenance were collected, placed in plastic and sent to Biotechnology and Biosafety Research Center of Agricultural Research Corporation (ARC) of Sudan, where they were washed with distilled water and dried.

DNA extraction and DNA quality determination

DNA extraction was carried out according to Ferreira and Grattapaglia's (1998) protocol modified for *M. brauna* because highest DNA concentration and good quality were obtained. The dried leaf tissue were placed in 2 µL microtube milled using a mortar and pestle. Genomic DNA was isolated using a cationic hexadecyltrimethylammonium bromide (CTAB) extraction buffer, 700 µL extraction buffer was added to each samples and samples were incubated at 65°C for 30 min, with occasional shaking. Chloroform/isoamylalcohol (24:1, vol:vol, 700 µL) was added to each microtube after cooling for 5 min. to remove proteins then shaken vigorously and left to stand for 5 min. at room temperature. Then the solution was centrifuged at 13,000 rpm for 7 min. The supernatant was pipetted into a new microtube, and two-third the volume of 95% ice-cold isopropanol was added and allowed to precipitate at 20°C for 12 h. Pellet DNA was obtained by centrifugation at 13,000 rpm for 10 min. by pouring off the top aqueous solution, leaving the precipitated DNA at the bottom of the tube. The pellet was washed first with 1 mL cold 70% ethanol, then cold 90% ethanol air dried. The DNA pellet was then resuspended in 50 µL 1xTE buffer (50 µL).

DNA quality and concentration were evaluated on a 0.8% agarose gel. The DNA samples were kept in (-20°C) until used.

RAPD genotyping

Twenty six random RAPD primers were tested for initial screening; among them only 10 primers successfully generated clear and reliable amplification products (Table 2). The ten primers which showed reproducible amplicons compromise eight Operon Technology, (USA) from groups A, B, C, O and Z and 2 A primer group intra- and interspecific variation were used for further analysis of all individuals.

Amplification reaction optimization was performed. The total volume of each reaction was 20 µL containing 1 µL genomic DNA, 4 µL master mix ready to load containing 12.5 $MgCl_2$ (Solis Biodyne) which contains a component of buffer, 1 U Taq polymerase, and 0.2 mM dNTPs, 0.5 µL RAPD primer, and the final volume was adjusted to 20 µL using double distill water.

Amplification conditions were adjusted according to the methodology proposed by Williams et al. (1990). The thermocycler (Biometra) was programmed for one initial denaturation step of 5 min at 95°C and denaturation in 94°C for 1 min primer annealing temperature for 1 min was done based on primer group and 2 min at 72°C for extension. The annealing step up to extension step was repeated for 40 cycles and final step for 5 min at 72°C for the final extension. The resulting DNA fragments from the amplification were separated by electrophoresis in 2% agarose gel for 90 min, and then transferred to solution containing 0.2 µg/mL ethidium bromide for staining for 30 min. The gels were visualized on transilluminator (Viberlourmat, Torcy company- France) in UV light and photographed.

Statistical analysis

The DNA amplification pattern obtained after amplification of the 36 genotypes was scored on the basis of presence (1) or absence (0) of amplicons on agarose gels. Clear, unambiguous and reproducible bands recovered through different techniques were considered for scoring. Each band was considered a single locus. Band size was estimated by comparing with 100 bp ladder (Solis Biodyne) using Gel Works 1D advanced gel documentation system

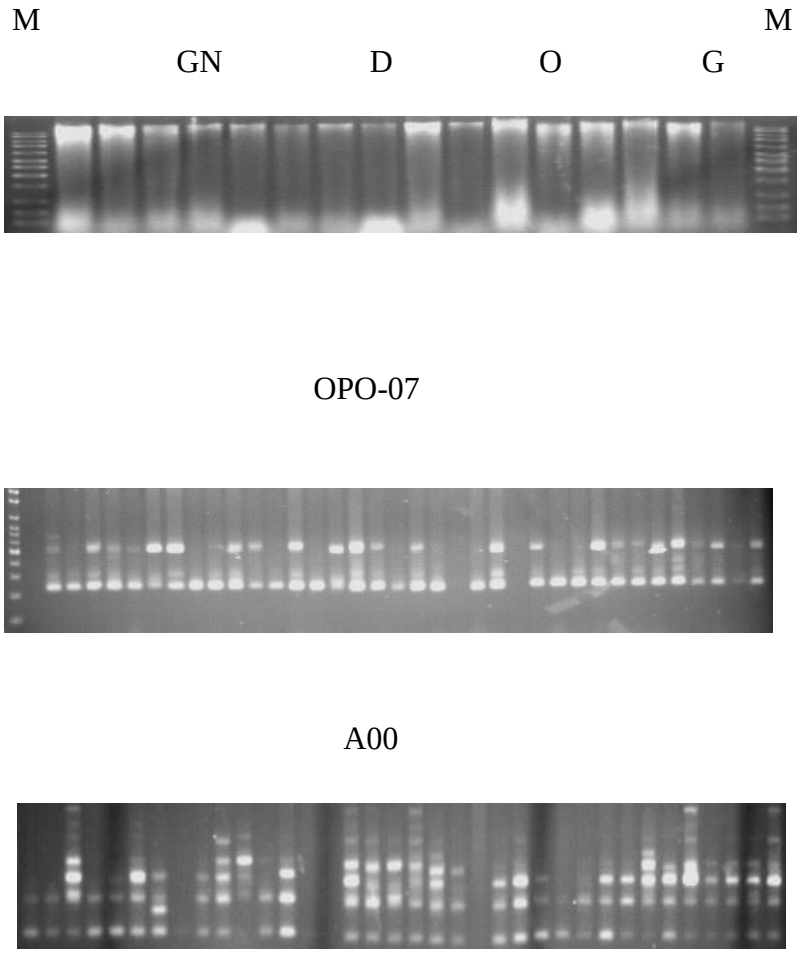

Figure 2. RAPD profiles of *B. aegyptiaca* collected from natural populations growing in different regions of Sudan (Representative DNA samples).

(BioCapt MW). The data was analyzed using NTSYS pc21 (version 2.11S). Dissimilarity coefficients were used to construct a dendrogram using un-weighted pair group method with arithmetic average (UPGMA) and sequential hierarchical and nested clustering (SHAN) routine.

RESULTS AND DISCUSSION

Genomic DNA extraction protocol success and genetic polymorphism

Over exploitation of forests for commercial purposes and other developmental activities have resulted in serious threat to tree species. Therefore, genetic diversity analysis is essential for both the long-term stability and short-term productivity of trees as diversity provides clues to the factors that direct the variation, inbreeding and gene flow.

The present investigation was undertaken to assess genetic diversity in Heglig (*B. aegyptiaca*) by using RAPD markers. In this study, the Ferreira and Grattapaglia (1998) protocol modified for *M. brauna* was used for the DNA extraction and significant amount with good quality was obtained. This result suggests that this method could be used successfully in genetic diversity studies in Heglig using CTAB extraction buffer to ensure both quantity and purity. Similar results were obtained by other authors (Chiari et al., 2009; Ginwal and Mawrya, 2009).

Figure 2 shows a representative DNA samples. The DNA amplification and polymorphism generated among various genotypes of *B. aegyptiaca* using random primers are presented in Table 3. The ten primers generated a total of 57 DNA banding patterns with an average of 5.7 bands per primer. OPC-11 and OBA-13 gave the highest number of bands (8), whereas OPZ-04 showed the fewest number (2). There were 42 polymorphic bands. The polymorphism level ranged from 50 to 87.5% with a mean of 71%. The high mean level suggests a high level of genetic diversity among populations. The same was observed in several other woody species for example those shown by Shah et al. (2008), Park et al. (2012) and Khamis et al. (2017).

Table 3. RAPD primers used and their total number of bands, total number of polymorphic bands and polymorphic band percentage.

Primer code	Total number of bands	Total number of polymorphic bands	Polymorphic band %
A00	7	4	57
A06	5	4	80
OPA-13	8	6	75
OPB-07	7	6	85.7
OPC-11	8	7	87.5
OPO-02	6	5	83.3
OPO-07	4	3	75
OPU-03	2	1	50
OPU-16	4	3	75
OPZ-04	6	3	50
Average	**5.7**	**4.2**	**71.7**

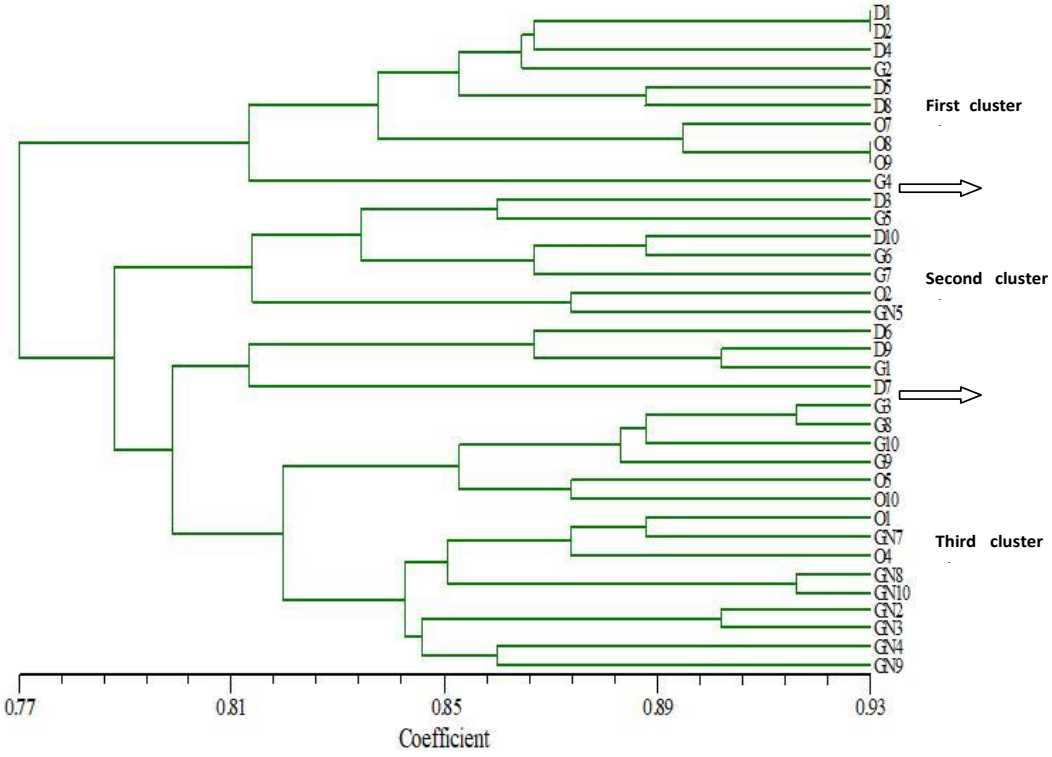

Figure 3. Cluster analysis derived from RAPD data to estimate the genetic dissimilarity for four populations of *Balanites aegyptiaca* growing in different regions of Sudan.

Genetic relationship and cluster analysis

RAPD analysis provides information that could help define the distinctiveness of species at a molecular level. Clustering components placed the 36 individuals from four provenances of *B. aegyptiaca* into three main clusters, using the software NTSYS-PC. The allelic diversity data were used for cluster analysis. 'Cluster Tree Analysis' sub-programme of the same software, revealed the genetic linkage and proximity among all the genotypes investigated. RAPD markers were used to produce a similarity matrix among individual species.

The dendrogram was constructed based on 57 fragments. Dissimilarity value varied between 77 and 93% (Figure 3). These wide ranges of genetic dissimilarity may emphasize the relatively wider diversity in gene pool of *B. aegyptiaca*.

A relatively high genetic diversity was obtained by Erfani et al. (2016) who tested the genetic variability among four species of Crataegus using morphological

traits and RAPD markers.

The first cluster contained five families from Damazin (D) provenance which was equal to 50% of the total number of trees, three families from Obied (O) provenance that equal to 37.5% and two families from Gedaref (G) that equal to 20%. In this cluster, genotypes D1 and D2 and O8 and O9 were genetically most variable with genetic dissimilarity value of 0.93. The second 50% of D provenance was included in the second main cluster in addition to 30% from G and 12.5% for both O and GN. Seven families out of eight of GN provenance (87.5%) were observed at the third cluster with 50 and 40% of O and G, respectively. The distribution of O, D and G genotypes was clear. This may be due to effective gene flow among the three populations (marketing and movements) and limited gene flow with GN or through foraging. Arif et al*.* (2009) obtained 0.56 to 0.93 similarity coefficient value in tree *Dalbergia sissoo*. Genetic variations among sources may have been achieved by out crossing and also by the spread of the seeds through various geographical areas (Khamis et al., 2017). The clustering results might indicate that the geographical separation is more important in the genetic diversity analysis.

The genetic diversity detected in the present study could be due to distance factor as the genotypes studied were widely distributed in different agro-climatic regions. Moreover, the heterozygous and heterogeneous structure of *Balanites* population driven by its out breeding behaviour might be one of the reasons for high degree of polymorphism variability (Shashidhara et al*.,* 2003).

Conclusions

The present study using RAPD displays some valuable information about Heglig diversity. In conclusion, RAPD markers are powerful tool for analyzing genetic relationships among *B. aegyptiaca* genotypes. Genetic diversity data obtained in this study can be further utilized in the conservation and development of improved varieties for further use in breeding programmes. Current study further concluded that the variability among and within sources indicates that seed movement between locations should be carefully examined so as to match planting site with seed source. Hitherto, this is the first report dealing with the pattern of molecular variation in those four provenances of *B. aegyptiaca* tree. As a result, the distribution of species is only related to its ability to disperse their pollen and/or seeds since no artificial acts are involved during the movement of wild seeds from one place to the other.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Genetic diversity of *Faidherbia albida* populations in the Sudano Sahelian region of Cameroon, using simple sequence repeat (SSR) markers

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***Faidherbia albida* (DEL.) A. Chev is widely grown in the northern region of Cameroon because of its many benefits to local communities. However, droughts and deforestation have led to decreases in the abundance of this species, increasing the need to identify improved genotypes for conservation, management, and breeding. The genetic diversity of *F. albida* in nine populations from the Sudano-Sahelian region of northern Cameroon was characterized using microsatellite (SSRs) markers. A total of 28 alleles were recorded across 8 loci and 255 samples. The effective mean number of alleles per locus was 2.3. Observed heterozygosity ranged from 0.24 to 0.30, while expected heterozygosities ranged from 0.22 to 0.26. For most loci, F_{IS} was negative. Higher variation was observed within than among the northern Cameroon populations, and principal component and admixture analyses did not reveal any population substructure. Phenotypic diversity in 3-month-old seedlings was also characterized and significant within population variation was found for most morphological traits. Although some populations differed significantly for one or more traits, in general the populations were phenotypically similar. These results suggest little barrier to gene flow between populations of *F. albida* in northern Cameroon, and that no single provenance is likely to be superior for selection and breeding purposes.**

Key words: Faidherbia albida, phenotypic, genotypic, variation, populations, breeding.

INTRODUCTION

The North and Far North regions of Cameroon are located in the Sahel, and are characterized by Sudano-Sahelian vegetation. This vegetation contains a variety of forest tree species, such as *Adansonia digitata* (Baobab), *Acacia senegal*, *Acacia nilotica*, *Faidherbia albida, Vitellaria paradoxa* (Shea butter), *Tamarindus indica* (Tamarind) and *Azadirachta indica* (Neem), with great multipurpose potential. Collectively, these trees are

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economically important sources of timber, building poles, and non-wood products such as gums and tannins, while in some cases also improve soil fertility through nitrogen fixation and organic matter production. However, the region is susceptible to droughts, degradation and desertification which has threatened, as well as caused loss of, species and genetic diversity. Additionally, reduction in land area available for cultivation due to population explosion and growing demand for forest products have resulted in the growth of trees under marginal conditions. The importance, demand and decline of these species calls for the breeding of improved and high yielding genotypes for future planting in forests, parks and farms.

Faidherbia albida (Del.) A. Chev is particularly important in this region because or its unique attribute of producing leaves and fruits in the dry season, when most other trees are leafless and dormant (World Agroforestry Center, 2009). *Faidherbia albida* also provides shelter for animals, whose dung is used as fertilizer for food crops (Boffa, 1999; Roupsard et al., 1999). Besides providing organic fertilizer and livestock fodder, *F. albida* acts as windbreak, supplies wood for fuel and construction, and reduces erosion by reducing soil compaction, which facilitates water absorption during the rainy season (Peltier, 1996). For all of these reasons, *F. albida* was selected as a priority species for domestication (Franzel, 1996) by the World Agroforestry Center (ICRAF) and by the FAO Panel of Experts on Forest Genetic Resources (FAO, 1974). To increase soil fertility in the Far-North region of Cameroon, in 1990 the Rural Development and Management Project (DPGT) lead by SODOCOTON encouraged the planting of *F. albida* on farm lands and the creation of parklands through natural and artificial regeneration (Gautier et al., 2002). Indeed, several reforestation projects in the region are currently ongoing. These projects would benefit from the availability of fast-growing genotypes resistant to biotic and abiotic stresses, but there has been little effort to identify improved genotypes of *F. albida* suitable for this region.

Forest tree genetic improvement programs typically begin with an assessment of the genetic diversity within the species of interest, to determine the amount of genetic and phenotypic variation available for selection (Muona, 1990; Wright, 1962; Franzel et al., 1996). Seed samples are collected across the entire natural range of the species, or within a geographic area of interest, with which seedling field trials are conducted, referred to as provenance tests (Zobel and Talbert, 1984). In the case of *F. albida,* genetic improvement studies started three decades ago with the morphological characterization of provenances from northwest, northcentral, east and south African populations, including field trials in Burkina Faso, Zimbabwe, Cameroon and Sudan (Sniezko and Stewart, 1989; Peltier and Eyog-Matig, 1988; Vandenbeldt, 1991, 1992; Abdelazim et al., 1997; Tchatchoua et al., 2019). These trials revealed two distinct ecotypes, one spanning the Sahel in North Africa, and a second typically found in riparian habitats in East and South Africa. Consistent with their habitats, provenances from the Sahel have small seeds, grow relatively slowly, and are drought tolerant, whereas provenances from East and South Africa have relatively large seeds, grow quickly, and are susceptible to drought (Vandenbeldt, 1991; Dangasuk et al., 2006). Analyses of isozyme variation support this distinction, and reveal that populations from northwest and northcentral Africa are genetically more diverse than populations from east and south Africa (Harris et al., 1997; Abdelazim et al., 1997; Joly et al., 1992).

Microsatellite (simple sequence repeat, SSR) markers have many advantages for analyses of genetic variation because of their high reproducibility, co-dominant inheritance, multi-allelic variation and abundance in the genome (Muchugi et al., 2008; Varshney et al., 2005). In addition, they can be evaluated relatively cheaply and at high throughput using automated platforms. In this study, the researchers took advantage of SSRs identified in *F. albida* by Russell et al. (2014) to characterize the genetic diversity within 9 provenances of *F. albida* from the Sudano-Sahelian region of Cameroon. The primary goals of this study were to determine if these markers could be used to differentiate populations and to identify populations with sufficient genetic variation to be useful for breeding programs.

MATERIALS AND METHODS

Germplasm collection

Seeds were collected indeterminately from well-spaced trees in nine populations in the distribution range of the species in the Sudano-Sahelian region of Cameroon (Table 1 and Figure 1). 50 to 100 fruit pods were collected from each of 20 trees in each population. The trees were at least 100 m apart to avoid sampling trees of the same genotype, and populations were separated by a distance of at least 100 km as proposed by Tchoundjeu et al. (1998). The seeds were extracted from the fruit by students of the National Advanced School of Engineering, University of Maroua during a practical session. Following inspection by the Plant Protection and Regulatory Services Directorate of Cameroon's Ministry of Agriculture and Rural Development (MINADER), the seeds were sent to the Schatz Center of Molecular Genetics USA (USDA import permit OMB No. 0579 - 0049) for DNA extraction, amplification and sequencing.

Phenotypic analysis

Seeds were nicked at the micropylar end using nail clippers, being careful to avoid damaging the radicle. They were germinated for two days on moist paper towels at 30°C, and viable seeds were then planted in a mixture of 1/3 bar sand and 2/3 Scott's Premium Topsoil in Stuewe TP38 pots. Four plants from each of 6 trees in each provenance were grown under fluorescent illumination in Conviron growth chambers on a diurnal cycle consisting of 12 h light (PAR 265 µm m^{-2} s^{-1}) at 30°C, and 12 h dark at 20°C. One-half teaspoon of Osmocote slow release fertilizer was added to each pot

Table 1. Populations of *Faidherbia albida* from northern Cameroon used in this study.

Population	Family code	Number of individuals	Geographical location
Adoumri	AD	24	09°16'N 13°50'E
Dinao	DIN	27	10°14'N 14°72'E
Kongola	KON	30	10°38'N 14°24'E
Mambang	MAM	30	10°65'N 14°28'E
Mokolo	MOKO	30	10°44'N 13°48'E
Moulvoudaye	MOU	30	10°23'N 14°50'E
Sirlawe	SIR	27	10°04'N 14°57'E
Tokombe	TOKO	30	10°52'N 14°09'E
Zamai	ZA	27	10°36'N 13°54'E

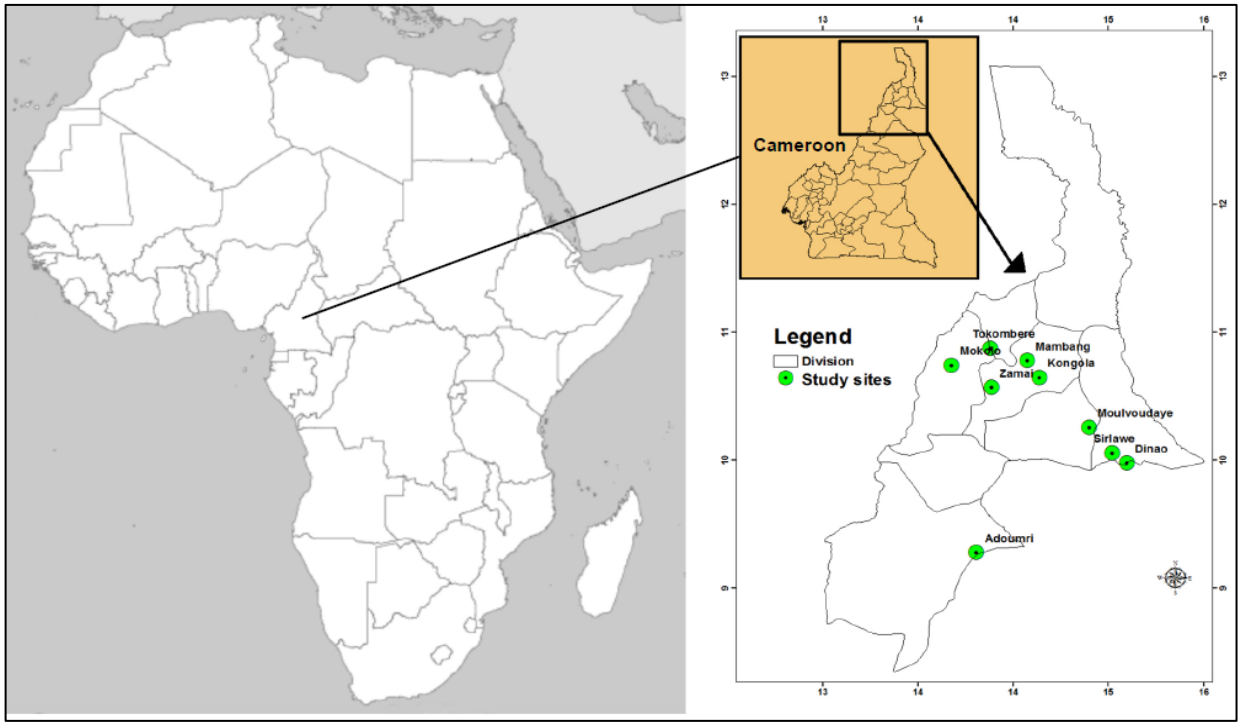

Figure 1. Maps of the geographical location of Cameroon and the study sites.

at planting and fertilized once a month with a dilute solution of 10:20:10 fertilizer. Plants were measured 3 months after planting with the help of a stereomicroscope. Plant height was measured from the soil surface to the tip of the last leaf. The number of all leaves larger than 5 mm was recorded at the same time. Stem width was measured in the third internode, using Vernier calipers. Fully expanded leaves near the tip of the shoot were attached to paper using double sided tape, scanned into a computer, and secondary leaflet area was then measured using Image J.

Growth of seeds and DNA extraction

A total of 255 seeds (that is, 3 seeds per family, 8 to 10 families per population and 9 populations) were nicked with a nail clipper and sown in polymix soil in 4 in plastic pots. Plants were grown in a growth chamber (25°C, 16 h. light: 8 h dark), and leaves were harvested for DNA extraction when plants were 4 weeks old. Approximately 100 mg of leaf tissue was macerated using a TissueLyser 11 (11.5 rmp for 1 minute), and genomic DNA was then extracted using DNeasy Plant Mini Kit (Qiagen), following the manufacturer's instructions. DNA concentrations were measured using a Qubit DNA kit (Qubit™ Assays) and a working DNA concentration of 1 ng/µl solution was prepared for all samples and stored in a -80°C freezer for further use.

Screening of SSR primers

Eight DNA samples, one from each of eight families were screened with 48 SSR primer pairs previously developed for *F. albida* (Russel et al., 2014). PCR was performed using 1 µl genomic DNA (1 ng/µl),

Table 2. Sequence of the PCR primers for the SSR markers used in this study.

Marker	Primer names	Primer sequences	Repeat motifs
5	comp19011_c0_seq1_120-F	CTTCGCAATAAGTTCTTCGT	AAG
	comp19011_c0_seq1_120-R	ATTCCTTGGTTGTGACTGAG	
8	comp15062_c0_seq1_145-F	CTACCACTGGTTCTCCTCAG	ACC
	comp15062_c0_seq1_145-R	TAGTTCGATACCAATCACCC	
11	comp20414_c0_seq1_450-F	TTGAGGTTCAAGCCAGTAGT	AGG
	comp20414_c0_seq1_450-R	TGTTTCTCAAATCTCCGTTT	
22	comp34406_c0_seq1_376-F	GATTTCATCGCAGGTAACTC	AAAG
	comp34406_c0_seq1_376-R	CTCTTATCATCGTCTCTGCC	
23	comp5175_c0_seq1_78-F	ACCCATATGAAACCCATACA	ACAT
	comp5175_c0_seq1_78-R	CTCGTGGAAATATCATTGGT	
24	comp5630_c0_seq1_260-F	CAAACTTCACACAACGTAGC	AAAT
	comp5630_c0_seq1_260-R	ATCCCAGACCCTAAAGGATA	
26	comp14514_c0_seq1_453-F	ACTCCAATTCTGGATCAGTG	AG
	comp14514_c0_seq1_453-R	GGGATTGATTCCTTCTAACA	
30	comp4810_c1_seq1_413-F	TCTTTTCCTCCTCATTCTCA	AAG
	comp4810_c1_seq1_413-R	ATGCAGATGGTTTAATCGAC	

2.5 µl each of 10 µM forward and reverse primers, 19 µl of Ultra-Pure water and 1 Edvo-tek bead. Samples were amplified in an Applied Biosystems thermocycler using the following conditions: 95°C for 15 mins, followed by 35 cycles of 94°C for 30 s, 55°C for 1.5 min and 72°C for 1 min, with a final extension at 72°C for 10 min. Eight primer pairs that produced clear, strong, polymorphic amplification products between 100 and 250 base pairs (bp) in length were chosen for further analyses (Table 2).

PCR amplification procedure

The eight selected markers were used to genotype 255 individuals from 85 families and nine populations. PCR was performed with a fluorescently labeled forward primer using the conditions described above, and the amplification products were then separated using an Applied Biosystems Illumina 3730 XL DNA sequencer at the Huck Genomic Core Facility of Pennsylvania State University, State College USA. Fragment analysis was performed with GeneMapper software at GS 500 (-200) LIZ standard size.

Data analysis

The phenotypic data were analyzed using SPSS v12 statistical package (IBM; https://www.ibm.com/products/spss-statistics) for data description and analysis of variance (ANOVA). All the measured traits assumed normal distribution and significance level for the ANOVA was at 0.05. Means comparison was conducted using the DUNCAN multiple range test (Duncan, 1955).

Population genetic analysis of the SSR marker results was performed within Excel using GenAlEx 6.5 (Peakall and Smouse, 2012). Summary statistics for allelic richness (the total number of alleles per locus and the effective number of alleles), unbiased observed and expected heterozygosities and the percentage of polymorphism in each population were calculated. The allelic richness (number of alleles), fixation index (F_{IS} = inbreeding) and geneflow information were calculated with F. Stat version 2.9 3.2 (February, 2002). GenePop (https://genepop.curtin.edu.au/) was used to conduct Hardy Weinberg Exact tests (HWE) and to estimate linkage disequilibrium at 0.05 Bonferroni corrections for pairwise comparison, genetic bottleneck, and Nei effective population size. The program ADMIXTURE v1.3.0 (Alexander et al., 2009) was used to cluster individuals into 2<K<10 clusters. The lowest cross-validation error occurred with K=2. Analysis of molecular variance (AMOVA) (Excoffier et al., 1992) was performed as a pairwise comparison of F_{ST} (genetic fixation index) values and *P*-values, using GenAlEx 6.5. Phylogenetic trees were constructed using Mega 7.027 software (Kumar et al., 1994).

RESULTS

Phenotypic variation in *F. albida* populations

A range of morphological traits was examined to determine the degree to which populations of *F. albida* in Cameroon are phenotypically distinct at the seedling stage. ANOVA revealed that there was significant variation for most traits among all 9 populations (Table 3). However, no single population was consistently different from other populations (Figure 2) and, in general; the populations were morphologically quite similar (Figure 3). At three months of age, the population means for the width of the third internode ranged from 1.5 and 1.8 mm, total leaf number ranged from 29 to 39, and plants were 19 to 29 cm in height. At this stage, populations had an average of 1 to 4.5 branches greater than 1 cm in length. The most recently formed leaves had one or two pairs of primary leaflets, each with 6 or 7 pairs of secondary leaflets, which were 4 to 5 mm^2 in area. Extrafloral nectaries (EFN) were typically absent from the first 4 to 10 leaves, but were present on later-formed leaves. One of the most obvious differences between populations was the frequency of glabrous (hairless) plants. Glabrous plants were either absent (Sirlawe) or a minor fraction of

Table 3. Analysis of variance for morphological traits.

Trait	SS	df	MS	F	P-value
Internode diameter (mm)	2.129	8	0.660	2.402	0.017
Total leaf number	1469.957	8	183.745	2.285	0.023
Plant height (cm)	2104.873	8	263.109	3.519	0.001
No. branches> 1 cm	174.667	8	21.833	2.628	0.009
No. of leaflet pairs	35.286	8	4.411	4.888	0.000
Leaflet area (mm^2)	32.184	8	4.023	1.239	0.280
Node with first extrafloral nectary	1592.439	8	199.055	2.644	0.009

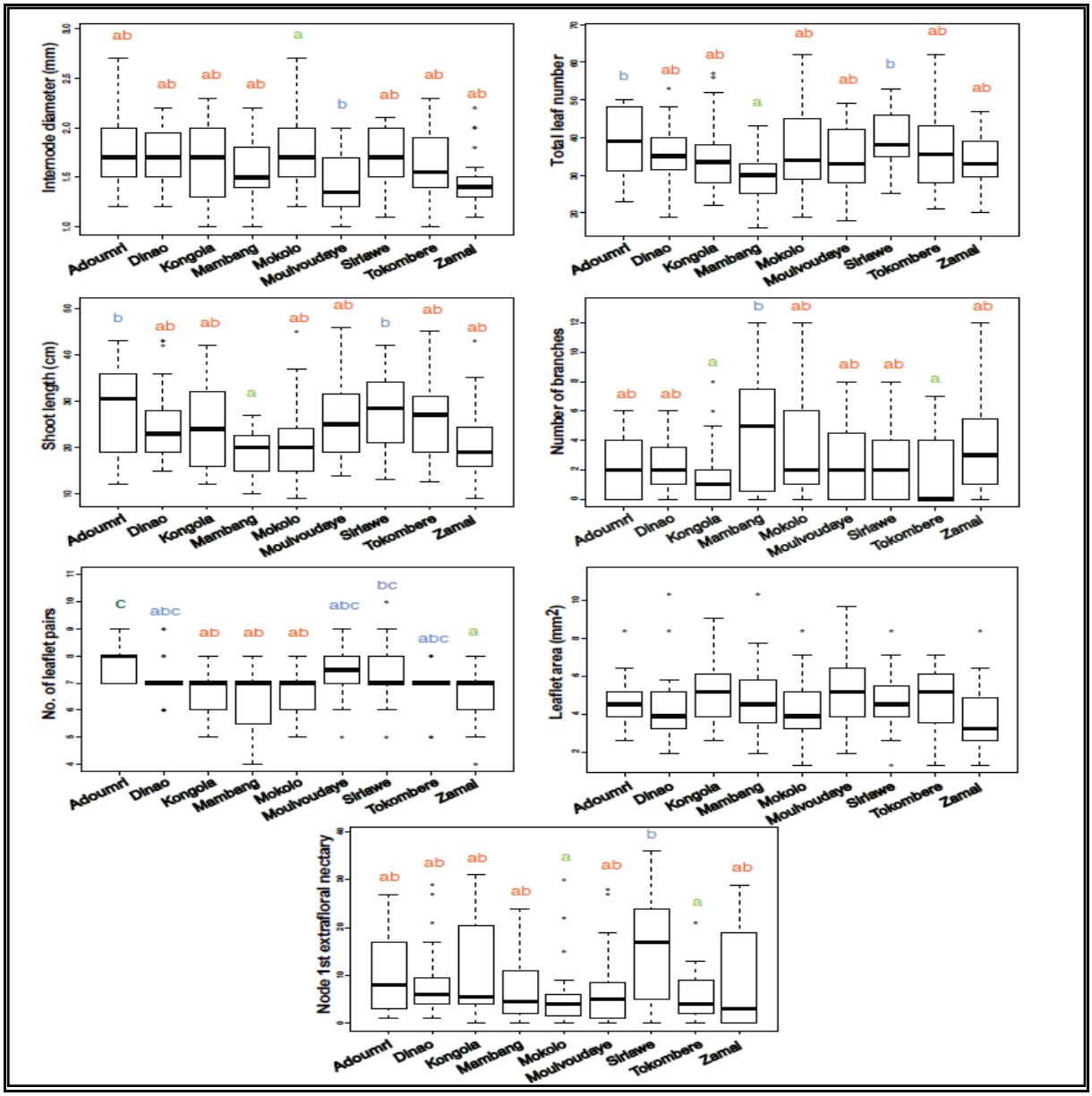

Figure 2. Comparisons of morphological traits in 3-month-old seedlings among *F. albida* populations. Center lines mark the median value, boxes outline the first and third quartiles, whiskers mark minimum and maximum values, and dots indicate outliers. Statistical significance was determined by a one-way ANOVA test followed by a family-wise Tukey HSD test. Populations with different letters are statistically different from each other at the 95% confidence level (P<0.05).

Figure 3. Three-month-old saplings of the provenances used for phenotypic analysis.

the plants in the Adoumi (11%), Dinao (22%), Kongolo (19%), Moulvoudaye (5%), and Tokombere (9%) populations, but were the major class in Mambang (87%), Mokolo (54%) and Zamai (79%) (Figures 2 and 3).

Genetic variation in *F. albida* populations

A set of 48 SSR markers were tested with DNAs from eight families to identify robust markers for assessing genetic variation in this study. Eight informative markers were selected as being most reproducible and informative based on agarose gel results, which were then used to genotype 255 individuals from 9 populations in Cameroon by high-resolution capillary electrophoresis. Seven of the SSR loci were polymorphic, while Locus_24 proved to be monomorphic in all of the populations studied based on GeneMapper analysis. The allele frequencies across all populations and loci are presented in Table 4 and the population genetic parameters calculated for these loci from the allele frequencies are presented in Tables 5 and 6. A total of 28 alleles were recorded across the 8 loci and 255 samples. The number of alleles observed at each locus ranged between 1 (marker 24) and 7 (marker 5), while the number of effective alleles ranged from 1 to 2.7 with a mean Ne of 1.5. The mean percentage of polymorphism was 73.6, while among populations polymorphism percentages ranged from 62 in Moulvodaye and Zamai to 87.5 in Sirlawe. Alleles 189 of Locus_22 and 195 of Locus_5 were only found in the Adoumri population. Alleles 180 and 198 of Locus_5 were only found in Moulvoudaye. Alleles 174 of Locus_30 and 153 of Locus_8 were unique to Kongola and allele 228 of Locus_11 was restricted to Dinao. As these alleles were only present at frequencies between 2 and 5%, further investigations should be conducted to determine if they can be used to discriminate between the Cameroon populations.

The highest number of alleles was found in the Sirlawe, Kongola and Adoumri populations, which also had the highest Shannon diversity indices (I). The Shannon diversity index ranged from 0.000 to 1.095 among the 9 populations, with a mean of 0.417. Families DIN 10, SIR 06, and TOKO 10 showed variation in 5 loci, while families AD 26, AD 36, DIN 07, DIN 11, KON 03, KON 01, KON 05, KON 08, KON 3, MOKO 08, SIR 05, SIR 28 and TOKO 05 varied at 4 loci (data not shown). These populations thus should be well-suited for selecting individuals for a tree improvement program. The average levels of observed and expected heterozygosities were 0.273 and 0.238 respectively. Observed heterozygosities ranged from 0.24 to 0.30 while expected heterozygosity ranged from 0.22 to 0.26. The highest heterozygosities were found in Sirlawe and the lowest in Moulvoudaye, but overall the heterozygosities values for all of the populations were very close to one another (Table 6).

The Hardy-Weinberg Equilibrium (HWE) values for the 9 populations ranged from 0.75 to 1.00, with an average value across populations of 0.93 and overall value of 0.67 for all 255 samples (Table 6). These HWE values indicate that the populations are largely in genetic equilibrium and that the allele frequencies can be expected to be stable in subsequent generations. The case for genetic equilibrium is supported by the lack of linkage disequilibrium (LD) observed among alleles of the 8 loci across all populations. Although LD is not necessarily expected among only 8 loci, the lack of LD does confirm that the selected loci are likely to be performing as neutral and independent loci and thus appropriate for use in genetic diversity estimates.

The sampled *F. albida* populations in Cameroon displayed higher within population variation (98%) than among population variation (2%), as shown by the Fst values for the individual SSR loci which ranged from

Table 4. Allele frequencies in populations of *F. albida* in northern Cameroon.

Primer	Alleles	AD	DIN	KON	MAMB	MOKO	MOU	SIR	TOKO	ZA	Mean
	180	0.000	0.000	0.000	0.000	0.000	0.050	0.000	0.000	0.000	0.006
	186	0.438	0.389	0.534	0.259	0.467	0.550	0.519	0.414	0.500	0.452
	189	0.292	0.167	0.207	0.431	0.217	0.117	0.037	0.207	0.148	0.202
Primer 5	192	0.250	0.389	0.259	0.276	0.317	0.250	0.370	0.379	0.352	0.315
	195	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
	198	0.000	0.000	0.000	0.000	0.000	0.033	0.074	0.000	0.000	0.012
	201	0.000	0.056	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.010
	153	0.000	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.004
Primer 8	165	0.250	0.192	0.233	0.133	0.200	0.117	0.222	0.167	0.278	0.197
	168	0.750	0.808	0.733	0.867	0.800	0.883	0.778	0.833	0.722	0.799
	209	0.348	0.333	0.267	0.167	0.283	0.333	0.327	0.328	0.333	0.300
Primer 11	217	0.152	0.130	0.183	0.167	0.133	0.133	0.038	0.190	0.296	0.159
	227	0.500	0.519	0.550	0.667	0.583	0.533	0.635	0.483	0.370	0.540
	228	0.000	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
	189	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
Primer 22	239	0.000	0.000	0.037	0.017	0.000	0.000	0.000	0.017	0.000	0.008
	243	0.958	0.979	0.963	0.983	0.931	1.000	0.960	0.983	1.000	0.973
	245	0.021	0.021	0.000	0.000	0.069	0.000	0.040	0.000	0.000	0.016
Primer 23	207	1.000	1.000	1.000	1.000	1.000	1.000	0.981	1.000	1.000	0.998
	210	0.000	0.000	0.000	0.000	0.000	0.000	0.019	0.000	0.000	0.002
Primer 24	202	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	182	0.083	0.000	0.033	0.000	0.017	0.000	0.038	0.017	0.000	0.020
Primer 26	184	0.833	0.885	0.867	0.950	0.917	0.933	0.885	0.917	0.963	0.907
	186	0.021	0.058	0.067	0.000	0.017	0.000	0.038	0.000	0.000	0.022
	188	0.063	0.058	0.033	0.050	0.050	0.067	0.038	0.067	0.037	0.051
	174	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000	0.000	0.002
Primer 30	177	0.979	0.980	0.931	0.850	0.933	0.850	0.840	0.933	0.981	0.918
	180	0.021	0.020	0.052	0.150	0.067	0.150	0.160	0.067	0.019	0.080

Table 5. Genetic diversity statistics for each microsatellite locus used in this study.

Loci	N	Na	Ne	I	Ho	He	uHe	F	F_{IS}	F_{IT}	F_{ST}	Nm
5	28	3.667	2.734	1.095	0.694	0.632	0.644	-0.096	-0.098	-0.060	0.035	6.961
8	28	2.111	1.483	0.506	0.325	0.319	0.325	0.001	#N/A	0.000	0.017	#N/A
11	28	3.111	2.422	0.969	0.811	0.581	0.591	-0.394	-0.396	-0.363	0.023	10.468
22	27	1.889	1.056	0.117	0.054	0.052	0.053	-0.035	-0.043	-0.020	0.022	11.150
23	28	1.111	1.004	0.010	0.004	0.004	0.004	-0.019	-0.019	-0.002	0.016	14.906
24	26	1.000	1.000	0.000	0.000	0.000	0.000	#N/A	#N/A	#N/A	#N/A	#N/A
26	28	3.111	1.219	0.374	0.142	0.174	0.177	0.170	0.180	0.194	0.017	14.536
30	27	2.111	1.179	0.262	0.153	0.142	0.144	-0.061	-0.080	-0.035	0.042	5.685
Mean	27.79	2.264	1.512	0.417	0.273	0.238	0.242	-0.069	-0.068	-0.041	0.025	9.772
SD	0.26	0.122	0.078	0.048	0.036	0.029	0.029	0.027	0.060	0.058	0.004	1.859

N = Total number of alleles observed, Na = Average number of alleles per population, Ne = Number of effective alleles per population, I = Shannon Diversity index, Ho = Observed heterozygosity, He = Expected heterozygosity, uHe = Nei's unbiased gene diversity corrected for sample size, F = Overall inbreeding coefficient, F*IS* = Inbreeding coefficient of individuals relative to their subpopulation, F_{IT} = Inbreeding coefficient of individuals relative to the total population, F_{ST} = Proportion of variation in subpopulations relative to all populations sampled, N = Estimated allele migration rates among sub-populations**.**

Table 6. Genetic diversity statistics from allele frequencies of 8 SSR loci in collections from 8 northern Cameroon populations of *F. albida*.

Population	N	Ar	Ho	He	uHe	F	F_{IS}	HWE	LD
Cameroon (total)	255	1.24	0.27	0.24	0.24	-0.04	**-0.11**	**0.67**	0.00
Adoumri	24	1.26	0.29	0.26	0.26	-0.07	-0.09	1.00	0.00
Dinao	27	1.24	0.25	0.23	0.24	0.06	-0.04	1.00	0.00
Kongola	30	1.26	0.28	0.26	0.26	-0.03	-0.07	0.83	0.00
Mambang	30	1.23	0.27	0.22	0.23	-0.15	-0.19	1.00	0.00
Mokolo	30	1.24	0.28	0.24	0.24	-0.12	-0.15	0.83	0.00
Moulvoudaye	30	1.23	0.24	0.22	0.23	0.06	-0.06	1.00	0.00
Sirlawe	27	1.26	0.30	0.25	0.26	-0.15	-0.17	1.00	0.00
Tokombere	30	1.24	0.27	0.23	0.24	-0.02	-0.15	1.00	0.00
Zamai	27	1.23	0.28	0.22	0.23	-0.17	-0.25	0.75	0.00
means		1.24	0.27	0.24	0.24	-0.07	-0.13	0.93	0.00
SE		0.014	0.017	0.013	0.014	0.084	0.066	0.095	0.000

N = sample size, Ar = Average number of alleles per individual, Ho = Observed heterozygosity, He = Expected heterozygosity, uHe = Nei's unbiased gene diversity corrected for sample size, F = Overall inbreeding coefficient, FIS = Inbreeding coefficient of individuals relative to their subpopulation, HWE = Hardy-Weinberg Equilibrium, LD = Linkage disequilibrium.

Table 7. Pairwise comparison of F_{ST} (genetic fixation index) values among Cameroon collections.

Collections	Adoumri	Dinao	Kongola	Mambang	Mokolo	Moulvoudaye	Sirlawe	Tokombere	Zamai
Adoumri	–	0.230	0.400	0.010	0.399	0.056	0.036	0.398	0.201
Dinao	0.005	–	0.429	0.008	0.420	0.098	0.162	0.418	0.098
Kongola	0.000	0.000	–	0.005	0.421	0.191	0.187	0.256	0.118
Mambang	0.033	0.028	0.031	–	0.022	**0.001**	**0.000**	0.019	**0.000**
Mokolo	0.000	0.000	0.000	0.023	–	0.324	0.205	0.407	0.089
Moulvoudaye	0.017	0.011	0.006	**0.040**	0.002	–	0.182	0.318	0.031
Sirlawe	0.024	0.007	0.006	**0.057**	0.006	0.007	–	0.039	0.008
Tokombere	0.000	0.000	0.004	0.023	0.000	0.003	0.019	–	0.397
Zamai	0.008	0.013	0.010	**0.066**	0.014	0.023	0.040	0.000	–

Mean = 0.02, range: 0.00–0.07, SD ± 0.02. Pairwise F_{ST} values below the diagonal, *P*-values above. Significant differences are in bold.

0.016 (1.6%) to 0.042 (4.2%) across the populations (Table 5). This finding was reinforced by the results of a pairwise comparison of F_{ST} values between the populations which resulted in a mean Fst value overall all comparisons of 0.02 and a range of Fst values between population pairs ranging from 0.00 to 0.07, with SD of 0.02. (Table 7). An analysis of population structure using admixture detected 2 types of genotypes which were both widely distributed among all populations sampled (Figure 4). A principal component analysis (Figure 4) based on allelic variation among all 255 individuals was also unable to differentiate among the 9 populations of *Faidherbia albida* sampled in Northern Cameroon. Also, the F_{IS} values were negative for most loci and ranged from -0.396 to 0.18.

DISCUSSION

Eight informative microsatellite markers (SSRs) were selected from the markers developed by Russell et al. (2014) for *F. albida* in tropical Africa to assess genetic diversity from populations in northern Cameroon for immediate application in seed collections for reforestation and agroforestry, and to initiate a breeding program. Phenotypic data were also collected and analyzed from the populations. It was found that there was higher within population than among population genetic variation, implying a high rate of gene flow between the Cameroon populations sampled. This is consistent with the extremely phenotypic variability found within individual populations, while overall the populations were morphologically quite similar. Genetic studies of other tropical fruit and timber species from Cameroon, using SSR markers, also indicated higher within population than among population variation (Benoit et al., 2011; Garcia et al., 2004), and similar results have been reported for A*cacia senegal* in Kenya (Omondi et al., 2010). Analyses of isozyme loci showed that populations of *F. albida* from across west and north Africa are

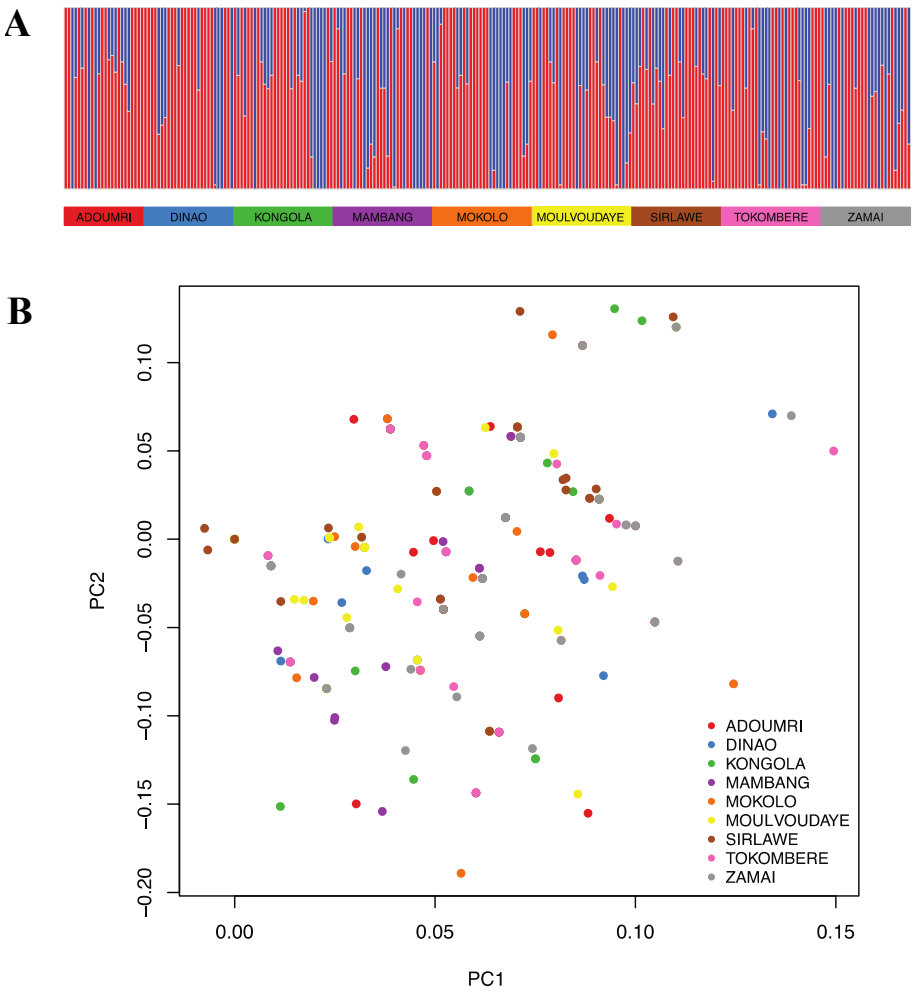

Figure 4. Results of hierarchical population structure analyses of genetic variation in populations of *Faidherbia albida* in Cameroon. (A) Admixture analysis of genotypic variation across individuals grouped by population; (B) Principal Component Analysis (PCA) based on allelic variation among all 255 individuals sampled from 9 populations of *Faidherbia albida* in Cameroon.

genetically diverse and are distinctly different from east African populations, which displayed a lower amount of genetic diversity (Joly et al., 1992; Harris et al., 1997; Dangasuk et al., 2002). In these previous studies, despite the high level of genetic diversity within the western and northern populations, within population diversity was found to be greater than among population diversity in this broad region (Harris et al., 1997). This may be attributable, in part, to exchange of seeds among farmers and transfer by animals during the nomadic life of herders in search of pasture (Hauser, 1994; Wickens, 1969).

The relatively low amount of genetic differentiation between populations of *F. albida* in the far north region of Cameroon has important implications for breeding studies. From a practical standpoint, this result means that it is impossible to predict from genetic data which populations are most likely to provide superior sources of breeding material. Given that all of the populations examined in this study are genetically similar, in principle any population is a useful starting point for the derivation of improved varieties. However, the relatively large amount of genetic variation and phenotypic variation within each of the populations reveals substantial opportunities for selection and breeding for *F. albida* improvement at the population level. Determining which populations and traits are best for breeding agronomically useful traits will require more detailed information, including which phenotypic traits segregate in families from different populations. In contrast, even with the large number of samples genotyped, the F_{IS} values were negative for most loci. Negative F_{IS} values indicate an excess of heterozygotes in populations (Guries and Ledig, 1981). This suggests that *F. albida* populations in Cameroon are either subject to high selective pressure,

or arose from genetic bottlenecks from a relatively small number of founding individuals (Hedrick and Kalinowski, 2000).

In this study, the initial steps in establishing breeding and reforestation programs for *F. albida* in Cameroon have been accomplished through the collection of seed from natural populations and assessing of genetic and morphological variation among seedlings from these populations. The high levels of variation observed within all of the source populations provides ample opportunity for making great strides in trait improvement, while retaining genetic diversity, during the next steps of selecting trees with desirable genotypes and establishing breeding orchards. The availability of the genome sequence of *F. albida* (Chang et al., 2018), will make it possible to identify many new polymorphic molecular markers useful for mapping quantitative trail loci linked to traits useful in agroforestry applications. The genetic diversity results coupled with the genome sequence should enable the use of genome-wide marker assisted selection in *Faidherbia* --an approach that is widely used in the breeding of crop plants and has the potential to dramatically accelerate the discovery and improvement of trees useful for agroforestry (Alkimim et al., 2020; Grattapaglia, 2017; Ribaut et al., 2010; van Nocker and Gardiner, 2014; Watson, 2019; Zargar et al., 2015). *F. albida* needs up to 12 years to flower (Barnes and Fagg, 2003), so breeding requires a long-term commitment on the part of both researchers and funding agencies. But, given the importance of *F. albida* in African agroforestry systems, it is an approach worth considering. Previous studies have shown that populations of *F. albida* from east and south Africa grow faster than populations from west Africa, but are also less drought tolerant than west African populations (Vandenbeldt, 1992; Dangasuk et al., 2006). This suggests that populations derived from crosses between Cameroonian and east/south Africa populations could be an important source of novel, and potentially useful, varieties with improved growth and stress-tolerance attributes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Readiness of the Nigerian public for the introduction of genetically modified crops into the food market

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Genetically modified crops (GM crops) have gained wide attention over the years some GM crops have been undergoing field trials in Nigeria, but they have not proceeded to commercial cultivation due to the absence of regulatory law. The biosafety act was passed into law in 2015 and the presence of this law, can see to the progression of the GM crops from field trials to commercialization. This study investigated the readiness of potential consumers for the possible introduction of these GM crops into the food market. The survey was designed to investigate among other factors their willingness to consume GM crops and to identify their concerns, if any. The results obtained indicated that most of the respondents have medium level knowledge about GMO\GM crops. The desire to consume GM crops among respondents varied and many respondents indicated that they have concerns about GM crops. Their concern was primarily related to potential health risks. Participants also indicated the need for further information about GMOs and stated the factors that influence their attitude towards GM crops. The internet and the media (newspapers, TV etc.) were stated as the means of previous knowledge about GMOs and the internet was again requested as a means of further information about GMOs. Based on this study, regulatory authorities and relevant stakeholders can understand the position and concerns of the consumers prior to the commercialization of GM crops in Nigeria.

Key words: Genetically modified organisms (GMOs), crops, food, biotechnology, biosafety, risk perception, policy.

INTRODUCTION

Genetically modified organisms (GMOs) are defined as organisms (e.g., plants, microorganisms, or animals) whose genetic material (DNA) has been altered beyond its natural state either by mating or natural recombination (Information, 2010; Rzymski and Królczyk, 2016). Crops produced by genetic modification (Genetically modified crops (GM crops)) are crops whose DNA has been modified using genetic engineering techniques to introduce new traits to the crop, precisely trait(s) which do not naturally occur in the crop (Fraley et al., 1986). The introduced traits can be intended at improving the nutritional value of the crop, prevent pest infestation,

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provide tolerance to pesticides/herbicides or increase adaptability to weather and growth conditions (Ojo and Adebayo, 2012). The introduced traits are mostly obtained from non-related organisms or wild relatives. An example is an insecticidal trait (*Cry* gene) obtained from the bacteria *Bacillus thuringiensis (Bt)*. Vectors such as bacteria (e.g., Agrobacterium) or viruses (e.g., retrovirus, Lentivirus) are used as delivery agents for the introduction of the new gene (Rogers et al., 1987). Chemical or radiation mutagenesis can also be employed to induce random mutations in the crop, and such mutations can also alter the crop's genetic components to produced GM crops (Avery et al., 1944; Matagne, 1969). Emerging gene-editing technologies such as the CRISPR technology can also be employed in the editing of the plant genome. The CRISPR/Cas9 gene-editing system has revolutionized research in plant and animal with its genome editing ability first demonstrated in 2012 in mammalian cells (Aerni, 2005).

The use of genetically modified crops in reducing poverty and solving food security problems has is not without debate. Nevertheless, policymakers from developing countries have considered GM crops as a possible tool for increasing crop productivity (Aerni, 2005). Debates over their benefits and concerns of application have, however, mired its implementation (Aerni and Bernauer, 2006; Kikulwe et al., 2011). Currently, 29 countries of the world are cultivating GM crops, of which 19 are developing countries, and only three of the developing countries are from Africa (Adenle, 2014). Commercial cultivation of GM crops has only been approved in South Africa and Burkina Faso (Adenle, 2001). According to the estimate by the World Bank statistics, (“Population, total - Nigeria Data,” n.d. 2018), the population of Nigeria stands at 195,874.74. Traditional agricultural practices can be complemented by plant biotechnologies such as genetic modification to ensure food security for such a populous country (Datta, 2013).

The use of GM crops is influenced by the evaluation of her safety to consumers, farmers, and the environment. This principle is described in biosafety, which is defined in this context as the precautions taken to control the cultivation and distribution of GM crops and products (Prakash et al., 2011). To ensure precautionary safety measures in plant biotechnology, Nigeria signed and ratified the Cartagena Protocol on biosafety in 2002 and 2003, respectively. However, the absence of a national biosafety law was a limiting factor for the commercialization of GM crops in the country. A biosafety bill was passed into law in April 2015. The law is poised to regulate the application of modern biotechnology techniques, management, and the use of obtained products (genetically modified organisms) that may pose potential risks about conservation and sustainable use of biodiversity (Li et al., 2014). The Nigerian food and agricultural landscape can accommodate domestic and international biotechnology, agricultural\seed companies, and research organizations to engage in commercial activities after due approval by the nations' biosafety regulatory agency. The National Biosafety Agency manages the Nigerian biosafety law, and the agency is responsible for implementing and regulating biosafety activities in Nigeria.

Currently, the following crops are undergoing field trials: Maruca -Resistant Cowpea (Bt Cowpea), Africa Bio-for-tified Sorghum Nitrogen Use Efficient, Water Use Efficient and Salt Tolerant (NEWEST) Rice and Bt cotton. A comprehensive database of crops undergoing field trials in Nigeria is provided in Table 1. Other important food crops in the county are also being considered for genetic alteration for desired and improved traits. For example, planned alteration of local tomato varieties (Animasaun et al., 2020) has gotten to an advanced stage, and also, the virus-resistant cassava enhanced with Zinc and Iron (Cassava plus) (Ivase, 2019). After successful approval by the biosafety regulatory agency, these crops may be cultivated for commercial purposes. It is important to note that most of the crops mentioned above are staple crops. Agricultural practices of staple foods are essential for both household self-sufficiency and income generation. The livelihoods and economic wellbeing of the nation can be affected by factors that impact staple food production; therefore, adequate attention must be paid to ensure the sustainability of these crops (Sawicka et al., 2020).

Globally, the cultivation, use, and commercialization of GM crops have been surrounded by many controversies and (negative) attitudes from many sectors, including the consumers (Adeoti and Adekunle, 2007; Aerni and Bernauer, 2006; Kikulwe et al., 2011). Factors responsible for these attitudes include limited knowledge of the scientific principles behind the gene modification technologies, minimal or absence of known potential benefits of GMOs, religious, moral or ethical beliefs and inability to accurately define what constitutes a GMO (Aleksejeva, 2014; Costa-Font and Gil, 2012; Pino et al., 2016). The expression of the views and opinions of the pro and anti-GMO groups in the media has also contributed to misinformation and confusion of potential consumers, users, and growers of GM crops (Rzymski and Królczyk, 2016). The existence of a biosafety law will enable the regulation of biological entities, including GMOs in Nigeria. It is, however, crucial to study the preparedness of the consumers for the introduction of GM crops as they present the potential end-user. It is also imperative to identify the expectations and perceptions of the subject matter. This study, therefore, evaluates the current knowledge about GMOs among the Nigerian public and the preparedness for the potential commercialization of genetically modified in the Nigerian food chain. Decision and policymakers, potential seed companies and research agencies will be able to benefit from this study as it will provide them insight into the concerns and expectations of the consumers (Table 1).

Table 1. Database of crops approved for field trials.

Name of crop	Trait	Developer	National collaborating institute	Regulatory status	Status as of December 2016
Maize	Stacked genes for insect resistance and glyphosate herbicide tolerance	Monsanto Nigeria LTD	Institute for Agricultural Research Zaria	CTF approved	Yet to commence
Cotton	Insect resistance	Monsanto Agriculture Nigeria LTD	Institute for Agricultural research Zaria	General release	Ongoing
Rice	Stacked with nitrogen use efficiency, water efficiency, and salt tolerance	African Agricultural Technology Foundation	National cereal research institute Baddegi	CTF	Ongoing
Cassava	Bio cassava plus (pro-vitamin A, protein, iron) cassava mosaic, virus resistance, and brown streak virus resistance	Danforth plant	The national root crop research institute, Umudike	CTF	Concluded
Sorghum (ABS)	Bioavailability of protein, zinc and iron	Africa Harvest	Institute for Agricultural research Zaria	CTF	Ongoing
Cowpea	Maruca insect resistance	CSIRO, Australia	Institute for Agricultural research Zaria	Multi-locations Trait	Ongoing

Adapted from Ivase (2019); CTF: Confined Field Trial, CSIRO: Commonwealth Scientific and Industrial Research Organisation, ABS: Africa Biofortified Sorghum.

MATERIALS AND METHODS

Participants and survey

The study design contained sections covering respondent's knowledge, attitude and concerns about GMO, willingness to consume GM crops; GM crops food labeling, GM food benefits and possible application of GM technology to food security and national development, and respondent's demographic characteristics. The sections are described below:

(i) Prior knowledge about GMO/GM crops: Participants were asked if they had previous knowledge about GMOs and to indicate their level of understanding if applicable. Participants who had knowledge about GMOs were also asked to indicate the GM crop they know.

(ii) Attitude towards acceptance of genetically modified crops: Since some genetically modified crops are undergoing field trials in Nigeria, participants were asked if they are willing to accept these crops when\if they are eventually commercialized following approval by the biosafety regulatory agency.

(iii) Safety concerns about genetically modified crops: Participants were asked if they have any concern about genetically modified crops and to state these concerns. They were also asked about the factors that influence their attitude towards genetically modified crops/foods.

(iv) Food labeling: Participants were asked to indicate if they read labels of food products during purchase and to indicate if they would want GM crops or food products containing genetically modified elements to be labeled as such.

(v) Price advantage of genetically modified crops and meeting the nation's food demands: Considering the economic capability of an average Nigerian, participants were asked if they would consider a price advantage between a GM crop and its unmodified counterpart. The participants were asked if they think the potential benefits of genetic modification should be applied to meet the nation's food demands.

(vi) Further information about GMO/GM crops: According to the participants' current level of information and previous source of information, participants were asked if they required more information about GMOs and their preferred medium of further information.

(vii) Demographic characteristics of the participants were collected, including their sex, age group, level of education, geographical location, and profession.

The full list of the survey questions is provided in Table 2.

Mode of dissemination

The Internet was chosen as a platform to reach participants because it cuts across all geographical regions of the nation. The questionnaire was made accessible for 11 months. Participants were invited to complete the questionnaire through platforms such as Facebook, WhatsApp (personal and group chats), blogs, and email.

Table 2. : List of questions asked participants.

What is the highest level of education you have completed?
Do you know what a genetically modified organism is?
What is your level of understanding about Genetically modified organisms/crops?
Which of the following genetically modified crops do you know about?
How did you know about genetically modified organism/crops?
Genetically modified crops are currently considered for field trials in the country. Are you ready to accept and make use of Genetically Modified organisms/crops in Nigeria when eventually commercialized?
Do you have any concern about Genetically Modified organisms/crops?
If yes,what is your major concern about genetically modified organisms/crops?
Have you ever knowingly eaten a genetically modified food?
Would you eat a Genetically modified crop when eventually introduced in Nigeria market?
Do you read labels carefully before buying food or crops ?
Would you like a Genetically Modified crop to be distinguished from its non-Genetically Modified crop counterpart in Nigerian markets (Clear labelling)?
For processed food products, Would you like them to be labelled if they contain Genetically modified elements?
Would you consider a price advantage between a Genetically Modified crop/food and its non-Genetically Modified counterpart?
Do you think Nigeria should harness the potentials of Genetically Modified crops to meet national food security and economic/commercial demands?
In addition to resistances to insects and disease, crops can also be modified to have a better flavor, increased shelf life and nutritional value. What features are important to you when shopping for food?
Which of the following factors influence your attitude towards Genetically modified crop/food
Do you need more information on genetically modified organism/crops?
Where would you prefer to get (further) information on Genetically modified organisms/crops from?
What suggestions do you have for the use of genetically modified organisms in Nigeria
What is your age group
What is your gender
Where is your current geographical location
Occupation

Data analysis

A total number of 335 responses were collected, and the data obtained were analyzed using the IBM SPSS statistics for windows, version 22 (SPSS Statistics, 2013), and Microsoft Excel 2016. The Chi-Square test of dependency was conducted to find relationships between variables and the level of significance (p). p values of <0.05 were considered statistically significant.

RESULTS

Demographics characteristics

A total of 226 of the respondents were male (67.5%), and 88 were female (26%), 21 respondents did not indicate their sex. The majority of the respondents (43%) live in the Southwest region of the country, and the age range of 25-39 years was predominant among the respondents (58%). The educational background of the respondents showed that 90% was educated beyond high school, 47% had a bachelor's degree, and 40% had a post-graduate qualification. A significant percentage (41%) of the respondents was working-class professionals (that is, bankers, civil servants, public servants, etc.), 24% are in business, and 26% were students. The overall demographics statistics of survey respondents are presented in Table 3.

Prior knowledge of GMO/GM crops

In this study, 88% of respondents had previous knowledge (Figure 1A) of GMOs, and 56% self-rated their knowledge to be medium while 23 and 19% rated their knowledge as high and low, respectively. The low level of knowledge was predominant among respondents who do not have previous knowledge about GMOs, and a medium level of knowledge was predominant among respondents that have previous knowledge (Figure 1B). The genetically modified crops known by the respondent varied, but 28% of the respondents knew only corn. Media sources and the Internet accounted for the primary source of prior knowledge of GMO/GM crops (48%). Based on the results of the Chi-Square test of dependency, the knowledge about GMOs by the respondents is not dependent on the gender ($p=0.117$)

Table 3. Demographic characteristics of respondents.

Demographic characteristics	Frequency (%) n=335
Age group	
18-24 years old	61(18.2)
25-39 years old	195(58.2)
40-59 years old	61(18.2)
No response	18(5.4)
Gender	
Female	88(26.3)
Male	226(67.5)
No response	21(6.3)
Current geographical location	
North Central	65(19.4)
North East	8(2.4)
North West	35(10.4)
South East	19(5.7)
South-South	39(11.6)
South West	143(42.7)
No response	91(27.2)
Occupation	
Applicant	4(1.2)
Business/trade/self-employed	81(24.2)
Professional	138(41.2)
Student	87(26)
No response	25(7.5)
Highest level of education completed	
Bachelor's degree	157(46.9)
HND/OND	26(7.8)
Postgraduate degree (Masters, Ph.D., Post-doc)	133(39.7)
SSCE)/ (GCE)	14(4.2)
No response	5(1.5)

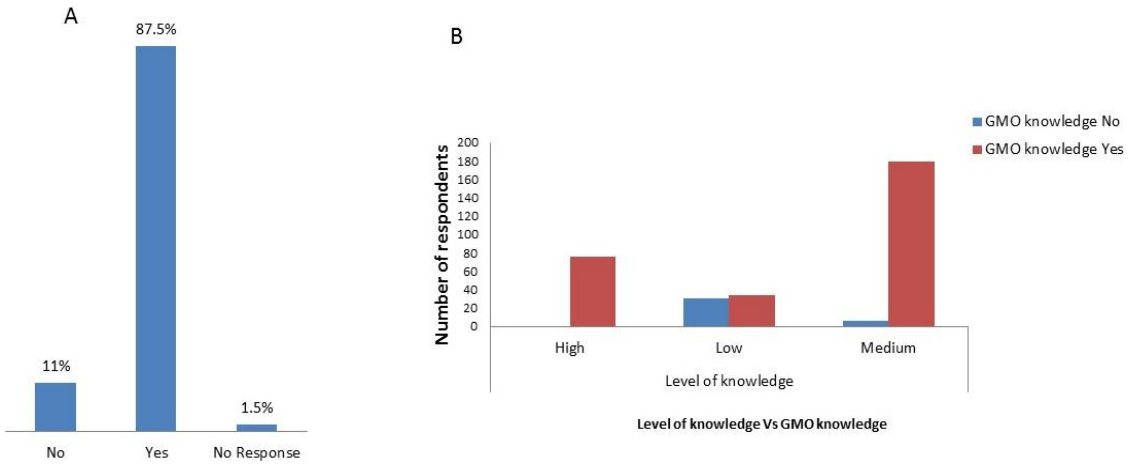

Figure 1. (A) Prior knowledge about GMOs and GM crops as indicated by respondents, (B) Comparison of prior GMO knowledge Vs Level of knowledge.

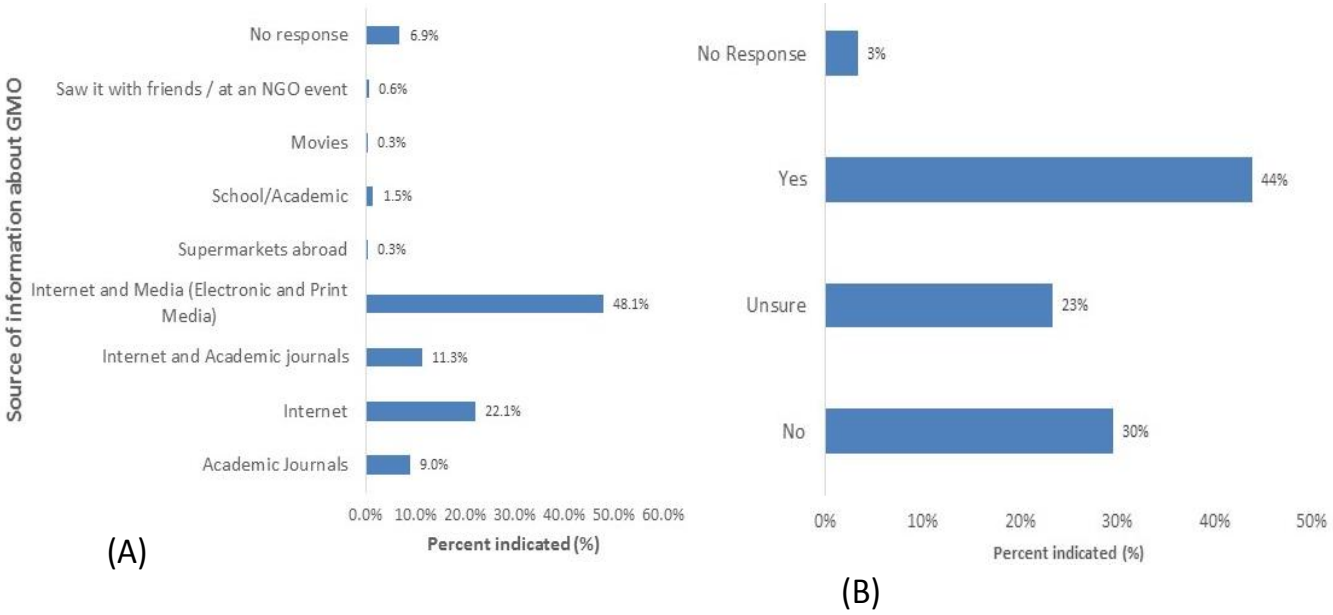

Figure 2. (A) Source of previous information about GMO by the respondents, (B) Willingness to consume GMO after its introduction to Nigerian food market.

nor on their educational qualification (p=0.002). According to this study, knowledge of GMOs is regardless of sex or the level of education. The knowledge of GMOs by respondents is, however, dependent on their occupation (p=0.002). The media and the Internet jointly accounted for the primary source of previous information about GMOs (48%) and closely followed by the Internet and academic journals, as seen alongside other sources in Figure 2A.

Attitude towards acceptance of genetically modified crops

According to this study, a total of 44% of the respondents were willing to consume GM crops when eventually introduced, 30% were not willing to consume, and 23% were uncertain (Figure 2B). The acceptance of GMOs was observed to be age and occupation dependent (p=0.001 respectively) but was independent of location (p=0.326) and educational qualification (p=0.484). Respondents with medium knowledge about GMO/GM crops were more willing to consume GM crops compared to respondents with high and low knowledge. The highest number of respondents willing to accept GMOs was observed with well-educated respondents. A similar response was observed for respondents that would not accept and those that were not sure. About 44% of the respondents indicated that they had never consumed GM food compared to 24% who had consumed it while 29% were not sure if they had ever consumed GM food (Data not shown).

Safety concerns about genetically modified crops

Generally, 80% of the respondents indicated that they have concerns about the potential use of GM crops, and more than 65% of this concern was attributed to perceived potential risks on human health, as shown in Figure 3A and B respectively. Respondents mostly expressed concerns about GMOs/GM crops with more than a bachelor's degree, and the majority of the respondents with such concerns were in the Southwest region of the country (Data not showed). The concern for the environment was indicated by only 10%. Based on the respondents in this study, minimal concerns were attributed to religious or ethical beliefs and biosafety regulation.

Food labeling and price advantage of genetically modified crops

A substantial percentage of the respondents (76%) claim that they read product labels during the purchase (Figure 4A), and 90% would want GM crops and food products containing GMO elements to be labeled accordingly (Figure 4B). Inclination towards buying GM crops based on price advantage between GM food/crops varied among the respondents (Figure 4C); about 39% would consider a price advantage while 36% would not, and 25% were not sure yet if they would consider a price advantage.

Desired crop/food feature

Considering many modifications that crops could undergo

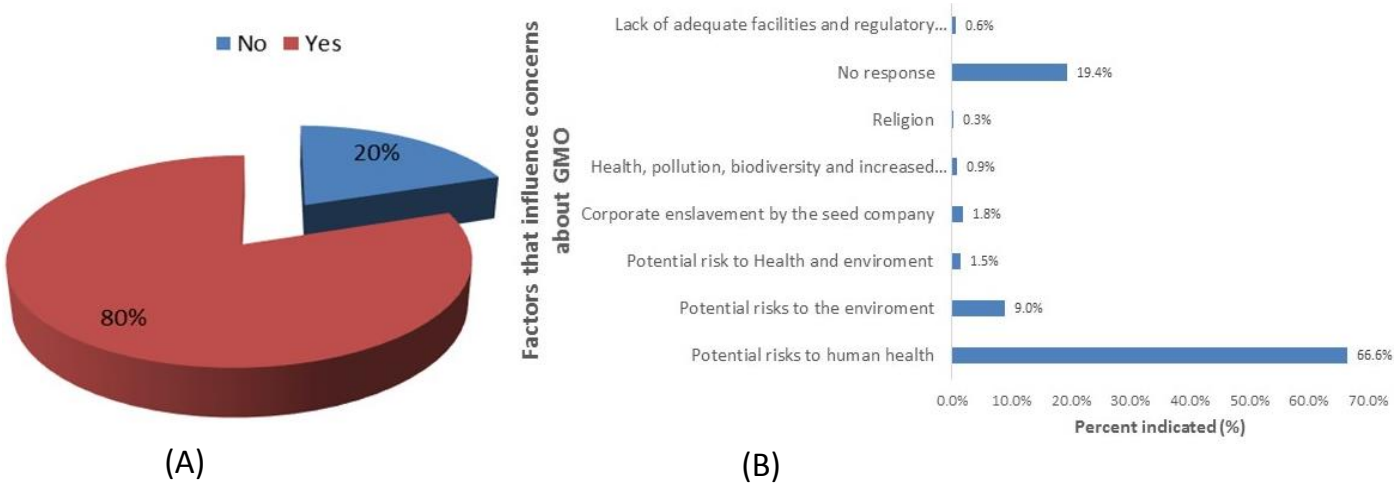

Figure 3. (A) Factors that influence respondent's attitudes towards GMO when it is eventually introduced, (B) Participants indication of their specific concerns/attitude about GM crops when eventually introduced.

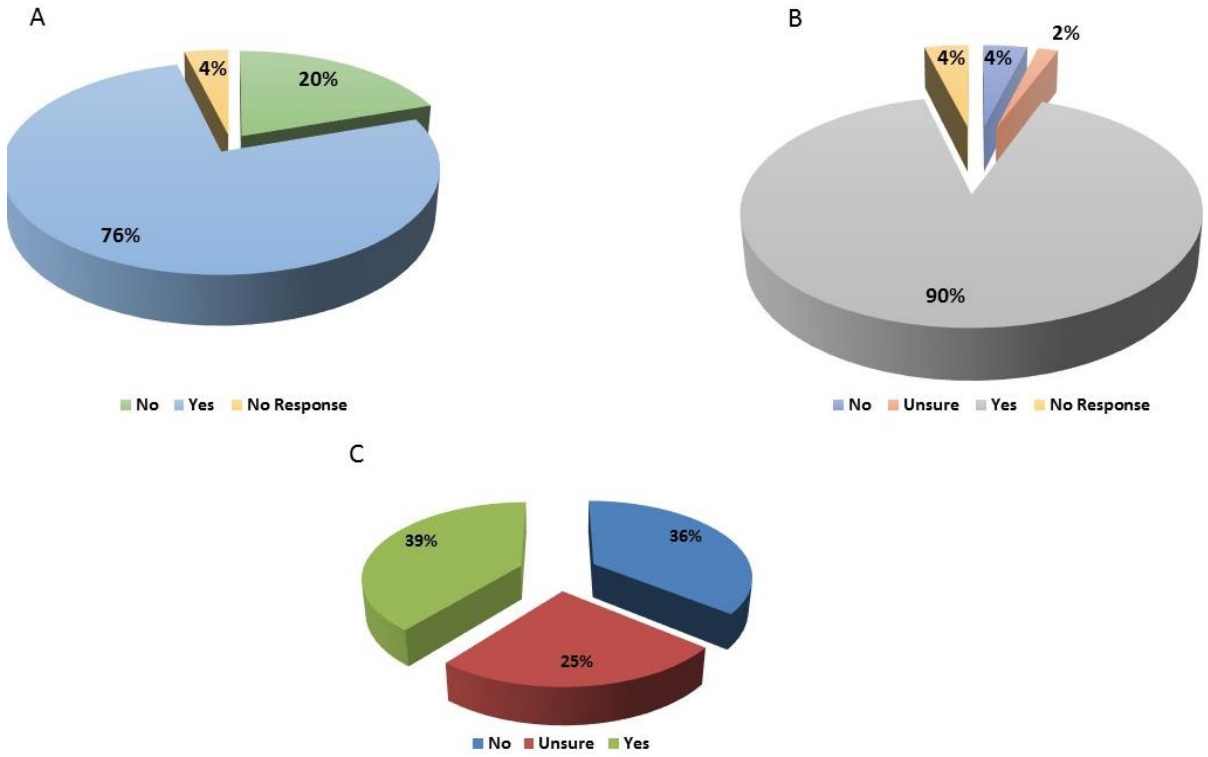

Figure 4. (A) Indication of reading of food label by respondents, (B) Participants desire for GMO crops or Food items containing GMO be labelled, (C) Participants consideration for price advantage of GMO crops.

genetically, respondents indicated the features of food products that they consider essential during purchase. Physical appearance, nutritional content, safety, price, and quality were observed to be dominant, as shown in Figure 5.

Further information about GMO/GMC

Respondents indicated their desire for further knowledge (83%), awareness, and information about GMO/GM crops (Figure 6A), and they indicated their preferred

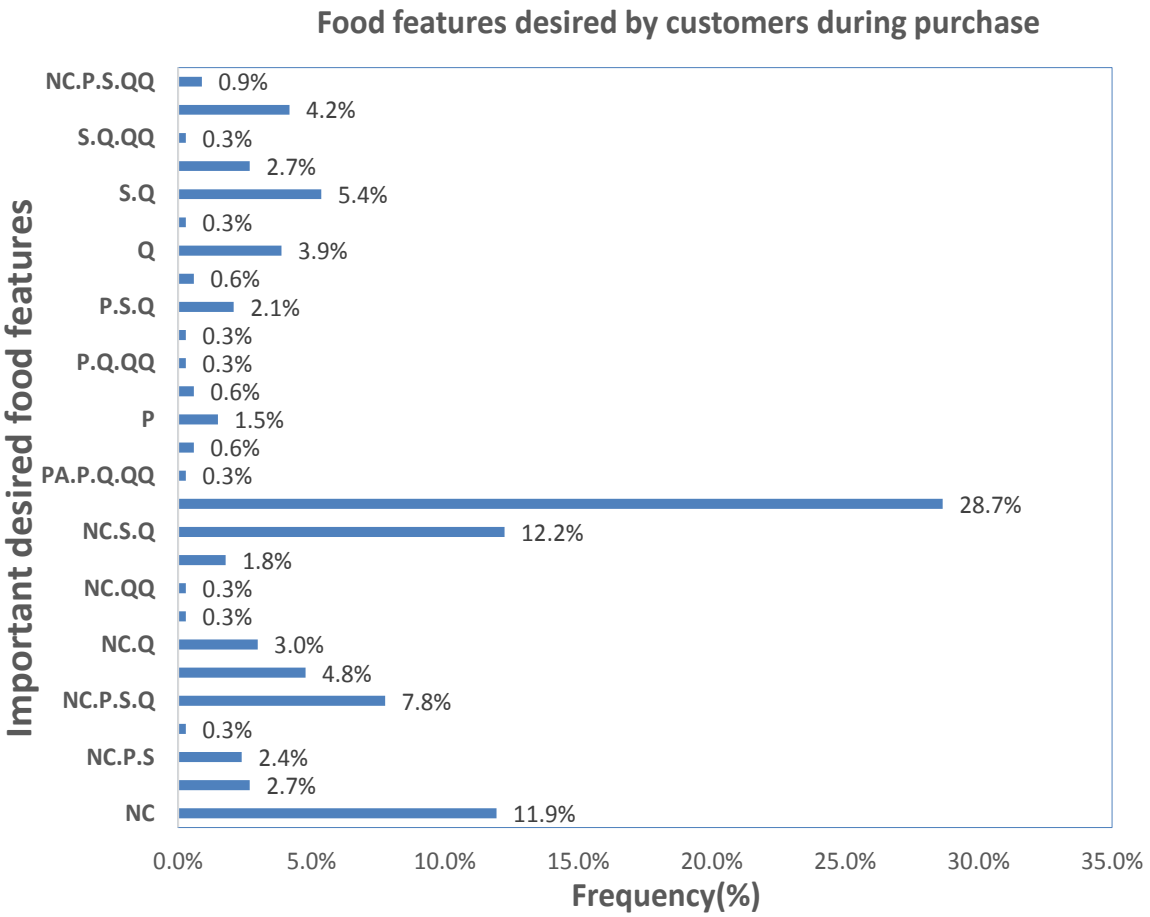

Figure 5. Important food features desired by respondents. NC=Nutritional content, P=Price, Q=Quality, QQ=Quantity, PA=Physical appearance, S=Safety.

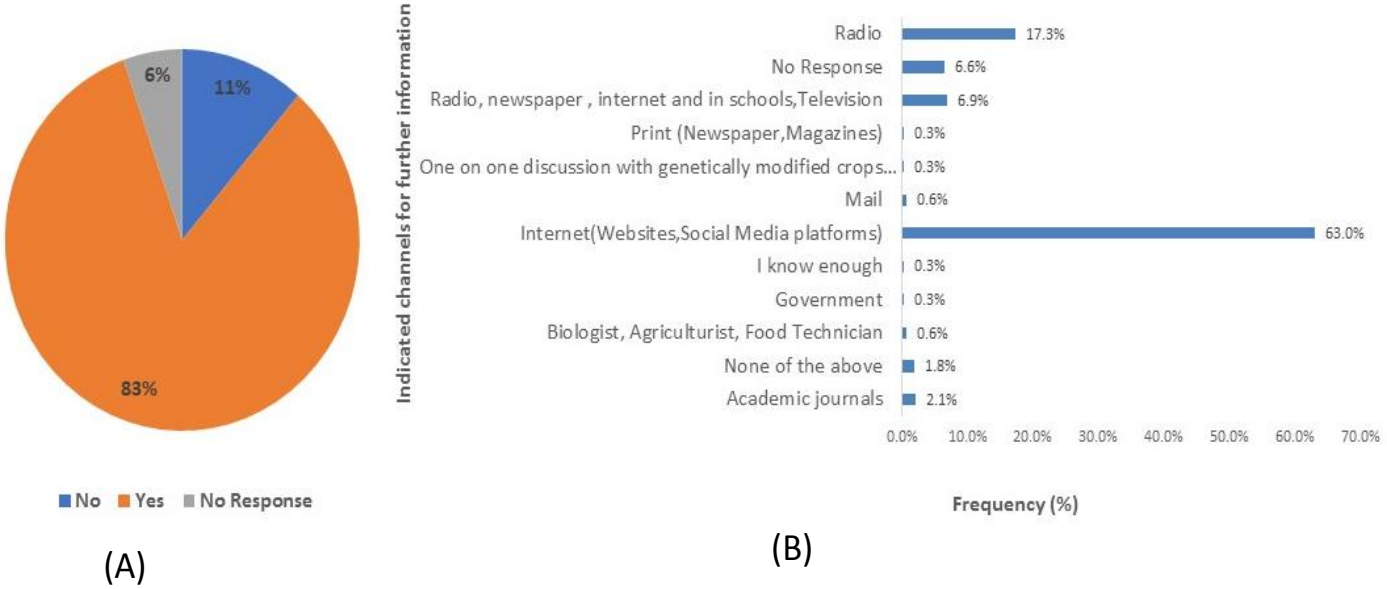

Figure 6. A. Indication of desire for more information by respondents B. Indication of source of further information about GMO by respondents.

source of further information (Figure 6B). The preferred source of information is through social media and the Internet.

DISCUSSION

The use of genetically modified organisms (GMOs) is mostly associated with controversies that have divided public opinion. This study accentuated a high degree of concern towards GMOs among the respondents. The study was carried out using the internet and social media tools such as blogs, email, Facebook, WhatsApp, and Instagram as a means of dissemination of the questionnaire. The use of the Internet as a survey tool is mostly characterized by lower data collection costs and qualitative questionnaire designs and administration, and this tool was applied in this study for these reasons mentioned above (Internet Statistics, 2020). Overall, the sample respondents represent an educated segment of the public and only cuts across people that make use of the social media/internet due to the method of dissemination of the survey. It was observed that more than half of the respondents had educational qualifications above the secondary school certificate. Thus, this study is not representative of the general population. The results also indicated a high interest in the survey by the majority of male respondents. Culturally, Nigerian females are more involved in the family's domestic affairs, such as cooking and purchasing food items. Therefore one would expect more females to be more interested in the debate about GMO crops in the country. This observation could have been influenced by many factors, such as lack of interest or limited knowledge of the subject matter or possibility of limited access to the Internet.

The participants self-reporting knowledge about GMOs is high based on the results observed in this study. Although the level of knowledge is mostly medium (63%) amongst the respondents, there is no clear indication of how the level of knowledge can be measured within the scope of this study. The indication of the desire for further information on GMOs by more than 83% of the respondents can attest to the fact that the medium level of knowledge is not deemed significant by the respondents. There is, therefore, the need to increase awareness and level of knowledge about GMOs. Increasing awareness and providing relevant information about genetic modification techniques and its associated biosafety components will provide valuable, well-balanced information on biotechnological processes and final products. Previous studies have shown that attitude towards GMOs is a crucial factor in understanding the public's perception of associated risks and benefits (Verdurme and Viaene, 2003). The process of modifying these perceptions requires accurate communication of science to mass-media and relevant stakeholders to prevent media hype (Rodríguez-Entrena and Salazar-Ordóñez 2013). Choosing an efficient means of communication for this purpose is also very important, and the respondents in this study attributed their primary source of previous knowledge to the print and electronic media (TV, radio, newspaper), Internet, and academic journals. Stating their preferred source of further information, about 68% of the respondents would prefer the use of social media platforms and the Internet.

In principle, all available mass media can be applied in communicating scientific information to the public. However, the limitation of this general approach is that some media channels may be dominated by sensationalism (Ransohoff and Ransohoff, 2001). The Internet has gained popularity as a source of information on safety assessment and approval procedures to the public. However, while considering the different media of information, the value should be placed on credible sources of information as information from credible sources is more likely to influence the perception of the public rather than information from sources that lack credibility. Factors that determine the credibility of such sources include trustworthiness, fairness, recognized competence or expertise, and lack of bias. Terms such as factual, knowledgeable, expert, public, welfare, responsible, truthful, and excellent track record are mostly associated with high credibility by the consumers (Aung and Chang, 2014). Trust and credibility should be cherished; otherwise, they can be lost through ineffective or inappropriate communication (Aung and Chang, 2014).

The respondents in this study also indicated academic journals as their source of information, and about 21% still desired further information via this channel. This quest can be fulfilled by increasing the rate of peer-review papers published in this field of study and any related discipline. The availability of such articles should be considered by ensuring that these publications are in open access (OA) mode. The use of academic publications, in this case, would only be useful for educated persons and, most likely, students, researchers, and scientists. It should also be noted that valuable free, full-text, online resources increase the chance of instant and accurate science communication to the public while contributing to the avoidance of media hypes and miscommunication (Rzymski and Królczyk, 2016). The role of academicians and school teachers in the public understanding of GMO risks and benefits should not be underestimated. Educational programs can be modified for increased biotechnology content. The teachers can also be given specialized training for this purpose, and this can be achieved by using specialized training materials such as the intersectional training developed by UNESCO in 2004. This kit is known as the "GMO Teaching Kit," is aimed at empowering secondary school teachers to educate and communicate developments and potential uses and risks of new technological advances (UNESCO, 2004).

Based on Internet usage in Nigeria, the Internet offers an excellent platform for the dissemination of information about GMOs. An efficient internet information dissemination strategy can be applied to achieve maximum impact. The Internet can host a massive repository of dynamic information that can be made available to everyone anywhere at any time (Vermesan et al., 2011). The social media platforms are also useful in reaching a target audience and stakeholders for scientific communication. Social media should, however, be engaged credibly to enhance the acceptability of the information being passed across. Other communication channels, such as public debates and information meetings organized by the government, churches, and other stakeholders, should also be considered. Although GMOs possibly remain controversial to some people, there is a need that the public has a balanced and evidence-based opinion rather than hysteric or reliant on populist views and debates (Rzymski and Królczyk, 2016). The relevant stakeholders in Nigeria, such as the ministry of health and the biosafety regulator, can fortify their efforts in communication strategies concerning health and safety assessment of food/feed derived using the inclination of the respondents in this study.

Almost half (45.3%) of the respondents were willing to accept the introduction of GM crops in Nigeria, and 24.4% were uncertain, and 30.3% were not willing to accept. Respondents with medium knowledge about GMO/GM crops were more willing to accept GM crops compared to respondents with high and low knowledge. Numerous factors could have influenced the decision of participants about the acceptance of GMOs. Significant determinants of human behavior include emotional needs, experience, and knowledge, and knowledge, in this case, includes regulations and principles that dominate individual and social life. Internalizing these markers and molding of attitudes contribute to decision making and undertaking of actions according to personality and temperamental traits (Lachowski et al., 2017). The acceptance of GMOs is observed to be occupation dependent in this survey as the Business/Trade/Self-employed, Professionals, and students were more willing to accept GM crops. Educational qualifications did not influence the attitude towards GMOs. The percentage of respondents that have concerns about GM crops was observed to be high in this study, and the results indicate the perceived risks associated with GMO products within this sample population. Comparable results were observed in (Rzymski and Królczyk, 2016), in which the study group generally perceived GM foods as unsafe for humans and the environment.

Based on the Eurobarometer survey, the level of worry about GMOs was indicated to have increased over the years since 2005 (European Commission, 2010). Generally, perceived health risks of GMOs have been observed as one of the major deterrents to public acceptance of GMO products. Fears have been expressed about the possibility of carcinogenesis, allergenicity, and the threat to biota (Rzymski and Królczyk, 2016). However, it should be noted that these health concerns about GMOs expressed by respondents are perceived and most probably not based on any scientific knowledge. There is there for a need to recognize further exact public apprehension behind their concerns about GM crops in Nigeria (Hilbeck et al., 2015; Krimsky, 2015). The use of GMO products as food or feed products is, in many cases are met by public resistance based on health concerns.

In contrast, the use of GMOs for medical and pharmaceutical purposes, for instance, in vaccine production or lifesaving medical procedures, is met with little resistance from the public (Amin et al., 2013). GM crops can also be considered for other fields of application in Nigeria. Activists and anti-GMO campaigners have built a significant percentage of their campaign on the potential (negative) environmental impact of GMOs, but the respondents in this survey attached little emphasis on the potential impact of GMOs on the environment. There was also less emphasis on religious or ethical/moral concerns about GMOs. Some participants indicated concerns about seed monopoly by the biotechnology companies. Because companies developing GMOs own the intellectual property right on their modifications, farmers must purchase the products annually from them. This requires continuous re-investment in seed purchase, increased financial commitment, and risks in case of a low yield farming season.

For the different levels of knowledge declared by the respondents, their attitude towards acceptance of GM crops could have also been influenced by their level of knowledge. Expectedly a low level of knowledge would account for limited knowledge of the technology behind these GM crops and the inherent inability to decide if they want the crops or not (Rodríguez-Entrena and Salazar-Ordóñez, 2013). The respondent with medium knowledge levels might also be skeptical because of the level of their knowledge. The respondents with high knowledge were willing to accept GM crops, and this could be rated as an informed choice. Because if they have excellent knowledge, then they possibly have good knowledge about gene modification technology and associated risks and benefits. Respondents within the age group 25-39 were the most willing to accept GM crops, followed by the 18-24years age group. The varying willingness of the age groups can be taken into consideration when planning GMO communication campaigns in the country. This strategy could determine the type and mode of commutation methods to be used based on the different age groups in a particular audience. The age group that showed limited willingness can first be introduced to the basics during initial campaigns. The geographical location did not affect this attitude and therefore suggests

that based on this study, the general attitude towards GMC in Nigeria cannot be rated based on geographical locations.

Food labels are designed to provide and communicate information about production techniques, ingredients used for the production, and quality of food/feed products. These product attributes reflect consumer's interests intentionally in making purchase decisions (Gautam, 2017). Labels can, however, be confusing, misleading, and improperly placed on the product (e.g., place in such a manner that they can be easily ignored or written in a clumsy or tiny text font) and thereby, consumers can ignore them. To find out the label reading habits of respondents, participants in this study were asked if they read food labels before making a purchase. A sizable percentage of the respondents (76%), read food labels of GMO foods, and 88% of respondents believed GMO foods and food products containing GMO entities should be clearly labeled. Many countries have adopted labeling policies for genetically modified foods in recent years, and it is currently mandatory in 64 countries. The European Union was the first to introduce these policies in 1997. However, many countries have followed, including all developed countries that have adopted some types of labeling policy for GM food. In the EU, products containing at least 0.9% of GM ingredients should be labeled as containing GM ingredients (Information, 2010). The appropriate authorities in Nigeria will also be required to identify and sate the benchmark for GM content for labeling. In the United States of America (USA), labeling policy is mostly unimplemented and often criticized due to additional cost and potential call for caution by anti-GMOs. It is mostly suggested that most people who are interested in GMO labelling would avoid buying GM foods (Kling, 2014), but this study cannot establish this. Although labeling policies may differ in their nature, scope coverage, exceptions, and level of enforcement (Gruère and Rao, 2007), there is a need for the relevant stakeholders in Nigeria to consider the desire for GMO labeling as indicated by respondents in this study. Key features that respondents look for while purchasing food items are quality, safety, and nutritional content of GMOs and derived food/feed products. These features can be considered for future crop improvement, and modification plans strategies should take note not to impart on the state features negatively.

The influence of price and physical appearance on the purchasing power was indicated by 45% and 30% of the respondents, respectively. Genetically modified crops are cheaper in terms of production, and this would eventually affect the retail price of food products (Gaisford, 2001). Only 37% of the respondents are ready to consider a price advantage, 34% would not, and the rest were not sure they would. The exact effect of a cheaper GMO food can only be established when the food gets to the market because this will be influenced by many factors such as earning power, knowledge, and perception about GMOs. A section of the public with limited financial resources may be persuaded by quantity (cheaper prices) rather (Gaisford, 2001) than quality and might not be bothered if the crop is a GM crop or not. Many factors can affect food security in a developing country like Nigeria; genetic modification can offer profitable solutions regarding disease protection, drought resistance, postharvest sustainability, etc. This is in resonance with the respondents as 63% (data not shown) support the idea that GMOs can be applied to meet food demand in the country.

Conclusion

This study represents research on the attitude and readiness towards GMOs in Nigeria by a sampled segment of the population. It provides an empirical analysis using descriptive statistics to determine the willingness of the public to accept GM crops when they are eventually introduced in Nigeria. This study is also able to provide a broader understanding to readers, policy makers, regulatory agencies, and the government about the public opinion and attitudes about GMOs. Based on the results obtained, there is a reasonable level of awareness of GMOs in Nigeria. However, participants still indicated the need for the provision of further substantive information on GMOs. The outcome of this study shows that Nigerians have divided opinions about the willingness to accept GM crops when eventually introduced. A high level of concern about the potential health implications of these crops was expressed. Although a small sample size limits the study, there is a need to implement evidence-based educational programs to increase the public understanding of potential applications and limitations of GMOs. The outcome of this study can also be employed by relevant stakeholders to address issues of inadequate information, non-evidence-based perceived risks, and general apathy towards GMOs.

Limitations of the study

This study was limited through its inability to capture members of the public that do not have access to the Internet. People living in remote locations may have limited possibilities to use the Internet. We, therefore, look forward to future research on public perception during which we will capture persons located in rural areas with limited possibilities for education in the field of modern plant breeding and the use of modern information technologies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Genetic diversity analysis among soybean genotypes using SSR markers in Uganda

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The assessment of genetic diversity among improved crop germplasm can facilitate the expansion of the genetic base for crop improvement in breeding program. However, little effort has been made to assess the level of genetic relatedness among released varieties and elite soybean [*Glycine max* (L.) Merr.] genotypes. The objective of this study was to determine the degree of genetic diversity that exists among released and elite soybean genotypes in Uganda. In this study, 21 polymorphic simple sequence repeat (SSR) molecular markers were used to determine the degree of genetic diversity and varietal identification among 34 soybean genotypes. A total of 59 alleles with an average of 2.85 alleles per locus were detected. The polymorphic information content (PIC) values ranged from 0.208 on BE806308 to 0.741 on Satt411, with an average of 0.5870. The expected heterozygosity varied from 0.208 on BE806308 to 0.725 on Satt411, with an average of 0.548 per marker. The dendrogram constructed based on Jaccard's genetic similarities among 34 soybean genotypes identified three major clusters, with six of the released varieties belonging to cluster I. The majority of elite genotypes including three recently released cultivars; Maksoy 4N, Maksoy 5N and Maksoy 6N were grouped in cluster II and III. The results showed moderate genetic variation among the soybean genotypes, which could accelerate genetic vulnerability. Therefore, there is need to widen the genetic base of the working germplasm through the use of techniques such as pre-breeding and novel biotechnology techniques such as mutation breeding and CRISPR to create genetic variation necessary to cope with the dynamics of biotic and abiotic stresses that affect soybean production in Uganda.

Key words: *Glycine max,* genetic relationship, microsatellite or simple sequence repeat (SSR), molecular markers, genetic vulnerability.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is a grain legume crop of great potential in Sub-Saharan Africa. It is an excellent source of protein and oil (approximately 40% protein and 20% oil content) for human food and animal feed (Ibanda et al., 2018). In Uganda and some other parts of Sub-Saharan Africa, soybean is increasingly becoming a

popular food and cash crop (Tukamuhabwa et al., 2016). This is evidenced by the rapid increase in number of industries involved in processing soybean in the region for food in the last decade (Tukamuhabwa and Obua, 2015). Due to the high protein content, soybean flour is often used to competent cereal flours mainly sorghum and maize lacking essential amino acids to boost their nutritional value (Tukamuhabwa and Oloka, 2016). Despite the importance of soybean in Uganda, its production is very low in some parts of the country probably due to limited number of commercial varieties. The northern region of Uganda is the highest producer of soybean with mean dry grain yield of 1804 kg/ha while the west nile region reported the least mean yield of 247.1 kg/ha (Tukamuhabwa et al., 2016).

Soybean being a self-pollinated crop has narrow genetic base (Kumawat et al., 2015). In the recent past, the soybean breeding program in Uganda focused on targeted hybridization using few selected genotypes as parental lines such as Duiker, GC001, UG05 and MNG. This in consequence led to narrowing the genetic base of the Ugandan germplasm, increasing vulnerability to changes in biotic and abiotic stresses (Ssendege et al., 2015; Tukamuhabwa and Oloka, 2016). For instance, the released soybean varieties in Uganda are susceptible to groundnut leaf miner (Namara, 2015; Ibanda et al., 2018) and bruchids (Msiska et al., 2018).

Although the national soybean breeding program in Uganda has been actively involved in developing improved varieties to meet the ever-changing biotic and abiotic factors as well as needs of farmers and processors in the diverse environments of the country (Tukamuhabwa et al., 2012). The extent of genetic relatedness among newly developed elite material and released soybean varieties has not been fully understood. Therefore, the assessment of germplasm genetic diversity among available released varieties and elite genotypes is essential to ensure efficient selection and recommendation of diverse lines as parents, for advancement, commercialisation and designing future breeding efforts to improve soybean yield, quality and resistance to pest and diseases. The knowledge of genetic diversity in the available soybean genotypes could help the breeder to understand the structure of germplasm and predict which parental combinations would produce the best progeny and facilitate to increase the genetic variation of breeding material for selection. Furthermore, the candidate varieties should always possess some genetic distinctiveness from other commercial varieties. In addition, assessment of genetic diversity among the genotypes helps the breeder for variety protection (Bisen at al., 2015).

Several methods have been used to assess genetic diversity among different soybean accessions including the use of morphological and agronomic traits, isozymes, pedigree information and DNA markers (Chakraborty et al., 2018). However, the use of morphological and agronomic traits for assessing genetic diversity are highly affected by environmental factors, makes examination of distinctiveness difficult (Gupta and Manjaya, 2017; Chauhan et al., 2015; Ghosh et al., 2014; Chakraborty et al., 2018). In addition, the use of pedigree information is also affected by uncertain or incomplete data and possible errors in data capture (Oda et al., 2015). The limitation of data provided by the use of isozymes permits the use of DNA markers in genetic diversity studies (Chauhan et al., 2015). The use of DNA markers has been considered more informative, reliable and reproducible compared to the commonly used conventional methods like phenotypic descriptors and pedigree analysis (Chakraborty et al., 2018).

Among the different DNA markers are; Amplified Fragment Length Polymorphisms (AFLPs), Single Nucleotide Polymorphisms (SNPs), Restriction Fragment Length Polymorphisms (RFLPs), Microsatellites/ Simple Sequence Repeats (SSRs) and Randomly Amplified Polymorphic DNAs (RAPDs) have been widely used in studying genetic diversity in soybeans, each with its own merits and demerits (Khare et al., 2013; Chakraborty et al., 2018). For molecular characterization and genetic diversity studies in soybean, SSR markers have been considered as the molecular markers of choice because of their abundance, codominance, high reproducibility (Kujane et al., 2019; Koutu et al., 2019), high polymorphism compared to RFLPs, AFLPs and RAPDs (Kumawat et al., 2015; Chakraborty et al., 2018; Moniruzzaman et al., 2019) and have much greater ability to identify unique alleles in parental and elite soybean germplasm than any other markers (Tantasawat et al., 2011). Therefore, the objective of this study was to determine the level genetic diversity that exists among released and elite soybean genotypes in Uganda based on the SSRs molecular markers.

MATERIALS AND METHODS

Plant materials

The study was carried out at Makerere University Agricultural Research Institute Kabanyolo (MUARIK) screen house and MaRCCI Biotechnology Laboratory. Thirty four genotypes were used in this study comprising of 23 elite genotypes and 11 released varieties (Table 1). These elite genotypes have desirable yield, percentage protein and oil content as well good agronomic traits for Uganda's diverse agro-ecological zones.

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Table 1. Description of genetic material which were used in the study.

Entry	Genotype	Pedigree	Source	Released
1	Duiker × 3N-5	Duiker × 3N-5	MakSoy	AYT
2	GC × 2N-1	GC × 2N-1	MakSoy	AYT
3	BSPS 48A-27-1	BSPS 48A-27-1	MakSoy	AYT
4	BSPSS 48A-28-1	BSPSS 48A-28-1	MakSoy	AYT
5	NGDT8.11× 14.16B	NGDT8.11× 14.16B	MakSoy	AYT
6	NII × GC 13.2	NII × GC 13.2	MakSoy	AYT
7	BSPS 48A-25-1	BSPS 48A-25-1	MakSoy	AYT
8	Nam II GC 17.3	Nam II GC 17.3	MakSoy	AYT
9	NII × GC 35.3-2	NII × GC 35.3-2	MakSoy	AYT
10	NG 14.1 × UG5	NG 14.1 × UG5	MakSoy	AYT
11	Nam 4M × 2N-2	Nam 4M × 2N-2	MakSoy	AYT
12	NII × 35.3-3	NII × 35.3-3	MakSoy	AYT
13	G8586 × UG5	G8586 × UG5	MakSoy	AYT
14	NDGT 8.11× 3N-1	NDGT 8.11× 3N-1	MakSoy	AYT
15	BSPS 48A-28	BSPS 48A-28	MakSoy	AYT
16	Bulindi 18.4B	Bulindi 18.4B	MakSoy	AYT
17	Maksoy 4N	Duiker x GC 00138-29	MakSoy	2014
18	BSPS 48A-24-1	BSPS 48A-24-1	MakSoy	AYT
19	Bulindi 24.1A	Bulindi 24.1A	MakSoy	AYT
20	NII × GC 35.3-1	NII × GC 35.3-1	MakSoy	AYT
21	NDGT 8.11 × 3N-2	NDGT 8.11 × 3N-2	MakSoy	AYT
22	2N × GC	2N × GC	MakSoy	AYT
23	Mak 3N × 1N	Mak 3N × 1N	MakSoy	AYT
24	NG 14.1 × NII-1	NG 14.1 × NII-1	MakSoy	AYT
25	Maksoy 3N	GC 00138-29 x Duiker	MakSoy	2013
26	Maksoy 6N	-	MakSoy	2017
27	Kabanyolo1	Mutant of Clark 63	Uganda	1987
28	Maksoy 1N	TGX 1835-10E	IITA	2004
29	Maksoy 5N	Nam 2 x GC 00138-299	MakSoy	2013
30	Maksoy 2N	Maksoy 1N x Duiker	MakSoy	2008
31	Namsoy 4 M	Nam2 x GC00 139-29	NaCRRI	2004
32	Nam 1	ICAL 131	NaCRRI	1990
33	Namsoy 3	Kabanyolo 1 x Nam 1	NaCRRI	1995
34	Nam 2	TGM 79	IITA	1992

AYT= Advanced yield trial elite genotypes (not yet released).
Source: Namara (2015).

DNA isolation and quantification

Total genomic DNA was extracted from two week old plants following the cytel trimethyl ammonium bromide (CTAB) method (Maughan et al. 1995). Prior to use, DNA quality and concentration were determined using NanoDrop ND-1000 spectrophotometer and the final concentration was adjusted to 50 ng/µl as described by Bisen et al. (2015).

Polymerase chain reaction

A total of 31 SSR markers that were previously mapped and evenly distributed on the 20 linkage groups by Cregan et al. (1999) were selected for initial screening. Gradient PCR was carried out for each primer with six randomly selected soybean DNA samples to standardize the annealing temperature for final amplification. Twenty-one SSR primers (Table 2) showed good amplification were used for further study across 34 soybean genotypes. Most of the SSR markers used had an AAT motif due to their abundance and highly polymorphic nature in soybean genome (Narvel et al., 2000). A PCR reaction volume of 12 µl comprising 2 µl genomic DNA (50 ng/µl), 5 µl of liquid premix, 4 µl of distilled water and 0.5 µl of each primer (10 nmol) was prepared. Amplification process was carried out in a thermocycler with the following conditions; Initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 2 min, annealing temperature at 48 to 60°C for 50 s, extension at 72°C for 50 s and a final extension at 72°C for 7 min. The PCR products were fractionated by gel electrophoresis on 2% metaphor agarose gel stained with Gel RedTM Nucleic Acid Stain (10 µl /100 µl of 1X TAE buffer) run at 100 volts for 1 h along with a 100-bp ladder as a size standard. Gel images were taken

Table 2. SSR primers and their linkage groups used for genotyping 34 soybean genotypes.

SSR primer	Forward primer	Reverse primer	Linkage group
Satt126	GCTTGGTAGCTGTAGGAA	ATAAAACAAATTCGCTGATAT	B2
Satt329	GCGGGACGCAAAATTGGATTTAGT	GCGCCGAATAAAACGTGAGAACTG	A2
Sat_409	GCGGAGGTTTGTGCATTTCTAGGTCTTC	GCGACGCGTATGTACATAAAATATGCTGTT	A2
Satt717	GCGTTTTGTGATTTGTTTTCCTCATTTACT	GCGGCTATCAAACATTTTTACATGATGGTTA	A1
BE806308	GCGATTTGACCCCGTTCATACAT	GCGGCAGAAATCCGCTCTCTTTA	B1
Satt173	TGCGCCATTTATTCTTCA	AAGCGAAATCACCTCCTCT	O
Satt185	GCGCATATGAATAGGTAAGTTGCACTAA	GCGTTTTCCTACAATAATATTTCAT	E
Satt409	CCTTAGACCATGAATGTCTCGAAGATA	CTTAAGGACACGTGGAAGATGACTAC	A2
SOYHSP176	TTTTTGTTTAAGTTACTGTACTGT	GCTAGTCTTCTACAACCTTCTA	F
Satt411	TGGCCATGTCAAACCATAACAACA	GCGTTGAAGCCGCCTACAAATATAAT	E
Satt431	GCGTGGCACCCTTGATAAATAA	GCGCACGAAAGTTTTTCTGTAACA	J
Satt245	AACGGGAGTAGGACATTTTATT	GCGCCTCCTGAATTTCAAAGAATGAAGA	M
Satt264	CCTTTTGACAATTATGGCATATA	GCATAGAAGGGCATCATTCAGAT	K
Satt373	TCCGCGAGATAAATTCGTAAAAT	GGCCAGATACCCAAGTTGTACTTGT	L
Satt440	TGAGAACGTTTGAAAAGAGAT	GAAGAGATTAAGCATAAAGAATACTT	I
Satt406	GCGTGAGCATTTTTGTTT	TGACGGGTTTAATAGCAT	J
Satt216	TACCCTTAATCACCGGACAA	AGGGAACTAACACATTTAATCATCA	D1b
Sat_084	AAAAAAGTATCCATGAAACAA	TTGGGACCTTAGAAGCTA	N
Satt211	GAAAAAGCCCACATCCAA	CATGGGCATGCAGTAACA	A1
Satt126	GCTTGGTAGCTGTAGGAA	ATAAAACAAATTCGCTGATAT	B2
Sat_366	GCGGCACAAGAACAGAGGAAACTATT	GCGGACATGGTACATCTATATTACGAGTATT	J

Source: Cregan et al. (1999).

using a Bio Doc-ItTM Imaging System.

Data scoring and analysis

The PCR products were analyzed by scoring the presence and absence of a band based on allele size for all 21 polymorphic primers. The SSR primer band appearing without ambiguity was scored as 1 (present) and 0 (absent) across 34 genotypes. Genetic diversity parameters, such as number of effective alleles, heterozygosity, fixation index, Shannon's information index were estimated using GenAlEx 6.51 (Peakall and Smouse, 2012). DARwin 6.0.21 Perrier and Jacquemoud-Collet (2006) was used to determine genetic similarity among genotypes by estimating dissimilarity coefficients using Jaccard's genetic similarity coefficient. The hierarchical cluster analysis was performed using dissimilarity coefficients with unweighted neighbor joining clustering algorithm. The effectiveness for cluster analysis was evaluated using 1000 bootstrapped (not displayed on Figure 1) replicates. The allelic diversity at each locus was determined as polymorphic information content (PIC) based on equation of Anderson et al. (1993):

$$PIC = 1 - \sum P_i^2$$

Where, P_i is the frequency of i^{th} allele in the set of genotypes analyzed, calculated for each SSR locus.

Furthermore, DARwin 6.0.21 was used to perform principal coordinate analysis (PCoA) to depict multiple dimensions of the distribution of released and elite genotypes in a scatter plot (Hipparagi et al., 2017) to complement the information obtained from hierarchical cluster analysis (Tantasawat et al., 2011).

RESULTS

Genetic diversity parameters

The results of expected genetic diversity parameters obtained at each locus across 34 soybean genotypes are presented in Table 3. A total of 59 alleles with an average of 2.85 alleles per locus were amplified among the genotypes. The fragment size of these 59 alleles varied from 100 to 375 bp with Satt406 recording the largest amplicon range (250 to 375 bp), while Satt411 had the smallest amplicon range of 100 to 160 bp. The number of alleles varied from 2 (Satt126, Sat_409, Satt717, BE806308, Satt185, Satt264 and Sat_084) to 4 (Satt216, Satt431 and Satt411) and the frequency of major allele ranged from 0.324 for primer Satt431 to 0.882 for BE806308 with an average of 0.532 among genotypes. The PIC value ranged from 0.208 (BE806308) to 0.741 (Satt411) with an average of 0.5870 and the number of effective alleles varied from 1.658 (Satt717) to 3.642 (Satt411) with an average of 2.362. Shannon's information index ranged from 0.362 (BE806308) to 1.337 (Satt411) with an average of 0.894 across all the primers used. The observed heterozygosity varied from 0.000 to 0.088 with an average of 0.010, while the expected heterozygosity (gene diversity) ranged from 0.208 (BE806308) to 0.725 (Satt411), with an average of

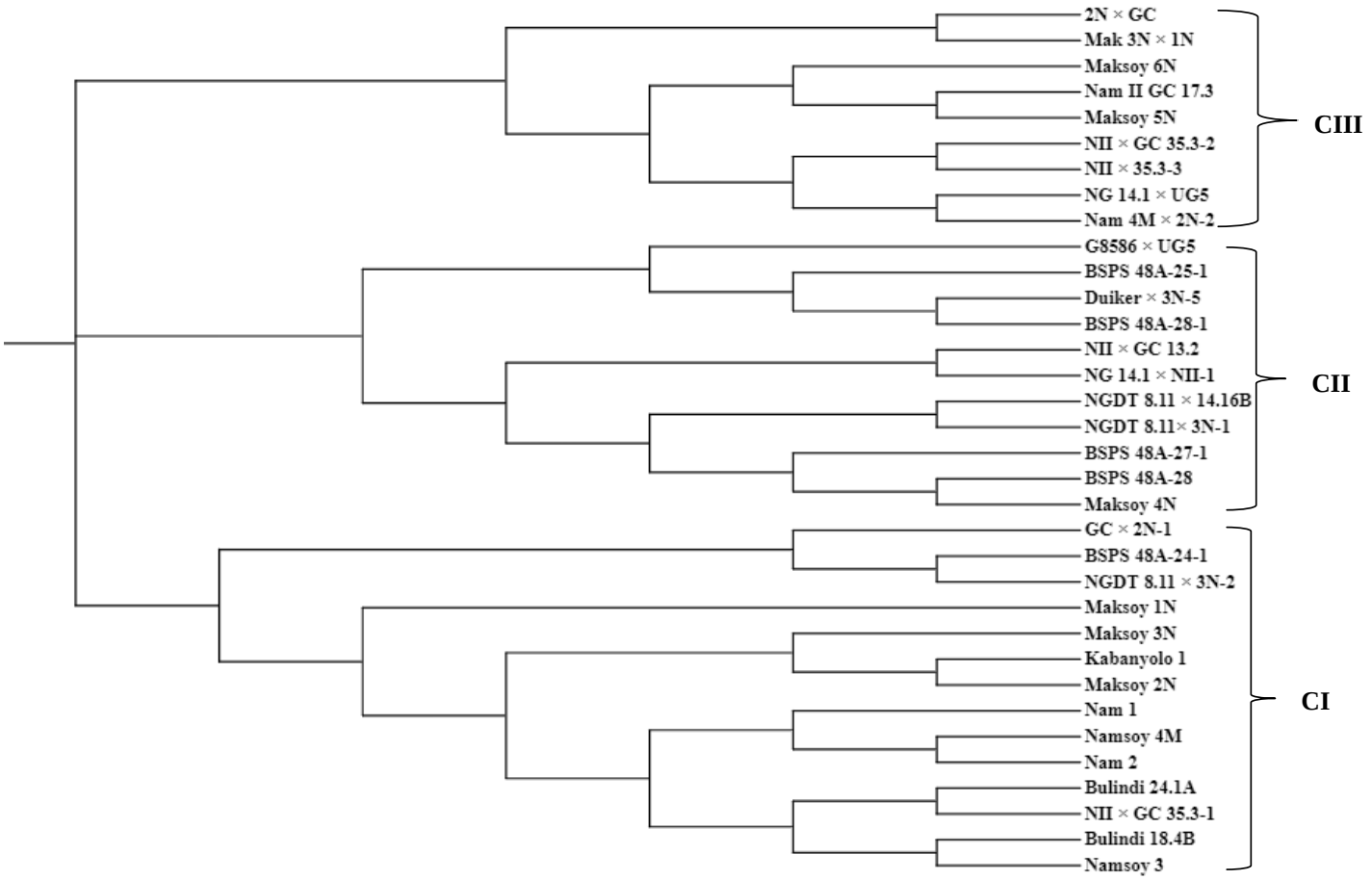

Figure 1. Dendrogram showing genetic diversity among 34 soybean genotypes based on unweighted neighbor joining clustering of Jaccard's similarity coefficients.

0.548. Furthermore, the fixation index varied from 0.862 to 1.000 with an average of 0.983. Figure 3 and Figure 4 shows the gel electrophoresis DNA bands amplified by primers Satt373 and Satt216 across the 34 soybean genotypes; with genotype 1-17 on the upper lane, while the bottom lane for genotype 18-34 and M denotes the 100 bp DNA ladder.

Hierarchical cluster analysis

Hierarchical cluster analysis which showed the genetic relationship among the genotypes using a dendrogram constructed from genetic distance dissimilarity matrix (Figure 1). The results showed that all the 34 genotypes were grouped into three major clusters, CI, CII and CIII, with cluster I comprising of 14 genotypes, cluster II comprising of 11 genotypes and cluster III comprising of 9 genotypes, respectively. Cluster I was further partitioned into two distinct sub-clusters with 11 and 3 genotypes, respectively. The majority of released varieties by Makerere University Soybean Research Centre Kabanyolo (MakSoy) and National Crop Resources Research Institute (NaCRRI) soybean breeding program belonging to CI. Cluster II formed two distinct two sub-clusters with 7 and 4 genotypes respectively. Most of the elite genotypes fell in cluster II, with only one released variety Maksoy 4N. Cluster III also consisted of two sub-clusters with 7 and 2 genotypes, respectively, with genotypes from Makerere University Soybean Research Centre Kabanyolo and only with two recently released varieties Maksoy 6N and Maksoy 5N belonging to this category.

Principal coordinate analysis (PCoA)

The results of the Principal Coordinate Analysis (PCoA) performed to further assess genetic relationship among released varieties and elite soybean genotypes showed three groups with PCoA explaining 28.9% of total variation (Figure 2). The first two axes discriminated the released varieties and elite soybean genotypes with PC1 and PC2 (the first and second principal coordinates) accounted for 17.2 and 11.7% of the total variation, respectively.

Table 3. Estimated genetic diversity parameters obtained at each locus across 34 soybean genotypes.

Marker	Na	Allele size range (bp)	Major allele frequency	Ne	I	Ho	He	F	PIC
BE806308	2	200 - 210	0.882	1.262	0.362	0.000	0.208	1.000	0.208
Sat_084	2	151 - 180	0.618	1.895	0.665	0.000	0.472	1.000	0.472
Sat_366	3	185 - 205	0.471	2.762	1.058	0.088	0.638	0.862	0.587
Sat_409	2	150 - 200	0.559	1.867	0.657	0.000	0.464	1.000	0.583
Satt126	2	120 - 148	0.647	1.832	0.647	0.030	0.454	0.933	0.457
Satt173	3	210 - 297	0.441	2.689	1.036	0.000	0.628	1.000	0.638
Satt185	2	250 - 270	0.500	2.000	0.693	0.000	0.500	1.000	0.500
Satt211	3	100 - 180	0.618	1.934	0.760	0.000	0.483	1.000	0.493
Satt216	4	150 - 210	0.382	3.051	1.184	0.000	0.672	1.000	0.693
Satt245	3	180 - 211	0.559	2.439	0.991	0.000	0.590	1.000	0.590
Satt264	2	200 - 220	0.559	1.973	0.686	0.000	0.493	1.000	0.493
Satt285	3	200 - 250	0.647	1.984	0.855	0.000	0.496	1.000	0.525
Satt329	3	250 - 300	0.559	2.355	0.958	0.030	0.575	0.947	0.587
Satt373	3	225 - 290	0.441	2.847	1.073	0.000	0.649	1.000	0.649
Satt406	4	250 - 375	0.353	3.501	1.312	0.031	0.714	0.956	0.733
Satt409	3	183 - 200	0.441	2.604	1.027	0.000	0.616	1.000	0.721
Satt411	4	100 - 160	0.353	3.642	1.337	0.000	0.725	1.000	0.741
Satt431	3	205 - 250	0.324	2.985	1.096	0.033	0.665	0.950	0.720
Satt440	3	185 - 215	0.588	2.067	0.883	0.000	0.516	1.000	0.598
Satt717	2	240 - 250	0.706	1.658	0.586	0.000	0.397	1.000	0.431
SoyHSP176	3	100 -170	0.529	2.256	0.913	0.000	0.557	1.000	0.607
Total	59	-	11.177	49.601	18.778	0.213	11.513	20.648	12.026
Mean	2.81	-	0.532	2.362	0.894	0.010	0.548	0.983	0.573
SE	0.15	-	-	0.134	0.055	0.005	0.026	0.008	-

Na=Number of alleles, Ne=Number of effective alleles, I=Shannon's information index, Ho= Observed heterozygosity, He=Expected heterozygosity (gene diversity), F= Fixation index, PIC= Polymorphic information content, SE= Standard error.

DISCUSSION

The reliability, reproducibility and authentic results obtained from using SSR markers have made them widely preferred in genetic diversity studies. Out of 31 SSR primer pairs screened, only 21 primer pairs, distributed on 15 of 20 linkage groups of soybean (Cregan et al., 1999), amplified scorable bands and a total of 59 alleles detected with a range of 2 (Satt126, Sat_409, Satt717, BE806308, Satt185, Satt264 and Sat_084) to 4 (Satt216, Satt431 and Satt411) alleles per locus. The lower allele number indicated low allelic diversity/ richness in the present set of soybean genotypes evaluated. Allelic richness (average number of alleles per locus) is an effective index for diversity evaluation but it is largely dependent on the sample size (Hipparagi et al., 2017), suggesting that to improve the allelic richness more genotypes need to be introduced into the breeding program to be able to enhance genetic diversity (Widaningsih et al., 2014). The number of alleles observed in this study is comparable to those reported by Kumawat et al. (2015) on 82 indigenous and exotic soybean accessions of different maturity groups and source in India where 2.9 alleles were detected per locus with an average polymorphic information content (PIC) value of 0.58. Similarly, Hipparagi et al. (2017) also reported 2.61 alleles with an average PIC value of 0.36 among 75 soybean genotypes assayed by 21 SSR markers in India. Bisen et al. (2015) detected 2.22 alleles per locus with an average PIC value of 0.199 using 16 SSR markers on 38 soybean varieties in active seed multiplication chain together with all varieties developed and released by JNKVV in India.

The average polymorphic information content (PIC) (a measure of the allelic diversity of SSRs) observed in this study was 0.573 which was consistent with previous studies (Widaningsih et al., 2014; Kumawat et al., 2015; Ghosh et al., 2014; Wang et al., 2010). A total of 20 markers excluding BE806308 had the PIC values greater than 0.4 indicating that these markers were highly informative for discriminating and distinguishing released varieties and elite genotypes, and these markers with high PIC values occurred on 14 separate linkage groups indicating that molecular polymorphism was spread across different regions of the genome (Song et al., 2010). The PIC was highest for the SSR primer Satt411

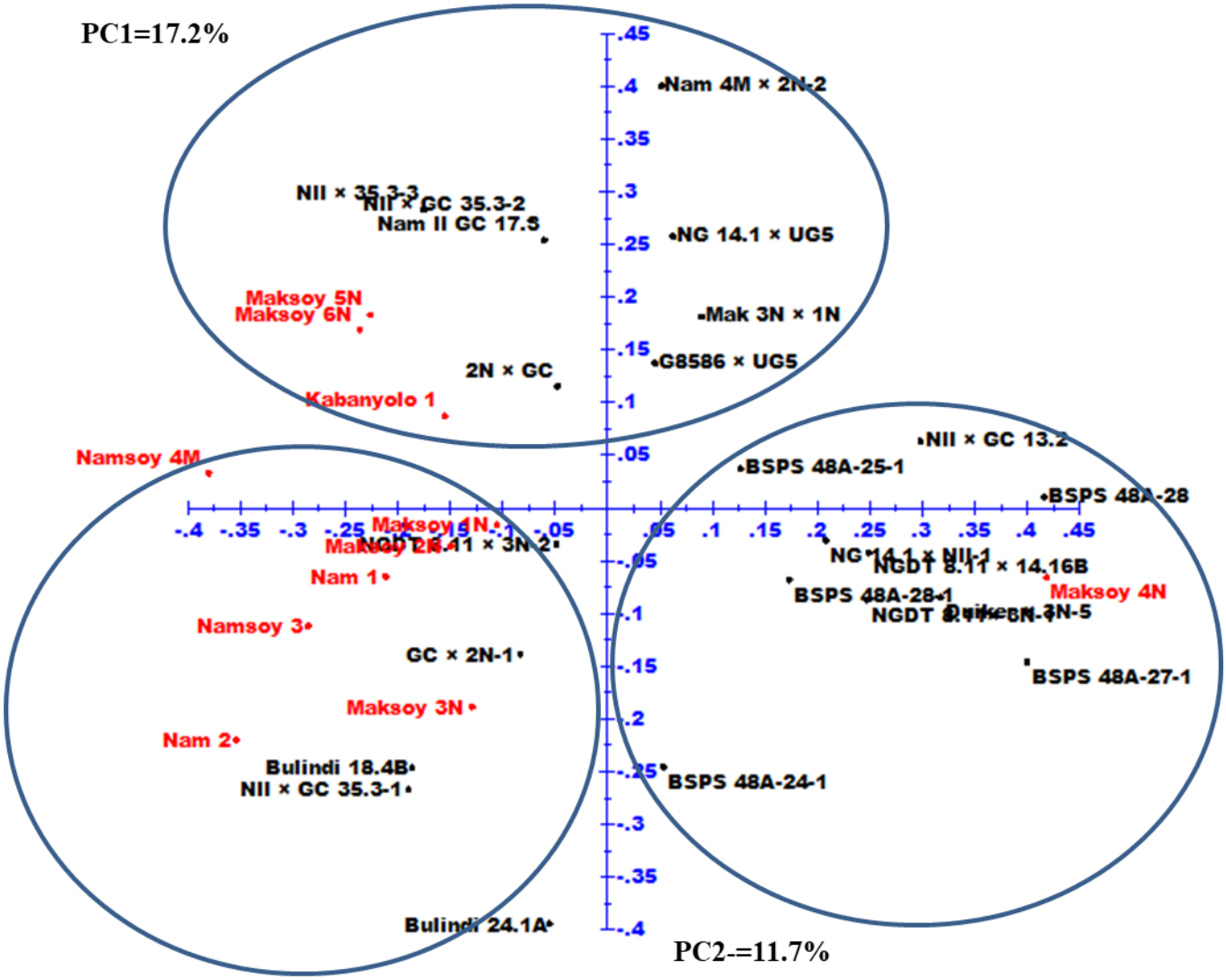

Figure 2. Principal coordinate analysis (PCoA) for 34 soybean genotypes on the basis of SSR marker data (Red labels = released varieties, and black labels = elite genotypes).

(0.741) and was lowest for the primer BE806308 (0.208) indicating that primer Satt411 was highly informative, effective and useful tool to determine the genetic differences among the released varieties and elite soybean genotypes and to study the phylogenetic relationship. Previous study by Kumawat et al. (2015) reported the PIC value of 0.50 for SSR primer Satt411 and 0.41 for primer BE806308 on genetic diversity analysis of 82 soybean accessions in India.

The gene diversity/expected heterozygosity (He) varied from 0.208 (BE806308) to 0.725 (Satt411) with an average of 0.548 which implied that there was moderate genetic variation existing among released varieties and elite genotypes. The presence of low - moderate genetic variation is not favorable in soybean breeding to mitigate dynamic pests, diseases and abiotic stresses that could not be handled by closely related cultivars. The moderate genetic variation observed among soybean genotypes in this study suggested the need for broadening genetic diversity through targeted hybridization of exotic germplasm with locally adapted elite germplasm. The gene diversity observed in the present study was lower than the previous studies reported by Widaningsih et al. (2014) (0.66); Song et al. (2013) (0.65); Zhao et al. (2018) (0.88) and Wang et al. (2015) (0.80) further indicating the need for introduction of more germplasm in the soybean breeding program.

The observed heterozygosity obtained from this study was 0.010. The reason for low heterozygosity is due to the fact that soybean is a highly self-pollinating crop which is expected to have low heterozygosity than mostly cross breeding crops (Zhang et al., 2013). Similar studies reported by Hipparagi et al. (2017) gave 0.11; and Zhao et al. (2018) reported 0.11 heterozygosity, so

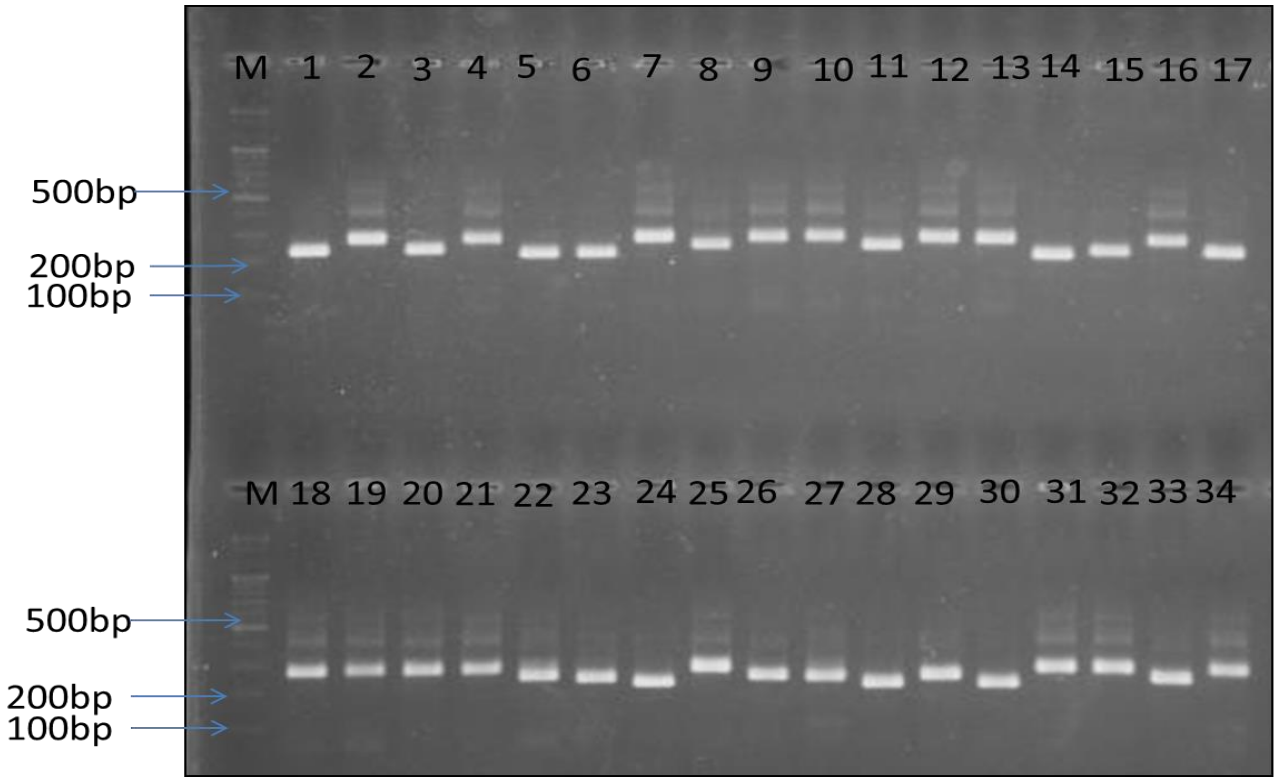

Figure 3. The gel electrophoresis DNA bands amplified by Satt373 marker across 34 soybean genotypes.

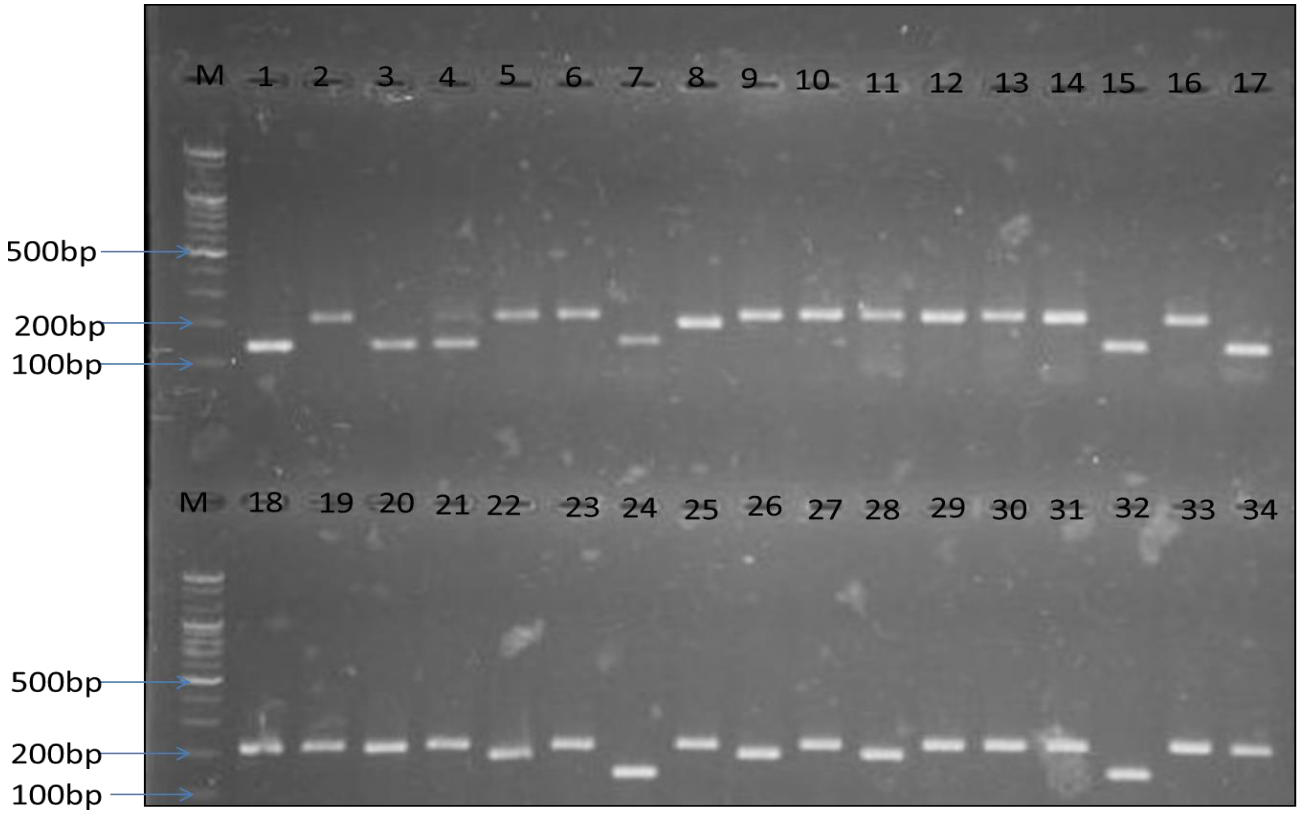

Figure 4. The gel electrophoresis DNA bands amplified by Satt216 marker across 34 soybean genotypes.

the heterozygosity observed in the present study was much lower than the value reported by the other researchers but some others studies reported low heterozygosity in the crop, for example Song et al. (2013) (0.045) and Li et al. (2008) (0.014). The Shannon's information index of 0.894 in the present study was lower than the results obtained by Zhao et al. (2018) who reported Shannon's information index of 2.528, but also reported fixation index of 0.987 which was comparable to the one obtained in this study. The results of Shannon's information index and fixation index revealed a shift from Hardy-Weinberg equilibrium, again indicating the presence of moderate genetic variation among the genotypes which could be attributed to the fact that these genotypes are sharing GC001 and Duiker as common parental lines (Table 1).

The hierarchical clustering divided the soybean genotypes into three distinct groups, with more than half of the released varieties grouped together (cluster I) indicating that most released varieties were developed from common parents. The clustering of large number of released varieties together in a single cluster implies that most of the released varieties will lose stability as well as resistance in case of changes in pests, diseases and abiotic stresses. Similarly, Ssendege et al. (2015) reported that Maksoy 1N, Maksoy 2N and Maksoy 3N were grouped in the same cluster which indicated that they are genetically related. The findings of this study are supported by the pedigrees on Table 1, which shows that these released varieties shared the common parents such as Duiker and GC001. Cluster II and III comprised of 17 elite genotypes with only three released varieties, that is, Maksoy 4N, Maksoy 5N and Maksoy 6N. The results revealed that most released varieties are genetically dissimilar from the elite material. Cluster analysis depicted that elite materials can be utilized in the breeding program due to its genetic diversity and uniqueness from many of the released varieties. This is crucial in plant breeding as the only sustainable way of widening and maintaining genetic dissimilarity among genotypes. Also, candidate varieties, that is, elite material should possess some genetic distinctiveness from other commercial varieties for future variety protection as suggested by Bisen at al. (2015). Further, the clustering analysis in the present study helps the breeder in understanding the genetic structure of the breeding material for effective and efficient selection, advancement and crossings in future which must be carried out between the clusters and avoiding within the clusters as way of widening and maintaining genetic dissimilarity among genotypes. The results of both the dendrogram and Principal Coordinate analysis (PCoA) were consistent with each other in grouping 34 soybean genotypes into three distinct classes which implied that most of the released varieties were related. While moderate genetic diversity identified among released and elite soybean genotypes of Uganda in the present study necessitates the need of broadening genetic diversity by introducing more exotic diverse germplasm along with utilization of wild relatives. The diverse genotypes identified in this study may serve as source of new alleles in soybean breeding program of Uganda in order to cope with ever-changing outbreak of pests, diseases and abiotic stresses.

CONCLUSION AND RECOMMENDATIONS

There is moderate genetic variation among released and elite soybean genotypes of Uganda. The SSR marker Satt411 was highly informative and could be an effective and useful tool to determine the genetic differences among the soybean accessions and the phylogenetic relationship. As a long term breeding strategy, the Makerere Soybean Breeding program can consider the use of pre-breeding and other novel biotechnology techniques such as mutation breeding or CRISPR that are known to enhance genetic diversity.

CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

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Evaluation of poultry waste medium and light quality for lipid accumulation in fresh water green microalgae isolate

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The global quest for production of biofuel at a competitive price with fossil fuel has driven research to the exploration of organic waste as a cost-effective growth medium for microalgal biodiesel production. In this study, poultry waste extract (PWE) was explored as growth medium for efficient biomass and some intracellular content accumulation by *Chlorella* sp. isolate for eight days. The analysis of the poultry waste (PW) revealed high concentrations of nitrogen (5.785%) and phosphorus (6.786 mg/L) minerals alongside magnesium, iron, zinc, potassium, manganese etc. Under phototrophic culture condition, 200 g/L PWE among the various PW concentrations tested elicited higher biomass and productivities than other PWE concentrations tested. PWE (200 g/L) yielded comparable cell biomass and lipid productivity (2.61±0.002 g/L) (0.143±0.0006 g/L/day) with the control (BG 11 medium) (3.55±0.0008 g/L) (0.152±0.004 g/L/day). White light or white+yellow lights were the most efficient in stimulating cell growth and lipid production. However, yellow light triggered significantly ($P < 0.05$) higher accumulation of chlorophyll-a content (9959 µg/ml) than white+yellow light (6479 µg/ml) at 200 g/L PWE concentration and more than white light at 400 g/L PWE concentration. The outcome of these research holds high prospect for simultaneous production of biolipid and chlorophyll-a by *Chlorella* sp using a cheap growth medium for cost-effective biodiesel production.

Key words: *Chlorella* sp, biodiesel, poultry waste extract, biomass, lipid content, light quality.

INTRODUCTION

Fossil fuels are valuable one-time energy gift to the human race and which are subject to depletion. Because energy is an indispensable need of life evidenced on world energy consumption reports (Brennan and Owende, 2010), man has resorted to finding an alternative source of energy for its continued existence. The adverse effect of fossil fuels on the environment such as greenhouse gasses emissions (Zhu et al., 2015) and climate change has triggered global concern and necessitated the search for an environmentally friendly

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alternative energy resource which cannot be depleted as the fossil fuels. Therefore, renewable energy which is environmentally friendly has become the global focus in terms of energy security to replace the current fossil fuels. The renewable energy such as wind energy, solar energy and bioenergy are environmentally friendly energy sources and help to reduce the greenhouse gas emission (Lam and Lee, 2012; Huang et al., 2010). Biodiesel- a common example of bioenergy resource- has received greater attention in recent years globally. Biodiesel is non-toxic, renewable and biodegradable and is considered as one of the most alternative fuels for diesel engines. Biodiesel is prepared from various sources including edible oils, non-edible oils, animal fats, legume plants, microalgae etc. The commercialization of biodiesel has not been achieved due to high cost of production which makes the product more expensive than fossil fuel. Hence, research has focused on cost-effective biodiesel production to make it competitive with fossil fuels. One of the effective ways to reduce the cost of biodiesel production is to use inexpensive and non-edible oils as feedstock. Currently, microalgae are considered as one of the most promising feedstock for biodiesel production. This is because microalgae can reproduce rapidly due to their simple cellular structure and high lipid productivity (Lam and Lee, 2012, 2014), and potential to grow in waste-formulated medium that contains nitrates and phosphates. Cultivation of microalgae in waste-formulated medium helps to minimize the cost of energy needed and as such reduces the cost of biodiesel production by microalgae. Waste such as compost contains high concentration of nutrient such as nitrate and phosphate (Lam and Lee, 2012; Ma and Hanna, 1999), which promote the growth of microalgae and economically improve microalgae biodiesel production (Zhu et al., 2015; Zhou et al., 2014). Cultivation of microalgae cells are usually influenced by pH, amount of nutrient added and types of compost. Therefore, this research is undertaken to investigate the potential of compost nutrient derived from poultry waste to grow microalgae isolate and to optimize isolate's biomass production as well as accumulation of some intracellular contents via batch cultivation method and varying qualities of light illumination.

MATERIALS AND METHODS

Medium composition and preparation

Blue-Green (BG) 11 medium

BG 11 growth medium was composed of (per litre): 0.25 g $NaNO_3$; 0.04 g K_2HPO_4; 0.075 g $MgSO_4 \cdot 7H_2O$; 0.027 g $CaCl_2 \cdot 2H_2O$; 0.006 g $C_6H_8O_7$ 0.006 g $C_6H_8O_7$.nFe.nNH_3; 0.001 g EDTA; 0.02 g $NaCO_3$; and 1.0 ml A_5 + Co stock solution. The A_5 + Co stock solution was prepared by dissolving (per litre): 2.860 g H_3BO_3; 0.222 g $ZnSO_4 \cdot 7H_2O$; 1.81 g $MnCl_2.4H_20$; 0.079 g $CuSO_4 \cdot 5H_2O$; 0.390 g $Na_2MoO_4 \cdot 2H_2O$; and 0.0494 g $Co(NO_3)_2.6H_20$.

Poultry waste extract (PWE) medium

Dried chicken manure was collected from Phinomar farm at Ngwo in Enugu state. Foreign objects were removed from the sample and sun dried for easy grinding into powder. Using Han et al. (2017) protocol; A slurry of 50, 100, 200 and 400 g/L respectively of the poultry waste sample powder was made using distilled water and refrigerated at 4°C using a beaker sealed with foil for 12 h. The slurry was filtered each using Whatman no 1 filter paper and the filtrate stored at 4°C for use.

Inoculum preparation

About 10% stock culture of microalga isolate (*Chlorella* sp) was inoculated in 500 ml Erlenmeyer flasks containing 200 ml BG 11 medium and incubated under continuous white light illumination for 10 days.

Cultivation of microalga isolate

Effect of different poultry waste extract (PWE) concentrations and light qualities on the growth of microalga isolate

Erlenmeyer flasks (500 ml) each containing 200 ml BG-11medium (pH = 7.3) were grouped according to the poultry waste extract concentrations as follows: Group 1, 2, 3, 4, and 5 for the 50, 100, 200 and 400 g/L PWs and control (containing BG 11 medium) respectively. Each group was in triplicates and the flasks were covered with foam plugs for aeration.

Approximately 10% of the inoculum volume of isolate was aseptically transferred into the growth medium in triplicates for each of the culture groups. They were incubated in a rectangular box at 28°C under continuous illumination by white light, white light+yellow light, yellow light or without light (flasks were wrapped with aluminum foils) for eight days and agitated manually thrice a day. The distance of the culture flasks from the light source (15 Watts lamp) was about 1.5 cm. Cell growth and pH were measured in replicates using UV/VIS spectrophotometer and digital pH meter respectively for eight days. The lipid content, carotenoid content, and chlorophyll content were determined at the end of cultivation.

Determination of cell growth and dry biomass

An aliquot (5.0 ml) of culture broth was sampled on two-day intervals under sterile condition in a clean bench. The cells were washed twice in distilled water and cell optical density was determined by measuring the samples at 680 nm using UV-Vis spectrophotometer (OD_{680}). The values were compared with standard calibration curves of optical density versus cell dry weight for the isolate.

Cell growth rate

The specific growth rate was calculated by the equation: μ (day^{-1}) = 1/t ln (X_m/X_0) Where X_m and X_0 (g/L) were the dry biomass at the end and beginning of the exponential phase, t (day) was the time between the two measurements, respectively.

Dry biomass

The dry biomass accumulated after cultivation was determined by the equation: Dry biomass (g/L) = X_m-X_n where X_m and X_n (g/L)

were the final biomass at the end of cultivation and initial biomass at the beginning of cultivation respectively.

pH determination

A homogenous sample was obtained using a 5 ml syringe from each flask, and transferred into a labelled glass test tube. The pH was determined using a pH meter at 2 days interval for 8 days and recorded accordingly for each sample.

Measurement of some intracellular contents

Determination of pigment content

An aliquot (5.0 ml) of culture broth was sampled and centrifuged at 5000 × g for 5 min, and rinsed twice with distilled water. The pellet was extracted with 5.0 ml 90% (v/v) methanol in a test tube wrapped with aluminum to prevent photo-oxidation at room temperature, and centrifuged at 5000 × *g* for 5 min. The supernatant was decanted into a separate test tube and the pigment content of the supernatant was determined by UV-VIS spectrometer (GENESYS 10S UV-Vis, Thermo Fisher Scientific, MA, USA) using the method of Lichtenthaler (1987) as follows:

Total carotenoid (mg/L) = $(1000A_{470} – 44.76A_{666} / 221)$
Ch-a = 16.72A645 – 9.16A645 (µg per ml solution)
Ch-b = 34.09A645 – 15.28A645 (µg per ml solution)
A = Absorbance, Ch-a = Chlorophyll a, Ch-b = Chlorophyll b. where 1000, 44.76, 221, 16.72, 9.16, 34.09 and 15.28 were constants (Lichtenthaler, 1987).

Measurement of lipid production

Drying and powdering biomass

The dry biomass obtained after drying the harvested wet biomass in a hot air oven at 70°C for 24 h was pulverized using mortar and pestle. The total lipid content of the microalgae sample was determined gravimetrically using method of Bligh and Dyer (1959). The total lipid content (g/g cell) was calculated thus:

Total lipid content (g/g cell) = (weight of lipid) g / (weight of powdered cell) g

Lipid productivity

The lipid productivity is a measure of the quantity of lipid accumulated by microalgae cell per day of cultivation and calculated thus:

Lipid productivity (g/L/day) = total lipid content (g/g cell) × dry biomass (g/L) / days of cultivation.

Poultry waste elemental analysis

Elemental analysis was conducted using Varian AA240 Atomic Absorption Spectrophotometer and standard method 4500-P B.5 and 4500-PE according to the method of American Public Health Association (APHA, 2005). Phosphate was measured using standard method 4500-P B.5 and 4500-PE (APHA, 2005). Nitrogen was measured using the method of Association of Analytical Chemists (AOAC) (1984).

Statistical analysis

The data obtained from comparison of poultry waste media concentration and the qualities of light illuminations in shake flask cultures were analyzed statistically using one-way analysis of variance (ANOVA), and the means were separated using Least Significant Different methods.

RESULTS

The mineral contents of fresh poultry waste

The elemental analysis of poultry waste revealed high concentrations of minerals such as nitrogen, phosphorus, potassium and some trace elements as shown in Table 1.

The effect of different concentrations of poultry waste (PW) extract on the cell biomass of microalga isolate

White light illumination produced the highest cell biomass of 2.60±0.002 g/L in 200 g/L PW medium, though lower than control (3.54±0.0008 g/L), while the least cell biomass (0.31±0.002 g/L) was produced by the cultures without illumination across the PW media concentrations tested (Figure 1). Also, the white+yellow light illumination produced higher cell biomass than the yellow light illumination only (Figure 1). Each of the PWE concentrations tested elicited comparable growth rates under white light and white+yellow light illuminations except 50 g/L PWE concentration which yielded the least cell growth rate under the different light qualities tested (Figure 2). Heterotrophic condition elicited the least cell growth rates in all the different PWE concentrations tested.

The effect of different concentrations of poultry waste (PW) under different qualities of light illumination on some intracellular contents of microalga isolate

The chlorophyll-a content of cells in 200 g/L PW cultures illuminated by white light (11849±1675 µg/ml) is higher than the values produced by other quality of light illuminations across the PW concentrations and the controls (Figure 3). Yellow light illumination elicited higher chlorophyll-a content in the isolate more than white+yellow light illumination across the PW concentrations except in 50 g/L PW medium (Figure 3). The culture without illumination (heterotrophic) yielded lower chlorophyll-a content than the illuminated cultures while the control and 100 g/L PW cultures produced higher contents of chlorophyll-a than the values obtained under white+yellow light illumination (Figure 3).

The accumulation of chlorophyll-b in the isolate was higher under white light illumination than other types of

Table 1. The mineral contents of poultry waste.

Contents	Concentrations (ppm)
Magnesium	29.506
Manganese	14.337
Iron	74.698
Copper	7.722
Zinc	11.639
Nitrogen (%)	5.785
Phosphorus (mg/L)	6.786
Calcium	9.675
Potassium ppm	8.343
Sulphur (mg/L)	4.967
Chlorine (mg/L)	15.7
Sodium	14.782
Molybdenum	0.0028

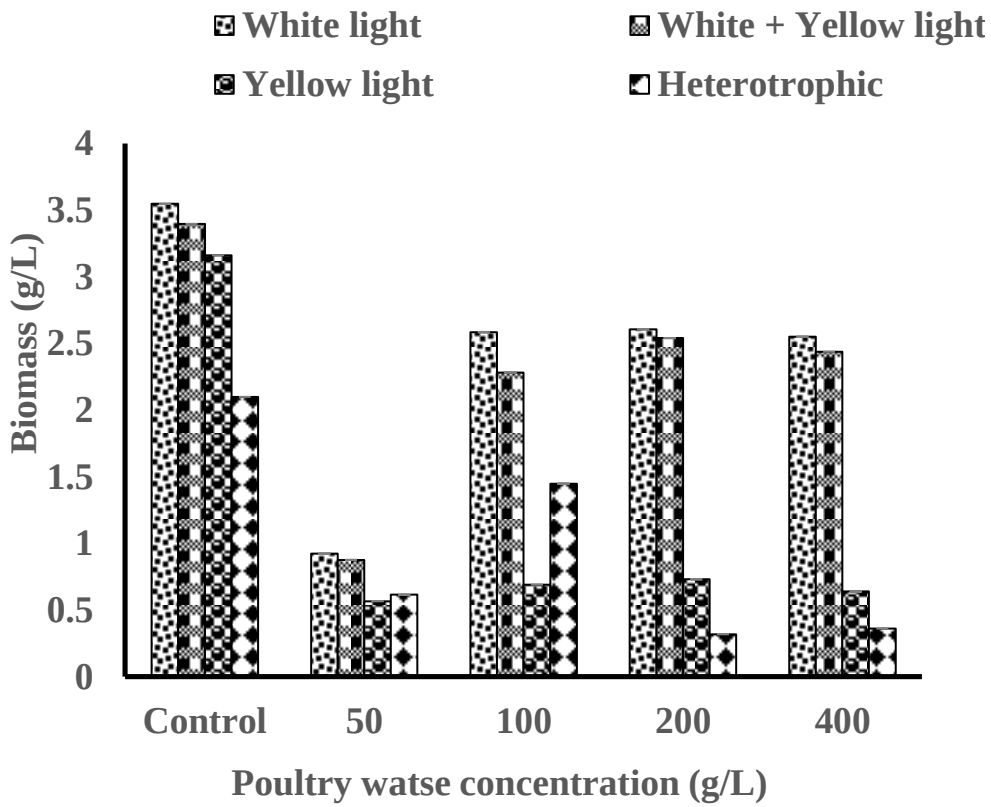

Figure 1. Effect of different qualities of light illumination on the cell biomass of microalga isolate cultivated in different concentrations of poultry waste for eight days at 28±0.2°C.

illuminations across the PW concentrations (Figure 4). The content was highest in 100 g/L PW medium (24034±1376 µg/ml) across the PW concentrations, although it was lower than the value produced by the control (28089±93 µg/ml). The cell cultures without light illumination yielded the least chlorophyll-b contents across the PW concentrations and the controls (Figure 4). The carotenoid content elicited by white light was higher than the values by other qualities of light illuminations for all the concentrations of PW media (Figure 5). The values produced by white+yellow light were higher than the value produced by yellow light only.

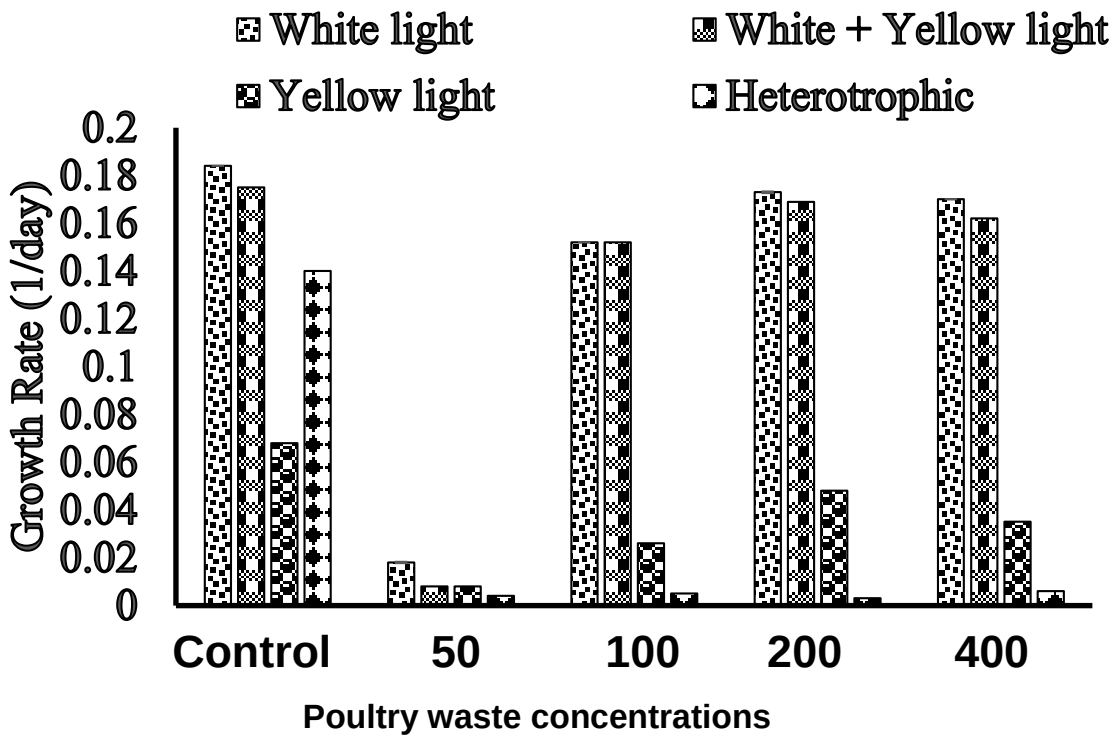

Figure 2. Effect of different qualities of light illumination on the cell growth rate of microalga isolate cultivated in different concentrations of poultry waste for eight days at 28±0.2°C.

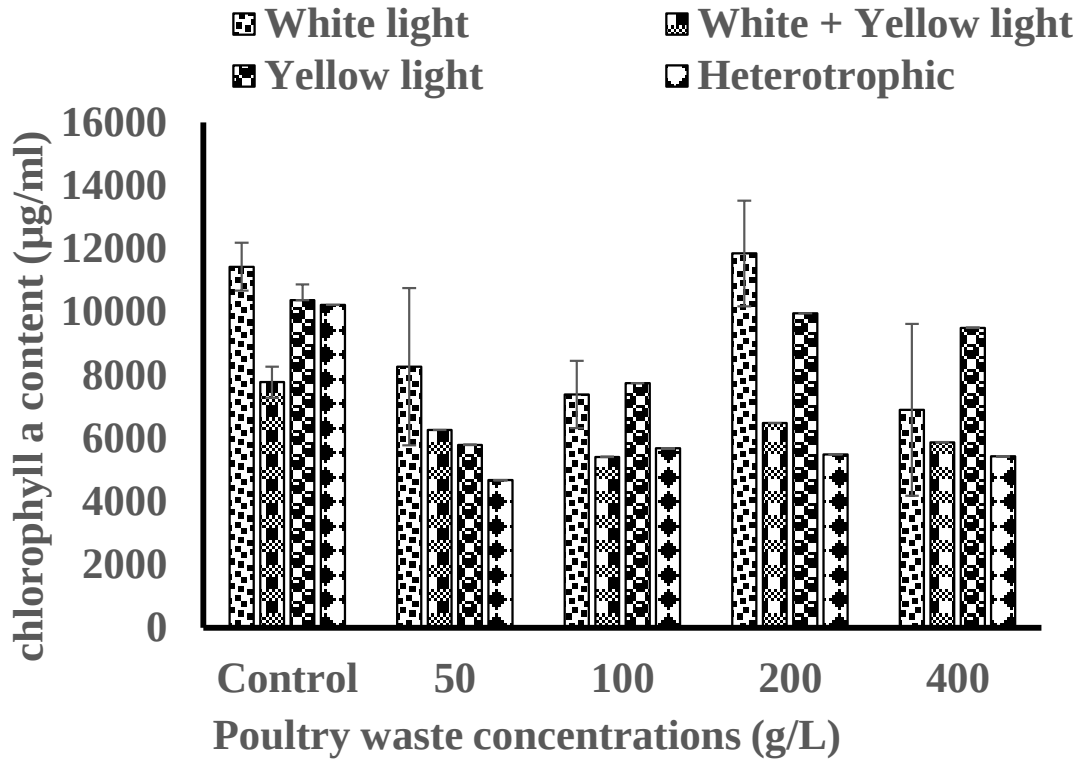

Figure 3. Effect of different qualities of light illumination on chlorophyll-a content of microalga isolate cultivated in different concentrations of poultry waste for eight days at 28±0.2°C.

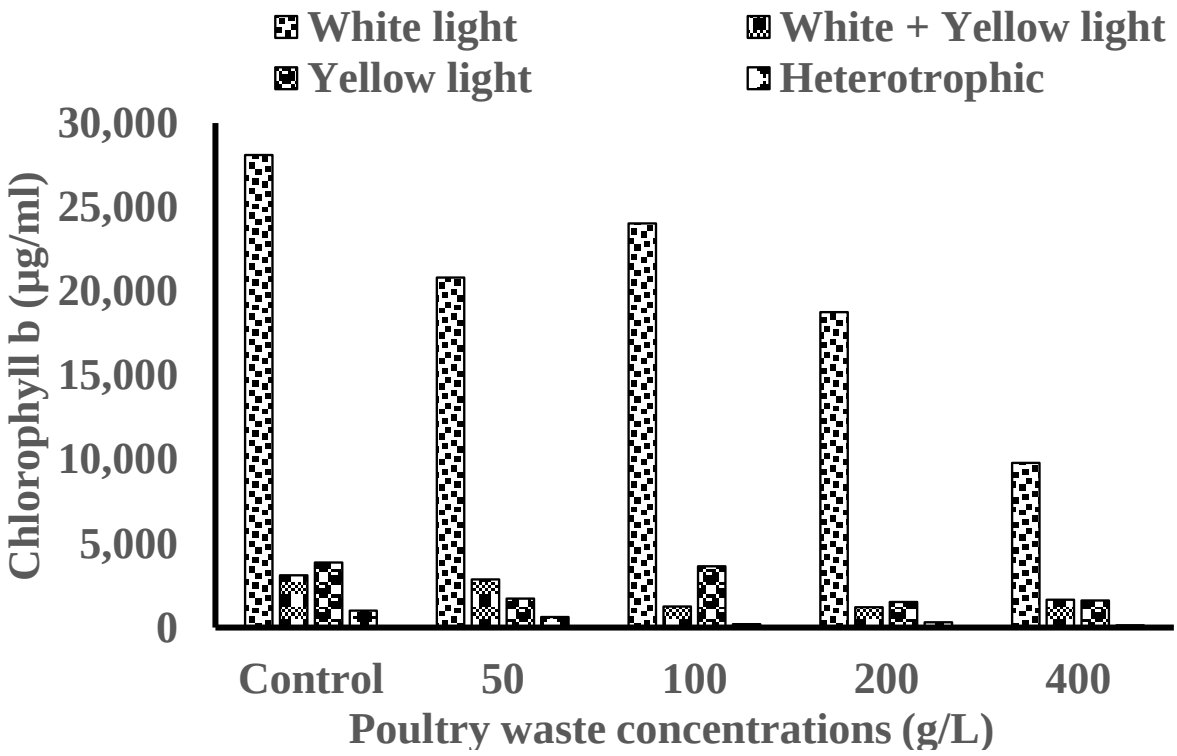

Figure 4. Effect of different qualities of light illumination on the chlorophyll-b content of microalga isolate cultivated in different concentrations of poultry waste for eight days at 28±0.2°C.

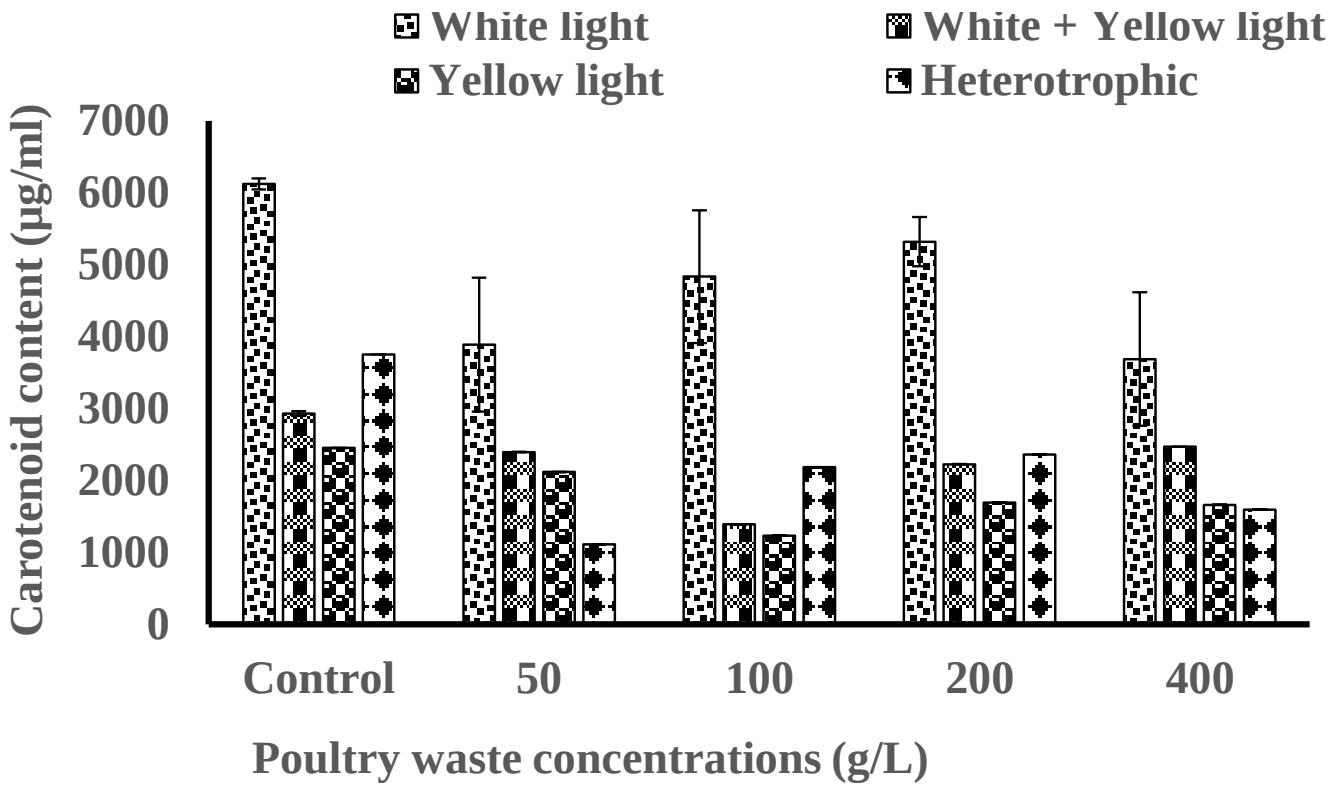

Figure 5. Effect of different qualities of light illumination on the carotenoid content of microalga isolate cultivated in different concentrations of poultry waste for eight days at 28±0.2°C.

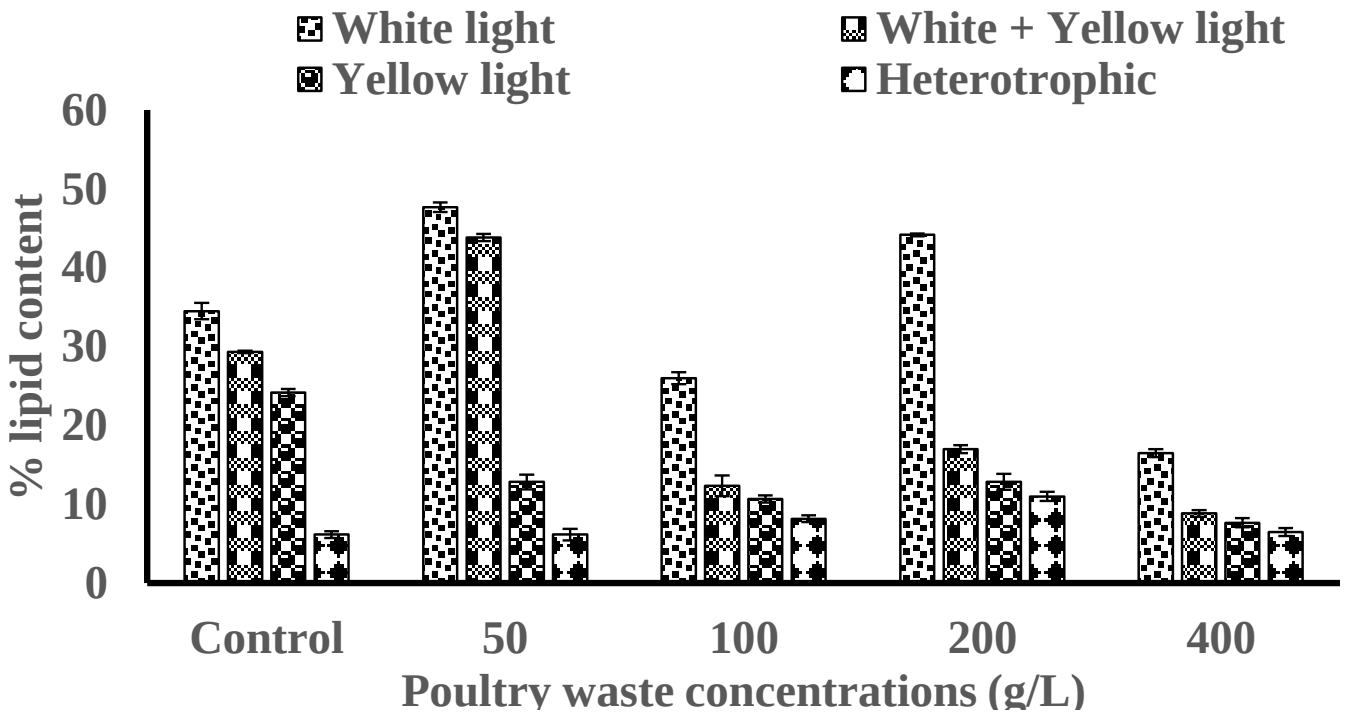

Figure 6. Effect of different qualities of light illumination on the lipid content of microalga isolate cultivated in different concentrations of poultry waste for eight days at 28±0.2°C.

The 50 g/L PW medium without light illumination produced the least carotenoid content (1113±0.66 µg/ml) while the 200 g/L PW medium under white light illumination produced the highest carotenoid contents (5320±341 µg/ml) (Figure 5).

The lipid content varied across the different PW media concentrations and light illuminations. The value produced by white light illuminations was higher than the values produced by other type of illuminations across the PW media concentrations (Figure 6). The cultures without light produced the least lipid contents, while the values produced by white+yellow light was higher than that of yellow light only (Figure 6). The 50 g/L PW medium under white light illumination produced the highest lipid content of 47±0.60%.

The lipid productivity by white light was higher than the values by other light types. The 200 g/L PW medium yielded the highest lipid productivity of 0.143±0.0006 g/L/day although lower than the value produced by the control (0.152±0.004 g/L/day) (Figure 7). The white+yellow light yielded higher lipid productivity than yellow light only. The culture without light illumination yielded the least lipid productivity across the PW media concentrations and the control (Figure 7).

DISCUSSION

The high concentrations of nitrogen and phosphorus in the poultry waste suggest the potential of poultry waste for cultivation of microalgae. Han et al. (2017) also affirmed that Chicken manure contains rich amount of nitrogen and phosphorus and has been used as crop fertilizer. These nutrients are responsible for eutrophication in the aquatic environment following the discharge or run-off of the poultry waste into aquatic environments, and resulting in algal blooms and hypoxic conditions in aquatic ecosystems (Han et al., 2017). These nutrient elements such as nitrogen, phosphorus, magnesium, iron, copper, etc as contained in the poultry waste extract (Table 1) are known to support the growth of microalgae. Chisti (2007) reported that growth medium for algal cell cultures must constitute essential micronutrients such as nitrogen and phosphorus. According to Mobin and Alam (2014), three primary nutrients (carbon, nitrogen, and phosphorus) and a number of micronutrients such as silica, calcium, magnesium, potassium, iron, manganese, sulphur, zinc, copper, and cobalt are required for producing algal biomass.

The growth of the microalga isolate in the different concentrations of poultry waste extract for the period of eight days confirms the wide reports on the potential of organic wastewater as alternative growth medium for the cultivation of microalgae (Renuka et al., 2015; Bhatnagar et al., 2011; Frampton et al., 2013). Hence, poultry waste extract is viewed as a potential cost effective medium for cultivation of microalgae biomass. Algal companies can

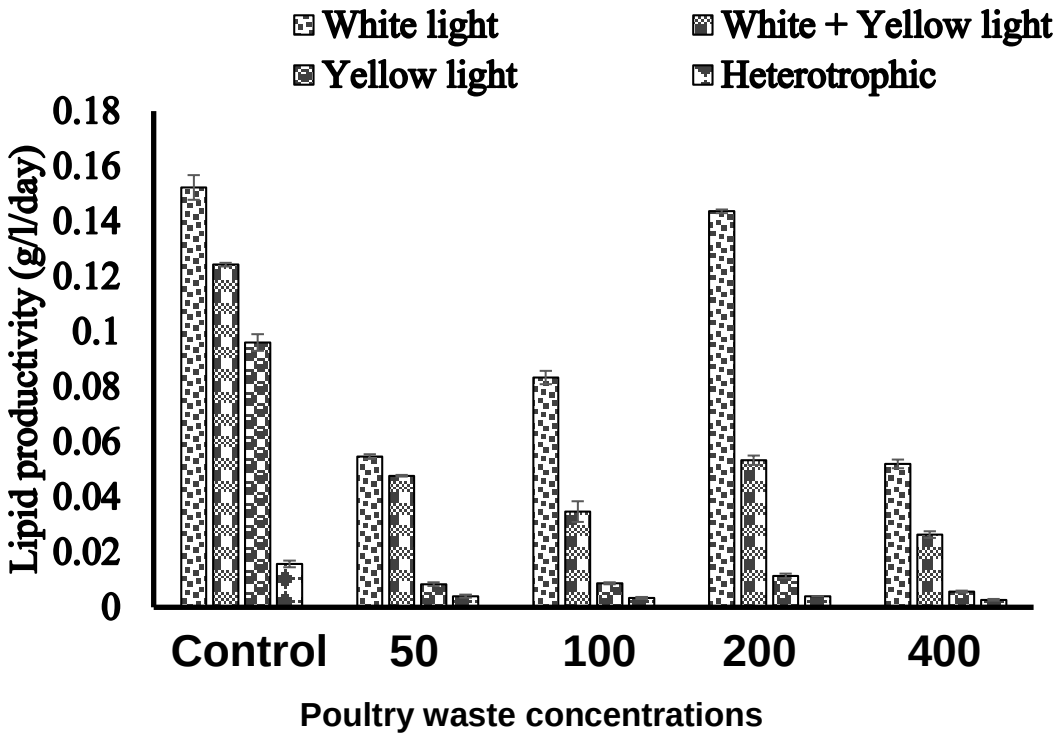

Figure 7. Effect of different qualities of light illumination on the lipid productivity of microalga isolate cultivated in different concentrations of poultry waste for eight days at 28±0.2°C.

save money and the environment if commercial fertilizer is replaced by Chicken manure (Han et al., 2017). Also, the growth of the microalga isolate in the poultry waste extract indicates the potential of the isolate for phycoremediation of wastewater since nutrients are removed from the medium. However, the quantities of nutrients were not assayed to determine the rate and extent of nutrient removal in this study. The variations in the cell biomass produced by different concentrations of the poultry waste (Chicken manure) extract are probably due to variations in the concentrations of nutrients contained therein. The increased concentration of PWE resulted in decreased dry cell biomass although there was no significant difference ($p > 0.05$) in the cell biomass produced by 100, or 400 g/L PWE. This report seems to differ from the report by Badawy (2008) that cell dry weight increased with waste concentration. However, the range of concentrations of waste tested in the studies, (0.5 - 4.0% against 0.2 - 0.6%) as well as the species of microalgae (*Scenedesmus dimorphus* against *Chlorella* sp) could account for the variation in the two reports. The control culture (BG 11) whose cell biomass was higher than the PWE concentrations tested may be due to optimum concentrations of the available nutrients (optimal N to P ratio) in the culture medium. However, this report differs from the report by Han et al. (2017) that chicken manure medium resulted in higher algal biomass than the standard medium BG11. The variation in the reports may be due to the difference in the physicochemical characteristics of the chicken manure media which directly or indirectly affect algal growth (Renuka et al., 2015). The difference in the microalgae species used as well as other environmental parameters such as light intensity may be implicated.

The reason for the significant increase in chlorophyll-a content ($p < 0.05$) by 200 g/L PWE above the values produced by other PWE concentrations is not known. However, it is widely known that phototrophy enhances the accumulation of pigments in microalgae (Eze et al., 2017). The significant increase in carotenoid content by the control above the test experiments ($p < 0.05$) is probably as a result of variations in light penetration into the cultures. The PWE media were colored which may have limited the quantity of light penetrating into the cultures thereby impacting negatively on carotenoid induction by the microalga isolate.

The different qualities of light impacted on the isolate differently in terms of cell growth and intracellular content accumulation. The reason for the non-significant difference in the cell growth rate and biomass of isolate by white light or white+yellow light is not known. However, the higher cell growth rate and biomass provided by white light ($p< 0.05$) agrees with the report of Wong (2016). The report of maximum dry biomass of 1353.33 mg/L at cool white light of 110 µmol m^{-2} s^{-1} during the 7th culture day (Wong, 2016), was lower than

the value obtained in this study across the PWE media concentrations by white light, and white+yellow light illuminations except for 50 g/L PWE medium. However, the light intensities were not determined in this study. The non-significant difference ($p > 0.05$) in cell growth and intracellular contents by yellow light and no light illuminations as observed in the present study probably suggest that the yellow light intensity was very poor and was not able to penetrate the culture to impact on the algal cells. Nwoba et al. (2016) also acknowledged that the lower cell density under the blue spectra would be due to reduced irradiance from the blue filter. However, significant decrease in the cell biomass and productivities of the *Chlorella* sp. grown in the dark (no light) was not unexpected because all photoautotrophic microalgae would require light as a source of energy for their growth and productivities. The significant positive impact of yellow light illumination only on the chlorophyll-a content of the isolate more than yellow+white light or no light illumination probably suggest that specific productivities by microalgae could be specific to the spectral quality of illumination. The economic implication of this finding is that when yellow spectrum is filtered for the production of chlorophyll-a, the remaining portions of the spectrum can be collected and redirected to a highly efficient photovoltaic cell with little or no loss of energy (Nwoba, 2017). The report of maximum lipid productivity using white light (Wong, 2016) is in agreement with the present report although the value reported was lower than what was obtained in the present study (0.14367 g/L/day).

Conclusion

This study demonstrates that nutrients extracted from chicken manure (poultry waste) contain sufficient nitrogen and phosphorus to support fast growth, high biomass and lipid production by microalga *Chlorella* sp. The *Chlorella* sp. isolate has the potential for cost-effective biodiesel production using poultry waste medium. Also, white light was the most efficient in stimulating the production of biomass, carotenoid and lipid in phototrophic condition while yellow light was the most efficient in stimulating chlorophyll-a content in phototrophic condition by the *Chlorella* sp. isolate.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Next generation sequencing as a method to verify virus elimination using heat treatment and meristem tip culture in the five most widely used sweet potato varieties in Ethiopia

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Sweet potato (*Ipomoea batatas* L. Lam) has become one of the staple crops in Africa in the last 20 years. In Ethiopia, sweet potato is the second most widely grown root crop and is the first regarding the production per hectare. Thus, there is a great demand of planting material throughout the country. Currently, planting material is usually obtained from own previous season harvest, local markets or from the neighboring fields since no certified clean planting material production scheme has been established in Ethiopia yet. Unfortunately, this practice has contributed to the spread of viral diseases throughout the country. Elimination of viruses from infected plants is a tedious job, which requires efficient methods to eliminate the virus and also to verify that the plants are indeed virus-free. In the case of sweet potato, it was observed that heat treatment, combined with meristem tip culture is an efficient method for virus elimination. Previous findings indicate that reverse transcription (RT) PCR is more efficient than ELISA to verify the efficiency of virus elimination. In this study, the use of next generation sequencing (NGS) was explored as a verification method and compared with RT-PCR. The results show that NGS seems to be more efficient than RT-PCR, although also prone to inconclusive results.

Key words: Viruses, next generation sequencing (NGS), sweet potato, reverse transcription (RT) PCR, badnavirus.

INTRODUCTION

Sweet potato [(*Ipomoea batatas* L. Lam)] is the seventh most economically important crops in the world with total annual production of 12.3 million tones (FAO, 2017). China leads the production followed by Nigeria, Tanzania, and Uganda and Ethiopia. Sweet potato is grown and consumed mainly by small-scale farmers in

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Ethiopia. Nevertheless, sweet potato demand, as a food source and as planting material has increased substantially over the last 10 years. In Ethiopia, the Southern Nations Nationalities Peoples Region (SNNPR) is the dominant sweet potato production area. Unfortunately, this area is also where sweet potato viral diseases cause severe reduction of crop yields (Tesfaye et al., 2011, 2013).

In order to increase the production of sweet potato, it is necessary to devise a strategy to produce clean planting material that can supply the market. An initial step for the establishment of a certified clean planting material production scheme is to identify the most widely used varieties in the country, identify the virus that are infecting these varieties, and device methods by which these varieties can be "cleaned" from the infecting viruses. During the last five years, all of these goals were accomplished. Nevertheless, true development also requires the incorporation of novel techniques, which would contribute to the scientific development of the country.

During the last 10 years, next generation sequencing (NGS) has proven to be a reliable robust method for screening for viruses in plants (Kreuze et al., 2009; Kashif et al., 2012; Jones et al., 2017). This method allows the detection of viruses infecting a given host without prior knowledge on the existence of the virus, provides sequences data that can be used to classify the viruses into taxonomical categories and more sensitive than RT-PCR in detecting viruses in symptomless plants (Maliogka et al., 2018). NGS has been used to screen for viruses in sweet potato, revealing the presence of many viral species in this crop (Kashif et al., 2012; Mbanzibwa et al., 2014; Kreuze et al., 2017; Nhlapo et al., 2018). Nevertheless, NGS has not been previously used for virus screening in Ethiopian varieties of sweet potato.

Previous findings indicate that reverse transcription (RT) PCR is more efficient than ELISA to verify the efficiency of virus elimination in sweet potato varieties. Thus, in this paper, the efficiency of using reverse transcription PCR (RT-PCR) was compared with NGS as a method to verify efficient elimination of viruses utilizing heat treatment combined with meristem tip culture in the five most widely used sweet potato varieties in Ethiopia. Results showed that NGS seems to be more reliable than RT-PCR, although it can also lead to unexpected results. In addition, by using NGS as a detection method, the occurrence of two sweet potato viruses was also identified previously not known to be present in Ethiopia.

MATERIALS AND METHODS

Plant materials

Five sweet potato varieties ('Hawassa-83', 'Berkume', 'Tola', 'Kulfo' and 'Guntute B') were obtained from research and academic institutions in Ethiopia. Four varieties ('Hawassa-83', 'Berkume', 'Tola', and 'Kulfo') were symptomless, whereas one ('Guntute B') showed severe virus symptoms. Shoot tips of each variety were planted in two sets. One set was subjected to heat treatment followed by meristem culture for virus elimination and the other set was used as the control initial plants. Following heat treatment, the *in vitro* plants were regenerated from meristem cultures.

Heat therapy

Heat-therapy treatment, followed by meristem tip culture was applied according to the method described for sweet potato (Dennien et al., 2013), with some modifications described as follows. Five vine cuttings from each variety were potted and grown in a growth chamber at a daily temperature cycle of 25/18°C under a 12/12 h photoperiod with a light intensity of 150 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Osram L 58W/840 Lumilux). After four weeks, the potted plants were divided into two sets each consisting of three potted plants of each variety. One set continued to be grown under same conditions and used as a meristem donor for meristem culture alone (non-heat treated control). The other set was transferred to a different growth room where it was subjected to heat therapy for 8 weeks at daily temperatures cycle of 39 ± 1°C / 25 ± 1°C day/night, 12/12 h of photoperiod and light intensity of 150 µmol$m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Osram L 58W/840 Lumilux).

Meristem culture and plant regeneration

Meristems were excised from both experimental conditions (non-heat treated and the heat-treated donor plants) and cultured *in-vitro* on nutrient media optimized for the sweet potatoes (Dereje and Hvoslef-Eide, 2020). The cultures were placed in the dark for a week at 25 ± 1°C and then moved to a growth condition of more intensity of light (28 µmol$m^{-2}s^{-1}$) provided by cool white fluorescent tubes (Osram L 58W/840 Lumilux). After five weeks, surviving meristems were counted and sub-cultured into test tubes containing a fresh medium of the same composition, for further shoot initiations. After initiation, plantlets were elongated and multiplied on plant growth regulators-free 1/2 basal MS media for subsequent virus indexing using combinations of methods described in Dereje et al. *(submitted*). The virus elimination efficiency of meristem culture alone and combined heat therapy were compared based on the percentages of plantlets that were cured of infecting viruses by each method.

Virus testing

All plants were tested by RT-PCR and NGS before and after heat treatment-meristem tip culture. RT-PCR was used to test for SPVC, SPVG, SPV2, SPFMV, SPCSV and SPMMV as follows. Briefly, total RNA was extracted from 0.1 g fresh leaf samples. These samples were grounded to a fine powder in liquid nitrogen using mortar and pestle. RNA extraction was performed using Trizol reagents (Invitrogen, Life Technologies) following the manufacturer's procedures. Total RNA was quantified and the quality verified using Nanodrop (Thermo Fisher Scientific, USA), Agilent 2100 Bio-analyzer (Agilent Technologies, USA) and with a 1% agarose gel electrophoresis stained with ethidium bromide.

First strand cDNA was synthesized using 2.5 µg total RNA template, random primer (Invitrogen) and Super Script™ II Reverse Transcriptase (Invitrogen) and all other reaction components and reaction conditions were according to the manufacturer's recommendations (Invitrogen, California ©2010 Life Technologies).

PCR reactions were carried out using the first strand cDNA as a template, virus-specific primers to SPVC, SPVG, SPV2 (Li et al., 2012) and SPFMV, SPCSV, and SPMMV (Kathurima et al., 2011)

Table 1. Small RNA reads generated per samples before virus elimination treatment (A) and after virus elimination (B).

Sample	Varieties	Total no. of small RNA reads per sample (million)	No. of small RNA reads (size; 18-26 bp)	% of small RNAs reads size (18-26 bp)
A. Before heat treatment-meristem tip culture				
1	Berkume	9.13	6.28	68.74
2	Guntute B	9.16	7.78	84.78
3	Kulfo	10.03	8.91	88.85
4	Hawassa-83	9.44	7.30	77.36
5	Tola	10.02	7.52	75.06
B. After heat treatment-meristem tip culture				
1	Berkume	10.35	8.24	79.61
2	Guntute B	9.97	7.72	77.43
3	Kulfo	8.36	7.42	88.81
4	Hawassa-83	9.07	8.12	89.44
5	Tola	8.26	6.92	83.69
Average		**9.35**	**7.42**	**79.40**

and TIF DNA polymerase (Invitrogen) following the procedures of the manufacturers and the appropriate controls. Amplification was performed under the reaction conditions of 94°C for 2 min, 35 cycles of 94°C for 20 s, 52°C (for SPVC, SPVG, SPMMV and SPCSV) or 53°C (for SPFMV) for 20 s, and 72°C for 30 s. A final elongation was carried out at 72°C for 5 min.

For NGS, total RNA was extracted from 100 mg leaf tissue using Trizol (Invitrogen, CA, USA). Extracted RNA samples were purified using PureLink RNA Mini purification kits and DNase treated using the TURBO DNA-free kit (Invitrogen by thermofisher scientific) according to the manufacturer's instructions. The RNA samples quantity and purity were evaluated using a Nanodrop (NanoDropTechnologies, Wilmington, DE, USA). Furthermore, the RNA integrity was confirmed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA (3 µg) was sent to Fasteris genome sequencing services center (Geneva, Switzerland) where the sample were sequenced.

Data analysis

Raw RNA sequence data sets obtained from Fasteris were downloaded and analyzed using the Virus Detect software (Zheng et al., 2017). For the virus detect analysis, reads in the size range of 10 to 50 nucleotides were used.

RESULTS AND DISCUSSION

Next generation sequencing

Small RNAs reads were generated using small RNA (sRNA) molecules of less than 30 nucleotides in size. sRNA deep sequenced data of the 5 varieties before and after virus elimination treatments were analyzed by denovo assembly, using the Virus Detect software package. Between 8.26 and 10.35 million sRNAs (1 to 50 bp) reads were generated per sample (Table 1). On average, 9.35 million reads were generated per sample and 79.4% of the sRNAs reads generated are between the size ranges of 18 to 26 bp (Table 1). The size distribution of the sRNAs read of the 10 samples libraries had slight differences. For all the libraries, reads with size of 21 and 24 bp accounted for the majority of sRNAs reads.

NGS reveals the existence of sweet potato viruses previously unknown to be present in Ethiopia

Table 2 shows viruses known to infect sweet potato detected in the samples before and after heat treatment-meristem tip culture. The following sweet potato viruses were detected: SPFMV, SPVC, SPCSV, SPVG, *Sweet potato symptomless mastrevirus-1* (SPSMV-1) and three sweet potato badnaviruses (SPBV-A, SPBV-B, SPBV-C) collective called *Sweet potato papakuy virus* (SPPV). Among these SPFMV, SPVC, SPCSV and SPVG have previously been detected in Ethiopia. However, and to our knowledge this is the first report of SPSMV-1 and SPPV in Ethiopia.

SPPV (family Caulimoviridae; genus *Badinavirus*) is the most commonly detected virus in sweet potato (Mbanzibwa et al., 2014; Kreuze et al., 2017) and has been reported to occur in many accession belonging to the sweet collection at International Center for Potato (CIP) (Kreuze et al., 2017). SPPV was initially identified in sweet potato plants showing no visible symptoms (Kreuze et al., 2009). In this study, SPPV was detected in 5/5 of the initial plants samples and 3/5 of plants regenerated after heat-treated and meristem culture. Thus, heat therapy and meristem tip culture have 50% chance of eliminating the virus. Whether SPPV is endemic to Ethiopia or was introduced into the country

Table 2. List of commonly found viruses infecting sweet potato detected by RT-PCR and NGS before (A) and after (B) heat treatment combined with meristem tip culture.

Variety	A. Before heat treatment-meristem tip culture		B. After heat treatment-meristem tip culture	
	RT-PCR	NGS*	RT-PCR	NGS*
Berkume	-	SPPV	-	-
Guntute B	SPFMV, SPVC, SPCSV	SPFMV, SPVC, SPVG, SPCSV, SPPV, SPSMV-1	-	-
Kulfo	SPFMV	SPFMV, SPVC, SPPV, SPSMV-1	-	SPPV
Hawassa 83	SPFMV	SPFMV, SPVC, SPVG, SPPV, SPSMV-1	-	SPFMV, SPVC, SPVG, SPPV, SPSMV-1
Tola	SPFMV	SPFMV, SPVG, SPPV	-	SPVG, SPPV, SPSMV-1

*Viruses which are underlined were only detected by NGS since no primers were available for RT-PCR detection.

through infected material used for breeding purposes, is unknown. Perhaps the wide use of CIP accessions in breeding programs has contributed to the spread of this virus in Ethiopia.

Viruses, which occur in in apparently healthy-looking plants have a higher chance of dissemination between farms when planting materials, are exchanged. Although Badinaviruses do not cause visible damage in sweet potato, they are serious pathogens of tropical horticultural crops: Banana, black pepper, cocoa, citrus, sugarcane, taro and yam (Bhat et al., 2016; Bömer et al., 2018). Thus, NGS can be a potential detection tool in rigorous certification schemes in nations where susceptible crops to these viruses exist.

SPSMV-1 (family *Geminiviridae*, genus *Mastrevirus*) has previously been reported to occur in sweet potato plants in Peru, Tanzania, Central America, China and Korea (Kreuze et al., 2009; Kwak et al., 2014; Mbanzibwa et al., 2014; Wang et al., 2015; Bhat et al., 2016; Kreuze et al., 2017; Bömer et al., 2018). The virus usually occurs as a symptomless infection and does not result in a severe disease. SPSMV-1 was detected in three varieties (‘Guntute B’, ‘Kulfo’, and ‘Hawassa-83’) before virus removal treatments. Based on the NGS results from the treated plants, SPSMV-1 was efficiently removed from ‘Guntute B’ and ‘Kulfo’, but not from ‘Hawassa-83’. However, surprisingly SPSMV-1 was also detected in ‘Tola’ (which was negative to SPSMV-1 before treatment).

Next generation sequencing (NGS) screening is more sensitive than RT-PCR

Throughout this study, two independent methods (NGS and RT-PCR) were employed to test viruses in the plant material. All plant material was tested by RT-PCR and NGS before and after heat treatment-meristem tip culture. Thus, by using these two independent methods, it is believe that our results are more reliable.

RT-PCR was initially used to test for SPVC, SPVG, SPV2, SPFMV, SPCSV, and SPMMV in all plants before undergoing virus elimination treatment. Of the six viruses tested only SPFMV, SPVC and SPCSV were detected. The results showed that 4 out of the 5 varieties were infected with SPFMV, and these results were verified by NGS. Furthermore, RT-PCR results indicated that the only one plant (‘Guntute B’) was infected with SPCSV, and these results were verified by NGS. However, RT-PCR results also indicated that only one plant (‘Guntute B’) was infected by SPVC whereas NGS indicated that this virus also infected two other varieties ‘Kulfo’ and ‘Hawassa-83’. Moreover, RT-PCR did not detect SPVG in any of the plants, whereas NGS revealed that three varieties (‘Guntute B’, ‘Hawassa 83’ and ‘Tola’) were infected with SPVG.

RT-PCR was also used to test for SPVC, SPVG, SPV2, SPFMV, SPCSV and SPMMV in all plants after undergoing virus elimination treatment. None of the viruses tested for were detected in any of the plants.

Thus, based only on RT-PCR results, the virus elimination treatment was successful in all varieties; efficiently eliminating the viruses (SPFMV, SPVC, SPCSV and SPVG) previously found infecting the untreated material. However, NGS results revealed that none of the viruses found infecting ‘Hawassa-83’ (SPFMV, SPVC and SPVG) was eliminated, whereas only SPFMV had been eliminated from ‘Tola’, which was still infected with SPVG. Surprisingly, NGS results also revealed that ‘Tola’ was also infected with SPSMV-1, even though this virus had not been detected in the plant before virus elimination treatment. More experiments are required to verify that the initial material was indeed free of SPSMV-1 or that the viral concentrations are below the

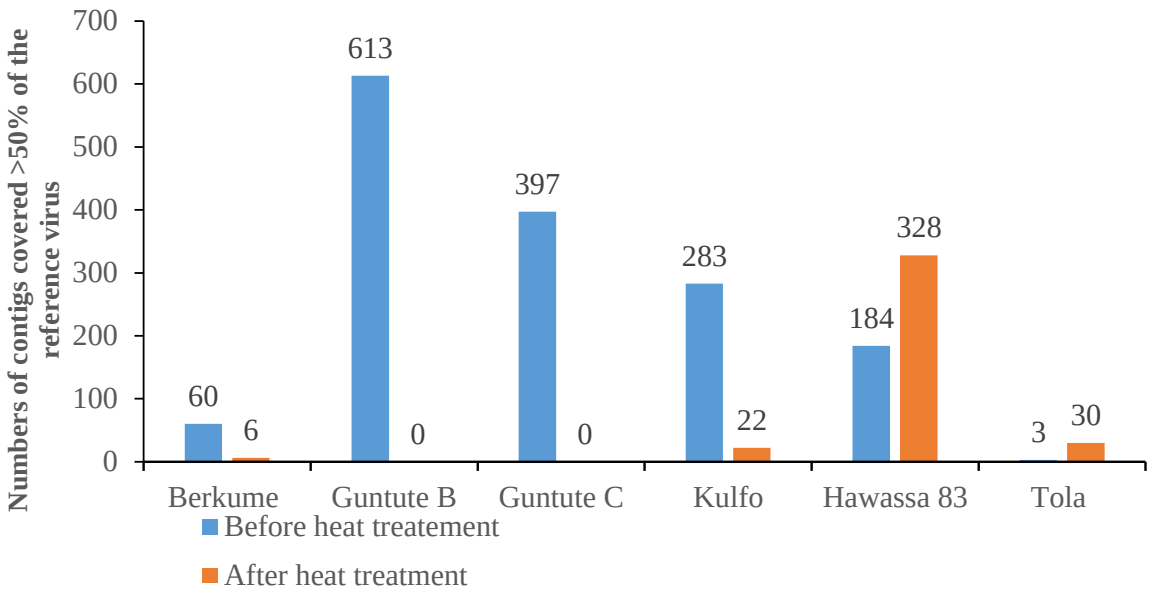

Figure 1. VsiRNAs contigs assembled in plants before and after virus elimination treatments.

detection threshold of NGS.

Abundances of homology based assembled vsiRNAs contigs differs between non-treated and treated plants and correlated with virus elimination treatments

Viral-derived siRNA (vsiRNAs) contigs are contigs which are assembled from siRNA molecules that belong to viral sequences. Thus, in theory, in the absence of viral infection, no vsiRNA contigs should be obtained. In this study, the number of viral-derived siRNA (vsiRNAs) contigs assembled from non-treated plants was higher in most of the plants (with the exception of one plant) than that of the treated plants (Figure 1). Heat treatment followed by meristem tip culture is an efficient method for eliminating viruses. Thus, a lower number of vsiRNAs derived contigs in the plants after undergoing heat treatment-meristem tip culture, might be due to the lower viral population present in the plant after the treatment. Nevertheless, and surprisingly, one variety ('Hawassa-83') showed a higher number vsiRNA derived contigs in the plant that had gone through heat treatment-meristem tip culture. The initial, non-treated "Hawassa-83" plant was infected with SMFMV, SPVC, SPPV, SPVG and SPSV-1 as determined by NGS. However, none of these viruses where eliminated from the plant by heat-treatment followed by meristem tip culture. Among the viruses found infecting 'Hawassa-83', SPPV belong to the genus *Badnavirus*.

Within the genus *Badnavirus*, *Banana streak virus* (BSV) occurs in two stages (endogenous and episomal). BSV which is present as an integrated sequence in the host's (banana) genome is named endogenous eBSV. On the other hand, when the virus is present as an episomal agent, capable of producing viral particle, it is named BSV. The functional episomal stage of BSV is triggered when the plant goes through some kind of stress, such as meristem tip culture (Gayral et al., 2010). Indeed, it is widely known that meristem tip culture in banana results in an increase of BSV in this host. Therefore, it is tempting to suggest that higher number of vsiRNA-derived contigs found in 'Hawassa-83' after the treatment corresponds to an increase in SPPV caused by the activation of the episomal stage of the virus due to the stress induce by procedure of meristem tip culture. Nevertheless, SPPV was also found in varieties 'Kulfo' and 'Tola' after heat-treatment followed by meristem tip culture. In both of these varieties the number of vsiRNAs derived contig was lower than that of the untreated plants. Thus, further experiments are required to determine if these results are indeed correct or an experimental mistake.

Conclusions

In this study the inclusion of NGS as means to screen viruses in plants before and after virus elimination treatment (heat treatment and meristem culture) helped verify the efficiency of this method. This study is the first to use deep sequencing to characterize siRNAs derived from viruses infecting sweet potato from Ethiopia. Using NGS the presences of previously reported viruses: SPCSV, SPFMV, SPVC and SPVG in sweet potato

plants from Ethiopia were confirmed. Moreover, this study reports new, but less important sweet potato viruses: SPPV and SPSMV-1 for the first time. This study demonstrated using the more recent molecular methods (NGS) enables the detection of previously unidentified viruses, without the need of utilizing virus-specific primers or antibodies. Moreover, this study shows that NGS is more sensitive than RT-PCR, although it can also give unpredictable results. Thus, standardizing the method is required before it is used in large scale or in rigorous certification schemes. Although the cost for NGS are still very expensive for routine testing in developing countries (such as Ethiopia), it was envision that in the near future NGS will be cheap enough to become the standard testing utilized in certification schemes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Stabilisation potentials of the essential oils of *Thymus vulgaris* L., *Cinnamomum zeylanicum* B. and *Mentha piperita* L. on palm olein at accelerated storage

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This study aims at bringing to light the preservative potential of essential oils of *Thymus vulgaris, Cinnamomum zeylanicum* and *Mentha piperita* in the stabilisation of lipids against oxidation. The essential oils were extracted by hydrodistillation and chemical composition were analysed by gas chromatography coupled with mass spectrometry. *In vitro* antioxidant potentials of the essential oils were tested by the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and hydrogen peroxide (H_2O_2) methods. The effects of essential oils on palm olein during accelerated storage for 20 days were carried out using the Shaal Oven test and indices of oxidation were measured. Essential oil of *T. vulgaris* was the most active oil with respect to the DPPH and H_2O_2 tests. *T. vulgaris* and *C. zeylanicum* showed varying degree of inhibition to palm olein oxidation within 20 days of storage at 60°C. Essential oils of *T. vulgaris* and *C. zeylanicum* at a concentration of 300 ppm showed comparable results to that of tert-butyl hydroquinone (TBHQ) applied at the recommended dose of 200 ppm. Palm olein samples treated with the essential oils did not show rancid characteristics at the end of storage. These essential oils significantly stabilised palm olein during accelerated storage conditions and may be exploited for use as preservatives in food products.

Key words: Antioxidant, essential oils, *Thymus vulgaris, Cinnamomum zeylanicum* and *Mentha piperita,* palm olein, food preservatives.

INTRODUCTION

The presence of oxygen is a key factor in the susceptibility of lipid food to quality loss. Independent of the mechanism, the spontaneous reaction of lipids with atmospheric oxygen causes oxidation (Johnson and Decker, 2015), resulting in undesirable changes in flavour, texture, appearance and nutritional quality of food products (Waraho et al., 2011). This degradation does not only result in considerable loss of foodstuffs, but

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consumption of such foods may induce certain degenerative diseases and mortality due to the presence of products of oxidation (Addis and Warner, 1991; Wasowicz et al., 2004). Synthetic antioxidants used as food preservatives, have been found to be hypersensitive, allergenic and mutagenic (Anand and Sati, 2013).

Plants are continually being exploited for their biological virtues and can be used to resolve food insecurity, as well as the health of the consuming population. The biological properties of aromatic plants are attributed to essential oils (Hulin et al., 1998). These essential oils are natural molecules, generally recognised as safe and have broad spectrum of action and represent an undeniable hope in the resolution of these problems. It is widely reported that essential oils possess antioxidant potentials (Jazet et al., 2008, 2010; Goudoum et al., 2009; Dabire et al., 2012).

Thymus, Mentha and *Cinnamum* species are essential oil producing plants, and have been exploited for different applications. For instance, several studies have revealed the antioxidant, anti-viral, anti-inflammatory and antimicrobial potential of the essential oil of *T. vulgaris* (Amiri, 2012; Sessou et al., 2012). Essential oils of *Mentha* species are generally employed to flavour liquors, salads, soups and cheese, as well as ingredient in cosmetic products (Yadegarinia et al., 2006). While *C. zeylanicum* is known to lower blood low density lipo-protein concentration and hence cholesterol (Aynur and Sedef, 2008).

In a bid to search for alternative to synthetic food preservatives with local plants, this study was set up to exploit the preservative potentials of the essential oils of *T. vulgaris, C. zeylanicum* and *M. piperita* in the stabilisation of lipids against oxidation at accelerated storage conditions. This will contribute to food safety by using the essential oils of some Cameroon flora to preserve food.

MATERIALS AND METHODS

The essential oils were extracted from fresh leaves and stem of *T. vulgaris* which was harvested in Dschang and fresh leaves of *C. zeylanicum* and leaves and stem of *M. piperita* were harvested in Mbouda. Dschang and Mbouda are found in the West Region of Cameroon. Palm Olein was purchased from an industrial producer.

Chemical composition analysis of essential oils

The essential oils, extracted by hydrodistillation, was analysed by gas chromatography coupled with mass spectrometry as reported by Ambindei et al. (2017).

In vitro antioxidant activities of essential oils

DPPH (2, 2-diphenyl-1-picrylhydrazyl) activity

It is known that DPPH readily produces free radicals as $DPPH^*$, which are then neutralised by the test substance, RH in this case, the essential oil. Scavenging of the DPPH* is visualised by the disappearance of the purple colour of the DPPH solution to yellow as they are being neutralised to a stable form. The scavenging rate is read at 517 nm wavelength. The scavenging rate of the test substance was evaluated with respect to a molecule of reference, butylated hydroxytoluene BHT, which is a monophenolic synthetic antiradical (Jazet et al., 2010).

Antiradical potential of BHT:

1. DPPH solution (Solution A, SA): With the aid of an electronic balance, 19.7 mg of DPPH granules were weighed and dissolved in 50 mL ethanol. This solution served as the mother DPPH solution. The solution to be used for experimentation was prepared by the removal of 5 mL from the mother solution and was dissolved in 45 mL ethanol, giving a $^1/_{10}$ dilution.

2. BHT solution: In the 50 mL methanol, 10 mg of BHT crystals were dissolved to give the mother BHT solution of concentration 1 g/l or 4.5 mmol/L.

3. Preparation of standard: From the BHT mother solution, standard solutions were prepared with the following concentrations: 0, 0.1, 0.2, 0.3 and 0.4 mg/mL.

From each concentration of standard, a fixed amount of the solutions was reacted with a given quantity of DPPH solution A. The reaction mixtures were left in the dark at room temperature and the absorbance read after an hour.

Antiradical potentials of samples

As in the BHT test, varying concentrations of essential oil were prepared: 0, 0.5, 1.5, 2, 2.5 and 3 mg/mL.

From each tube, 50 µL of essential oil solution was reacted with 950 µL DPPH solution A. The mixture from SA served as a negative control since no essential oil was added. A BHT mother solution and DPPH solution A were prepared, and this served as a positive control. The reaction mixtures were left in the dark at room temperature and the absorbance read after an hour on a multi-tube PC SPECTRONIC GENESYS 2PC spectrophotometer.

The blank solution was made of 950 µL ethanol and 50 µL methanol. The percentage scavenging capacity (SC) was calculated using the formula:

$$\mathbf{SC}\ (\%) = \frac{\mathbf{A_{ref}} - \mathbf{A_{sam}}}{\mathbf{A_{ref}}} \times \mathbf{100}$$

Where A_{ref} = Absorbance at t = 60 min of control (without inhibitor); A_{sam} = Absorbance at t = 60 min of sample and A_{100} = Absorbance at the end of reaction for total entrapment (positive control).

Hydrogen peroxide (H_2O_2) activity

The ferrous oxidation-xylenol orange (FOX) assay as described by Meisner and Gebicki (2009) was used with some modifications.

In an acidic medium, and in the presence of hydrogen peroxide (H_2O_2), ferrous ion (Fe^{2+}) is oxidized to ferric ion (Fe^{3+}). When the mixture is put in the presence of xylenol orange (3,3' - bis [N, N - di (Carboxymethyl) - aminomethyl] - o - cresolsulfone - phthaleine tetrasodium salt) ferric ions are fixed, forming a complex with a stable violet colouration which can be measured at 560 nm. The

colour intensity of the complex is proportional to the quantity of ferric ions; the latter being proportional to the quantity of hydrogen peroxide present in the medium. This reaction can be represented thus:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$
$Fe^{3+} + XO \rightarrow [Fe^{3+} - XO]$

The [Fe^{3+}-xylenol Orange] complex has an extinction coefficient of 1.5×10^4 $M^{-1}cm^{-1}$ at 560 nm when ferric ions are in direct contact with xylenol orange. In the presence of an antioxidant in the medium, non-specific oxidation of iron is prevented, thereby causing an increase in the extinction coefficient of the purple complex, and hence a better stability of the complex.

Procedure: In clinical centrifugal propylene tubes containing 50 µL of hydrogen peroxide, 50 µL of each essential oil concentration was added. The mixture was then homogenised with the aid of a vortex then incubated in the dark for 30 min. After incubation, 900 µL of FOX reagent (prepared according to the protocol suggested by Gay and Gebicki (2002)) was introduced in each tube. The resulting mixture was then homogenized and incubated in the dark for 30 min. A negative control was prepared in the same conditions, but essential oil was replaced with 50 µL HPLC grade methanol. The blank used for spectrophotometric measurements was 10 µL methanol (HPLC grade). Control essential oils were prepared under the same conditions as the tests with the only difference that hydrogen peroxide was replaced by 50 µL distilled de-aerated water. The absorbance of all the prepared and incubated tubes was read in a spectrophotometer at 560 nm with a test specimen of 400 µL, and stabilization temperature of 37°C. The percentage inhibitions of each concentration of essential oil were calculated using the formula (Meisner and Gebicki 2009):

$$I_{H_2O_2}(\%) = \left(\frac{OD_c - (OD_{EOt} - OD_{EOc})}{OD_c}\right) \times 100$$

Where $I_{H_2O_2}$ is percentage inhibition of hydrogen peroxide; OD_c is optical density (absorbance) of the negative control; OD_{EOt} is optical density in the presence of the test essential oil and DO_{EOc} is optical density of the control essential oil in the absence of hydrogen peroxide

Stabilisation of palm olein by essential oils at accelerated storage conditions

The Schaal oven test initially described by Slater (1984), reported and modified by Przybylski et al. (2013) was modified in terms of storage temperature, duration and sampling frequency. Essential oils and palm olein were blended at varying concentrations of EOs (0, 100, 200 and 300 ppm) and put in an oven at 60°C. Two control samples were prepared: One in which essential oil was replaced with a standard antioxidant, Tertiary butyl hydroquinone (TBHQ) at a concentration of 200 ppm and stored at 60°C. The second sample contained no antioxidant and was stored at room conditions to ascertain the effect of temperature on storage. The peroxide and *p*-anisidine values of samples were determined after every five days. The acid value and percentage free fatty acid (%FFA) were determined at the beginning and at the end of the experiment.

The peroxide value, expressed as milli-equivalent oxygen per kilogram oil (meq O_2/kg), gives an indication of primary oxidation products (hydroperoxides).

The anisidine value gives a measure of secondary oxidation, that is, when hydroperoxides decompose to carbonyl compounds notably aldehydes. These secondary products give the rancid characteristic smell of oils.

The Totox value indicates oil's overall oxidation state. The lower the PV, the lower the AV and hence the lower the Totox value, the better the quality of oil (AOAC, 1990).

The acid value, which is approximately twice the % FFA value, measures how many fatty acids are cleaved from their parent molecules, the triglycerides or phospholipids. Cleavage of a free fatty acid from a parent molecule shows hydrolytic breakdown and is often used in whole biological systems as an indication of stress (Miller, 2015).

The peroxide, *p*-anisidine and acid values were determined according to the AOAC official methods (1990).

Fatty acid profile of refined palm olein

Palm Olein was first methylated to fatty acid methyl esters (FAMEs) prior to fatty acid profile analysis.

The FAMEs were prepared by dissolving the oil sample (20 mg) in 1 mL hexane and refluxed for 3 h with 2% methanolic sulphuric acid (2 mL). Extraction was then done with 1 mL hexane three times. The hexane layer was washed 2 to 3 times with 3% sodium bicarbonate and then with distilled water. The resulting FAME solution was transferred into graduated stoppered vials and stored in the refrigerator

The fatty acid composition of oil was determined by subsequent analysis of the fatty acid methyl esters (FAMEs) using gas chromatography (GC-2010, Shimadzu Japan) equipped with nitrogen flame ionization detector, in a DB23 capillary column of 30 m length, 0.32 mm id wide bore, 0.25 µm film thickness (Agilent technologies USA). From an initial temperature of 80°C the oven was programmed to 120°C, for 2 min, then to 240°C at a rate of 5°C/min and held at 280°C for a further time. The fatty acid composition was analysed comparing with standard FAMEs and were expressed as relative area percentages.

Peroxide value

AOAC official method 965.33: PV of oils and fats (1990) was employed.

Reagents

1. Acetic acid–chloroform solution: 3 volumes of glacial acetic acid with 2 volumes of chloroform were mixed.
2. Potassium Iodide (KI) solution, saturated: Excess KI was dissolved in freshly boiled water. Excess solid must remain (Saturated KI: approximately 10 g KI in 6 mL water).
3. Sodium thiosulphate standard solutions: 0.1 and 0.01N was prepared with freshly boiled and cooled water.

Determination

Five grams of palm olein sample were weighed in a 250 mL glass-stoppered flask. To this, 30 mL of acetic acid-chloroform was added and the flask swirled to enable dissolution. With occasional shaking, 30 mL of water was then added, giving an almost yellow colouration.

The resulting solution was slowly titrated with 0.1N sodium thiosulphate with vigorous shaking, until the yellow colour was almost gone. About 0.5 mL of 1% starch solution was added, changing the colour of the solution to blue. With vigorous shaking so as to release all the iodine from chloroform layer, titration was continued until the blue colour turned colourless. When titrated volume was less than 0.5 mL, 0.1 N $Na_2S_2O_3$ was used. A blank

experiment was conducted daily and must be ≤ 0.1 mL 0.1 N $Na_2S_2O_3$ which was subtracted from sample titration.

The peroxide value in milli-equivalent peroxide per kilogram sample was calculated using the formula:

Peroxide value (meq peroxide / kg sample) = $(\mathbf{S \times N \times 1000})/\mathbf{w}$

S = mL of $Na_2S_2O_3$ (blank corrected); N = Normality of $Na_2S_2O_3$; W = weight of sample in g.

p-Anisidine *value*

Preparation of anisidine reagent

For 50 mL solution, 0.125 g of *p*-anisidine was weighed and dissolved in glacial acetic acid in a 50 mL volumetric flask, avoiding strong light.

Preparation of test sample: To the nearest 1 mg, a sufficient mass of the prepared test sample (palm olein) was weighed directly into a 25 mL volumetric flask. The sample was dissolved in iso-octane and the volume completed with the same solvent.

Unreacted test solution: By means of a pipette, 5 mL of the test solution were transferred into a test-tube and 1 mL of glacial acetic acid was added. The tube was then stoppered and well shaken. The test-tube was kept in the dark for eight minutes, and within a further two minutes the absorbance was read.

Reacted test solution: With the aid of a pipette, 5 mL of the test solution were transferred into a test-tube and 1 mL of freshly prepared *p*-anisidine reagent was added. The tube was stoppered and well shaken. The test-tube was kept in the dark for eight minutes, and within a further two minutes the absorbance was read.

Blank: The blank was prepared in the same way as the reacted test solution but the test solution was replaced with an equi-volume of iso-octane.

Spectrophotometric measurements

The spectrophotometer was adjusted to zero absorption with iso-octane at 350 nm, and the following measurements were carried out against iso-octane:

A1: Reacted test solution which should be between 0.2 and 0.8 or adjust test sample
A0: Unreacted sample
A2: Blank sample which should be ≤ 0.2 or purify anisidine reagent

Results were recorded to one decimal place per the formula:

$$AV = \frac{100\,QV}{m}\,[1.2\,(A1 - A2 - A0)]$$

AV = *p*-anisidine value; m = mass of test portion in grams; V = volume in which the test sample was dissolved in mL (V = 25 mL); Q = sample content of the measured solution based on which the anisidine value is expressed, in grams per mL as Q = 0.01 g/mL; 1.2 = correction factor for the dilution of the test solution with 1 mL of glacial acetic acid.

Totox value

The Totox value was calculated using the formula AV + 2PV to indicate the oil's overall oxidation state.

Acid value and percentage free fatty acid

The acid value is the number of mg of KOH/NaOH required to neutralize the free fatty acids in 1g of fat. The acid value was determined by the AOAC Official Method 940.28 (1990).

Procedure

Approximately 2 g of palm olein, (the test sample) were weighed in a 250 mL beaker. To this, 30 mL of chromatographic grade methanol was added, and the resulting solution heated till boiling. A drop of phenolphthalein indicator was added, and the solution titrated against 0.1 N alkali (potassium hydroxide).

The acid value was calculated using the formula:

$$\mathbf{Acid\ value} = \frac{\mathbf{M \times N \times V}}{\mathbf{W}}$$

M = molar mass of KOH; N = normality of KOH; V = volume of KOH used; W = weight of sample
The percentage free acid, as oleic acid, was calculated from the acid value using the relation:

Percentage FFA (%) = Acid value / 1.99.

RESULTS AND DISCUSSION

Chemical composition of the essential oils

The compounds and their respective percentages present in the different essential oils as analyzed by GC/MS are shown in Table 1.

Antioxidant activities of the essential oils

The DPPH test

BHT activity: Figure 1 shows the percentage inhibition of BHT at varying concentrations on the DPPH*. The degree of inhibition of the DPPH radical is dependent on the concentration of BHT. Graphically, the minimum concentration of BHT needed to inhibit or scavenge 50% of DPPH* (SC_{50}) was 0.07 ± 0.01 mg/mL of BHT.

Essential oils activity

The result of the antiradical properties of the essential oils of *T. vulgaris, C. zeylanicum* and *M. piperita* are presented in Figure 2.

Percentage inhibition of DPPH* increases as concentration of essential oil increases. The SC_{50} of essential oil and the reference antioxidant, BHT are shown in Table 2.

The SC_{50} values for the essential oils were respectively 0.75±0.07, 0.88 ± 0.04 and 1.08 ± 0.04 mg/mL for *T.*

Table 1. Chemical composition of essential oils.

No.	RI	Compounds (In order of elution)	Relative percentage		
			T. vulgaris	*C. zeylanicum*	*M. piperita*
		Non-Terpenes	**0.14**	**1.06**	**0.18**
1	-	Heptanal*	0.08	0.15	-
2	989	3 – Octan-2-one	0.06	-	0.18
3	956	Benzaldehyde	-	1.06	-
		MTH	**45.00**	**17.74**	**26.30**
4	922	α – Thujene	0.94	0.17	0.23
5	930	α – Pinene	0.89	6.31	8.21
6	945	Camphene	0.99	2.76	0.32
7	971	Sabinene	0.52	-	-
8	974	β – Pinene	0.19	2.24	0.69
9	985	Myrcene	1.38	0.42	1.24
10	1002	α – Phellandrene	0.11	1.25	-
11	1008	δ – 3 – Carene	-	0.16	0.90
12	1014	α-Terpinene	1.09	-	0.21
13	1021	*p* – Cymene	25.36	1.75	0.28
14	1025	Limonene	0.46	2.50	13.36
15	1031	(Z)-β-Ocimene	-	-	0.09
16	1056	γ – Terpinene	12.48	-	0.34
17	1063	p – Menthe-3,8-diene	0.57	-	-
18	1085	Terpinolene	-	0.19	0.44
		MTO	**46.91**	**50.96**	**68.458**
19	1028	1,8 Cineole	0.12	0.56	1.28
20	1095	Linalool	4.72	2.80	0.40
21	1122	(Z)-Epoxy-ocimene	-	-	0.44
22	1137	E-Pinocarveol	-	-	0.22
23	1143	Camphor	2.38	-	0.23
24	1151	Nerol oxide	-	0.50	-
25	1154	Menthone	-	-	18.47
26	1159	Z-Isocitral	-	0.80	-
27	1163	Isoborneol	-	-	3.50
28	1169	Borneol	1.14	0.95	-
29	1174	Terpinen-4-ol	0.97	0.23	-
30	1176	Menthol	-	-	33.59
31	1177	Isomenthol	-	-	0.60
32	1187	α – Terpineol	-	0.49	0.55
33	1195	Myrtenol	-	0.19	-
34	1226	Cis-Carveol	-	0.31	-
35	1249	Geraniol	-	0.28	-
36	1253	Piperitone	-	0.29	4.66
37	1270	E – Cinnamaldehyde	-	13.03	-
38	1287	Safrole	-	0.21	-
39	1291	Menthyl acetate	-	-	3.73
40	1293	Thymol	35.12	-	-
41	1294	Carvacrol ethyl, ether	0.30	-	-
42	1299	Carvacrol	2.01	-	-
43	1355	Eugenol	0.16	12.15	-
44	1446	E-Isoeugenol	-	-	0.80
45	1456	E-Cinnamic acid	-	3.26	-

Table 1 Contd.

46	1525	Eugenyl Acetate	-	4.30	-
47	1765	2-Hexyl-(Z)- Cinnamalehyde	-	10.66	-
		STH	**6.68**	**27.91**	**2.44**
48	1346	α-Cubebene	-	-	0.21
49	1371	α – Copaene	-	0.93	-
50	1378	β – Cubebene	-	4.22	-
51	1385	β – Bourbonene	0.21	-	-
52	1408	Z-Caryophyllene	-	-	0.30
53	1420	β-Cedrene	-	-	0.31
54	1423	E-B-Caryophyllene	4.72	21.82	-
55	1464	(E)- 9-Epi-Caryophyllene	-	-	0.76
56	1477	D – Germacrene	0.21	-	0.24
57	1483	α – Murolene	0.78	-	-
58	1498	α – Selinene	-	0.95	0.26
59	1515	γ – Cadinene	0.27	-	-
60	1524	δ – Cadinene	0.49	-	0.35
		STO	**1.25**	**2.18**	**0.00**
61	1587	Caryophyllene oxide	0.98	1.94	-
62	1613	Guaiol	-	0.23	-
63	1621	Iso Caryophyllene	0.03	-	-
64	1763	14-oxy-α-Muurolene	0.23	-	-

*Identified tentatively; - = Absent, MTH = Monoterpenes hydrocarbons; MTO = Oxygenated monoterpenes; STH = Sesquiterpenes hydrocarbons; STO = Oxygenated Sesquiterpenes.

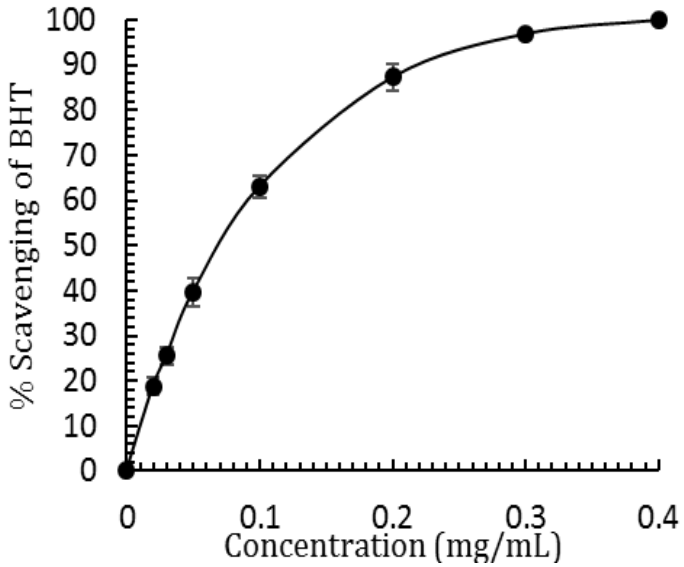

Figure 1. Percentage scavenging capacity of DPPH* by BHT.

vulgaris, C. zeylanicum and *M. piperita* as against 0.07±0.01 mg/mL for BHT. The *T. vulgaris* essential oil was more active than *C. zeylanicum* which in turn was more active than *M. piperita* as revealed by their SC_{50} values. These differences in activity are a function of the chemical composition of the different essential oils.

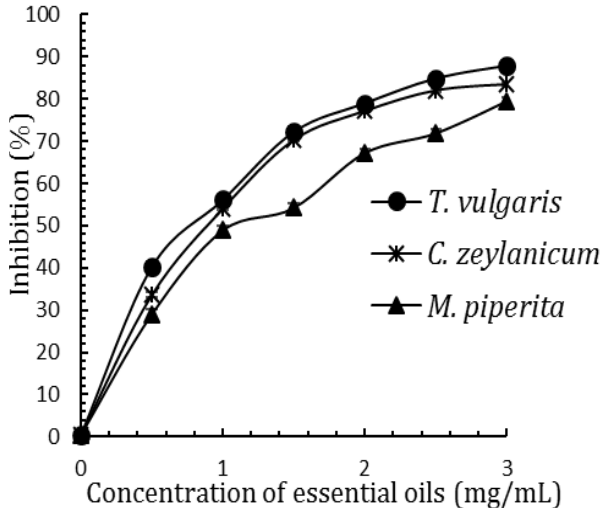

Figure 2. Percentage scavenging of DPPH* against concentration of essential oils.

Table 2. SC_{50} values of essential oils compared to BHT.

Essential oils and referent	SC_{50} (mg/mL)
T. vulgaris	0.75 ± 0.07^{b}
C. zeylanicum	0.88 ± 0.04^{c}
M. piperita	1.08 ± 0.04^{d}
BHT	0.07 ± 0.01^{a}

Values on the same column with different indices are significantly different ($p<0.05$).

Compared to BHT, the three essential oils were less active, as BHT had the smallest SC_{50} value of 0.07±0.01 mg/mL. In fact, BHT was 10 times more active than *T. vulgaris*, about 13 times more active than *C. zeylanicum* and about 15 times more active than *M. piperita*.

The hydrogen peroxide test

The result of the inhibition of hydrogen peroxide by the essential oils of *T. vulgaris, C. zeylanicum* and *M. piperita* are shown in Figure 3.

Inhibition of peroxide ions increased with increased concentration of essential oil. At a concentration of 2.5 mg/mL, *T. vulgaris* showed the highest inhibition (94.35 ± 2.29%) followed by ascorbic acid (90.47 ± 2.02%), *C. zeylanicum* (89.19 ± 0.71%) and by *M. piperita* (56.80 ± 2.96%). At all concentrations, the essential oil of *T. vulgaris* showed a higher antioxidant activity than the reference antioxidant, ascorbic acid. The concentrations of antioxidants necessary to scavenge 50% of the peroxide radicals are as recorded in Table 3.

Within the test concentration range of 0 to 2.5 mg/mL, all the three essential oils scavenged at least 50% of the peroxide radical. The *T. vulgaris* essential oil recorded the least IC_{50} value of 0.85 ± 0.05 mg/mL. There was no statistical difference ($p < 0.05$) between the IC_{50} values of *C. zeylanicum* (1.19 ± 0.03 mg/mL) and ascorbic acid (1.12 ± 0.08 mg/mL). The *M. piperita* essential oil was the least active (2.31 ± 0.09 mg/mL) of all three essential oils, and also had a lower activity than ascorbic acid.

In relative terms, *T. vulgaris* was about 1.3 times more active than ascorbic acid and *C. zeylanicum*, and about 3 times more active than *M. piperita.*

The high antioxidant activity of *T. vulgaris* compared to the other essential oils may be due to the presence of thymol and eugenol that have been shown to be very effective compared to cinnamaldehylde (in *C. zeylanicum*) and menthol and menthone (in *M. piperita*) (Wei and Shibamoto, 2010).

Stabilisation of refined palm olein

After subjecting palm olein to accelerated oxidation conditions, the potential of the essential oils of *T. vulgaris* and *C. zeylanicum* to stabilise or prevent oxidation was evaluated using them. The two essential oils were

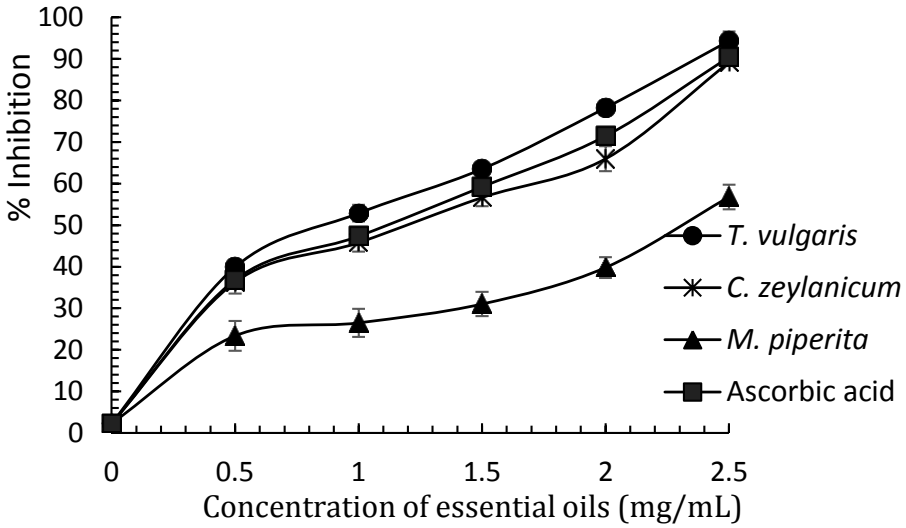

Figure 3. Percentage inhibition of peroxides by essential oils.

Table 3. IC_{50} values of essential oils compared to ascorbic acid.

Essential oils and referent	IC_{50} (mg/mL)
T. vulgaris	0.85 ± 0.05^{a}
C. zeylanicum	1.19 ± 0.03^{b}
M. piperita	2.31 ± 0.09^{c}
Ascorbic acid	1.12 ± 0.08^{b}

Values on the same column with different indices are significantly different ($p<0.05$).

Table 4. Fatty acid profile of palm olein.

Fatty acid	Lauric acid	Myristic acid	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid
Lipid number	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
%	Tr	1.0	37.0	4.0	46.0	11.5

considered due to their relative high antioxidant activities with respect to the DPPH and hydrogen peroxide tests.

Fatty acid profile

The relative percentage of the different fatty acids in the Palm Olein oil used is shown in Table 4. The higher the degree of unsaturation of a fatty acid, the more prone the fatty acid is liable to oxidation. The most abundant fatty acid is the mono unsaturated oleic acid (18:1) constituting 46% of the total oil composition. Palmitic acid, a 16-carbon saturated fatty acid, constitutes 37% of the total oil composition. The double unsaturated fatty acid, linoleic acids (18:2) and occupied 11.5%. The palm olein under study had about 57.5% unsaturated fatty acids. The relative high amount of saturated fatty acids of about 42%, notably palmitic acid confers a more stable structure when stored at room temperature. Linoleic acid (11.5%), with double unsaturation will be more liable to oxidation, followed by oleic acid. This level of unsaturated fatty acids makes the palm olein a suitable substrate for oxidation studies.

Peroxide value (PV) of test samples within storage duration

Within the 20 storage days under accelerated storage conditions, the PV of the different samples are shown in Figures 4 and 5.

Generally, the peroxide values increased with the storage duration time but a fluctuation in the peroxide values of the negative control was noticed. The PV

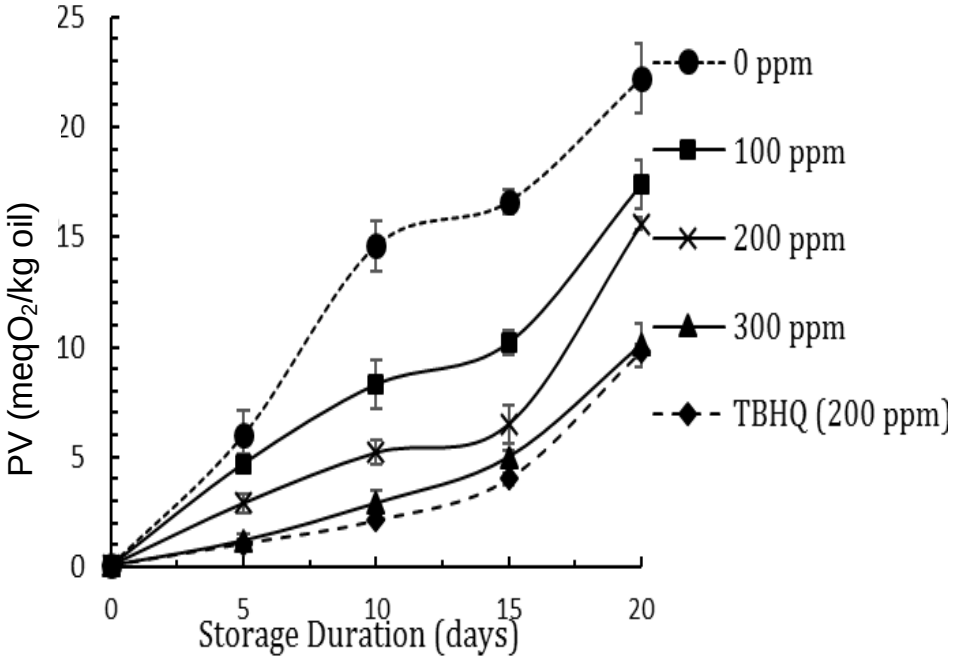

Figure 4. Peroxide values of palm olein at 60°C for 20 days treated with *T. vulgaris* essential oil compared to TBHQ.

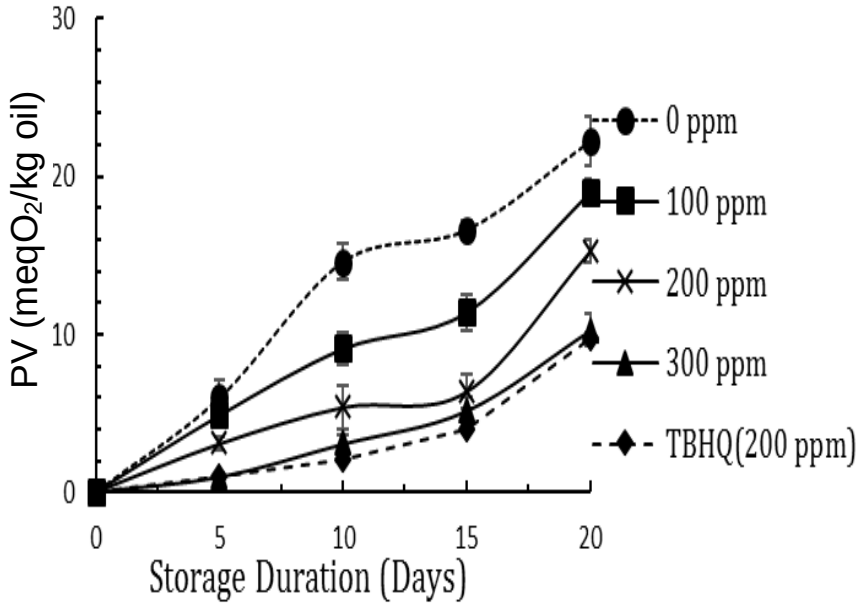

Figure 5. Peroxide values of palm olein at 60°C for 20 days treated with *C. zeylanicum* essential oil compared to TBHQ.

increased rapidly up to the 10th day, then a reduction in the rate of increase was noticed between the10th and 15th day. The slow increasing rate in PV between the 10th and 15th day may probably be due to an increase in the rate of secondary oxidation. Peroxide radicals, which are products of primary oxidation, are further oxidised to secondary oxidation products such as aldehydes, ketones, acids among others, thereby leading to a drop in PV as reported by Gordon (2001).

Statistical analysis showed significant differences between the concentrations of each essential oil, and in some cases, the same concentrations of the two

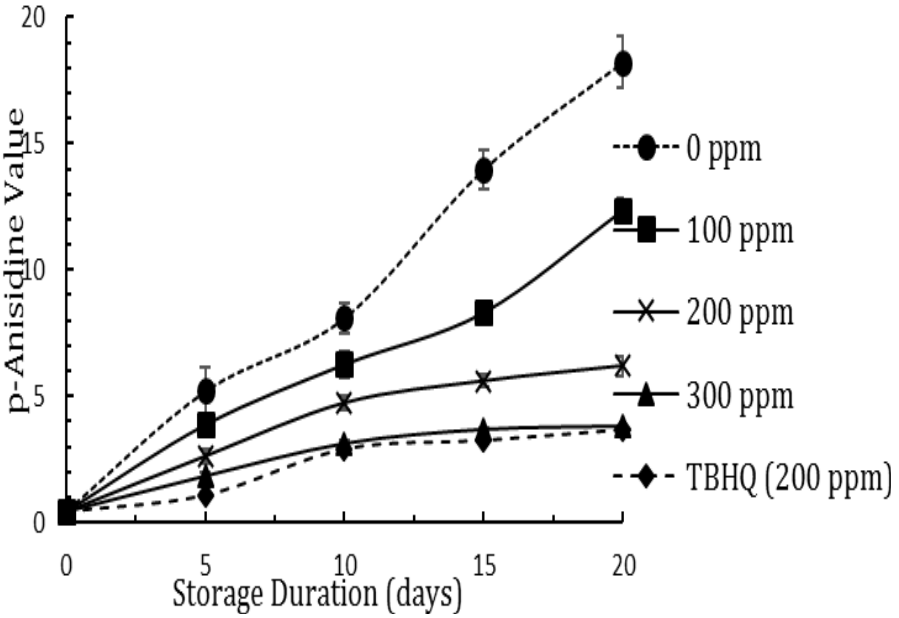

Figure 6. p-Anisidine values palm olein at 60°C for 20 days treated with *T. vulgaris* essential oil compared to TBHQ.

essential oils had statistically similar results ($p < 0.05$). Thus, the stabilisation potential of TBHQ at 200 ppm was generally comparable to that of the essential oils at 300 ppm (Figures 4 and 5). Throughout the experiment, palm olein without essential oil stored at room temperature showed very little variation, with its PV being less than 3 meq.O_2/kg oil. According to AOAC (1990), a PV below 10 meq.O_2/kg oil is acceptable, and higher values are indication of deterioration. From the results obtained in this study, peroxide values were more or less in the acceptable range after 15 days of accelerated storage. At a concentration of 300 ppm, the essential oils prevented oxidation significantly as the treated palm olein still had peroxide values within the acceptable range.

The decrease in PV upon the addition of essential oil in the present study concords with the findings of Sana et al. (2012) who worked on the essential oils of *Thymus*, *Salvia* and *Rosemarinus* on the stability to autoxidation of refined oils. Dabire et al. (2012) used essential oil of *Ocimum basilicum* as a natural antioxidant at concentrations of 500 and 1000 ppm to prevent deterioration of cottonseed oil. Also, *Cinnamon* essential oil was found to significantly lower peroxide values of hazelnut and poppy oils (Özcan and Arslan 2011).

Para-Anisidine value (*p*-AV) of test samples within storage duration

Para-AV is a reliable measurement of the amount of secondary oxidation products (Zhang et al., 2010) giving a characteristic off-flavour, the state of rancidity at AV>10. The results of *p*-AV values of the samples within the test period are shown in Figures 6 and 7.

The *p*-AV of palm olein samples stored at 60°C increased with increase in storage duration, but reduced with increasing concentration of the essential oil. palm olein not treated with antioxidant and stored at room temperature was stable as all AV were statistically not different ($p < 0.05$). The essential oil of *T. vulgaris* at 300 ppm had similar results with TBHQ at 200 ppm. All samples treated with the essential oils had AV less than 10 by the 15th storage day. This implies a strong antioxidant effect of the essential oils with respect to the negative control. By the 20th day, at concentrations of 200 and 300 ppm, the essential oils had acceptable AV, that is, less than 10, implying a strong antioxidant effect of the essential oils as the peroxides were prevented from oxidation as reported by Zhang et al. (2010).

The variations in AV were like those of Turan (2013) who found *T. vulgaris* essential oil as the most active with respect to *S. officinalis*, *R. officinalis* and *L. nobilis* on the stability of canola oil during accelerated storage conditions.

Totox value (TV) of test samples within storage duration

The variation in TV is shown in Figures 8 and 9. The lower the TV, the better the oil quality (AOAC, 1990). Generally, TV decreased with increasing concentration of the essential oils. The essential oils at 300 ppm especially *T. vulgaris*, showed comparable results with

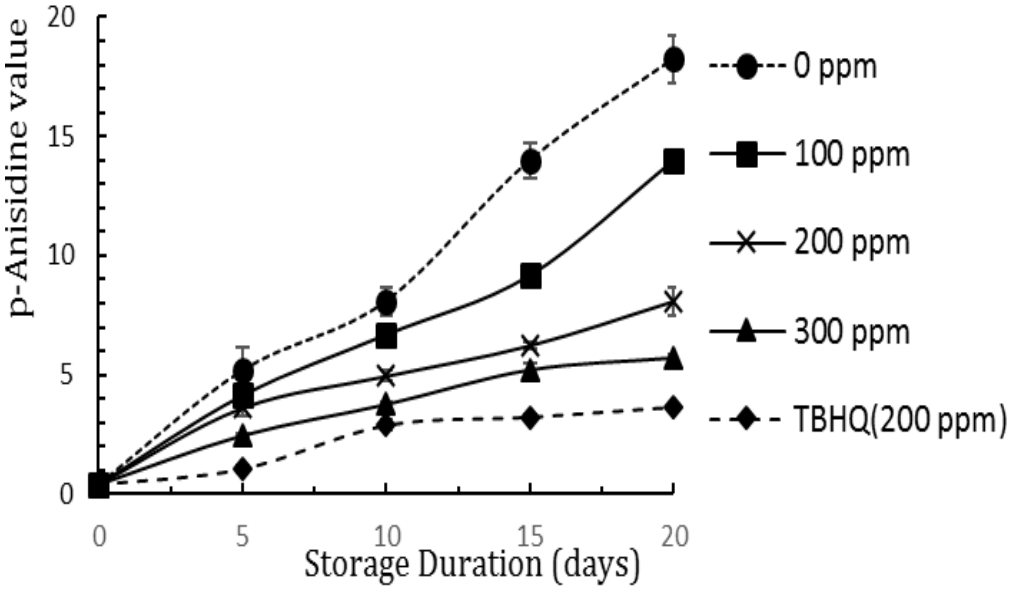

Figure 7. *p*-Anisidine values of palm olein at 60°C for 20 days treated with *C. zeylanicum* essential oil compared to TBHQ.

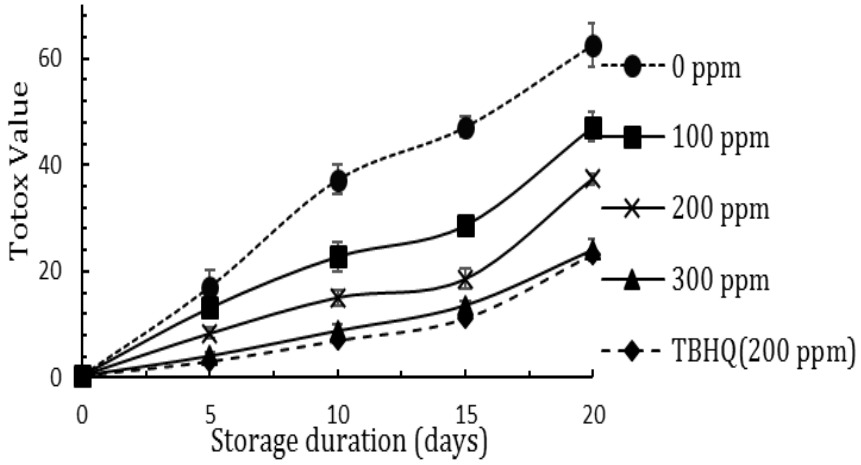

Figure 8. Totox values of palm olein at 60°C for 20 days treated with *T. vulgaris* essential oil compared to TBHQ.

that of the reference, TBHQ. After 20 days of storage, the TV of *T. vulgaris* at 300 ppm and TBHQ at 200 ppm were not statistically different ($p < 0.05$). The highest TV value was obtained with the palm olein sample without essential oils and stored at 60°C, while palm olein without essential oils stored at room temperature had the least TV. This implies that high temperatures during storage accelerate oxidation and hence hasten the deterioration of vegetable oil. The relatively high percentage of phenolic compounds and reactive benzene rings such as thymol, cavarcrol and cinnamaldehyde found in these essential oils could explain their strong antioxidant activity.

Acid value and percentage free fatty acid (%FFA)

Before storage, the acid value was evaluated to be 0.82 ± 0.06 mg/kg while the percentage free fatty acid (FFA) was 0.41 ± 0.3 mg/kg. Based on these two parameters, the refined vegetable oils should have an acid value not more than 4 mg/kg and a % FFA not more than 1% (AOAC, 1990). At the end of the 20 days accelerated storage period, the variation of the acid values and % FFA of the various test samples are as shown in Figures 10 and 11, respectively.

All samples subjected to accelerated storage conditions showed an increase in the acid values and

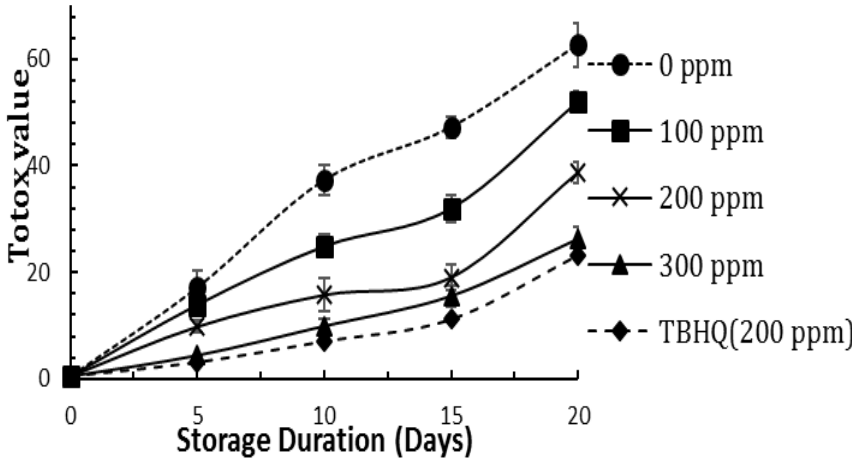

Figure 9. Totox values of palm olein at 60°C for 20 days treated with *C. zeylanicum* essential oil compared to TBHQ.

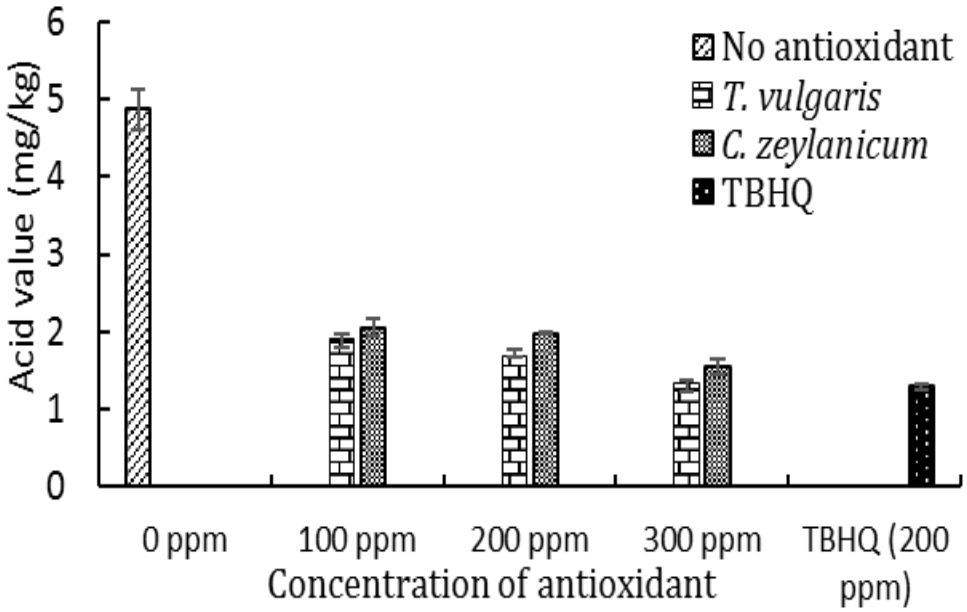

Figure 10. Acid values of palm olein treated with antioxidants and stored at 60°C for 20 days.

hence the %FFA. These values increased with decreasing concentration of the essential oils. The negative control sample was not consumable at the end of the experiment, with an acid value of 4.88 ± 0.26 mg/kg and a %FFA of 2.45 ± 0.13%. These values are higher than the stipulated standards of 4 mg/kg and 1% respectively (AOAC, 1990). All the other samples were below 4 mg/kg, hence were consumable. However, the sample incorporated with *C. zeylanicum* essential oil at 200 ppm had a %FFA of 1.04 ± 0.06%, which is at the limit of the maximum required value.

Generally, *T. vulgaris* was the most active essential oil, with its acid value and % FFA at 300 ppm not significantly different from the reference antioxidant TBHQ. Palm Olein sample stored at room temperature without antioxidant showed minor variation in its acid value and %FFA at the end of the experiment compared to the initial values. Therefore, increased temperature during storage accelerated deterioration, and the presence of essential oils inhibited oxidation.

According to IUPAC (1987) as well as AOAC (1990), refined vegetable oil is considered edible if its PV is less than or equal to 10 meq of O_2/kg, and its AV is less than 10, its acid value is less than or equal to 4 mg/kg, and its acidity or FFA is less than 1%. Taking into consideration the PV, AV, acid value and %FFA, a fat may have a high

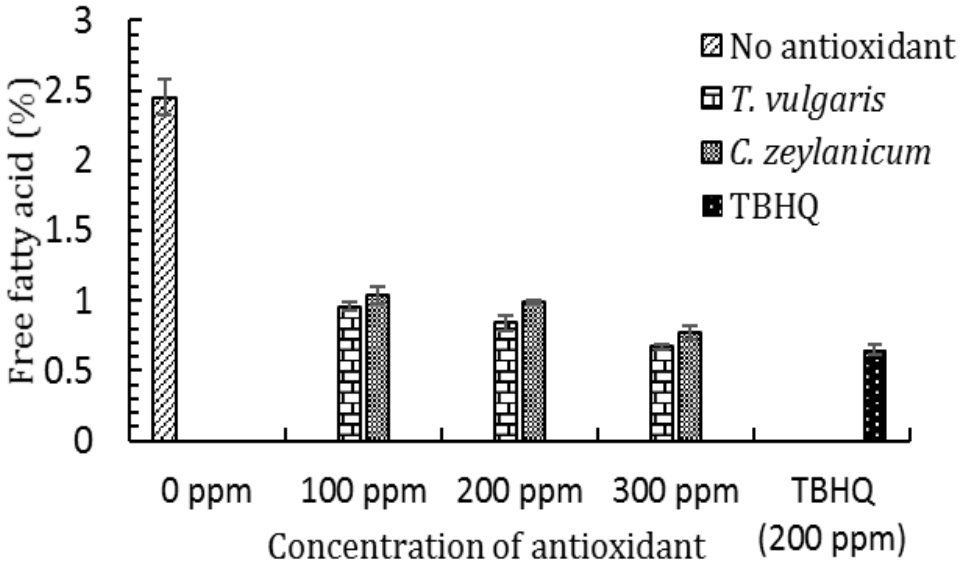

Figure 11. Percentage FFA of palm olein treated with antioxidants and stored at 60°C for 20 days.

value of PV without any characteristic of rancidity. The PV is related to peroxidation and not to rancidity, as the latter is highlighted by the determination of secondary oxidation products (Dabire et al., 2012). Therefore, despite values of PV greater than 10 meq O_2/kg, all samples treated with the essential oils could be considered consumable by the 15th day, and even at the end of the experiment as their AV, acid values and %FFA fell below the maximum values of 10, 4 mg/kg and 1% respectively.

The antioxidant potential of these essential oils is due to the presence of compounds such as thymol, eugenol, carvacrol, safrole, menthol, 1,8-cineole, *α*-terpineol, *p*-cymene, cinnamaldehyde that endow these essential oils as powerful antioxidants (Zeghad and Merghem, 2013). The phenolic group and / or benzene ring or reactive functional group they possess renders them very reactive and susceptible to bond formation. These essential oils reported in this study can be used in agro-food as natural antioxidants to conserve fatty foods or in all formulations containing fats.

Conclusion

The essential oils of *T. vulgaris* and *C. zeylanicum* remarkably stabilised palm olein against oxidation at accelerated storage conditions. Despite values of PV greater than 10 meq O_2/kg, all samples treated with the essential oils could be considered consumable at the end of the experiment as their *p*-anisidine values, acid values and percentage FFA, which are true indicators of rancidity, were below the maximum values of 10, 4 mg/kg and 1%, respectively. These essential oils can, therefore, be exploited as suitable alternatives to synthetic chemicals as food preservatives. They can not only be exploited in food storage, but can as well be exploited as food additives for their antioxidant properties both in the food and eventually in the body of consumers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Response of catalase to drought in barley (*Hordeum vulgare* L.) seedlings and its purification

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Drought induced oxidative stress triggers anti-oxidative system in cell to minimize oxidative damage where catalase plays vital role to neutralize H_2O_2. In this work, catalase activity was evaluated to examine the role of catalase in barley (*Hordeum vulgare* L.) seedlings under drought stress. As compared to control, catalase activity increased with decreasing soil moisture where 219% higher activity were recorded at 10% soil moisture of field capacity (FC) compared to control (75% FC), but was reduced below 10% FC. Four different catalase isozymes that specifically accumulated in barley leaves in response to drought (10% FC) which nominated catalase, particularly CAT4 and CAT2, as key players for H_2O_2 scavenging were identified. However, for future study, one catalase was purified from barley leaves with an apparent molecular weight of 54 kDA and specific activity of 871.32 µmol min-1 mg-1 protein. Therefore, in this study, it was found that four CAT isozymes in barley leaf under drought, and the purified catalase needs characterization at molecular level for further biotechnical use.

Key words: Catalase, isozymes, barley, drought, purification.

INTRODUCTION

The fast growing population is facing difficulty to feed the people worldwide due to reducing situation of arable land. Moreover, increased stress due to climatic change has caused higher risk for agricultural production. Bangladesh is one of the most over populated countries in the world with ranking 8th for population and 92nd for area. Moreover, about 30% of cultivable land in the southern coastal belt is affected by salinity (Rohman et al., 2019a); salinity has affected crop production in 100 million ha. On the other hand, about 0.28 million ha of land is dry (locally, Charland) and often faces drought (Sattar and Islam, 2010). Such types of problematic soils need tolerant crop species. Barley (*Hordeum vulgare ssp. vulgare*) inherently exhibits a higher level of abiotic stress tolerance than other crops (Baik et al., 2011; Nevo et al., 2012; Powell et al., 2012), and it has potential role as human food in different salinity and drought affected areas in the world (Zhou, 2010). It ranks the fourth most important cereal crop on a global scale (FAOSTAT, 2018), which predicts its future prospects for food production in problem areas like salinity and drought affected ones. Moreover, because of its relatively simple

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diploid genetics along with tight relationship between other cereals, barley has gained importance as a resistance source (Wiegmann et al., 2019). Under abiotic stress, a plant, tolerant or susceptible, undergoes a series of morphological, biochemical, physiological and molecular changes (Gill and Tujeta, 2010). However, the tolerance mechanism to abiotic stress, particularly saline and drought stress is very complex and still not clear.

Drought is the most detrimental stress that inhibits the growth and yield of crops. In plant species, drought causes osmotic stress resulting in oxidative stress in plants through declining stomatal conductivity that limit CO_2 entry into the leaves which reduces the leaf internal CO_2 resulting in the formation of reactive oxygen species (ROS) (Foyer and Noctor, 2012; Choudhury et al., 2013). ROS are highly cytotoxic in different ways: firstly, through lipid peroxidation resulting in increasing membrane leakage and reducing membrane fluids that damages ion channels, membrane proteins, enzymes and receptors; secondly, through oxidizing proteins that hinder or change activities and make plants more susceptible to proteolytic attack; and thirdly, through DNA damage (base deletions and modifications, strand breaks, cross-links and pyrimidine dimers) which ultimately reduces or injures protein syntheses, damages cell membrane, unstabilizes DNA replication, genomic stability and transcription (Filiz et al., 2019). ROS constitute oxidant molecules like superoxide radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$), and cause cellular damage by oxidizing organelles like enzymes, proteins, DNA and lipids (Gill and Tujeta, 2010).

Plants have very well-organized ROS scavenging system consisting of both enzymatic and non-enzymatic antioxidants. Among enzymatic antioxidants are superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione peroxidase (GPX; EC 1.11.1.9), glutathione reductase (GR; EC 1.6.4.2), peroxidase (GPX; EC 1.11.1.7), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), dehydroascorbate reductase (DHAR; EC 1.8.5.1) and glutathione *S*-transferase (GST; EC 2.5.1.18). On the other hand, ascorbic acid (ASA), glutathione (GSH), alkaloids, tocopherol some amino phenolic compounds, carotenoids and flavonoids are non-enzymatic components (Gill and Tujeta, 2010). ROS generation or accumulation is an unavoidable consequence of normal metabolic processes in plants, and in normal growth condition, there is a balance between ROS production and its scavenging; whereas any stressful condition imbalances the equilibrium by increasing cellular ROS (Sharma et al., 2012).

Under stressful condition, SOD provides first line protection from $O_2^{\bullet-}$ mediated oxidative damage through its dismutation to H_2O_2 (Apel and Hirt, 2004). H_2O_2 is also produced in other enzymatic and non-enzymatic metabolic pathways. In peroxisomes, the generation of H_2O_2 is catalyzed by glycolate oxidase involving glycolate oxidation, the β-oxidation of fatty acids and catabolism of lipids (Halliwell, 2006). H_2O_2 that accumulates in cell can be then metabolized by CAT, POD, APX, GPX and GST (Gill and Tujeta, 2010; Sharma et al., 2012). It is important that metabolism of H_2O_2 by CATs is different from other enzymatic H_2O_2 metabolisms, as they do not require any substrate (Mhamdi et al., 2010). Therefore, CATs are the most important tools for mitigating oxidative stress in plants. Considering those, the experiments were designed to examine the role of catalase in barley under saline stress, and a CAT was purified from barley leaves.

MATERIALS AND METHODS

Plant materials

Seedlings of BARI barley-6 were used as plant materials. The leaves of seedlings were used to examine the regulation of CAT. For purification, 10 days old leaves of seedlings were used.

Stress treatment

Seedlings were grown in soil media (Soil: organic matter =3:1) in 30 L plastic bucket in the green house of Plant Breeding Division of Bangladesh Agricultural Research Institute. Fifteen days old seedlings were subjected to water withdrawal after attaining soil moisture of 30% (field capacity, FC) of experimental soil. Seedlings were observed until soil moisture attained 5% of FC. Data were taken from fully expanded leaves at 75 (as control), 25, 10 and 5% FC. Soil moisture level was monitored with a digital soil moisture meter (Lutron PMS-714, Taiwan). The experiment was repeated three times each containing three replications.

Enzyme extraction for CAT assay and isozyme analysis

Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 ml of 50 mM ice-cold potassium-phosphate buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM β-mercaptoethanol and 10% (w/v) glycerol. The homogenates were centrifuged at 11,500 × *g* for 10 min, and the supernatants were used for determination of enzyme activity. All procedures were performed at 0 to 4°C.

Determination of protein

The protein concentration in the leaf extracts was determined according to the method of Bradford (1976) using albumin from bovine serum (BSA) as a protein standard.

Assay of CAT activity

CAT (EC: 1.11.1.6) activity was measured as per description of Rohman et al. (2019b) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2. The reaction mixture contained 50 mM K-phosphate buffer (pH 7.0), 15 mM H_2O_2, and enzyme solution in a final volume of 0.7 ml. The reaction was initiated with enzyme extract, and the activity was

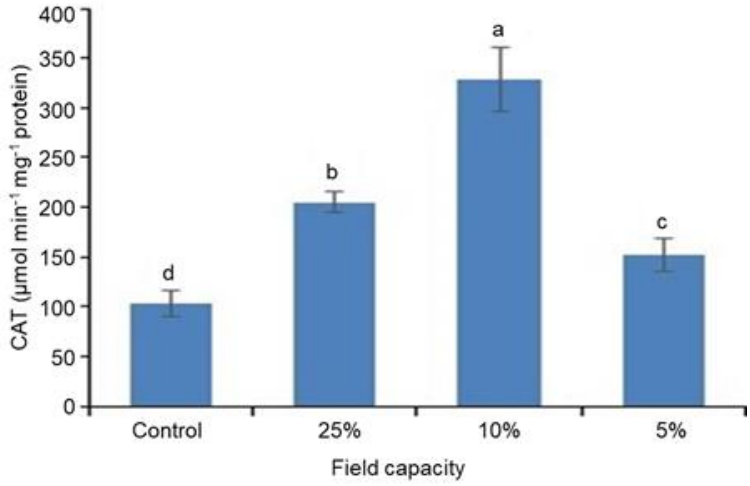

Figure 1. Specific activity of CAT in leaves of barley at different soil moisture levels under drought stress. The values of bar graph are mean of three independent experiments ± SE. Different letters on bars are significantly different among the treatments at p≤0.05.

calculated using the extinction coefficient of 39.4 M^{-1} cm^{-1}. CAT isozymes were analysed using SDS-PAGE under non-reduced and non-denatured conditions at 4°C according to Laemmli (1970). CAT isoenzymes were determined by using 10% separating gel implementing the method of Woodbury et al. (1971) with modification. The gels were treated with 0.01% H_2O_2 for 10 min. Then the gels were rinsed with distilled water, and stained with 1% $FeCl_3$ and 1% $K_3Fe(CN)_6$. Photograph was taken as the CAT bands appeared in the staining solution.

Protein extraction for CAT purification

Thirty grams of barley fresh leaves were extracted by homogenizing in an equal volume of 25 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA, 1% (w/v) ascorbate and 10% (w/v) glycerol with mortar pestle. The homogenate was centrifuged at 11500 × *g* for 15 min, and the supernatant was used as a soluble protein solution for CAT purification.

DEAE-cellulose chromatography

Proteins were precipitated by ammonium sulfate at 65% saturation from the supernatant and centrifuged at 11,500 × *g* for 10 min. The proteins were dialyzed against 10 mM Tris-HCl buffer (p^H 8) containing 0.01% (w/v) β-mercaptoethanol and 1 mM EDTA (buffer A) overnight to completely remove low molecular inhibitors. The dialyzate (crude enzyme solution) was applied to a column (1.77 × 20 cm) of DEAE-cellulose (DE-52, Whatman, UK) that had been equilibrated with buffer A and eluted with a linear gradient of 0 to 0.20 M KCl in 750 ml of buffer A.

Hydroxyapatite chromatography

The pooled sample of CAT, separated by DEAE-cellulose column chromatography, was applied on a hydroxyapatite column (1.5 × 5.5 cm) that had been equilibrated with buffer A. The column was eluted with a 300 ml linear gradient of potassium phosphate buffer (K-P buffer; 0-20 mM, pH 7.0) in buffer A. The high active fraction (5 ml) was found to elute which was collected and further purified on Phenyl Sepharose CL-4B chromatography.

Phenyl sepharose CL-4B chromatography

The pooled sample of CAT, purified by hydroxyapatite chromatography, was applied on phenyl sepharose CL-4B chromatography (1.5 × 5.0 cm) that had been equilibrated with buffer A. The column was eluted with a 200 ml linear gradient of 0-50 mM potassium phosphate buffer (pH 7.0) in buffer A. The high active fractions were collected and purity was tested in an SDS-PAGE.

SDS-PAGE and CBB dying

To check the purification, different fractions were run into a SDS-PAGE of 12.5% (w/v) gel containing 0.1% (w/v) SDS by the method of Laemmli (1970) followed by staining with Coomassie Brilliant Blue (G-250). Molecular weight was measured using Alpha Innotech Gel Imaging System.

Statistical analysis

Data obtained from drought stress were analysed by statistical software Statistix 10 following complete randomized design (CRD), and the mean differences were compared by least significant test (LSD), and p≤0.05 was considered to be significant.

RESULTS AND DISCUSSION

The activity of CAT increased surprisingly with decreasing soil moisture (Figure 1). At control condition, the activity was 101 µmol min^{-1} mg^{-1} protein. The activity increased significantly and continuously with decreasing

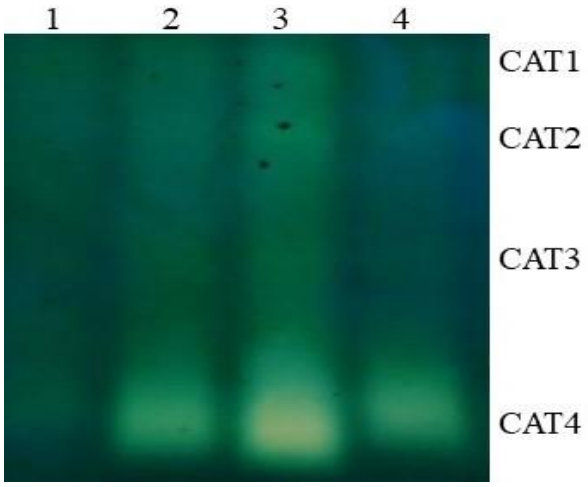

Figure 2. Isozymes of CAT in leaves of barley at different soil moisture level under drought stress. For each lane, 50 µg protein was applied in non-denatured native gel. Lane 1, control; lane 2, 25% FC, lane 3, 10% FC and lane5, 5% FC.

soil moisture (Figure 1). However, the activity decreased after 10% of soil moisture of FC. As compared to control, the activity was 99 and 219% higher at 25 and 10% FC, respectively.

CATs are heme-containing tetrameric enzymes with crucial role for de-toxification of H_2O_2 into H_2O in different stresses (Garg and Manchanda, 2009). They are located in all major sites of H_2O_2 production in the cellular environment (such as peroxisomes, mitochondria, cytosol and chloroplast) of higher plants. CAT have the highest turnover rate of detoxification of H_2O_2 to H_2O per one molecule per minute. Therefore, highly induced CAT activity played essential role in the removal of H_2O_2 (Figure 1). CAT activity has been reported to increase in drought stress in alfalfa (Rubio et al., 2002), *Arabidopsis thaliana* (Koussevitzky et al., 2008), pea (Türkan et al., 2005), citrus (Balfagón et al., 2018; Zandalinas et al., 2017), *Coffea canephora* (Lima et al., 2002), cotton (Ratnayaka et al., 2003; Zhang et al., 2016), (Safronov et al., 2017), maize (Jiang and Zhang, 2002; Rohman et al., 2016), *Populus przewalskii* (Lei et al., 2006), rice (Guo et al., 2006), tobacco (Badawi et al., 2004; Jung, 2004) and wheat (Cheng et al., 2016; Shan et al., 2018).

Recently it has been established that catalase is present as multiple isoforms encoded by multiple genes expressed in organelle, temporal and stress specific manners. For further confirmation, the enzymatic protein extracts were subjected to isozyme analysis (Figure 2). Surprising, increment in intensification of CAT activity bands up to 10% FC as compared to control. Four CAT isozymes were appeared at 10% FC, although CAT 2 and CAT4 were clearer than CAT1 and CAT3.

The modulation of H_2O_2 by the catalase isozymes within specific cells or organelles at specific time and developmental phases directly or indirectly interferes with signal transduction in plants, and the expression of *CAT* gene shows time, species and stress specificity (Sharma and Ahmad, 2014). Previously, Azevedo et al. (1998) reported two isozymes in barley root. In this study, four isozymes in barley leaves (Figure 2) particularly, under drought condition were found. Therefore, the isozymes that appeared under drought condition have important role in H_2O_2 metabolism. Previously, Mallik et al. (2011) investigated CAT activity in diverse groups of plants, such as a unicellular alga, *Chlorella* sp., an aquatic macrophyte, *Najas graminea*, and a mangrove plant, *Suaeda maritima* under saline stress, and reported highly induced CAT activity with formation of new isoforms under only severe saline stress. CAT isozymes have also been studied in many higher plants like 3 isoforms (CAT1, CAT2 and CAT3) in maize (Scandalios, 1990), in sweet potato (Sharma and Ahmad, 2014) and in *Arabidopis* (Filiz et al., 2019). On the other hand, four isozymes have been reported in *Helianthus annuus* cotyledons (Azpilicueta et al., 2007), 12 isozymes in Brassica (Frugoli et al., 1996) and only one prominent CAT in the leaves, stems and roots of *Broussonetia papyrifera* with different responses to salinity stress (Zhang et al., 2013). Polidoros and Scandalios (1999) showed positive relationship of two isozymes CAT1 and CAT2 with H_2O_2 metabolism in maize. Filiz et al. (2019) evaluated expression profile of two gene *CAT2* and *CAT3* coding for CAT under salt, cold, heat and light stress in natural *Arabidopsis* ecosystem through micro array system, where high light, salt and cold stresses substantially up-regulated the expression of the genes, but down regulated in cold stress. They also reported one *CAT1* gene coded for SOD. On the other hand, Skadsen

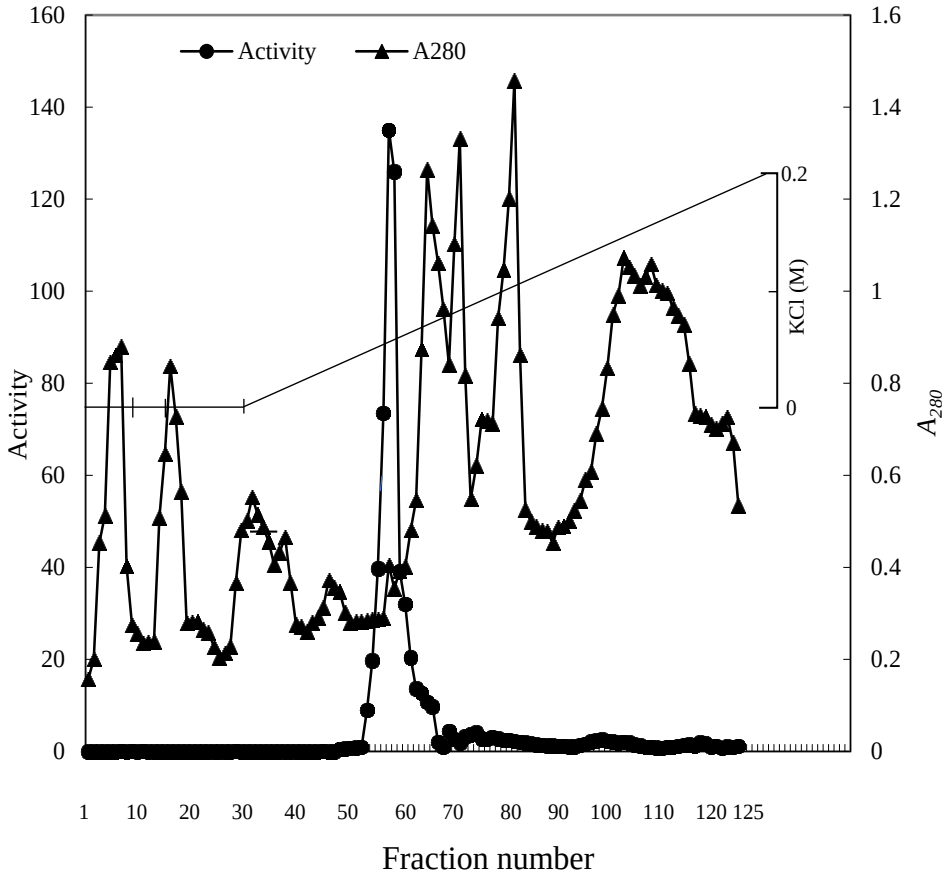

Figure 3. A typical column chromatography of DEAE-cellulose of soluble proteins prepared from 30 g leaves from 10 days-old barley seedlings. For each fraction, absorbance at 280 nm (▲) and CAT activity (●) were determined. Activity is expressed as mmol min^{-1} ml^{-1}. Bars indicate the high active peak fractions of barley CAT. The fractions under the bar of CAT peak were pooled for subsequent purification. The curve shows the concentration of KCl (0-0.2 M).

et al. (1995) characterized *CAT1* and *CAT2* gene in barley, but their expression was not reported under abiotic stress. Jeong and Kim (2004) reported two CAT isozymes in barley root with differential expression in aluminium stress. On the other hand, one isozyme was reported under salinity (Mohammad et al., 2015) and drought (Salekjalali et al., 2012), where highly expression of the activity was observed under drought. Therefore, CAT isozymes varied with different research group probably due to use different genotypes of barley. In this study, the variety used showed the presence of four isozymes under drought. Therefore, we believe that this study bears importance to study the four isozymes under different abiotic stress.

Purification of catalase from barley leaves

Catalase always draws the attention of researchers due to its efficient catalytic and regulatory properties among all antioxidant enzymes of the plant system. Thus, it was purified from barley leaves in this study for future characterization at the genetic, biochemical, and molecular level.

The soluble protein fraction was prepared from 30 g fresh leaves. The soluble protein was precipitated by $(NH_4)_2SO_4$ at 65% saturation and dialyzed overnight and the dialyzate was applied on DEAE-cellulose column chromatography (i.d. 1.7 × 20 cm) and eluted with a linear gradient of KCl (0-0.2 M) (Figure 3). A total of 125 fractions, each containing 5 ml, were collected. The CAT activities and absorbance at 280 nm were measured. A high active peak was eluted at 67 mM of KCl.

The fractions showing high CAT activity were pooled and applied onto a hydroxylapatite column chromatography (Figure 4). A total of 300 ml gradient solution containing 0 to 20 mM potassium-phophate (K-P) buffer, pH 7 was passed. A total of 70 fractions were collected and CAT activity and absorbance at 280 nm was recorded for each fraction (Figure 4). An active CAT

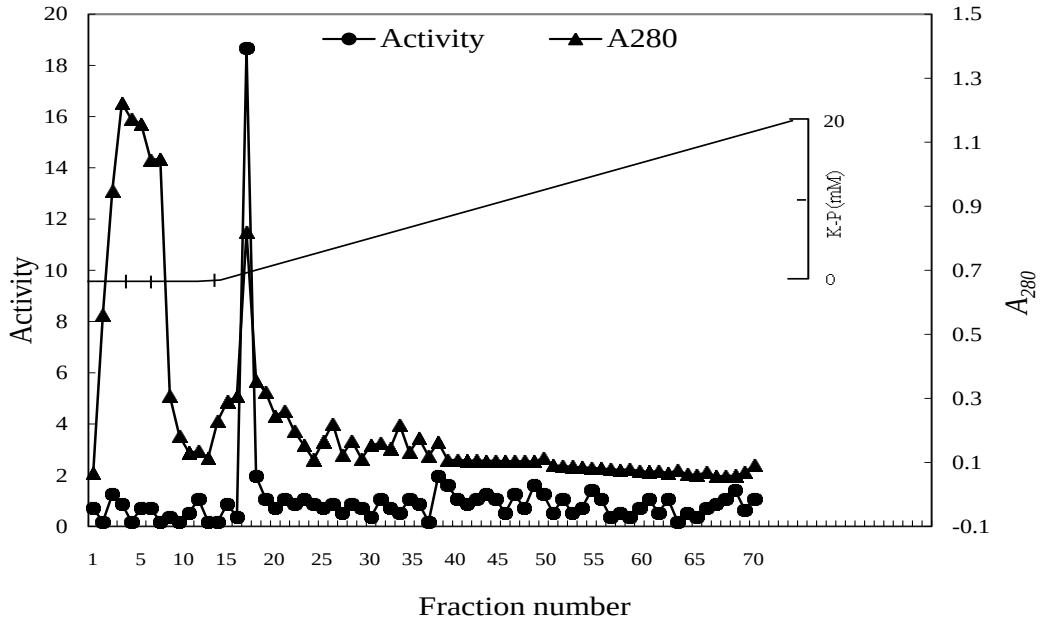

Figure 4. Elution profile of CAT from hydroxyapatite column chromatography. For each fraction, absorbance at 280 nm (▲) and CAT activity (●) were determined. Activity is expressed as mmol min^{-1} ml^{-1}. Bars indicate the high active peak fractions of barley CAT. The fractions under the bar of CAT peak were pooled for subsequent purification. The curve shows the concentration of K-P buffer (0-20 mM).

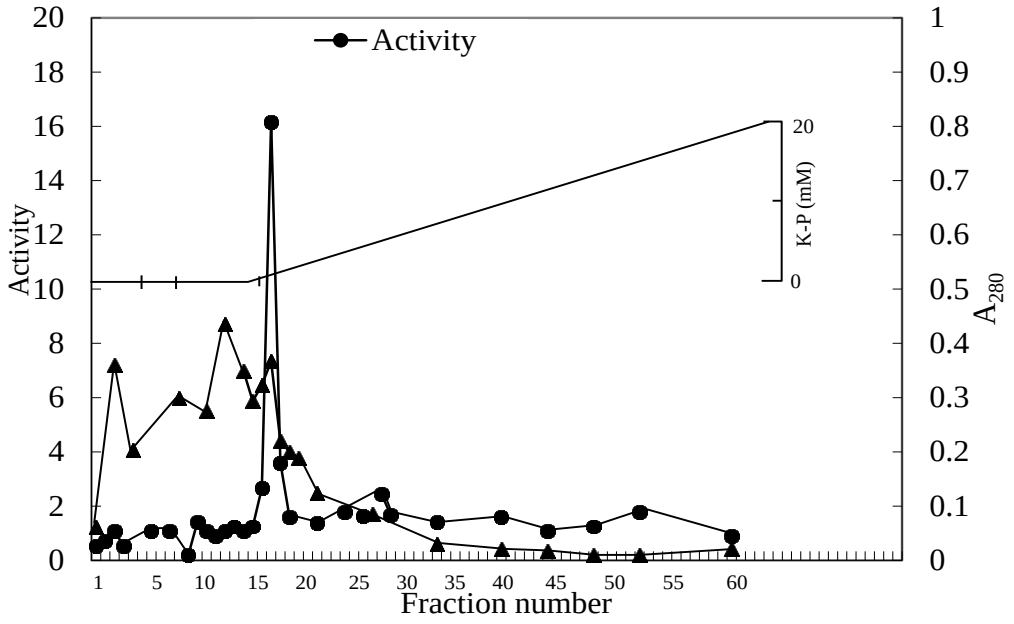

Figure 5. Elution profile of CAT from phenyl sepharose CL-4B and eluted with 50 mM K-P buffer. For each fraction, absorbance at 280 nm (▲) and CAT activity (●) were determined. Activity is expressed as mmol min^{-1} ml^{-1}. The curve shows the concentration of K-P buffer (0-20 mM).

peak was found.

Finally, the active fractions were applied to phenyl sepharose CL-4B and eluted with 50 mM K-P buffer. The CAT activity and absorbance were taken and presented in Figure 5. Only one active peak of CAT activity was found. Therefore, all the fractions were applied on a

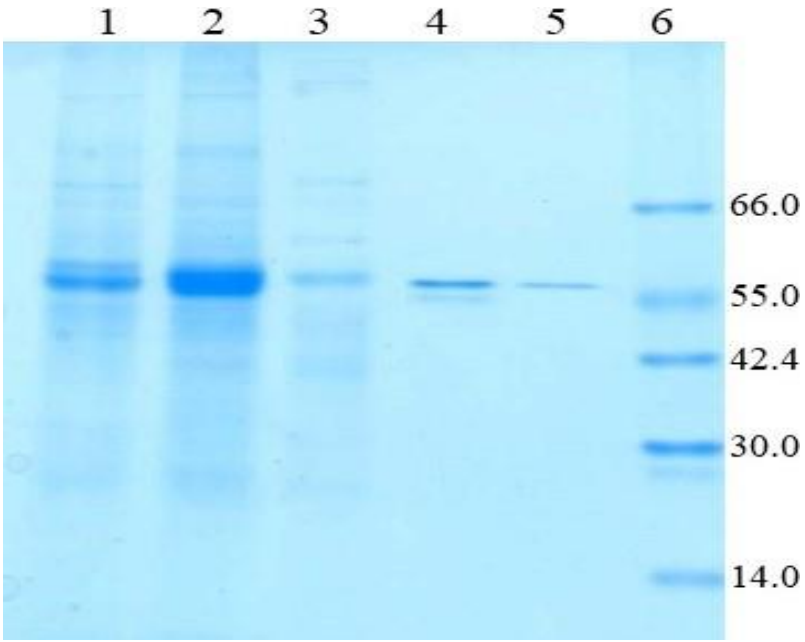

Figure 6. CBB staining of purification fractions in SDS-PAGE of barley CAT. Lane 1, Homogenous; 2. $(NH_4)_2SO_4$ ppt; 3, DEAE fraction; 4, Hydroxylapatite fraction; 5, Phenyl Sepharose CL-4B fraction and 6, Molecular weight marker (KDa)

Table 1. Summary of CAT purification from barley seedling.

Fraction	Total activity (mmol min^{-1})	Specific activity (µmol $min^{-1}mg^{-1}$ protein)	Total protein (mg)	Recovery (%)	Purification fold
Homogenous	67717.5	180.58	375.00	100	1.00
$(NH_4)_2SO_4$ ppt	40925.3	239.57	170.83	60.4	1.33
DEAE-cellulose	3346.92	446.17	7.50	4.94	2.47
Hydroxylapatite	798.32	528.68	1.51	1.18	2.92
Phenyl sepharose CL-4B	351.45	871.32	0.43	0.52	4.83

SDS-PAGE and stained with coomassie brilliant blue (CBB) (Figure 6).

The CBB staining showed that the subsequent purification method eliminated the undesirable protein gradually. Finally, CAT protein was purified and moved with a single band showing the apparent molecular weight of 54 kDA. The summary of the purification is shown in Table 1.

It was found that the purified protein contained activity of 351.45 mmol min^{-1} (Table 1). The specific activity and amount of protein were 371.32 and 0.43 mg, respectively, with 0.52% recovery and 4.83 purification fold. In purification of CAT from barley, it was apparently observed in SDS-PAGE that the purified CAT protein is a polypeptide of 58 kDa (Figure 6). Beulah and Ramana (2013) reported a CAT of 51.3 kDa in *Phyllanthus reticulates.* The CAT purified from cotyledon of germinating pumpkin seed was 55 kDa (Yamaguchi and Nishimura, 1984) while CAT purified from *Zantedeschia aethiopica* had a molecular weight of 54 kDa (Trindade et al., 1988).

Conclusion

From the above data, it was found that CAT activity increased with increasing droughts, and after 10% FC, the activity decreased. Strong evidence was provided by analysing isozymes where four isozymes CAT1, CAT2, CAT3 and CAT4 were visualized in drought stress. Therefore, it is very clear that in barley, new CAT isozymes are synthesized under drought, and likely to have essential role in H_2O_2 scavenging. On the other hand, a CAT from barley leaf was purified, and the purified catalase had an apparent molecular weight of 54 kDA. Therefore, further study is required for its molecular

and biochemical characterization.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Diversity of arbuscular mycorrhizal fungi (AMF) and soils potential infectivity of *Vachellia nilotica* (L.) P.J.H. Hurter & Mabb. rhizosphere in Senegalese salt-affected soils

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***Vachellia nilotica,* a multipurpose tree useful to rural populations, is often used to rehabilitate Senegalese salt-affected soils due to its salt-tolerance probably related to arbuscular mycorrhizal fungi (AMF) symbiosis. This work aimed to determine the AMF communities associated with *V. nilotica* rhizosphere in salt-affected soils and their potential infectivity. Soils were sampled from six sites in the central region of Senegal. Soil chemical properties and total fungal communities were analyzed. Spores number was estimated and morphotypes were identified. Soil-borne AMF were trapped using *V. nilotica* plants to analyze their infectivity potential and their effect on plant growth and biomass production. Results showed the capacity of *V. nilotica* to grow in a heterogeneity of soils and revealed that rhizospheric soils are rich in AMF infectives strains. Data showed that salinity was the main factor that influenced the fungal structure and has a negative effect on AMF development, hence the highest number of spores in high salinities soils. This negative effect is greater in soils collected in dry season than in wet season. The highest density of spores was found in soils beneath the canopy, indicating the influence of plant rhizosphere on AMF diversity. Morphotypes identified are related to the genus *Glomus, Gigaspora, Acaulospora.* AMFs in these soils can establish a good mycorrhization with *V. nilotica* and increase plant biomass and height.**

Key words: *Vachellia nilotica*, arbuscular mycorrhizal fungi, diversity, salt affected soils, rhizosphere, infectivity.

INTRODUCTION

Soil salinization is a worldwide problem amplified by global warning, especially in arid and semiarid regions. Salinization reached about 7% of total areas and 20% of cultivated soils in the world (Zhu, 2001; Abbas et al., 2013).

In Senegal, about 45% of cultivated soils are affected by salinization (LADA, 2009) and more than 8% in Fatick and Kaolack, center region of the country (PAPIL, 2013). Consequences of soil salinity are harmful especially in arid lands ranging from plant diversity lost to total lack of plants (desert) including damaging soil properties and decline of fertility. One of the sustainable strategies to rehabilitate these salts affected soils and control salinity is reforestation with adapted perennial plants like leguminous. *Acacia* species such as *Vachellia nilotica* are often used in Senegal for reforestation (Sambou et al., 2010) because of their tolerance to salinity (Singh and Thomson, 1992; Giri et al., 2007). *V. nilotica* is a multipurpose tree species for rural populations: fuel-wood, timber, gum, therapeutic use (Bargali and Bargali, 2009). However, its symbiotic association with both arbuscular mycorrhizal fungi (AMF) and rhizobia could contribute to plant adaptation to harsh environments such as saline soils because they could supply nitrogen, water, phosphorus and other nutrients. AMF contributed to crop productivity and ecosystem preservation through many ecosystemic services including plant nutrition, soil structure, and plants protection against abiotic stresses by improving drought tolerance, protection against pathogens, stimulation of synthesis of plant secondary metabolites beneficial to human health (Gianinazzi et al., 2010; Hanin et al., 2016; Begum et al., 2019).

Several studies have reported beneficial effect of AMF on *Acacia* spp. plant growth and adaptation to harsh environments (Raghuwanshi and Upadhyay, 2004; Diouf et al., 2005; Ashraf et al., 2008; Fall et al., 2017; Manga et al., 2017). Thus, some previous studies also showed that *V. nilotica* is mycotrophic and inoculation with AMF can enhance plant productivity (Dommergues et al., 1999; Giri et al., 2007; Chandrasekaran et al., 2014). But few studies addressed indigenous communities of AMF associated to *V. nilotica* in salt-affected soil in this part of the country where settlements of this tree were well established. However, it is established that there is variability in the response of plants to AMF depending on the nature of the soil (Bâ et al., 1996; Diop et al., 2015). Moreover, rhizospheric soil of *V. nilotica* trees established in salt-affected soils could harbor indigenous communities of AMF that could be identified as inoculum to enhance *V. nilotica* plants growth and to contribute to their survival and their establishment in saline soils. This work aimed to describe morphological diversity of indigenous AMF associated to *V. nilotica* rhizosphere in salt affected soils and their effect on plant growth, in order to identify potential AMF inoculum strains able to optimise *V. nilotica* plant nursery development used for reforestation programs of degraded salt affected soils.

MATERIALS AND METHODS

Soils sampling and chemical properties analysis

Soils samples were collected from six sites in Fatick and Kaolack regions, center of Senegal, and their geographic positions are recorded by Global Position System (Figure 1). Climate in this area is characterized by the alternation of two seasons: a long dry season which is spread over eight months (October-June) and a short rainy season up to four months (June-October). The temperature fluctuates between 21-24°C in December-February and 35-42°C particularly in May-June (SES, 2016) and annual precipitations are about 400-800 mm.

Soils were sampled in the rhizosphere of mature *V. nilotica* trees from six salt affected sites in Fatick and Kaolack regions (Djilass, Nguessine, Fatick, Ngane, Kahone and Sadioga) to recover more areas. The same sites are sampled in both wet and dry seasons, except Nguessine 2 which was inaccessible in wet season. For each site, two samples soil mixtures were collected, beneath the canopy of *V. nilotica* (SC) and outside the canopy (as control, T). Each sample is a mixture of three samples (replicates) from three rhizospheric trees. Four soil subsamples were collected in each sample point (1 m from the trunk in all cardinal directions) at a depth of 0-25 cm and pooled to one homogeneous rhizospheric soil sample.

The chemical properties analysis of the soils samples were carried out at the LAMA lab (Laboratoire des Moyens Analytique) certified International Standard for Organization 9001, version 2000; Institut de Recherche pour le Développement, IRD-Bel Air, Dakar-Sénégal (www.lama.ird.sn)

Soils DNA extraction

DNA extraction of soil microbial communities was performed using the Power Soil DNA isolation kit (MoBio Laboratories, Inc.) according to the manufacturer's instructions. DNA extracts were stored in 50 µl of sterile water and kept cold for PCR uses later.

Nested-PCR (PCR-DGGE)

The analysis of total fungal community of soils was performed using "Nested PCR" technique. A first PCR amplification was carried out with the primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns, 1993, 1996) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The following thermocycle programme was used for the PCR amplification: 94°C (5 min) initially, followed by 30 cycles of 94°C (30 s); 57°C (30 s); 72°C (1 min) and 72°C final (10 min). PCR products were migrated in a 1% agarose gel stained with ethidium bromide and visualized in UV light.

PCR products with 700 to 900 bp length were then used as template DNA for a second PCR with the primers ITS1F-GC (Muyzer et al., 1993) and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') (White et al., 1990). This PCR reaction yielded DNA fragments

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Figure 1. Geographical location of soils sampling sites.

length of about 400 bp with the initial PCR cycle of 94°C (5 min), followed by 30 cycles of 94°C (30 s); 60°C (30 s); 72°C (30 s) and 72°C final (10 min). The reaction mixture contained per tube, 1 µL of primers (25 µM), 0.5 µL DNA (2.5 ng / µL); Taq Ready To Go bead (Amersham Biosciences, USA) (contains 2.5 µg *Taq* DNA polymerase, 200 µM dNTP, 10 mM Tris-HCl pH 9, 50 mM KCl and 1.5 mM $MgCl_2$) for a final volume of 25 µL.

DGGE gel migration

DGGE is based on the electrophoretic separation of double stranded DNA molecules depending on the difference in their melting behaviour in a gradient of either a denaturing agent or temperature (Muyzer et al., 1993). The DGGE resolution distinguishes two different PCR bands from a single base (Muyzer and Smalla, 1998).

The rDNA fragments obtained by Nested PCR were separated on an 8% polyacrylamide gel [40% acrylamide-bisacrylamide (37.5: 1)] with a gradient of denaturants (23 to 58%) for the fungal community (100% denaturant contains 7 M urea with 40% formamide).

The migration was carried out at 100 volts for 18 h in a TAE buffer (1X) at 60°C. Visualisation of rDNA fragment profiles was done at UV (254 nm) after staining the gel with BET (1 mg / ml). Images capture of rDNA profiles was done using Bio-Capt software (Vilber Lourmat, France). Profiles were analysed with the Phoretix 1D tutorial version 10 (TotalLab Ltd) tape detection, based on presence-absence, intensity of bands. After analysis and validation, a dendrogram was generated.

Spores extraction and morphotypes identification

Spores of arbuscular mycorrhizal fungi were isolated from soil samples using the wet sieving and decanting method as described by Gerdemann and Nicolson (1963). An aliquot of 100 g of each soil sample was used. The supernatant containing the spores was filtered through superposed sieves of decreasing size (500, 200, 100 and 50 µm) in order to recover the maximum number of spores. This operation was repeated at least 3 times. All particles retained in the sieves of 200, 100 and 50 µm were recovered in 30 ml tubes containing distilled water (spores suspension). The separation of the spores was performed using two sucrose solutions, 20 and 60%. At the end, the 50 µm sieve contents were collected with tap water and stored at 4°C for preservation.

The total number spores in 100 g of soil were observed and counting under the WILD M400 binocular loupe. Microscopic observation was used to identify and to photography the different morphotypes from the rhizospheric soils of *V. nilotica* and soils outside canopy.

Morphological spore characterization was made in comparison with genus descriptions provided by the International Culture Collection of Arbuscular and Vesicular Arbuscular Fungi (http://invam.caf.wvu.edu). Criteria for morphological spore characterization were mainly based on spore size, wall colour and structure and hyphal attachment (Cho et al., 2006).

AMF trapping in greenhouse

The experiment was carried out in greenhouse using *V. nilotica* plants to compare AMF infectivity of rhizospheric soils from six sites. Seeds were first treated with concentrated H_2SO_4 and thoroughly washed with running tap water and soaked in water overnight. Two germinated seeds were transplanted in plastic bag that contained 1 kg sterilised (by autoclaving 180°C for 4 h) soil of Sangalkam, a nutrients poor sandy soil with low P content (Bâ et al., 1999). A week after, one plant was taken off to have plants with homogeneous growth.

Experiment has compared 12 treatments inoculated using six rhizospheric soil samples and their controls collected outside canopy and one non inoculated as control. Each treatment has ten replicates. Inoculation was performed by adding 50 g per bag of each rhizospheric soil sample (as inoculum) before transplanting

the seeds. Non-inoculated pots received the same amount of autoclaved soil. After three months, the plants were harvested, biomass estimated and height also measured. Roots were sampled for observation of mycorrhizal colonization and estimation of mycorhization parameters.

Roots coloring

Roots colouring was performed as described by Philips and Hayman (1970). Samples roots were thoroughly cleaned with tap water to remove soil particles and then placed in a 10% KOH solution to discolour them and to empty the cytoplasmic contents of the root cells. The tubes were boiled in a water bath for 1 h at 90°C. The roots were thoroughly rinsed with water to remove the KOH, followed by roots dewatering and lightening with bleach for 3 min. The staining of the roots was carried out in a Trypan blue solution of 0.05% and boiled in a water bath at 80°C for 30 min. The stain was removed and roots rinsed for last time to remove the excess. The roots are finally soaked in tap water.

Estimation of mycorrhizal roots colonization

The intensity and frequency of mycorrhization were determined using the Trouvelot et al. (1986) method. For each sample, the coloured roots were cut into fragments of about 1 cm each and deposed between slides and lamellae which are parallels and coated with 90% glycerol for preservation. One slide contains 15 fragments, with 5 repetitions for each treatment. Slides were observed under a microscope (x 20) fragment by fragment. The colonization level of the roots was estimated by presence of hyphae, vesicles or arbuscules in the roots.

Statistical analysis

Analysis of variance (ANOVA) was performed on all data using XLSTAT (version 2010, Addinsoft) software. Mean values of all treatments were compared using Duncan test (Honestly significant differences, HSD) at the significance level ($p<0.05$). The correlation between the chemical properties of the soils was established with the PCA test (principal component analysis) according to the type of Pearson (n).

RESULTS

Chemical properties of soils

Results showed differences in salinity rate and pH for sampling soils sites. Samples of dry season showed high soils salinity in localities of Djilass beneath canopy (SC) and outside (T), Nguessine2T, Fatick T and Nguessine1 (SC and T) and low salinity soils such as Nguessine2 SC, Sadioga (SC and T), Ngane (SC and T) and Kahone (SC and T) and Fatick SC. Comparison of different soils chemical properties using PCA analysis allowed the distinguishing of three groups of soils (Figure 2): Group 1 correspond to soils with high salinity, acidic pH and high percentage of exchangeable sodium. Soils of this group are TNguessine 1 (TNGE1), Nguessine1 (NGE1), TDjilass (TDJI), Djilass (DJI), TFatick (TFAT); Group 2 correspond to soils with low salinity and alkali pH, an abundance of exchangeable bases (K, N, Ca, C), a high rate of organic matter (% MO): soils from Kahone (KAH), Kahone control (TKAH), Fatick (FAT), Ngane control (TNGA). The group 3 grouped soils from Nguessine2 SC, Sadioga (SC and T) and Ngane SC with low salinity and acid pH, a low level of mineral elements and organic matter. This demonstrated the capacity of *V. nilotica* to grow in a heterogeneity of soils.

Total fungal richness of soils

In order to analyze how salinity affect fungal population, soil richness in fungal communities was estimated by analyzing the ITS of 18S gene region of the total DNA extracted from the rhizosphere soils sampled beneath and outside canopy of the *V. nilotica.* The DGGE profiles were compared for their similarity and the resulting dendrogram (Figure 3) showed two main groups of soils (I, II).

Group I could be divided into three subgroups (IA, IB, IC). IA was composed of soils from Kahone (beneath and outside canopy), Sadioga (beneath and outside canopy) and Ngane outside canopy characterized by low salinity and pH that can be acidic or basic. It should be noted that the samples under cover have profiles similar to those from outside the canopy, indicated that low salinity did not affect fungal communities.

Group IB contains soils of Ngane, Nguessine 1 and Nguessine 2 and Djilass all having an acidic pH, and salinity that can be low or high. The IC group with low salinity and acidic pH was composed of the soils Fatick beneath canopy and Nguessine1 outside canopy.

Group II consists of soils outside canopy from Nguessine 2, Djilass and Fatick. They are characterized by heterogeneity of soils with high salinity and various pH rates. It means that salinity is the main factor that has influenced fungal communities structure, secondary supported by pH and perhaps others factors.

Diversity and richness of AMF morphotypes in *V. nilotica* rhizosphere

AMF spores in salt affected soils was estimated analyzing the number per 100 g of soil and identified morphotypes. Spores extraction by the wet sieving technique yielded mature spores in varying numbers depending on the soil sample origin (Figure 4). These spores are also varying in sizes and colors (yellowish, brown, black and white).

Spores richness is different according to soil origin and there was a high density on soils collected under cover compared to those from outside the canopy. This could be explained by the best development of AMFs in the presence of the host plant roots in favorable conditions

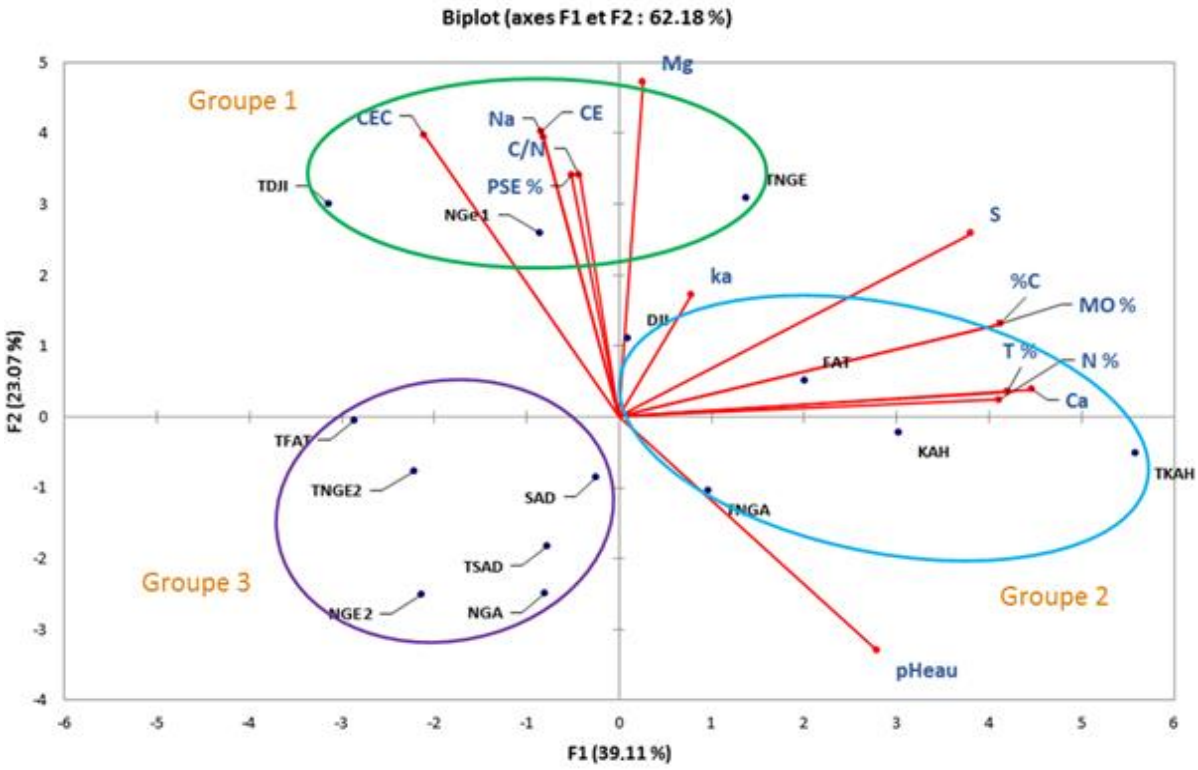


Figure 2. Comparative analysis of physio-chemical properties of soils from Ngane (NGA), Djilass (DJI), Sadioga (SAD), Fatick (FAT), Kahone (KAH), Nguessine (NGE) collected beneath canopy and outside canopy (T).

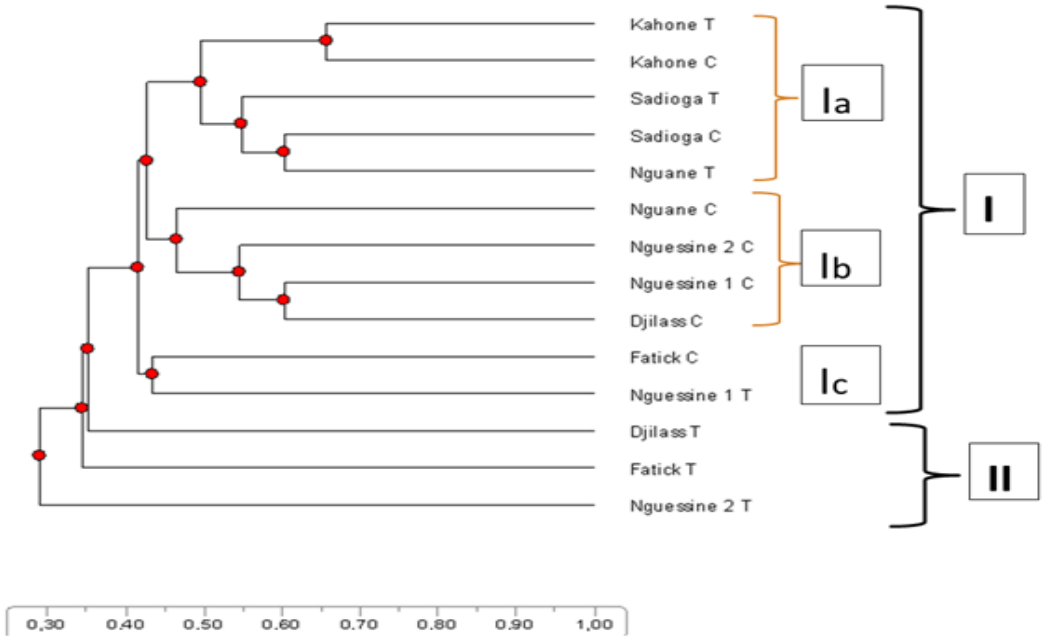


Figure 3. Dendrogram of similarity based on comparaison of the DGGE profiles of the ITS region of the 18S rDNA gene of the total soil fungal community, carried out with the Phoretix 1D tutorial version 10 software (Total Lab Ltd). Site name with C = beneath the canopy and with T = outside canopy.

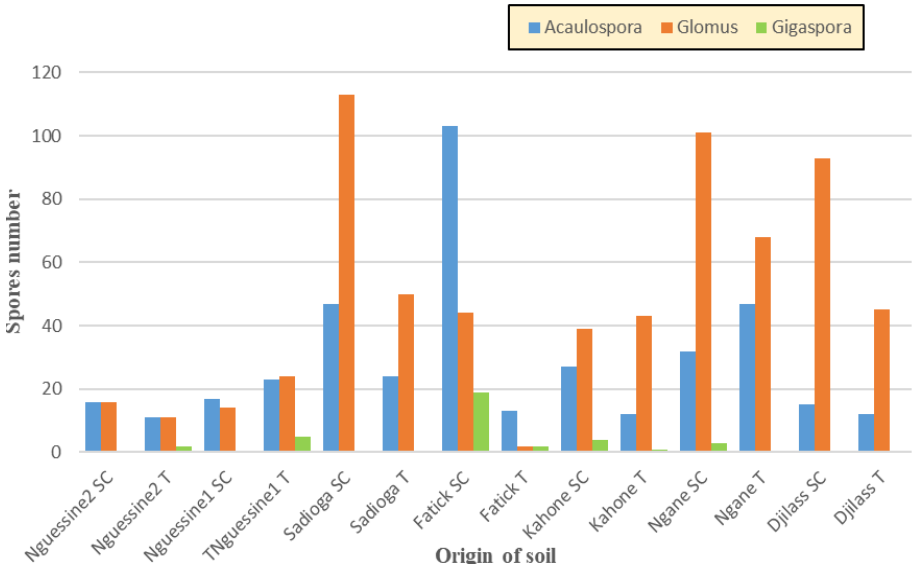


Figure 4. Numbers of AMF spores for each morphotype isolated per 100 g of rhizospheric soils beneath (SC) and outside the canopy (T) of *V. nilotica* in dry season.

and spores release nearly. Generally, the high salinities soils showed the highest number of spores indicating that spores are formed in adverse conditions.

Spores morphotypes identified in this study in all the sites appeared to belong to three genera: *Glomus (G), Gigaspora (Gi), Acaulospora (A)*; *Glomus* and *Acaulospora* are widespread in almost all soils whereas the genus *Gigaspora* was found on some soils (Figure 4).

Several spores morphotypes could not be identified at species level. Among identified spores, three species are related to the genus *Glomus* (*G. sp.1, G. sp.2, G. sp.3*), five species to the genus of *Gigaspora* (*Gi. rosea*, *Gi. aff. albida*, Gi. sp.1, sp.2, sp.3) and five species of the genus *Acaulospora* (*A. aff. colombiana* and *A. sp1, A. sp.2, A. sp3 and A. sp.4)*. *Gi. rosea* was found in Fatick and Nguessine, *Gi. aff. albida* was found in Kahone and *A. aff. colombiana* in Djilass (Photo 2).

Analysis of spores collected according to season showed that in dry season, soils under the canopy from Fatick, Ngane, Djilass and Sadioga showed high number of spores while soils outside the canopy of Kahone, Djilass, Nguessine, Fatick showed low spores density due to absence of host plant. In wet season, soils outside the canopy from Fatick, Sadioga, Kahone and Nguessine had a high number of spores while soils under capony of Kahone, Nguessine and Djilass showed low spore numbers. It can be noted that Sadioga had high spore number in both season; this could be explained by their low salinity and herbaceous development.

Comparative analysis of spores number from dry and wet seasons (Figure 5) revealed that spores number is highest in dry season in most of the soils, because under favourable conditions (wet season) the spores germinate and colonize the plants.

Mycorrhizal colonization

Microscopic observation of the colored root fragments of the different treatments showed a lack of mycorrhizal structures in un-inoculated plants roots. Vesicles and hyphae were observed in the plants inoculated with rhizospheric soils samples, meaning that these rhizospheric soils were rich in AMF strains infectives to *V. nilotica.* Because of many hyphae and vesicles observed in the roots (Photo 1), *V. nilotica* is a very mycotrophic plant.

Mycorrhization frequency and intensity estimated for soils collected in dry and wet seasons are presented in Figures 6 and 7. The intensity of mycorrhization is variable depending on the type of soil, season (wet or dry) and position to tree (beneath or outside canopy). The less salty soils showed the highest frequency and intensity of mycorrhization compared to high salty soils. This could be explained by the negative effect of salt on the AMFs multiplication. It is also noted that rhizospheric soils usually have significantly the highest values compared to samples outside the canopy showing the positive effect of the presence of the tree in mycorrhization. Plants with soil samples from Fatick, Kahone, Djilass, Sadioga, Ngane under the canopy have significantly higher colonization intensities and frequencies compared to uninoculated plants. The greater frequency is observed for Fatick in dry season and Ngane SC and Nguessing SC dominated in wet season.

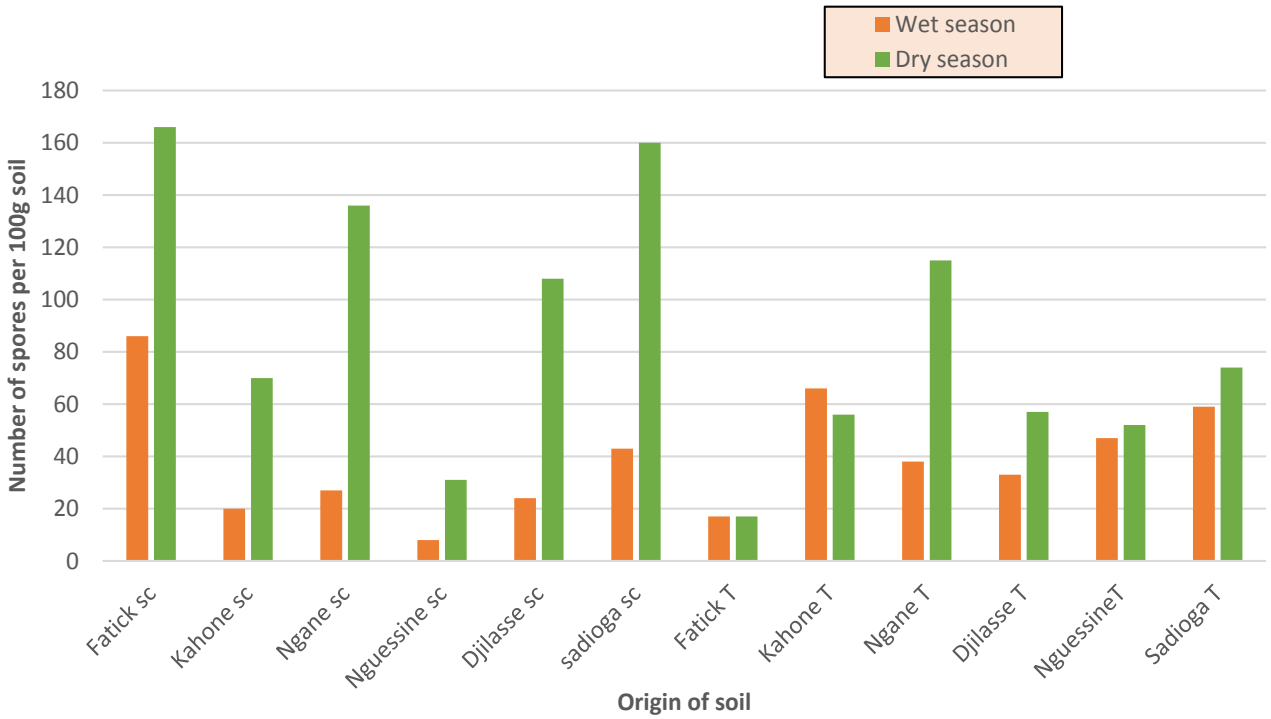


Figure 5.Spores number per 100 g of soil isolated from rhizospheric soils of *V. nilotica* from different sites, beneath (SC) and outside the canopy (T) in dry and wet season.

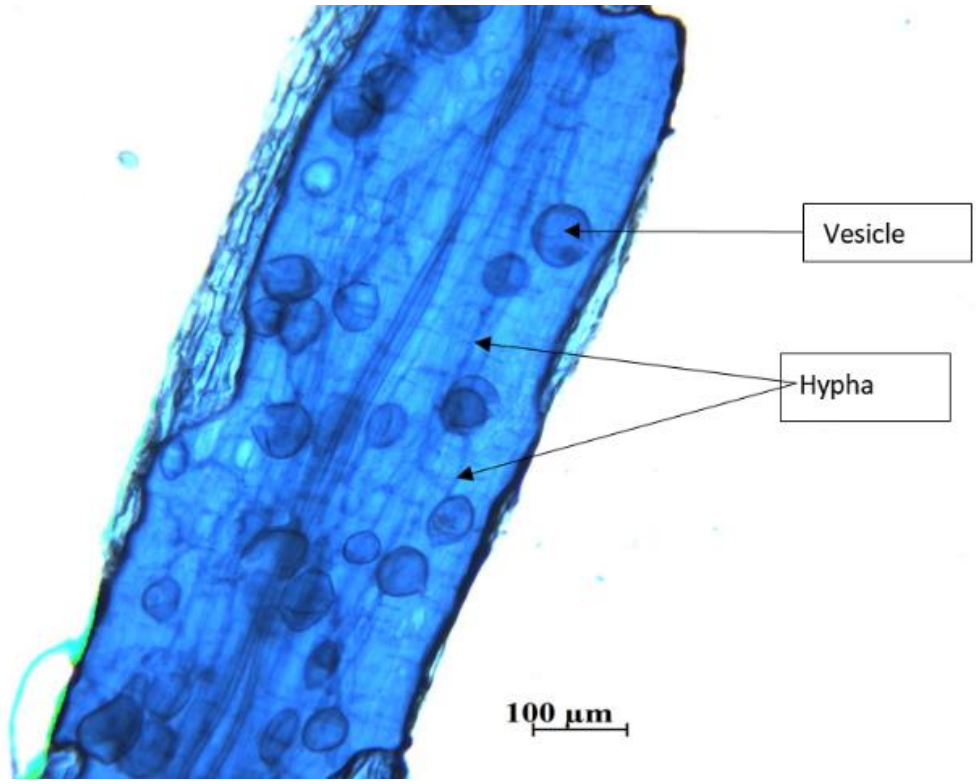
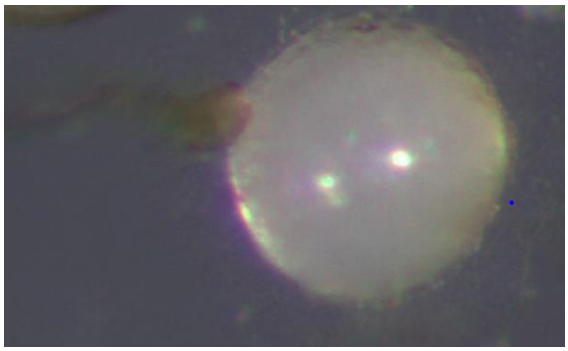
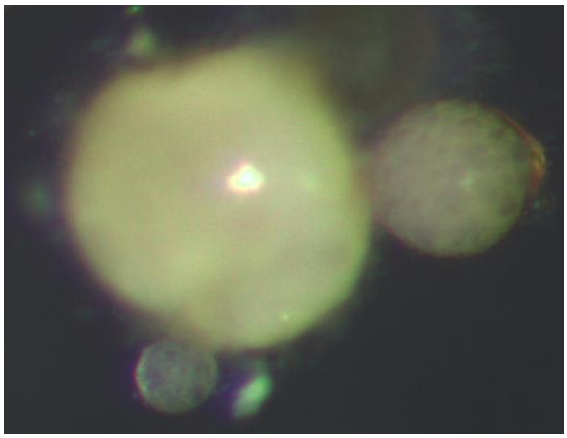


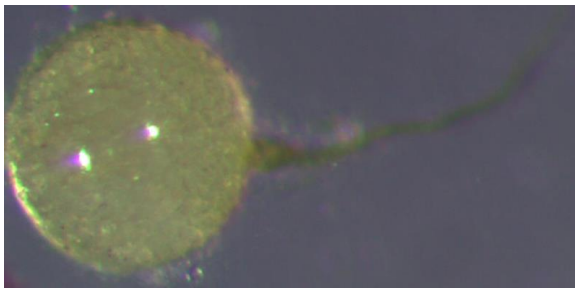
Photo 1. AMF Vesicles and hypha in *Vachellia nilotica* plant roots after trapping in greenhouse conditions.



***Gigaspora rosea* (Fatick)**



Acaulospora aff colombiana (Djilass)



Gigaspora aff albida (Kahone)

Photo 2. Spores morphotypes identified as known species in some soils.

Effect of mycorrhization on *V. nilotica* plant height and biomass

Our results showed mycorrhization have a positive effect on plant height (Figure 8), and soil samples collected beneath canopy lead to greater plant height compared to soils outside canopy. This effect varied according to soil origin and sampling period. In dry season (Figure 8A), soils beneath canopy from Nguessine2, Kahone, Fatick and NganeT showed a significant increase in the height of inoculated plants compared to plants control. DjilassT and Ngane SC soils showed no significant difference with plants control, because of their very low frequency and intensity of mycorrhization. In wet season (Figure 8B), soil beneath canopy from Nguessine, Fatick, Ngane, Djilass showed best results.

The impact of mycorrhization on plant total biomass is presented in Figure 9. Results showed a significant effect of inoculation on biomass of the inoculated plants which is greater than the control plants. However, increase of biomass varied according to the types of soil. Soils with high mycorrhizal frequencies and intensity are also those with high biomass ; this corroborated the positive effect of mycorrhization on plant biomass. The greater effect was

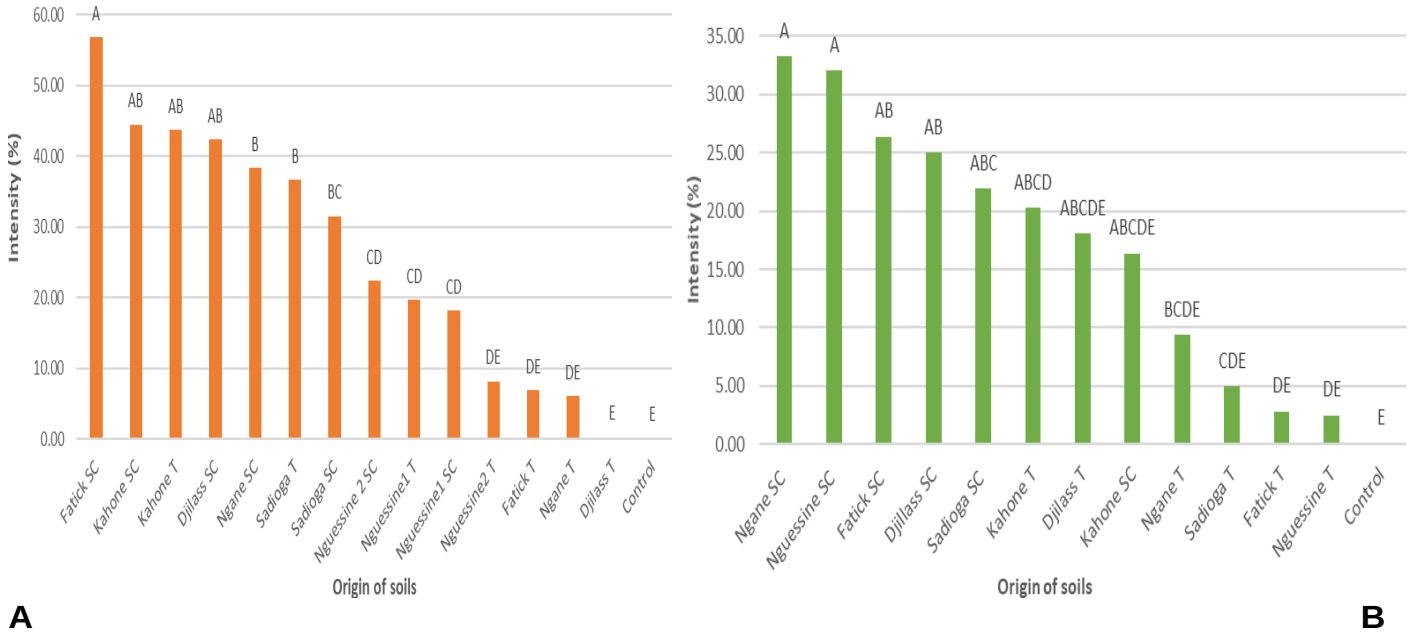


Figure 6. Mycorhizal intensities of *Vachellia nilotica* plants cultivated with rhizospheric soils samples collected beneath canopy (SC) and outside canopy (T) in dry (**A**) and wet (**B**) seasons. NB. Numbers with the same letter are not significantly different according to Newman et Keul's test. P< 0,0001.

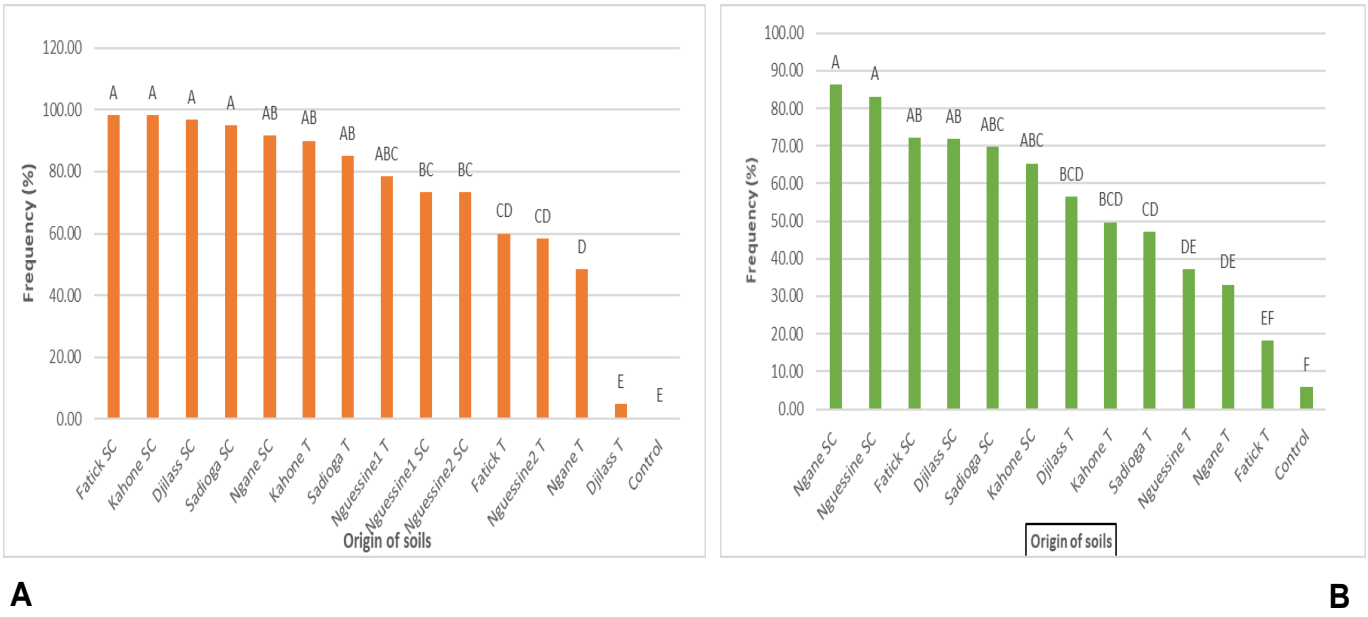


Figure 7. Mycorrhizal frequencies of *V. nilotica* plants cultivated with soils collected beneath canopy (SC) and outside canopy (T), in dry (A) and wet (B) seasons. NB. Numbers with the same letter are not significantly different according to Newman et Keul's test. P< 0.0001.

observed in dry season (9A) with soils from Sadioga, Kahone Fatick and Djilass and in wet season (9B) with those from Nguessine, Ngane, Kahone, Sadioga and Djilass, soils with the highest mycorrhizal colonization.

Otherwise, the positive impact of the mycorrhization on plant productivity (plant biomass and height) in all soils

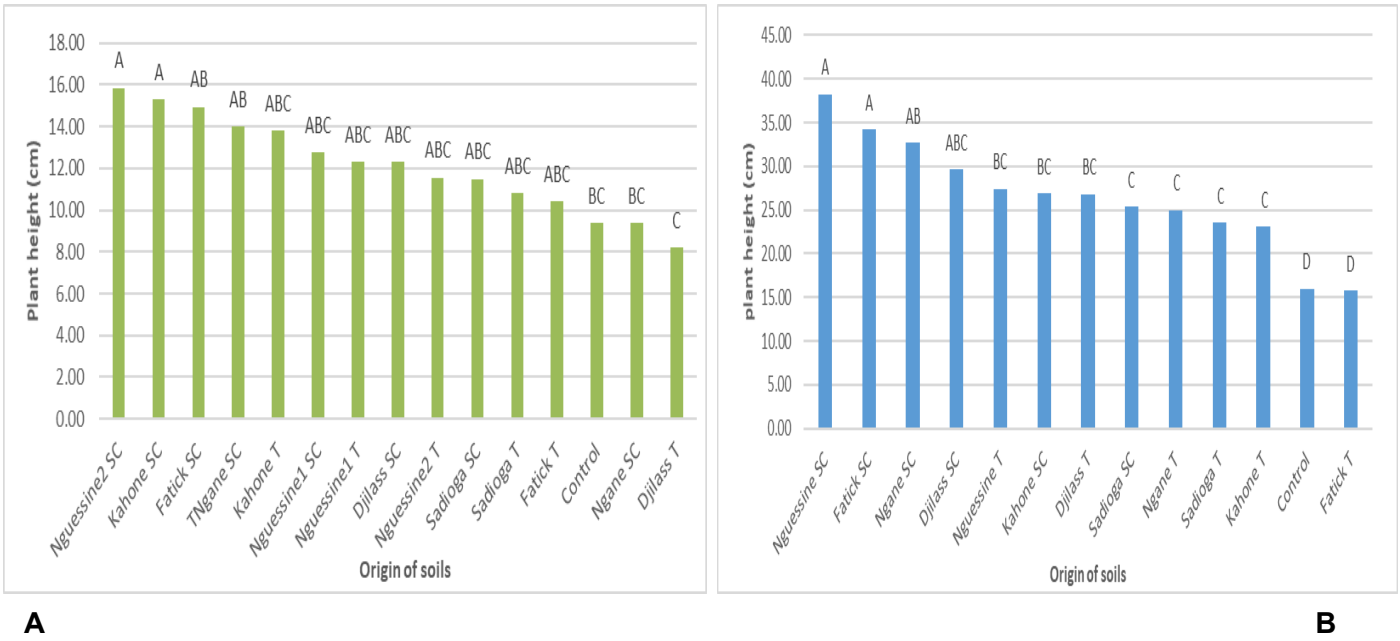


Figure 8. Effect of mycorhization on *V. nilotica* plant height cultivated in greenhouse with rhizospheric soil samples collected beneath canopy (SC) and outside canopy (T) in dry (A) and wet (B) seasons. Sites with the same letter do not differ significantly in plant biomass according to Newman Keul's test, P< 0.0001.

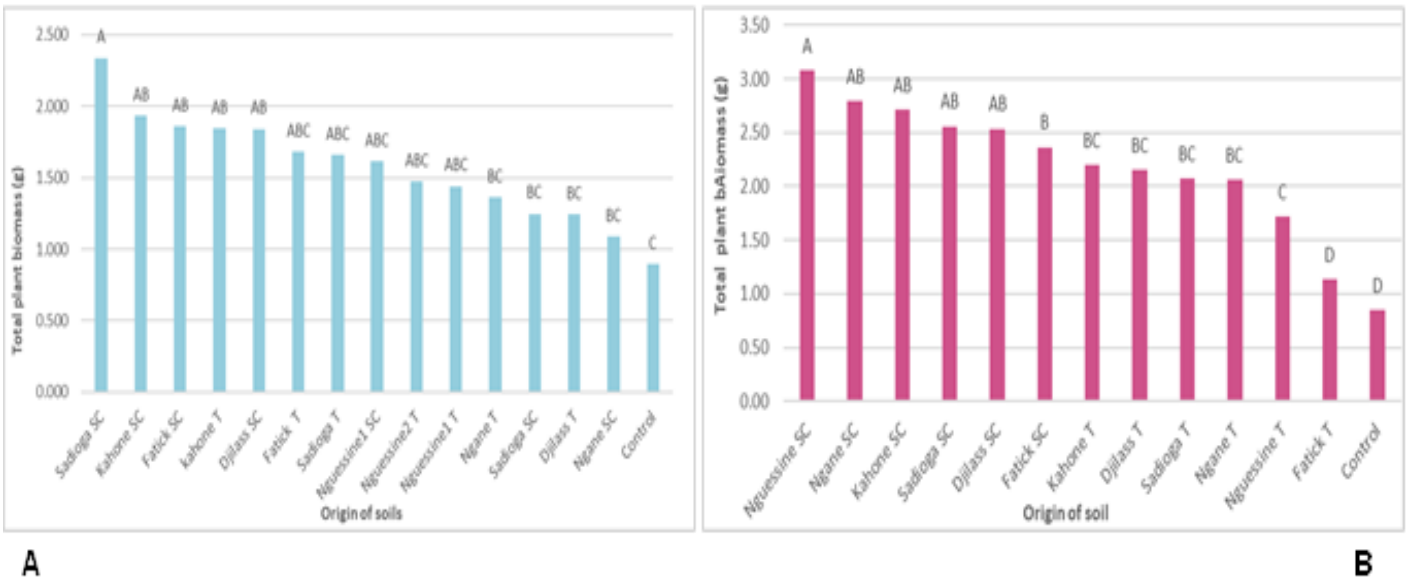


Figure 9. Biomass of *A. nilotica* plant cultivated with soils samples collected beneath canopy (SC) and outside canopy (T) in dry (A) and wet (B) seasons. Sites with the same letter do not differ significantly in plant biomass according to Duncan test, P < 0.0001.

especially in samples collected beneath canopy, as well in wet or dry season could mean that it has efficient AMF strains in rhizospheric soils of *V. nilotica* growing in salt affected areas.

DISCUSSION

Results show that AMF symbiotically associated with *V. nilotica* are present in the rhizosphere in salt affected

soils of Senegal as well as in dry and wet season. Our results revealed the presence of AMF viable spores related to the genus *Glomus* mainly, and to *Gigaspora* and *Acaulospora* in the *V. nilotica* tree rhizosphere grown in salt-affected areas.

Spores' numbers are more abundant in dry season compared to wet season. Silva-Flores et al. (2019) showed that there was a strong season effect of the soil in spores number at both sites, while physical-chemical parameters differed between sites.

In wet season, water availability would increase fungal mycelium growth for root colonization, leading to an increase of spore germination (Van Der Heijden et al., 1998; Ndoye et al., 2012). In dry season, soil salinity of most of our sites increases, but salinity has a negative effect on the development of AMFs (Bothe, 2012), hence the high number of spores was observed usually for the high salty soils. However, the effect is less marked beneath the tree canopy where the soil is influenced by the rhizosphere and where root exudates could mitigate the effect of salinity and allow a better development of AMF. Bargali and Bargali (2009) reported that *V. nilotica* improve soil fertility under its canopy because of increasing organic matter input, nutrient cycling through leaf litter and protection of soil from erosion. So, AMF diversity is not affected by salinity and only population size is affected as revealed in our results by the same AMF genus in all sites. Sene et al. (2012) reported the presence of *Glomus* only in rhizosphere of *V. nilotica* in non-saline soils. These AMF recovered are apparently widespread in many Senegalese soil because Sene et al. (2012), Ndoye et al. (2012) and Diop et al. (2015) described their association with other plant species. The presence of these AMF in salty soils could mean their adaptability in this harsh environment, as reported by many authors (Singh and Thomson, 1992; Giri et al., 2007; Bothe, 2012).

Only tree species were identified to known species: *G. rosea* was found in Fatick and Nguessine, *G. aff. albida* was found in Kahone and *A. aff. Colombiana* in Djilass and the others species were not affiliated to known species, so characterization will be continued to precise their taxonomical group.

Furthermore, soils factor such as salinity and pH appear to be decisive in structuring of AMF fungal communities as revealed in our results. Indeed, salinity has the main effect on diversity on total fungi, despite differences in soils properties. Analysis of total soil fungi targeted through the ITS region revealed that salinity decrease the total fungi diversity and soils with similar amount of salinity present similar profiles. In other words, salinity is the main factor that has influenced fungal community structure in salt affected soil. As revealed by Davidson et al. (2015), local environment conditions determine composition of AMF communities.

It appears that *V. nilotica* subsp *adansonii* can grow on different salt affected soils with different pH levels (acidic, basic) according to analysis of chemical properties of the rhizosphere soils. This corroborate the idea that *V. nilotica* tree is adapted to various edaphic conditions as reported by Giri et al. (2007) and by Chandrasekaran et al. (2014). Soils with a basic pH and low saline pH have the most important minerals (N, Ca, Mo, C, S, K, Mg) whereas acidic soils showed low mineral and organic matter contents.

Indigenous AMF in soils sampled in salt affected areas trapping using *V. nilotica* as trap plant indicated the infectivity of mycorrhizal strains present in rhizospheric soils as revealed by many propagules observed in plant roots. This corroborated the idea that *V. nilotica* is very mycotrophic and could be used as a trap culture plant for AMF associated with plants of the same genus, instead of the use of wheat as plant trapping.

Many authors such as Singh and Thomson (1992) and Giri et al. (2007) also reported that *V. nilotica* is a salinity-tolerant plant. However, mycorrhization is one of the factors contributing to this tolerance by improving hydromineral nutrition and soil nutrients release. This is also seen in our results that plants with high mycorrhizal colonization as observed for *V. nilotica* showed the highest plant biomass. Strains present in these salts affected soils might be efficient with *V. nilotica* and could therefore increase growth and biomass, corroborate the positive effect of AMF on plant development, especially in abiotic stress (Bothe, 2012). It should be noted that the soil of Kahone and Djilass present few nodules in the root system which could act in synergy with the mycorrhizae for the growth of the plant.

Conclusion

This work shows that rhizospheric soils of *V. nilotica* growing in salt affected areas have a great richness in indigenous AMF related to genus *Glomus, Gigaspora* and *Acaulospora*. Among AMF morphotypes identified, three are related to known species: *Gi. rosea*, *Gi. aff. albida* and *A. aff. Colombiana.*These strains can establish a good mycorrhization with *V. nilotica* and increase plant productivity (biomass and height), therefore could be used as inoculum for *V. nilotica* plants used to revegetalizise salt-affected soils. Also, AMF communities structure is correlated to soil salinity which has a negative effect on them. For further studies, it will be interesting to characterize genetically the real strains spp. that colonize plant roots and to determine the efficiency of each taxa separately on plant productivity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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