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Full Length Research Paper

Selection of *Lactobacillus* strains newly isolated from Algerian camel and mare fermented milk for their *in vitro* probiotic and lipolytic potentials

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The main objective of this study was the characterization of new lactobacilli probiotic strains belonging to lactic acid bacteria (LAB). Eighty-eight strains were isolated from different Algerian camel and mare fermented milks; three of them were pre-selected for their stability, fast growth and resistance to acidity and bile salts. Cell viability was assessed in simulated gastric and intestinal conditions. On the other hand, cell safety was checked by testing their hemolytic capacity. The *in vitro* tests revealed a good probiotic potential of selected strains. The majority of lactobacilli is resistant to cross-stress and persists beyond 4 h of incubation in contact with simulated gastrointestinal juices; a survival rate of over 80% was observed. All strains showed better lipolytic activity in the presence of natural substrates compared to Tween-80. Lipolysis zones diameters obtained in the presence of butter and olive oil were remarkable (between 20 and 27 mm respectively). Investigation of the cholesterol-lowering and the triglyceride-lowering properties revealed a cholesterol ratio degradation of 54.8% and a triglyceride ratio degradation of 80.3% for *Lactobacillus plantarum* NSC5C.

Key words: Probiotic, camel and mare fermented milks, cholesterol lowering, triglycerides lowering, *Lactobacillus plantarum.*

INTRODUCTION

Hyperlipidemia is the excess of lipids in blood, mainly cholesterol and triglycerides. This physical state is asymptomatic in many people. Nevertheless, it can have adverse consequences on human health. It is one of the most important risk factors associated with cardiovascular disease (Manson et al., 1992). The accumulation of these

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Abbreviations: LAB, lactic acid bacteria; **TG,** triglycerides; ***Lb,*** *Lactobacillus*; ***Lc,*** *Lactococcus*; **CFU,** colony forming unity; **CRD,** cholesterol ratio degradation; **TRD,** triglycerides ratio degradation.

blood lipids is mostly due to bad nutritional balance affecting many western countries resulting in obesity (Ferrières et al., 2004). Dairy products are also an important source of fat, however many studies have shown that some fermented products show a low cholesterol content such as fermented camel and mare milk (Pieszka et al., 2016). These low lipid levels are attributed not only to the composition of the milk but also to the bacterial flora that reside there (Konuspayeva et al., 2008; Raziq et al., 2008; Kamal and Salama, 2009). This flora is principally composed of LAB including lactobacilli; these microorganisms have the capacity to reduce blood lipids (Shah, 2007; Mansoub, 2010). Bacteria with beneficial properties for the organism are considering as probiotics (Lilly and Stillwell, 1965). To be designated as such they must meet several criteria mainly resistance to gastric and intestinal conditions, résistance to antibiotics, antagonism against pathogens, adhesion to intestinal epithelial cells and safety (Salminen et al*.,* 1998; Aarti et al., 2017). The pharmaceutical or agri-food industries are increasingly using probiotics as a dietary supplement (Liao and Nyachoti, 2017), as additives or as alternatives to antimicrobials (Aarti et al., 2018; Alagawany et al., 2018).

New indigenous probiotic strains isolated from dairy sources known for their many health benefits such as components of camel milk (Abdel Gader and Alhaider, 2016) or mare milk (Jastrzębska et al., 2017) could compete with commercial strains while being more effective and less expensive. Fermented milks are widely consumed in Algeria for their health benefits among them camel milk which is known for its cholesterol-lowering and hypotriglyceridemic effects, nevertheless the consumption of fermented raw milk must be very framed. The health of milk-producing animals must be tightly controlled, as must the hygiene of milking tools in order to prevent risks to the health of consumers. These data incited looking for these abilities on a set of lactobacilli from collection of our lab. Three strains were isolated from Algerian camel and mare fermented milks, and were preselected for their resistance to bile salts and acidity. This study was aimed at testing *in vitro*:

1. Strains whose resistance in stress conditions simulates the gastrointestinal conditions,
2. Strains with antagonistic and hemolytic power;
3. The lipolytic power of strains on different lipidic substrates, and finally the search for cholesterol-lowering and triglyceride-lowering power.

MATERIALS AND METHODS

Strains isolation, screening and identification

Different milk samples were collected from each animal, camel or mare, after washing the breast and udder and eliminating the first jets of milk. Samples (100 ml) were placed at 4°C and transported to the laboratory and then incubated at 30°C for 18 h. After an endogenous fermentation, 10 ml of camel or mare fermented milk were homogenized with 90 ml sterile physiological water (0.9% w/v NaCl). Serial decimal dilutions were prepared (from 10^{-1} to 10^{-6}), and 100 µl samples of appropriate dilutions were spread in duplicate on de Man, Rogosa and Sharpe medium plates (MRS, Fluka, Geneva Switzerland). After an incubation of 24 to 48 h at 30°C, distinct colonies were selected randomly and purified by re-streaking on MRS agar plates until only a single type of colonies was observed. The different pure isolates obtained were characterized by Gram staining, catalase production, and cell morphology. Only Gram positive and catalase negative bacilli were selected. Strains were conserved at room temperature after freeze-drying or by storage at -80°C either in 10% skimmed milk or in liquid MRS supplemented with 40% glycerol. All the isolated lactobacilli (88) were tested for their resistance to different acid pH (pH 1-pH6), to different bile salts concentrations (0.25, 0.5, 1, 2 and 10%) (Idoui, 2008), which is one of the most important criteria for the selection of probiotics strains. They were also tested for their lipolytic activity on MRS medium supplemented with butter or olive oil to target strains with liporeductive potential. The three strains presenting the most interesting results for the rest of our research were selected, conserved and then identified using the biochemical galleries API 50CHL (Biomérieux, France).

A molecular identification was also done by the Sanger sequencing of the full length 16S rRNA gene. Total DNA was extracted from overnight culture of the strain using the Phenol-chloroform method (Azcárate-Peril and Raya, 2001). An amplification was done by PCR using primers 16S-27F and 16S-1492R (27F5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-ACGGCTACCTTGTTACGACTT-3′) and also 16S-27F and 16S-19R (27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 19R 5′-GRG TAC CTT TTA TCC GTT G-3′ while R, A or G) (Lane, 1991) in order to amplify V1-V2 16S gene segments for the 3 strains. The PCR conditions were realized with the 5× HOT BIOAmp® Evagreen HRM Mix at 12.5 mM, 2 µl of Enhancer 10X and 4 µl of $MgCl_2$, using 1 µM of forward and reverse primers and 2 µl of genomic DNA template in a total volume of 20 µl. The PCR cycling conditions were as follows: A first denaturation step at 96°C for 12 min, 45 cycles of denaturation at 96°C for 20 s, annealing at 52°C for 20 s, extension at 72°C for 1 min 30 s, followed by an elongation step at 72°C for 5 min. The sequencing was performed in Biofidal laboratories (Lyon, France).

For comparative purposes, two probiotic reference strains *Lactobacillus plantarum* BH14 and *Lactobacillus brevis* CHTD27 isolated from Algerian camel milk of regions of Illizi and Tindouf, respectively were also used. Pathogenic strains used in this study and their origins are presented in Table 1. All strains belong to the LBMB collection (Laboratory of Biology of Microorganisms and Biotechnology, Oran, Algeria).

Resistance to simulated digestive conditions

The survival of the bacterial strains under conditions simulating those encountered during their passage through the digestive tract (stomach and intestines) was tested. This test was carried out in two steps following the method of Bahri (2014).

Resistance to simulated gastric conditions

For the execution of this test, an overnight culture of the LAB strains, obtained after 18 h of incubation in MRS broth at 30°C was used; these cultures were diluted to an optical density of 0.5 to 0.7 under a wavelength of 600 nm. The simulated gastric juice was prepared by mixing pepsin (Sigma) to 0.5% (w/v) NaCl (pH1.5) at a final concentration of 3 g/l. The enzyme was first dissolved in 0.02 M glycine-HCl buffer (pH1.5) and then sterilized using a Millipore

Table 1. Pathogenic strains.

Strains	Origin
Salmonella Thyphimurium	Laboratory of Biology of Microorganisms and Biotechnology (Oran, Es-Sénia)
Proteus mirabilis	
Klebsiella pneumoniae	
Citrobacter freundii	
Enterobacter cloacea	
Enterobacter aerogenes	
Staphylococcus aureus ATCC 25923	
Acinetobacter baumannii	
Pseudomonas aeruginosa ATCC 27853	
Escherichia coli ATCC 25922	
Bacillus cereus	
Staphylococcus aureus (II2) ATCC 433005	

filter (Millipore, MILLEX-GV, 0.22 µm, SLGV0130S). This solution was distributed in tubes at the rate of 9 ml, which have been supplemented with 1 ml of the overnight cultures of LAB strains previously obtained. One hundred microliters of each tube was taken at T_0= 0h, T_1= 2h and T_2=4h, to be counted by the agar plate method on MRS agar after 24 h of incubation at 37°C.

Resistance to simulated intestinal conditions

In order to simulate the hostile conditions of the human small intestine, a solution adjusted to a pH of 8 containing Pancreatin (Nature's plus, Warwickshire, UK) dissolved in buffer (0.013 M Tris-HCl, pH8) at a final concentration of 1 g/l and 0.3% (v/v) of filtered sheep bile (Millipore, MILLEX-GV, 0.22µm, SLGV0130S) was prepared. The prepared simulated intestinal juice was distributed into a tube then inoculated at a rate of 10% (v/v) with a young culture of LAB ($0.5<OD_{600nm}>0.7$, that is 10^9 cells/ml); 0.1 ml was taken from each tube at different exposure time intervals (T_0 = 0 h, and T_1 = 4 h) to inoculate the surface of the MRS agar. The colonies obtained were then counted after incubation at 37°C for 24 h.

Antibacterial activity against pathogenic strains

This antibacterial activity was researched using two methods.

Spot method

The purpose of this test is to determine the inhibitory effect of LAB on some indicator strains according to the method of Fleming et al. (1975). Overnight cultures of all strains (inhibitors and indicators) were inoculated respectively in MRS broth and Luria Bertani (LB) broth for the lactobacilli and pathogenic bacteria, respectively. LAB were inoculated in spots on MRS agar; after 24 h of incubation at 30°C, the obtained colonies were covered with 10 ml of 1% (v/v) soft agar MRS previously seeded with a fresh culture of the indicator strain (pathogens at an OD600 nm ≈ 1)) and then incubated for 24 h at 37°C. The size of the inhibition zones around the spot was measured.

Impregnated disc method

The selected lactobacilli were tested for their antibacterial potency using the impregnated disk method (Savadogo et al. 2004; Tadesse et al., 2004). Fifteen milliliters of LB soft agar were inoculated with 1% (v/v) of fresh pathogenic bacteria culture (OD_{600nm}≈ 1) poured in Petri dish and then allowed to dry at room temperature, 6 mm Whatman filter paper discs were impregnated with 10 µl of a fresh LAB culture and then placed on the surface of the LB soft agar. The size of the inhibition zones around the disks were measured after 24h of incubation at 37°C.

Lipolytic activity

The lipolytic activity of tested strains was investigated on MRS medium supplemented with different natural and artificial lipid substrates. The activity was sought on a solid MRS medium buffered to pH 7 (phosphate buffer Na_2HPO_4/NaH_2PO_4, 0.1 M) containing 1% (v/v) of butter, olive oil or tween 80 as the only lipid source. The medium was pacified by adding 0.5% calcium carbonate ($CaCO_3$) to clearly visualize an eventual lipolytic zone. Overnight cultures LAB strains were spot seeded on the surface of the enriched MRS medium. Two hours of drying at room temperature are necessary before the incubation at 30°C for 24 to 48 h. Lipolysis was then revealed by the appearance of opaque zones around lactobacilli colonies (Guiraud and Galzy, 1980).

Hypocholesterolemic and hypotriglyceridemic *in vitro* activity of lactobacilli

All strains presenting a lipolytic activity were then inspected for their hypocholesterolemic and hypotriglyceridemic properties using the modified method of Guo et al. (2011).

This test was done using MRS broth supplemented with 0.3% (v/v) of sheep bile. Cholesterol and triglycerides were sterilized by filtration (Millipore, MILLEX-GV, 0.22 µm, SLGV0130S, Perkin Elmer, Boston, MA) and then added individually to broth at a final concentration of 200 mg/ml; 500 µl of this solution were transferred to an Eppendorf and supplemented with the same volume of lactobacilli fresh culture (OD_{600nm} ≈1). The final concentration of cholesterol or triglycerides was then 100 mg/ml. This operation was

Table 2. Identification percentages of selected strains using molecular and biochemical methods.

Strain	Taxon	% by molecular identification	% by API 50 CHL identification	Origin
NSC5C	*Lactobacillus plantarum*	99	99.9	Camel milk from Naama, Algeria
NSC10	*Lactobacillus plantarum*	99	99.9	Camel milk from Naama, Algeria
JUMIII4	*Lactobacillus plantarum*	99	99.4	Mare milk from Saida, Algeria

carried out for all the selected lactobacilli. The cells were removed from the culture by centrifugation (12,000 rpm for 10 min at 4°C) after 24 h of incubation at 37°C. The supernatants were recovered, and the cells were washed three times with a volume of MRS broth containing 0.3% (v/v) of bile, identical to the original broth. After each washing, the suspension was centrifuged (12,000rpm for 10 min at 4°C) and the three supernatants were combined and represented the wash solution.

Cells obtained after the third wash step were suspended in MRS broth containing 0.3% of bile plus lysozyme at a final concentration of 4 mg/ml and placed in a water bath at 37°C for 1 h 30 min. Lysis buffer (10% SDS, pH12) was then added at a rate of 100 µl/ml (V buffer/V cells).

The lysed cell solution was centrifuged (12,000 rpm for 10 min) to recover the supernatant containing the cholesterol or triglycerides entrapped in the cells.

In all fractions, the cholesterol or triglyceride concentration was assessed using the colorimetric method described by Rudel et al. (1973) slightly modified.

The ratio of cholesterol degradation (CDR) was calculated from the equation:

$$\mathbf{CDR} = \frac{[\mathrm{C} - (\mathrm{C1} + \mathrm{C2} + \mathrm{C3})]}{\mathrm{C}} \times 100$$

The ratio of triglycerides degradation (TDR) was calculated from the equation:

$$\mathbf{TDR} = \frac{[\mathrm{T} - (\mathrm{T1} + \mathrm{T2} + \mathrm{T3})]}{\mathrm{T}} \times 100$$

Where C and T are the initial substrates concentrations: C1, C2 and C3; T1, T2 and T3 are substrate concentrations of cholesterol and triglycerides, respectively in the supernatant, wash solution, and solution of lysed cells.

Hemolytic activity

The hemolytic activity of lactobacilli was determined by the method of Maragkoudakis et al. (2006). Hemolyticity was examined by seeding strains of lactobacilli on blood agar (Columbia Medium). The type of hemolysis was examined after an incubation of 24 h at 30°C. The result can be α-hemolytic (green around colonies), β-hemolytic (lightening around colonies) or γ-hemolytic (the medium is unaffected).

Data analysis

Data were analysed with Statistica 5.5 software (1999 edition; Tulsa, OK, USA). One-way analysis of variance (ANOVA) with Duncan's multiple range test was performed to compare any significant differences. Values of $P<0.05$ were considered statistically significant. Differences among means were detected by paired Student's test.

RESULTS AND DISCUSSION

Isolation, screening and identification

Eighty-eight lactobacilli were isolated from the different fermented milks; the three most resistant to acidity, bile salts and presenting a good lipolysis activity were selected (NSC10, NSC5C and JUMIII4) to conduct this study in comparison with the two reference probiotic strains. The biochemical identification API 50 CHL revealed the belonging of the 3 selected strains to the *Lb. plantarum* taxon over 99% (Table 2).

Molecular identification

Identification results obtained by the API50 CHL galleries and the sequencing of the 16S gene are indicated in Table 2. Alignment and homology of the PCR amplified sequences were done in NCBI website (http://www.ncbi.nlm.nih.gov) using BLAST Software, which determine identity of the 3 strains NSC5c, JUMIII4 and NSC10 to the taxon *Lactobacillus plantarum.* The phylogenetic tree is represented on Figure 1.

Resistance of lactobacilli to simulated gastrointestinal conditions

Resistance to simulated gastric conditions

The tested lactobacilli had a similar starting concentrations with an optical density ranged between 0.5 and 0.7. Their survival in simulated gastric conditions (3 g/l pepsin, pH 1.5 and 0.5% NaCl) varies according to the strain (Figure 2). It is noted that the number of colonies decreases as soon as the cells are exposed to the solution, which explains the difference of Log10CFU/ml at T_0.

All strains show remarkable resistance after 2 h exposure to simulated gastric conditions with a survival rate of over 80%. After 4 h of gastric stress, NSC5c is the most resistant (3.56Log10CFU/ml at T_0 to 2.64 Log10CFU/ml at T_{4h}), regarding strains, BH14, CHTD27 and JUMIII4, despite a sharp decrease, they were quite resistant to cross-stress and persist even after 4 h of incubation in contact with stressors. The number of cells remained, even so, more important than the most

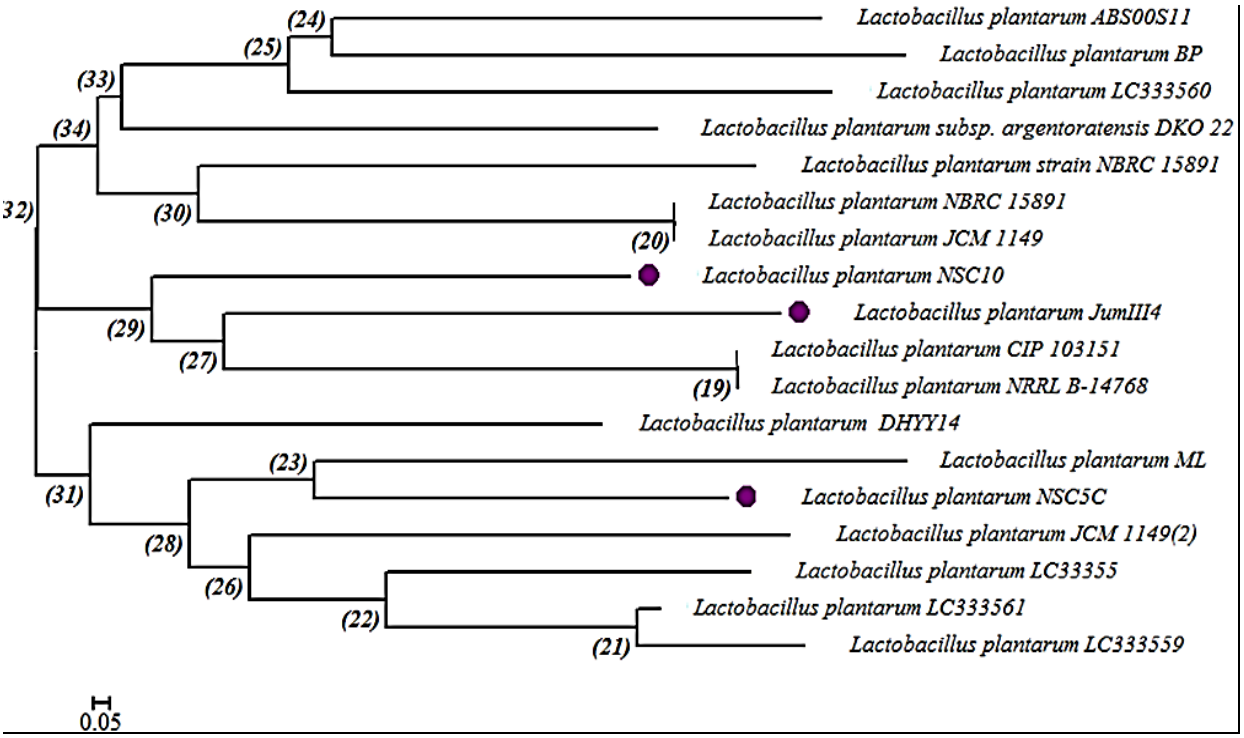

Figure 1. Concatenated phylogenetic tree of *Lactobacillus plantarum* (NSC5c, JUMII4 and NSC10) among neighbouring known species.

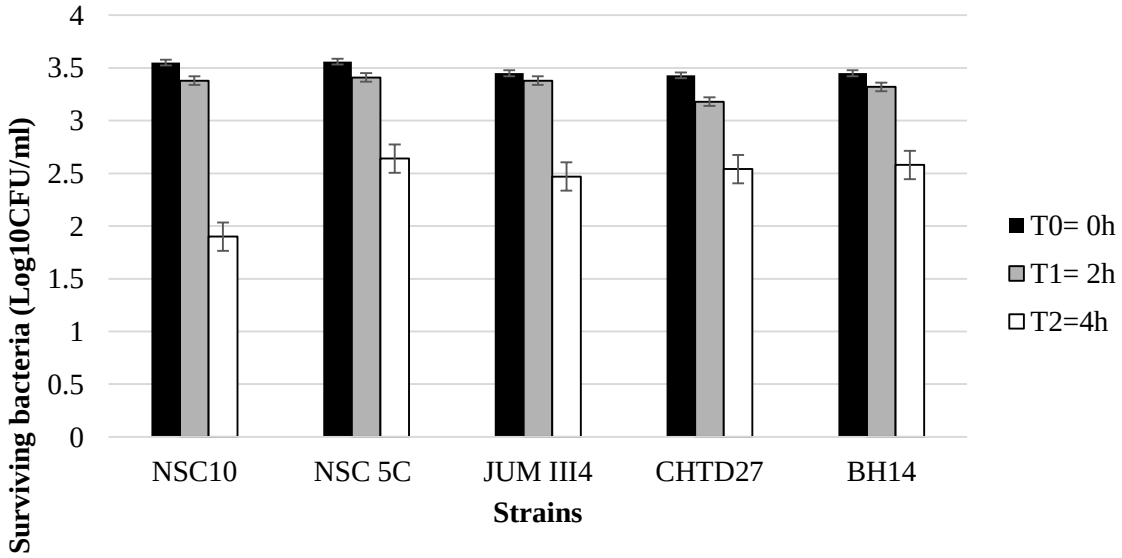

Figure 2. Resistance of lactobacilli in contact with simulated gastric juice.

sensitive strains, such as NSC10 which undergo an important decrease from an average of 3.55 Log10 CFU/ml at T_0 up to 1.9 Log10 CFU/ml after 4h in contact with the simulated gastric juice.

These results are consistent with those obtained by Bahri et al. (2014) who determined the resistance of some strains of *Lactobacillus* including *Lb. plantarum* in similar stress conditions. Maragkoudakis et al. (2005) showed that the tested probiotics resist pH 3 for 3 h, and most have lost their viability in 1 h in pH 1. Akalu et al. (2017)

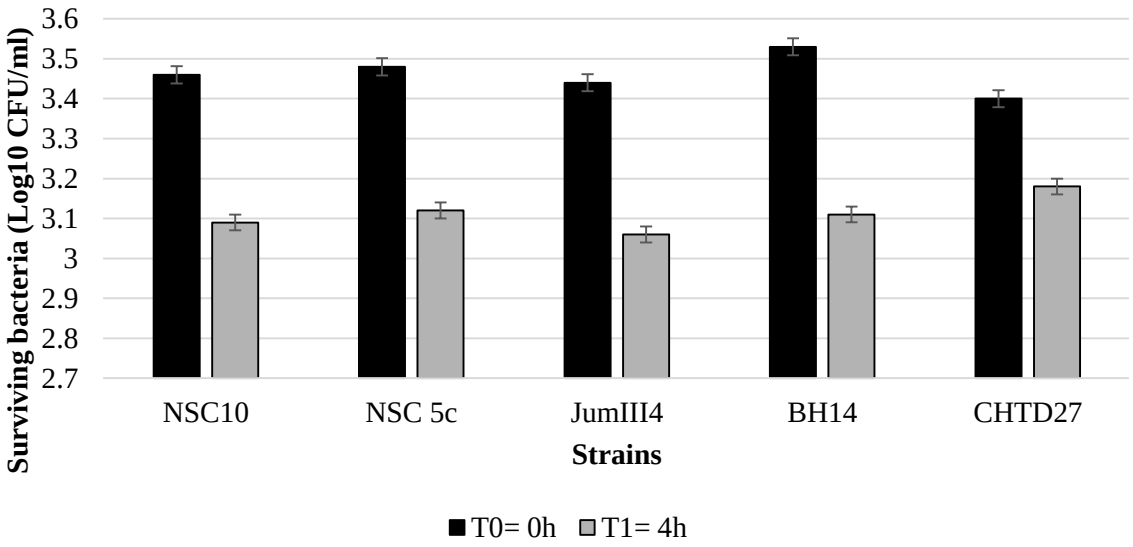

Figure 3. Resistance of lactobacilli in contact with simulated intestinal juice.

showed that 80 to 94% of the tested LAB survives after 6 h at pH 2.5.

Conway et al. (1987) and Lindwall and Fonden (1984) have shown that, unlikely to strains used in the study, *Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus* strains have a very low resistance to acidity and were destroyed very quickly at pH 1, and after about 1 h at pH 3.

Acid stress causes intracellular acidification, which decreases the activity of cytoplasmic enzymes (Even et al., 2002). Transcriptomic and proteomic studies have highlighted that many LAB enhance the levels of glycolytic enzymes under acid, thermal, and osmotic stresses, but without increasing the synthesis of lactic acid (Marceau et al*.,* 2002; Di Cagno et al., 2006a). LAB such as *Lb. plantarum, Lactobacillus. reuteri*, *Lactobacillus rhamnosus* and *Lactococcus lactis* modify pyruvate metabolism at the expense of lactic acid, and they increase the synthesis of basic compounds (e.g., lysine and diacetyl/acetoin) (Heunis et al., 2014; Zuljan et al., 2014). The level of lactate dehydrogenase (Ldh) which is responsible for the synthesis of lactic acid from pyruvate markedly decreases. Acetyl-CoA is rerouted toward the biosynthesis of fatty acids instead of butanoate (Di Cagno et al*.,* 2006b; Koponen et al., 2012), which may enhance the rigidity and impermeability of the cytoplasmic membrane (Cotter and Hill, 2003; Fernandez et al., 2008). Pyruvate oxidase and phosphate acetyltransferase, used to synthesize acetyl-coenzyme A (acetyl-CoA), which are induced in *Lb. delbrueckii subsp. bulgaricus* and *Lb. rhamnosus* under acid stress conditions (Koponen et al., 2012; Zhai et al., 2014).

Resistance to acid stress is an important factor for LAB since they acidify their environment during growth. Lactobacilli are generally more resistant to acid stress than lactococci (Siegumfeldt et al., 2000). In addition, acid-resistant strains also have good resistance to other stresses such as bile salts and NaCl (Collado et al., 2006).

Resistance to simulated intestinal conditions

After passing through the stomach, the bacteria reach the duodenum where the bile is secreted. At this level, some components of bile, especially bile acids such as colic acid, seriously compromise the viability of ingested bacteria. Bile tolerance is also a criterion for *in vitro* selection of probiotic bacteria; it is generally considered necessary to assess their ability to withstand intestinal tract conditions such as pancreatic enzymes and gives them the ability to colonize the intestinal environment (Bron et al*.,* 2006). As well, adaptation to bile can also protect bacteria against other stresses (acid, enzymes or thermal stress) (Saarela et al., 2004; Sanchez et al., 2006).

To investigate the effect of bile stress, *in vitro* experiments were conducted with a solution of 1 g/l of pancreatin and 0.3% (v/v) of sheep bile at pH 8, that is, similar to intestinal conditions. The results are shown in Figure 3. The Log10 CFU/ml of strains at T_0 reaches its maximum for BH14 and NSC5C strains with 3.53 Log10CFU/ml and 3.48 Log10CFU/ml, respectively. Nonetheless, all strains survive even after 4 h in contact with the bile solution. *Lactobacillus plantarum* JUMIII4 has the lowest rate of resistance and presented an important decreasing from 3.44 Log10CFU/ml at T_0 to 3.06 Log10CFU/ml at T_{4h}.

These results express a variable resistance according to the strains; it was reported that bacterial resistance to

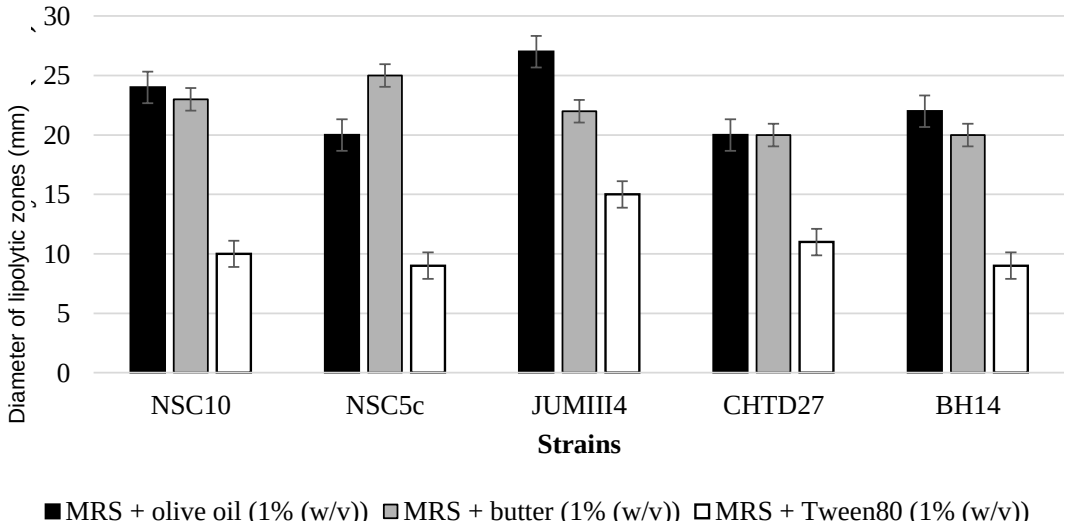

Figure 4. Lipolytic effect of lactobacilli on olive oil, butter and tween 80 (expressed in mm).

bile salts is determined genetically (Fang et al., 2009), so these variations may be explained by a different expression of stress resistance genes and a correlation between acid, saline, biliary and various digestive enzymes.

The stress caused by bile on bacterial cells can corrupt their ability to survive. In contrast to the acidity that fades after gastric passage, the bile that encounters surviving bacterial cells remains in contact with them for a longer time. Marteau and Shanahan (2003) and Izquierdo et al. (2009) clearly demonstrated *in vitro* that bile salts had a bactericidal effect. In the same way as for gastric acidity, their study demonstrated a difference in sensitivity to bile salts between bacterial species. *Lb. bulgaricus* and *Streptococcus thermophilus* have a very low survival percentage compared to *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. Bile salts have a detrimental effect on cell membranes resulting from an increase in cell permeability. The resistance to bile salts is likely due to BSH enzymes. Many strains of lactobacilli have the ability to reconvert via these enzymes (BSH, EC 3.5.1.24) (De Smet et al., 1995). According to Reyes-Nava et al. (2016) BSH functions are not yet clearly understood. These authors also concluded that many strains with BSH activity were particularly resistant to bile salts and then had the ability to modulate blood lipids in rats and protect their liver functions.

Wu et al. (2010) found that expression levels of 26 proteins were acutely stimulated and/or regulated by factor of bile salts. Transcription-PCR and bioinformatics analysis showed that the implicated pathways are involved with a complex physiological response under bile salts stress, particularly including cell protection (DnaK and GroEL), modifications in cell membranes (NagA, GalU, and PyrD), and key components of central metabolism (PFK, PGM, CysK, LuxS, PepC, and EF-Tu). Furthermore, Mathipa and Thantsha (2015) concluded that multi-stress pre-adaptation enhances viability of probiotics under simulated gastrointestinal conditions and formulations containing a mixture of multi stress-adapted cells exhibits enhanced synergistic effects against food borne pathogens.

Microencapsulation can be an effective means of increasing the resistance of certain strains used as probiotics to enable them to survive gastrointestinal conditions and reach their target in a viable form (Al-Furaih et al., 2016; Gonzalez-Cuello et al., 2017).

Lipolytic activity

Lipases have a broad spectrum of action on emulsion substrates. LAB which exerts efficient lipase activity could be interesting for use as a probiotic. The tested strains of lactobacilli showed a significant activity in the presence of natural substrates olive oil and butter compared to Tween 80 *(P<0.001).* The majority of the strains show similar results for the degradation of the two natural substrates (Figure 4). Nevertheless, the strain JUMIII4 has preferentially degraded olive oil than butter, with a lipolysis zone of 27 and 22 mm in diameter, respectively, unlike NSC5c that showed better degradation of butter with lysis zone of 25 mm, compared to olive oil with a degradation zone of 20 mm. Dinçer and Kivanç (2018) investigated this activity on 50 strains of LAB isolated from the Turkish pastırma. The lipolytic activity is observed in 25 of the tested strains where *Lb. plantarum* revealed the highest lipolytic activity.

Katz et al. (2002) found a wide variation in activity between strains of *Lb. plantarum, Lb. acidophilus* and

Table 3. Antibacterial activity of lactic acid strains against pathogenic bacteria.

Strain	1	2	3	4	5	6	7	8	9	10	11	12
JUMIII4	+	++	-	++	+++	+	++	-	-	++	-	-
NSC5C	-	-	+++	++	-	-	+++	+++	-	-	++	-
NSC10	++	+	++	+	++	-	+	++	+	+	++	++
CHTD27	+	+	-	++	+	+	+	+	-	+	+	-
BH14	++	++	++	++	++	+	+	+	++	+	++	++

Discs of Whatman papers (6 mm diameter) were soaked with 10 µL of a fresh bacterial suspension. (+++) Inhibition zone>20 mm; (++) Inhibition zone>15 mm; (+) Inhibition zone>10 mm; (−) Inhibition zone<10 mm. 1*: Proteus mirabilis;* 2*: Salmonella Thyphimurium;* 3*: Klebsiella pneumoniae;* 4*: Citrobacter freundi;* 5*: Enterobacter cloacea;* 6*: Staphylococcus aureus;* 7 *:Enterobacter aerogenes* ;8 *: Pseudomonas aeruginosa* ATCC 27853*;* 9*: Escherichia coli 25922*; 10*: Bacillus cereus*; 11*: Staphylococcus aureus* ATCC 433005; 12: *Acinetobacter baumannii.*

Enterococcus feacium. Shahab-Lavasani et al. (2012) also determine that the addition of *Lactobacillus lactis* had a significant (p<0.05) effect on the lipolysis characteristics of *Lighvan* cheese.

These results are in disagreement with those described in several studies, which reported that LAB have a lower lipolitic activity with natural lipids (De Moraes and Chandan, 1982; Kamaly et al., 1988; Papon and Talon, 1989).

Antibacterial activity against pathogenic strains

The presence of inhibition zones is the result of an antagonism exerted by the LAB against the pathogenic strains. Generally, the lactobacilli strains do not present the same spectrum of action towards the pathogens (Table 3). No significant difference was found between the activity of lactobacilli isolated from camel milk and that isolated from mare's milk (JUMIII4), which supports researches of Tremonte et al*.* (2017) who demonstrated that there is no relationship between the intensity of inhibition and the origin of inhibitory strains of *Lb. plantarum*.

Lb. plantarum BH14 inhibited the entire indicator strains tested, these performances are followed closely by the strains CHTD27 and NSC10 which showed a significant inhibitory effect (11 and 10 pathogeneses inhibited, respectively), unlike the NSC5C strains, which inhibited only 7 of the 12 pathogens tested.

Lactobacilli showed relatively similar antagonistic activity against Gram-positive and negative pathogens with a slightly more pronounced activity against Gram-negative pathogens. These results are in agreement with those found by other authors who have shown that LAB are able to prevent the growth of Gram-positive and negative pathogenic bacteria *in vitro* and *in vivo* (Lin et al., 2007; Balcázar and Luna-Rojas, 2007; Mahdhi et al., 2010; Okpara et al., 2014; Anyika et al., 2018; Digo et al., 2017).

Acinetobacter baumannii, Escherichia coli 25922 are the most resistant indicator bacteria, they were inhibited only by 2 LAB out of the 5 tested with a maximum inhibition zone not exceeding 16 mm in diameter. *Enterobacter aerogenes* and *Citrobacter freundii* strains were inhibited by all LAB with inhibition zones ranging between 13 and 21 mm in diameter. Antagonism of lactobacilli was also observed on *Bacillus cereus, Staphylococcus aureus* ATCC 433005 and *Enterobacter cloacae*.

The pathogenic microorganisms tested in this study are involved in toxi-infections or food poisoning such as the following species: *Staph. aureus*, *E. coli, Pseudomonas aeruginosa and Klebsiella pneumonia.* Values obtained for this test coincide for some strains with the work of Belyagoubi and Abdelouahid (2013), where the diameters of the inhibition zones of LAB isolated from Algerian traditional dairy products are of the order of 4 mm up to 34 mm on the same pathogenic bacteria.

García-Cayuela et al*.* (2009) reported that beneficial bacteria, mainly LAB and bifidobacteria, could be a useful and effective strategy for preventing or reducing the incidence of pathogens, thereby improving food safety and protecting consumer health. LAB producing antimicrobial agents have been used as an alternative to antibiotics for the treatment of gastrointestinal diseases (Soomro et al., 2002; Akpinar et al., 2011) and against infections by Candida (Aarti et al., 2018).

The antibacterial activity of a probiotic is essential for the successful colonization of the intestinal mucosa (Tejero-Sarinena et al., 2012). It provides a barrier and defense effect against pathogens (Vaughan et al., 1999). Lactobacilli can produce antimicrobial substances such as organic acids, which are active *in vitro* and *in vivo* on enterovirulent pathogens involved in diarrhea cases (Servin, 2004). Lactic and acetic acids are produced *via* the fermentation of hexoses by lactobacilli. In addition, in acidic medium, the bacterial competitiveness of lactobacilli is favored compared to other bacteria because of their tolerance to acidity (Servin, 2004). Inhibition of pathogens such as *Staph. aureus* and *Bacillus cereus* by LAB is related to several antagonistic factors including decreased pH after lactic acid production, competition for food, production of bacteriocins and hydrogen peroxide (Isolauri et al., 2004; Charlier et al*.,* 2009; Merzoug et al., 2016, 2018).

Table 4. Lactobacilli cholesterol lowering-activity in MRS broth.

Strain	C1 (g/l)	C2 (g/l)	C3 (g/l)	CDR (%)
CHTD 27	0.484	0.011	0.071	43.4
BH14	0.477	0.003	0.011	50.9
NSC5C	0.419	0.022	0.011	54.8
JUMIII 4	0.477	0.007	0.018	49.8
NSC10	0.496	0.003	0.026	47.5

CDR: Cholesterol degradation ratio; C1: Concentration of cholesterol in the supernatant; C2: Concentration of cholesterol in the wash solution; C3: Concentration of cholesterol in fragmented cells solution.

These organic acids can passively diffuse through the bacterial membrane in their undissociated form. They acidify the cytoplasm after dissociation and inhibit the cellular enzymatic activity of acid-sensitive pathogens (Deng et al*.,* 1999). This decrease in pH can therefore affect the viability of bacterial pathogens (Bruno and Shah, 2002; Servin, 2004). This activity is favored under certain *Lactobacillus* culture conditions. Tashakor et al. (2017) showed that the optimum conditions achieved at pH 6.0, 25°C temperature, 1.5% (w/v) Na_2HPO_4 and 0.5% (w/v) peptone. This indicates that the inhibition of pathogens is promoted under controlled conditions *in vitro* rather than in the intestinal tract where the temperature is higher and the nutritional sources variable.

Pathogens can also be inhibited by a nutrient restriction process. It is obvious that the ability of microorganisms to compete for limiting available nutrients is a significant factor in determining the composition of the microbiota. Hence, an increase in the number of lactobacilli obtained during a probiotic treatment would make it possible to reduce the substrates available for the implantation of pathogenic microorganisms (Fooks and Gibson, 2002).

The Fleming et al. (1975) method gave clear results for all the strains tested with significant inhibition diameters (from 15 to 45 mm), but these performances could not be confirmed after reiterations of the test using the same method.

Hypocholesterolemic *in vitro* activity of lactobacilli

Results presented in Table 4 reveal that all strains have a cholesterol-lowering activity. In the presence of bile salts, the cholesterol contained in the culture medium (1 g/l initially) was reduced to more than 50% for 2 strains of the 5 tested. Strains NSC5c is the most effective with a CRD of 54.8% as opposed to the strain CHTD27 which reduced cholesterol only at a ratio of 43.4%. These results are consistent with the studies of Bendali et al. (2017) which reported the effectiveness of LAB in reducing cholesterol *in vitro*. *Lb. pentosus* KF923750 was able to remove 62.4% of cholesterol in the growth medium after 24 h incubation. The hypocholesterolemic power of lactobacillus strains was also revealed by several studies (Mirlohi et al., 2009; Kondo et al., 2010; Huang et al., 2013; Liu et al., 2016; Zhang et al., 2017; Ding et al., 2017).

The concentrations of residual cholesterol in the 3 fractions (C1, C2 and C3) show a higher level in the initial supernatant C1 unlike the wash solution in which the cholesterol level is lower. It expresses that the cholesterol deduced from the supernatant of culture was not adsorbed to the bacterial wall, the low cholesterol level recorded in the fragmented cell solution proves that cholesterol has not been trapped inside the cells either, the hypothesis that can be emitted is that lactobacilli degrade cholesterol extracellularly.

Several hypotheses also have been put forward to explain cholesterol-lowering effect, such as the assimilation of cholesterol by bacteria or the hydrolysis of conjugated bile salts (Zhang et al., 2008). The deconjugation of bile acid by Bile-salt-hydrolase (BSH) was the most supported, the lactobacilli with this activity are preferred over the BSH-negative lactobacilli as selection criteria for probiotic strains with lowering cholesterol properties (Pereira et al., 2003). According to Jaspers et al. (1984), the organic acids produced by its bacteria are presumably cholesterol-lowering agents, hydroxymethyl and orotic acids lower serum cholesterol; on the other hand, uric acid inhibits the synthesis of cholesterol.

Another explanation relates to a decrease in cholesterol level, which would be solely due to the co-precipitation of cholesterol with the deconjugated bile salts, a phenomenon that cannot occur *in vivo* because the pH is higher than in a culture medium acidified by LAB (Desmazeaud, 1996).

Hypotriglyceridemic *in vitro* activity of lactobacilli

Lactobacilli strains showed variable triglycerides reduction (TRD) oscillating between 3% for strain JUMII4 and 80.3% for strain NSC5c (Table 5) which shows that the strains do not have the same abilities to reduce TG. From the observations made by comparing the residual concentrations in the culture supernatants and the fractionated cells solution, it can be seen that, unlike

Table 5. Lactobacilli triglycerides lowering-activity in MRS broth.

Strain	T1 g/l	T2 g/l	T3 g/l	TRD (%)
CHTD27	0.116	0.130	0.696	5.8
BH14	0.492	0.135	0.249	12.4
NSC5C	0.112	0.027	0.058	80.3
JUMIII4	0.192	0.136	0.669	3.0
NSC10	0.189	0.132	0.261	41.8

TDR: Triglycerides degradation ratio, T1: Concentration of triglycerides in the supernatant, T2: Concentration of triglycerides in the wash solution; T3: Concentration of triglycerides in fragmented cells solution.

cholesterol, triglycerides are found in the solution after cell lysis, although very low TRDs for certain strains such as CHTD27 and JUMIII4 (5.8 and 3%, respectively). Their triglycerides levels recorded in the fragmented cell solution (T3) are the highest (0.696 and 0.669 g/l) indicating the capture of triglycerides by these strains within the cells, thereby reducing the concentration of triglycerides in the external medium. NSC5C strain shows the highest TRDs (80.3%) for which the triglycerides concentrations in T3 are very low (0.058 g/l). These results reveal a difference between the mechanisms used by lactobacilli for triglyceride reduction.

Findings are consistent with those obtained by Gao and Li (2018) who also revealed this activity *in vitro* with triglyceride lowering rate for *Lb. acidophilus* L2- 73 and L2-16 and *Enterococcus faecalis* of 38.27% and 41.38% respectively.

As with cholesterol reduction, BSH activity may also be involved in triglycerides reduction *in vitro* and *in vivo*. Huang et al., (2013) results showed that BSH-active *Lb. plantarum* strains could reduce plasma total cholesterol, LDL-cholesterol and triglycerides in rats fed a high cholesterol diet.

When the organism overproduces cholesterol and triglycerides, the surplus is degraded to regulate their rate. Diet is also an important source of these two compounds; lactobacilli tested in this study can help the body reduce this excess in the intestinal lumen before absorption, thus preventing the risk of cardiovascular disease caused by excess lipids. The mechanisms by which triglycerides are degraded are not well known; there are currently very few studies on the elimination of triglycerides by lactobacilli *in vitro* (Gao and Li, 2018) or *in vivo* (Huang et al., 2013).

Hemolytic activity

In this study, none of the lactobacilli strains was able to hydrolyze human blood on Columbia medium, indicating that the strains are non-hemolytic. It means that strains do not possess the phosphatidyl-choline esterase enzyme that allows lysis of red blood cells, which indicates their safety on human health. It is well known that non-hemolytic bacteria are part of the microorganism group Generally Recorganized as Safe (GRAS), which is the case of LAB. Lactobacilli strains do not pose a health risk to animals or humans (Rychen et al., 2017; Olek et al., 2017; Chaves et al., 2017).

Conclusion

Among the 88 LAB isolated from camel milk or mare, only 3 strains of *Lactobacillus* show good *in vitro* probiotic and lipolytic capacities. The NSC5c strain of *Lactobacillus plantarum* isolated from camel milk shows the best performances during *in vitro* tests; indeed this strain is capable of surviving in gastrointestinal conditions, inhibiting pathogenic microorganisms and effectively degrading natural and synthetic lipids. The strain was also able to reduce *in vitro* cholesterol to more than 54% and triglycerides to more than 80%. Further studies are needed to elucidate these bacterial mechanisms in order to predict or specify lipid reduction mechanisms by probiotic strains observed in animal models or in clinical studies. The results certainly contribute to the knowledge of the potential to reduce lipid levels in rare strains of lactobacilli, which is an interesting property for probiotic strains that are candidates for use in food or feed.

This research is now proceeding with an *in vivo* study; they are actually testing the efficiency of the selecting lactic strains on Wistar rats receiving a high fat diet with and without addition of probiotic lactobacilli.

CONFLICT OF INTEREST

The authors Sabrina AMARA, Halima ZADI-KARAM and Nour-Eddine KARAM declare that they have no conflict of interest.

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ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

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Optimizing DNA isolation protocol for rosemary (*Rosemarinus officinalis* L) accessions

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Rosemary plant is in high demand due to its application in traditional health care, food flavoring, fragrance and pharmaceutical industries. It contains high level of secondary metabolites which are responsible for its beneficial activities. Application of molecular techniques would facilitate the production of these substances and screening of accessions. The isolation of polymerase chain reaction (PCR) amplifiable genomic DNA is a pre-requisite for taking advantage of these technologies. Even though several DNA isolation protocols for plants with high level of secondary metabolites were developed, they may not permit optimal DNA extraction due to chemotypic variation within species. Extracting DNA from different rosemary accessions is a challenging task due to its high level of secondary metabolites. Therefore, this research is conducted with the aim of optimizing a reliable and rapid method suitable for extracting DNA from rosemary plants. The optimized protocol avoids the use of repeated toxic phenols, liquid nitrogen and large polypropylene tube. It is appropriate for both fresh and dry leaf samples. The quality of the obtained DNA was excellent as evident by A_{260}/A_{280} ratio ranging from 1.7 to 1.89 and the concentration ranged from 195.8 to 2184 ng/µl. The success of this protocol indicated its applicability for other plants with high secondary metabolite contents.

Key words: DNA isolation, secondary metabolites, rosemary, gel electrophoresis, polymerase chain reaction (PCR) amplification.

INTRODUCTION

Rosemary (*Rosemarinus officinalis* L.) is an aromatic, medicinal and spice herb that belongs to the Lamiaceae family (Elhassan and Osman, 2014). The genus *Rosemarinus* also includes *Rosmarinus eriocalyx*, *Rosmarinus tomentosus*, *Rosmarinus lavandulaceus* and *Rosmarinus laxiflorus* (Zaouali et al., 2010; Rosselló et al., 2006; Upson, 2006; Angioni et al., 2004; Elamrani et al., 2000; Arnold et al., 1997). Among all *Rosmarinus* species, only *R. officinalis* had gained medicinal, pharmaceutical and industrial importance. It is the most exploited species due to its valuable essential oil and phenolic contents (Zaouali et al., 2010). It is known for the quality of its essential oils and polyphenols exhibiting antiseptic (Rampart et al., 1986; Bult et al., 1985), anti-

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rheumatic (Makino et al., 2000), antispasmodic and antioxidant activities (Zaouali et al., 2010; Almela et al., 2006; Del Bano et al., 2003). Moreover, rosemary oil is known to have antimicrobial antitumor, antiviral, antibacterial, anti-inflammatory and carminative activity (Peshev et al., 2011).

The plant is in high demand due to its accessibility and application in traditional health care, food flavoring, fragrance, perfume, pesticide and pharmaceutical industries (Mishra et al., 2009; Mulas et al., 2002). It has long been known to contain high level of bioactive secondary metabolites which are responsible for its diverse use (Peter, 2012; Hamedo and Abdelmigid, 2009). Some of the bioactive compounds found in rosemary are phenolic diterpenes (carnosic acid, carnosol or rosmanol), flavonoids (genkwanin, cirsimaritin or homoplantaginin), and triterpenes (ursolic acid) (Bai et al., 2010; Del Baño et al., 2004; Bicchi et al., 2000). Application of molecular techniques would increase and facilitate the production of these substances, screening of accessions, choosing of parents and selection of progenies as well as prevent biological privacy (Khanuja et al., 1999; Moyo et al., 2008). The isolation of pure, intact and high-quality DNA for polymerase chain reaction (PCR) amplification is a pre-requisite for taking advantage of these technologies. But the isolation and purification of high molecular weight DNA from aromatic and medicinal plants is compromised by excessive contamination by secondary metabolites (Sahu et al., 2012). This substance causes great problems in DNA isolation and isolated DNA that contains these metabolites is not suitable for PCR amplification (Puchooa and Venkatasamy, 2005). Degradation of DNA due to endonuclease, co-isolation of polysaccharides, polyphenols and other secondary metabolites are problems encountered during DNA isolation from these plants. The presence of polysaccharides and polyphenols inhibits enzyme activities, reduces yield, quality and maintenance time of extracted DNA (Khanuja et al., 1999).

Several genomic DNA isolation protocols for plants with high level of secondary metabolites were developed (Iqbal et al., 2013; Sahu et al., 2012; Khanuja et al., 1999). But most of the methods are lengthy and involved repeated use of toxic phenols. The repeated use of chloroform: isoamyle alcohol extraction makes the steps lengthy, costly and more dangerous for health. As chloroform is potentially dangerous for human health, it is important to reduce usage of it. Moreover, most of the procedure involves the use of liquid nitrogen freezing to preserve and grind the samples. Since liquid nitrogen is difficult to handle and could be dangerous in an open laboratory, it is important to find an alternative method. In addition, plant species belonging to the same or related genera will have wide variability in chemical composition. This chemotypic variation within species may not permit optimal DNA isolation from one protocol (Varma et al., 2007). Thus DNA isolation protocols need to be adjusted to each plant species.

Since rosemary is among the plants with high level of secondary metabolites, extraction of non-contaminated DNA from it is a challenging task. Therefore, appropriate extraction method should be optimized for it. Therefore, the objective of this study was to develop a simple, rapid and safe extraction method which yields DNA in desirable quantity and quality for molecular work.

MATERIALS AND METHODS

Plant

Leaves were taken from 10 rosemary accessions collected from different part of Ethiopia and grown at National Biotechnology Research Center experimental station. Leaf material was collected from actively growing parts of the plants and stored in -80°C until use. Leaf samples were also subjected to silica gel drying in order to check the applicability of the optimized protocol for dry sample.

Extraction methods

DNA extraction method developed by diversity array technology (DArT, 2019), cetyl trimethylammonium bromide (CTAB) extraction method by Doyle and Doyle (1990) and a methods developed by Khanuja et al. (1999) were employed for extracting DNA from rosemary accessions. Among the tested methods, Khanuja et al. (1999) method performs better in regard to DNA quality. Therefore, this method was taken and optimized for DNA extraction of different rosemary accessions by varying incubation and centrifuge condition, time and revolution per minutes (rpm), and by avoiding repeated steps of chloroform: isoamyl and high salt re-extraction. The modified method also avoided the use of large polypropylene tube and liquid nitrogen.

Standardized DNA extraction procedure

(i) 10 mg of -80°C frozen leaves were ground to fine powder by using pre chilled mortar and pestle (silica gel dried leaves were ground with mortar and pestle without ice cold condition).
(ii) The powdered materials were transferred into 2 ml sterile Eppendorf tube and 1000 µl of freshly prepared extraction buffer which contains 100 mMTris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 1.5 M NaCl, 2.5% CTAB (w/v), 0.2% β-mercaptoethanol (v/v) (to be added just before use) and 1% PVP (w/v) (to be added just before use) was added and mixed by inverting the tubes slowly.
(iii) After properly mixed, the samples were incubated at 65°C for 90 min in water bath (2 h incubation was employed for dry samples). The samples were mixed every 20 min by inverting the tubes.
(iv) 1000 µl of chloroform: isoamyl alcohol (24:1 v/v) mixture was added and mixed by inversion of the tubes for about 15 min, followed by centrifugation at 10,000 rpm for 10 min.
(v) After centrifugation, supernatant was taken carefully and transferred into another 2 ml sterile Eppendorf tube, followed by addition of 500 µl of 5 M NaCl and gentle mixing.
(vi) 0.5 volumes of cold isopropanol were added and the tubes were slowly inverted 5 to 10 times and stand at room temperature for about 1 h. After 1 h, the samples were carefully mixed by inversion of the tubes and then centrifuged for 30 min at 10,000 rpm.

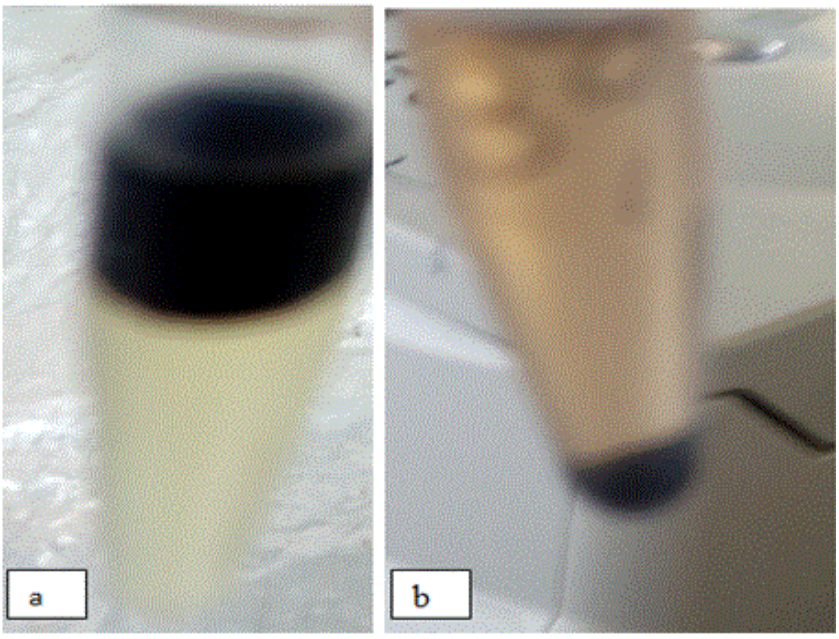

Figure 1. Very black supernantant (a) and pellet (b) obtained by using a method developed by diversity array technology.

(vii) After centrifugation, the supernatant was discarded and the pellet is washed in chilled absolute and 80% ethanol, followed by drying the pellet at room temperature for 20 to 30 min.
(viii) After drying the pellet, it is re-suspended in 100 µl of nuclease free water for about 1 h and 5 µl of RNase A was added and incubated at 37°C for 30 min.
(ix) After the DNA quantity and quality is checked, it is stored at 4°C until use and/or at -20°C for long term preservation.

Quantification and qualification of the extracted DNA

The quantification of genomic DNA was achieved using a Nanodrop spectrophotometer (ND-8000). The DNA purity was determined by the A_{260}/A_{280} absorbance ratio. DNA purity was further tested by running the extracted genomic DNA samples on 1% agarose gel in 1× TAE gel buffer. For gel preparation, agarose powder was dissolved in 1× TAE buffer, the mixture was boiled on microwave oven at 100°C. After agarose was dissolved completely and cooled to 50 to 60°C it is casted in a gel tray with comb. After solidifying, gel was placed in gel tank containing 1× TAE buffer. A DNA sample from each accession was taken, mixed with 2 µl of loading dye which contains gel red and loaded in the wells. Gel was run at constant voltage of 80 V for approximately 40 min. The gel was observed under UV light using gel documentation system (Bio Doc-IT Imaging system).

PCR amplification

PCR for amplification of DNA were carried out in a final volume of 12.5 µl. A reaction tube contained 50 ng of genomic DNA, 6.25 µl of master mix, 10 pmol of each SSR forward and reverse primer. The amplifications were carried out using the T100 thermal cycler for 35 cycles of 94°C for 4 min, 94°C for 1 min, 72°C for 1 min, followed by a final extension step of 72°C for 7 min (Segarra-Moragues and Gleiser, 2009). The amplified products were loaded in a 3% agarose gel and observed in gel documentation system.

RESULTS AND DISCUSSION

The DArT protocol did not show promising results for different rosemary accessions as evident by poor quality, very black supernatant and pellet (Figure 1). The quality of the extracted DNA was very low and the ratio of A_{260}/A_{280} is below 1.5 (Table 1). It also gave unclear and sheared bands during gel electrophoresis (Figure 2a). This showed that the extraction method developed by DArT is not optimal for extraction of non-contaminated DNA from different rosemary accessions.

CTAB extraction method by Doyle and Doyle (1990) also did not give desirable result for all the tested rosemary accessions. The obtained DNA was brownish in color, sticky to the wells and produced sheared bands in agarose gel electrophoresis (Figure 2a). The brownish pellet, sticky and sheared bands in the agarose gel obtained by this extraction method indicated contamination by polysaccharides and phenols (Moreira and Oliveira, 2011). The quality of the DNA was also very poor and ratio of A_{260}/A_{280} was below the optimal limit (Table 1). These make the DNA non-suitable for PCR amplification and further molecular work.

The extraction method developed by Khanuja et al., (1999) yielded better DNA quality compared to the two protocols. The obtained DNA was free from staining and not sticky to the wells during electrophoresis. Even though the method produced better quality DNA and relatively better in removing polysaccharides and other secondary metabolites, it is not applicable in the case of rosemary, because the amount of extracted DNA was very low (Table 2) and degraded within short time as

Table 1. Genomic DNA quality and quantity of rosemary accessions extracted by using DArT and CTAB extraction method by Doyle and Doyle (1990).

Accession code	DArT protocol				Doyle and Doyle (1990) protocol			
	Replication 1		Replication 2		Replication 1		Replication 2	
	A_{260}/A_{280} ratio	DNA quantity (ng/µl)	A_{260}/A_{280} ratio	DNA quantity (ng/µl)	A_{260}/A_{280} ratio	DNA quantity (ng/µl)	A_{260}/A_{280} ratio	DNA quantity (ng/µl)
1	1.44	20	1.21	78.53	1.43	206.9	1.43	38.46
2	1.4	22.35	1.29	93.04	1.16	109.5	1.34	59.42
3	1.28	8.12	1.27	42.92	1.45	86.93	1.55	46.85
4	1.45	58.55	1.28	68.33	1.49	82.09	1.42	54.34
5	1.22	27.4	1.28	85.27	1.23	71.91	1.29	45.14
6	1.51	17.47	1.27	71.26	1.28	112.4	1.42	111.1
7	1.47	58.68	1.43	61.32	1.46	85.13	1.41	134.4
8	1.47	30.84	1.5	59.24	1.1	111.9	1.42	156.3
9	1.21	81.79	1.14	38.79	1.08	168.5	1.29	117
10	1.32	80.51	1.15	45.07	1.16	174.6	1.19	117.6

evident by poor PCR amplification (Figure 2b). Similar problems were reported by Iqbal et al., (2013). When using this protocol for *Berberis* and *Mentha* species. The obtained DNA pellet by such protocol is subjected to high salt TE buffer dissolving and then re-extracted by chloroform: isoamyl alcohol. This reduced the quantity of the DNA obtained.

High quantity and quality DNA was extracted from both fresh and dry leaf samples by modified protocol. The absorbance ratio of A_{260}/A_{280} ranges from 1.7 to 1.89 and concentration of the DNA ranges from 195.8 to 2184 ng/µl (Table 3). For most of the accessions in all the three replication, the A_{260}/A_{280} ratio is above 1.75 and the quantity is above 300 ng/µl. Suggesting that the obtained DNA is free of proteins and polyphenols (Saghai-Maroof et al., 1984). The pellets were clear and white without visible discoloration indicating that the DNA isolated by this protocol is neither contaminated nor degraded. A clear band on agarose gel electrophoresis also showed the DNA is free of polysaccharides and secondary metabolites (Figure 1a). The DNA of all tested accessions were also run for PCR amplification, the result exhibited that the DNA obtained by the optimized protocol is suitable for PCR amplification and further molecular work (Figure 2b).

Increasing incubation temperature, avoiding high salt TE buffer dissolving and chloroform: isoamyl alcohol re-extraction steps in this protocol enables obtaining of high quality quantity DNA. The modification made on centrifugation condition also helps to obtain clear and easily separable supernatants and well precipitated DNA pellet. As it has been reported for other aromatic and medicinal plants (Sahu et al., 2012; Khanuja et al., 1999), the use of high concentration of PVP and β-mercapto ethanol also help to successfully remove the polyphenols from *R. offocinalis* accessions. Moreira and Oliveira (2011) and Paterson et al., (1993) also reported the addition of more than 0.5 M NaCl to remove polysaccharides during DNA extraction. The use of high concentration (1.5 M in extraction buffer and 5 M in supernatant) of NaCl in this protocol therefore successfully removed the poly-saccharides from all the tested rosemary accessions.

Conclusion

The optimized protocol modifies incubation time and temperature, centrifugation time and revolution per minute. It also eliminates elution of the pellet in high salt TE buffer and re-extraction by chloroform: isoamyl alcohol. All this modifications made the protocol optimum for obtaining DNA in desirable quantity and quality from rosemary accessions. The protocol also avoids the use of liquid nitrogen by using -80°C stored and silica gel dried leaf sample for DNA extraction. This makes the protocol applicable in areas where storage of liquid nitrogen is difficult. Moreover, the method avoids the use of large polypropylene tube and leaf sample. These make the method more applicable in modern

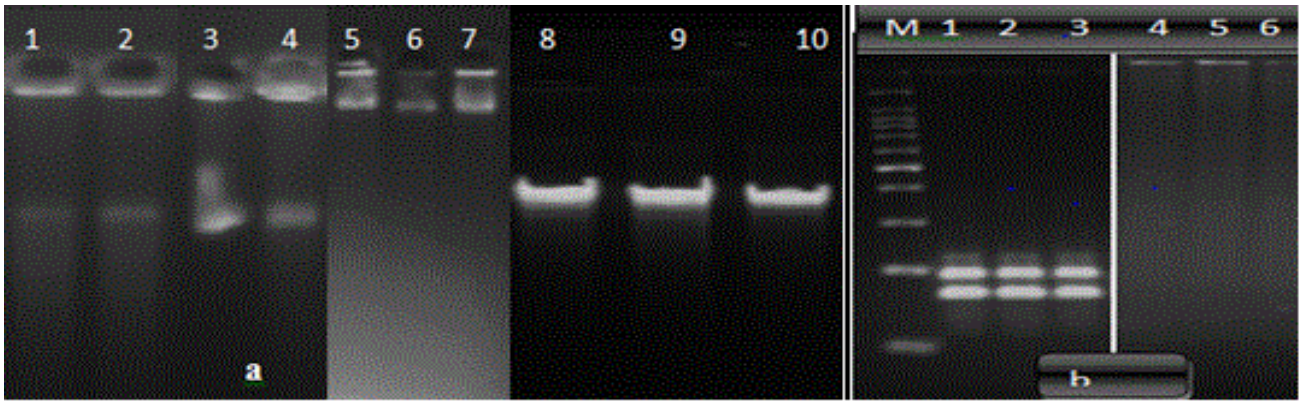

Figure 2. (a) Genomic DNA isolated from plant leaves resolved under 1% agorose gel. Lanes 1 and 2 show the DNA isolated by using DArT genomic DNA extraction protpcol. Lanes 3 and 4 show the isolated DNA by using CTAB extraction method developed by Doyle and Doyle (1990). Lanes 5 to 7 show the isolated DNA by Khanuja et al. (1999) protocol. Lanes 8 to 10 show the isolated DNA extracted by the present optimized protocol. (b) PCR amplified samples on 3% agorose gel by using primers: F:5'AGATGAAGATGGGTGAACTGAAG3'; R:5' TTGAAGGGTGCATTTGGATAGA3'. Lanes 1 to 3 show amplied DNA extracted by the optimized protocol and Lanes 4 to 6 show poor amplification of DNA extracted by Khanuja et al. (1999). M represents 100 bp ladder.

Table 2. Genomic DNA quality and quantity of rosemary accessions extracted by using Khanuja et al. (1999) protocol.

Accession code	Replication 1		Replication 2	
	A_{260}/A_{280} ratio	DNA quantity (ng/µl)	A_{260}/A_{280} ratio	DNA quantity (ng/µl)
1	2.07	63.24	1.94	59.06
2	1.6	63.97	1.96	31.3
3	2.01	71.29	1.89	62.74
4	1.98	65.29	1.83	79.7
5	1.97	57.51	1.85	61.08
6	1.95	99.88	1.9	68.22
7	1.95	61.98	1.71	40.19
8	2.04	69.33	1.88	82.28
9	1.92	94.93	1.94	43.37
10	2.05	58.7	1.9	94.27

Table 3. Genomic DNA quality and quantity of rosemary accessions extracted by using the current optimized protocol.

Accession Code	Replication 1		Replication 2		Replication 3	
	A_{260}/A_{280} ratio	DNA quantity (ng/µl)	A_{260}/A_{280} ratio	DNA quantity (ng/µl)	A_{260}/A_{280} ratio	DNA quantity (ng/µl)
1	1.83	766.8	1.88	1448	1.76	510.3
2	1.75	313.3	1.87	1583	1.87	384.9
3	1.7	270.1	1.86	1740	1.85	326.8
4	1.76	554.5	1.87	1950	1.75	376.2
5	1.86	454.9	1.85	1229	1.78	279.9
6	1.77	296.2	1.7	2184	1.78	195.8
7	1.7	552.7	1.89	1464	1.8	353.6
8	1.75	857.1	1.8	2053	1.86	344.9
9	1.73	828.5	1.81	1485	1.73	604.3
10	1.73	1577	1.85	1382	1.77	371

biotechnology laboratories which use eppendorf and micro centrifuge tube. Generally, the optimized protocol is time and cost efficient less hazardous and applicable for extracting DNA from both fresh and dry leaf samples of rosemary accessions. The success of this protocol also indicated its usefulness for extraction of DNA from other plants with high level of secondary metabolites.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Nutritional enhancement of cocoa pod husk meal through fermentation using *Rhizopus stolonifer*

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This study evaluated the effect of fermentation period on the proximate composition, anti-nutritional content, fibre fractions and amino acid profile of cocoa pod husk meal (CPHM). Cocoa pod husk was taken through a solid state fermentation process involving *Rhizopus stolonifer* as its starter culture for a period of two weeks. The fermented CPHM was dried and analyzed for its proximate composition, anti-nutritional factors, fibre fractions and amino acid profile. The results of the study revealed that the crude protein content of CPHM significantly (P ≤ 0.05) improved during fermentation by 48.59%, while crude fibre and crude lipid decreased significantly (P ≤ 0.05) by 14 and 22%, respectively after 2 weeks of fermentation. The theobromine, tannin and phytate of the fermented samples decreased by 77.3, 94 and 27% after 14 days fermentation, respectively. Also, the neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), hemicellulose (HEMM) and cellulose (CELL) decreased progressively as the days of fermentation increased and total esssential amino acid (TEAA) of the fermented CPH meal increased significantly (P ≤ 0.05) as days of fermentation increased showing that *Rhizopus stolonifer* may both enhance CPHM protein quantity and improve its quality. It could be concluded that fermentation with *R. stolonifer* for 14 days could improve the nutritive value of CPH and thus increase its inclusion in the formulation of diets for animals.

Key words: Cocoa pod husk meal, fermentation, *Rhizopus stolonifer*, nutritive, anti-nutritive composition.

INTRODUCTION

Countries in West and Central Africa account for 71.4% of the total world production of cocoa (*Theobroma cacao*) beans, primarily for the manufacture of chocolate and cocoa powder (International Cocoa Organization, 2012). An estimate of 6.7 million metric tonnes of cocoa pod husk, a by-product of cocoa cultivation is often generated from these cocoa products. According to Tijani et al., (2016), cocoa pod husk contains protein (6.8-10%), gross energy (10.7 $MJkg^{-1}$ DM), fibre (24-35.4%), fat (1.6-2.4%) and non cellulose carbohydrate (46.6%).

Given the critical shortfall in livestock production in most cocoa-producing countries in Africa, attributable largely to the prohibitive cost of animal feed, the utilization of the vast quantities of discarded cocoa waste

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products as affordable accessory animal feed would be of tremendous benefit to millions (Campos-Vega et al., 2018).

Previous works revealed their potential use as an unconventional low-cost feed ingredient for livestock nutrition, reducing feed costs by replacing some of the expensive conventional feed ingredients used in ration formulation (Ozung et al., 2017; Adeyeye et al., 2018). Ashade and Osineye (2013) also reported an increase in weight gain and profit margin when CPH was used to substitute 100% maize in the diet of Oreochromis niloticus. However, the replacement value for CPH in monogastric nutrition is limited by its poor nutrient composition which causes slow growth rate of livestock due to poor feed intake and digestibility (Adeyeye et al., 2018). Its low protein value coupled with the presence of high amounts of lignin as well as non-starch polysaccharides (NSPs) including hemicellulose and cellulose, which are poorly utilized by farm animals (particularly monogastrics) constitute major limitation to its replacement value in animal diets (Ozung et al., 2017).

The advent of biotechnological innovations, mainly in the area of enzyme and fermentation technology might offer potential economic utilization of cocoa pod husk. The application of effective bio-treatment approaches such as fungal biotechnology is worth considering for the improvement of the nutritional value of CPH as higher fungi have the ability to bio-transform fibrous agro-residues into value-added products through their extracellular enzyme activities (Oduro-Mensah et al., 2018).

Solid state fermentation was carried out on CPH using *Rhizopus stolonifer,* at the end of fermentation, the substrate (CPH meal) was studied for value-addition, in terms of improved nutritional qualities and reduction in theobromine content. The value-addition may expand the scope of the utilization of CPH in animal husbandry in regions where cocoa is produced on a large scale. This study was therefore carried out to assess the effect of a solid state fermentation treatment involving *R. stolonifer* on the composition of CPH.

MATERIALS AND METHODS

Experimental site

Microbial analysis of CPH meal was conducted at the Microbiology Laboratory and solid-state fermentation of CPH meal was carried out at the Nutrition Laboratory of the Department of Animal Production and Health, the Federal University of Technology Akure (FUTA), while all chemical analyses were carried out at the Centre of Excellence on Food Security, Professor Julius Okojie Central Research Laboratory, FUTA.

The study location lies between latitude 7°15' North and longitude 5°12' East of the equator of the Greenwich in the humid tropical rainforest region. It has an average annual rainfall of about 2378 mm with temperature ranging between 28 and 30°C and a relative humidity of about 80% (Climatedata, 2018).

Collection and processing of cocoa pod husk

Freshly discarded cocoa pod husks were collected during the harvest season from plantation around Idanre and Ondo town, Nigeria. The surface of the pods was cleaned, grossly chopped to pieces and sun-dried to a moisture content of ca. 10%. Dried cocoa pod husks were ground in a hammer mill (Model 912, Winona Attrition Mill Co., Winona, MN) to produce the CPH meal, which was later stored in polyethylene bags and kept under moisture free conditions pending solid state fermentation and chemical analysis of its nutrient.

Isolation of microorganism and preparation of inoculum

R. stolonifer commonly known as black bread mold and sometimes used in preparing fermented foods was isolated from decomposing bread using potato dextrose agar (PDA). Dark patches were scrapped with sterile scalpel to inoculate the medium and incubated at 30 ± 1°C for 5 days. The pure culture was subsequently stored in PDA slants at 4°C. It was identified conventionally according to its macroscopic and microscopic features following the scheme of Domsch et al., (1980). Inoculum was developed by transferring a loopful of mycelium into the inoculum medium (1% sucrose, 0.2% yeast extract, pH 5.50). The flasks were incubated at 30°C on a shaker at 100 rev/min for 24 h. For use as inoculum, the spore suspensions were standardized to 2×10^6 spores/ml. A hemacytometer (Neubauer-ruled Bright Line counting chambers; Hausser Scientific, Horsham, Pa.) was used to count the spores (n = 4). Spore suspension of *R. stolonifer* was prepared in distilled water after incubation for up to 5 days in Potato Dextrose Agar nutrient broth at room temperature (25-29°C) following the procedures of Wolk et al., (2000).

Fermentation of cocoa pod husk meal with starter R. stolonifer

Dried and finely ground CPH meal (100 g) placed in aluminium foil was sterilized by autoclaving. 1 g of urea was dissolved in 100 ml of sterile water which was used to moisture the sterilized CPH meal. 10 ml of the prepared inoculum of the starter culture *R. stolonifer* was used to inoculate the urea treated CPH meal and kept in an incubation room. The fermentation of the cocoa pod husk meal was terminated on the 3rd day, 5th day, 7th day and 14th day followed by sun drying the substrates for two days to inactivate the microorganism. The dried CPH meal was subsequently kept in air tight plastic container in readiness for proximate analysis (Aro et al., 2008; Laconi and Jayanegara, 2015).

Chemical analyses

The proximate composition of raw and *R. stolonifer* fermented CPH meal (moisture, crude protein, ash and fibre) was determined as described by AOAC (2012). Dry matter (DM) content was based on the weight loss after 24 h in an oven at 104°C; nitrogen (N) content by the macro Kjeldahl method, where crude protein (CP) was calculated as N×6.25. The ash content was determined as the residue left after incinerating the sample at 600°C for 3 h in a muffle furnance. The analyses for proximate fractions were done in triplicate for each sample. The metabolizable energy (ME) was calculated by methods described by Pauzenga (1985): ME = (37 × %CP) + (81.8 × %FAT) + (35.5 × % nitrogen free extract [NFE]) and amino acid profile was determined as described by Benitez (1989). 20 g of the sample was dried to constant weight, defatted, hydrolyzed, evaporated in a rotary evaporator and loaded into the Applied Biosystems PTH Amino Acid Analyzer. Theobromine was

Table 1. Nutrients composition of raw and fermented CPH Meal at various days of fermentation with *Rhizopus stolonifer* (g/100 g).

Proximate Composition (g/100 g DM)	Unfermented	Fermented				SEM	P value
	Raw	3rd day	5th day	7th day	14th day		
Crude protein	11.27[c]	15.58[b]	16.32[b]	18.20[b]	21.92[a]	0.28	0.05
Ash	11.37	9.73	9.52	9.23	9.03	0.15	0.67
Crude Fiber	9.60[b]	9.09[b]	8.93[b]	8.91[a]	8.19[a]	0.20	0.05
Crude Fat	7.15[a]	6.96[b]	6.65[a]	6.39 [b]	5.54 [b]	0.08	0.05
NFE	60.61	58.64	58.58	57.27	55.32	0.30	0.61
M.E (Kcal/kg)	3153.52	3227.51	3227.40	3229.19	3228.07	0.35	0.49

Values represent means of triplicate. [ab]Means within a row with different letters are significantly different (p<0.05). SEM: Standard error mean, ME: metabolizable energy = (37 × %CP) + (81.8 × %FAT) + (35.5 × %NFE). Source: Pauzenga (1985).

Table 2. Anti-nutritional composition of raw and fermented CPH Meal at various days of fermentation with *Rhizopus stolonifer* (%DM).

Anti-nutrient	Unfermented	Fermented				SEM	P value
	Raw	3rd day	5th day	7th day	14th day		
Theobromine (g/100 g DM)	1.32[c]	0.70[b]	0.33[a]	0.33[a]	0.30[a]	0.01	0.04
Tanin (g/100 g DM)	0.50[c]	0.37[bc]	0.10[b]	0.10[b]	0.03[a]	0.02	0.05
Phytate (mg/100 g)	30.49[c]	28.87[b]	23.90[a]	23.90[a]	22.25[a]	0.02	0.05

determined using the protocol developed by Janna, (2011), while the phytate content was determined by the method of Young and Greaves (1994) based on the ability of standard ferric chloride to precipitate phytate in dilute HCl extracts of fermented CPH meal. The Folin-Ciocalteu method according to Makkar et al., (1993) was employed to determine the levels of tannins in the CPH meals. Fibre fraction was determined as described by Van Soest et al., (1991). The levels of lignin was determined by solubilization of cellulose with sulphuric acid; neutral detergent fiber (NDF) assayed with a heat stable amylase and expressed as inclusive of residual ash and acid detergent fiber (ADF) also expressed as inclusive of residual ash fractions were determined according to procedures described by Robertson and Van Soest, (1981), Mertens (2002) and AOAC (1990) method 973.18, respectively. The values of these fiber fractions were subsequently used to estimate levels of hemicellulose (that is, difference between NDF and ADF values) and cellulose (that is, difference between ADF and lignin-sa values) in the samples.

Statistical analysis

Data generated from the trial were subjected to analyses of variance using SAS (version 9.2) where significant difference were observed, difference between means was tested using Duncan's multiple range test outlined in the SAS statistical package. All analyses were carried out in three replicates (n=3).

RESULTS

Proximate composition of unfermented and fermented cocoa pod husk meal

The proximate composition of raw and *R. stolonifer* fermented CPH meal summarized in Table 1 revealed that crude protein and metabolizable energy increased progressively as the days of fermentation increased from 11.27 (g/100 g DM), 3153.52 (kcal/kg) in the raw to 21.92 (g/100 g DM), 3228.07 (kcal/kg) at 14th day of fermentation with *R. stolonifer*, respectively. However, progressive decreases were observed in ash content (P≥0.05), crude fibre (P≤0.05), fat (P≤0.05) and nitrogen free extract (P≥0.05) as the days of fermentation increased (that is, ash: 11.37 (g/100 g DM) to 9.03 (g/100 g DM); CF: 9.60 (g/100 g DM) to 8.19 (g/100 g DM); crude fat: 7.15 (g/100 g DM) to 5.54 (g/100 g DM); NFE: 60.61 (g/100 g DM) to 55.32 (g/100 g DM).

The anti-nutrient composition summarized in Table 2 revealed a significant (P≤0.05) reduction in theobromine concentration of CPH meal during fermentation from 1.32 (g/100 g DM) to 0.33 (g/100 g DM) as the period of fermentation increased. Also, the tannin and phytate concentration of the CPH meal during fermentation significantly (P≤0.05) reduced from 0.50 (g/100 g DM) to a very minimal level of 0.03 (g/100 g DM) and 30.49 (g/100 g DM) to 22.25 (g/100 g DM), respectively.

Table 3 also shows a significant (P≤0.05) reduction in all fibre fractions measured. The nitrogen detergent fibre reduced significantly (p<0.05) from 91.89 to 65.89 g/100 g DM after 14 days of fermentation, while acid detergent fibre significantly (p<0.05) reduced after 14 days of fermentation from 45.25 to 61.29 g/100 g DM. Also, the lignin content of the fermented CPH meal significantly (p<0.05) reduced by 53.75% from 23.33 to 10.79 g/100 g DM. Cellulose significantly (p<0.05) reduced from 38.19 g to 29.79 g/100 g DM with a 22% reduction and the

Table 3. Fibre fractions composition of raw and fermented CPH meal at various days of fermentation with *Rhizopus stolonifer* (%DM).

Fibre fraction	Unfermented	Fermented				SEM	P value
	Raw	3rd day	5th day	7th day	14th day		
NDF	91.89^{e}	81.59^{d}	73.89^{c}	69.59^{b}	65.89^{a}	0.01	0.05
ADF	61.29^{c}	54.17$_{bc}$	49.95^{b}	48.45^{a}	45.25^{a}	0.01	0.05
ADL	23.33^{c}	21.81^{bc}	20.15^{b}	17.91^{b}	10.79^{a}	0.01	0.04
HEMM	30.59^{c}	27.41^{bc}	23.93^{b}	21.13^{a}	20.63^{a}	0.01	0.05
CELL	38.19^{c}	34.45^{bc}	33.35^{b}	30.53^{a}	29.79^{a}	0.01	0.02

Values represent means of triplicate, ^{ab}Means within a row with different letters are significantly different ($p<0.05$). SEM: Standard error mean, NFE: nitrogen free extract, NDF: neutral detergent fibre, ADF: acid detergent fibre, ADL: acid detergent lignin, HEMM: hemicellulose, CELL: cellulose.

hemicellulose content significantly ($p<0.05$) reduced from 30.59 to 20.63 g/100 g DM by 32.56%.

The quantitative composition of amino acids profile of CPH meal fermented with *R. stolonifer* at different periods is shown in Table 4. The duration of fermentation significantly ($p<0.05$) led to progressive increase in the values of Leucine and Valine while other amino acid profile improved numerically as the days of fermentation increased. Total amino acids increased significantly ($p<0.05$) as the days of fermentation increased from 0 to 14 days. The values recorded on the 7 and 14th days of fermentation were 50.72 and 59.71 g/100 g protein, respectively, while the value obtained from the untreated CPH meal was 44.06 g/100 g protein. The total essential amino acids (TEAA) increased significantly ($p<0.05$) and was the highest on days 14 (33.31 g/100 g protein), while the lowest value was obtained in the untreated CPH meal (25.04 g/100 g protein). The non-essential amino acids (TNEAA g/100 g protein) values were the highest on 14 days fermentation period (26.40 g/100 g protein), while the unfermented CPH meal had the lowest (19.02 g/100 g protein). However, the TEAA: TNEAA values were 57:43, 56:44 and 56:44 for 0, 7th and 14th fermentation period, respectively.

DISCUSSION

The result obtained from this study implied that the crude protein (CP) content of the *R. stolonifer*-fermented CPH meal was higher than the one in raw sample by about 27.66 to 48.59% and those earlier reported by Adeyeye et al. (2017) and Ozung et al., (2017). Implying that fermented CPH meal can be used to replace some conventional feed ingredient within the same protein content range. Adeyeye et al. (2017) reported 13.66 g/100 g CP in ash-treated cocoa pod husk meal while Ozung et al., (2017) observed crude protein values ranging between 7.70 and 8.94 g/100 g DM CP as against 21.92 g/100 g DM CP that was obtained in this current study. This is indicative of the efficacy of *R. stolonifer* and the fermentation process employed in this study to increase the crude protein content of the CPH and this confirms the studies reported by Balagopalan (1996), Leifa et al., (2001) and Alemawor et al., (2009) on the ability of fungi to enhance the nutritive values of agro-residue on coffee husk, cassava by-products and cocoa pod husk. The increase in growth/biomass of the fungus on the fermented cocoa pod husk (FCPH) might account for the increase observed in the protein contents with the fungal hyphae serving as single cell protein. The fungus in view contains a relatively high protein content of high biological value (Waliszewska et al., 1983).

The crude fiber content of the raw CPH meal obtained declined by 5.31 and 14.7% after fermentation (3 to 14 days), implying a better digestibility when used as ingredients in animal nutrition. This supports the intended aim of fermentation which was meant to improve the CP content and lower the crude fiber content of CPH meal so as to enhance the usability of the test ingredient for monogastric animal nutrition. The crude fiber contents reported by Nortey et al., (2015) and Adeyeye et al., (2017) after fermentation were 7.04 and 14.83%, respectively whereas Ozung et al., (2017) reported significantly higher values: 57.42 and 53.37% from fermentation and hot-water treatment of the CPH meal, respectively. Lateef et al., (2008) and Alemawor et al., (2009) reported 7.2 and 17.08% reduction of CF in CPH meal, respectively. The reduction in the crude fibre content may be an indication of *R. stolonifer* having enzymatic system, that is, secretion of cellulose/ hemicellulose-degrading enzymes for degradation of polmeric lignocelluloses of CPH (Alemawor et al., 2009). The results herein reported showed that the fungal strain in use can effectively reduce the crude fibre content of CPH and this may have positive effect on its digestibility by animals.

The values for ash and crude fat were partly in consonance with the reports of Ozung et al., (2016, 2017) and Adeyeye et al., (2017). The observed reduction in crude fat was in agreement with the report of Oliveira et al., (2011) who also observed that crude fat content of fungal fermented whole rice bran decreased significantly and might be as a result of lipid use by the filamentous

Table 4. Amino acid profile of raw and fermented CPH meal at various days of fermentation with *Rhizopus stolonifer* (g/100g protein) (DM).

Amino acid	Unfermented	Fermented		SEM	P value
	Raw	7th day	14th day		
Essential					
Arginine	3.87	4.13	5.16	0.29	0.65
Histidine	1.24	1.40	1.66	0.09	0.44
Isoleucine	3.53	4.14	4.51	2.60	0.35
Leucine	3.56^{c}	4.49^{b}	4.96^{a}	0.34	0.04
Lysine	3.02	3.63	4.69	0.36	0.22
Methionine	0.29	0.34	0.37	0.11	0.55
Phenylalanine	2.57	2.84	3.72	0.30	0.87
Threonine	3.11	2.99	3.38	0.13	0.68
Valine	3.59^{c}	4.15^{b}	4.44^{a}	0.23	0.04
Trytophan	0.26	0.26	0.42	0.04	0.38
TEAA	25.04^{c}	28.37^{b}	33.31^{a}	1.35	0.04
%TEAA	56.83	55.93	55.79	0.55	0.06
Non-Essential					
Alanine	1.78	2.05	2.39	0.13	0.24
Aspartic acid	4.00	4.59	5.27	0.38	0.96
Cysteine	1.09	1.21	1.21	0.03	0.10
Glutamic acid	4.24	5.30	6.36	0.44	0.41
Glycine	2.04	2.30	3.11	0.26	0.77
Proline	2.13	2.64	2.84	0.16	0.07
Serine	2.19	2.54	2.64	0.10	0.10
Tyrosine	1.55	1.72	2.58	0.23	0.49
TNEAA	19.02^{c}	22.35^{b}	26.40^{a}	0.10	0.04
% TNEAA	43.17	44.07	44.21	0.30	0.06
TAA, g/100 g DM	44.06^{c}	50.72^{b}	59.71^{a}	0.10	0.03
TEAA:TNEAA ratio	57:43	56:44	56:44	-	-

EAA = Essential amino acids; TEAA = total essential amino acids; NEAA = non-essential amino acids; TNEAA = total non-essential amino acids; TAA = total amino acids; SEM = pooled standard error of means. ^{a,b}Mean values within a row without a common lowercase superscript differ at $P < 0.05$.

fungi, possibly in the synthesis of phospholipid constituents of the cell membrane of fungal tissue. It has been reported that during fungal growth, some lipolytic strains assimilate lipids from substrates for biomass production leading to a general reduction of the overall lipids content of the substrate (Das and Weeks, 1979). The nitrogen free extracts value obtained in this study negates the report of both Ozung et al., (2016, 2017) and Adeyeye et al., (2017) who reported 14.56 and 39.31%, respectively for post-fermentation CPH meal.

The result from this current study also showed a remarkable decline in the theobromine content of the FCPH meal. There was a 46.97 to 77.2% decline in theobromine content of the cocoa pod husk meal post-fermentation which is indicative of the ability of *R. stolonifer* to degrade the methylzanthine backbone of theobromine in the substrate. This reduction coefficient in theobromine agrees with the reports of Adamafio et al., (2011), Bentil et al., (2015), Amorim et al., (2017) and Oduro-Mensah et al., (2018) who recorded significant decline in theobromine concentration after microbial fermentation. A significant reduction in coefficient by 72% was reported for theobromine by Oduro-Mensah et al. (2018) after employing a solid state fermentation of cocoa pod husk for 7 days using two strains of fungi, namely: *Aspergillus niger* and *Talaromyces* species which is still lower than 77% reported in this current study for the same fermentation period. The degradation of theobromine by *R. stolonifer* could have been made possible by using theobromine as a sole carbon and energy source via the demethylase oxidase, xanthine dehydrogenase, xanthine oxidase, urease and uricase pathway (Yamaoka-Yano and Mazzafera, 1999; Dash and Gummade, 2006; Huq, 2006). The reduction in theobromine content could lead to improved palatability with resultant better feed intake and utilization of FCPH

by animals.

The tannin and phytate contents were also observed to be highly reduced from 0.50 to 0.03 g/100 g DM and 30.49 to 22.25 g/100 g DM, respectively which translate into reduction coefficients of 94.00 and 27.00%, respectively after 14 days fermentation period. The tannin reduction coefficient agrees with the report of Adeyeye et al., (2017) while the phytate reduction coefficient agrees with the report of Bentil et al., (2015). The complexing of phytic acid with nutritionally essential elements and the possibility of interfering with proteolytic digestion have been suggested as responsible for antinutritional activity (Agbede et al., 2009a, b). The decrease in the phytate content of FCPH meal could possibly be attributed to the secretion of the enzyme phytase by the fungi. This enzyme is capable of hydrolyzing phytate (Oboh and Akindahunsi, 2003) and therefore could lead to better accessibility to phosphorus by the animals. The fibre fractions obtained in the FCPH meal in this study reveals a decline in values which disagrees with the report of Ozung et al., (2016) who recorded an increase in values of the fibre fractions after fermentation. Lignin, cellulose and hemicellulose fractions form the bulk of CPH fibre. During the fermentation process, the changes observed in the levels of these fibre fractions indicated the degree of lignocellulose biodegradation exhibited by *R. stolonifer* on CPH meal. The importance of dietary fibre fractions in animal feeding is shown by its ability to influence the rate of passage, mucosal functionality and its role as substrate for gut microbiota which in turn improves performance and digestive health (Gidenne, 2015). Minimal dietary fibre supply is essential to prevent digestive troubles. The cellulose degradation is mostly facilitated by the synergistic action of hydrolytic enzymes (cellulases) secreted by the filamentous fungus during the fermentation (Chesson, 1993; Datta and Chakravarty, 2001). Pothiraj and Eyini (2007) also observed that *R. stolonifer* showed the highest and fastest utilization of cellulose in the solid state fermentation of cassava waste which resulted in 51.24% utilization of cellulose on the 2nd day.

The degradation of the hemicellulose could be as a result of extracellular hemicellulases such as xylanases secreted by the fungus during the fermentation process (Chesson, 1993). This result strongly supports a study by Brimpong et al., (2009) who observed a 41% decrease in hemicellulose after complete colonization of corn cobs by the mycelia of the oyster mushroom. The degradation of lignin could be as a result of the production of lignin-degrading extracellular enzymes such as peroxidases and the laccases that oxidize both the aromatic rings and the aliphatic side chains to produce compounds more absorbable by the current fungi used (Youri, 2004).

The levels of essential amino acids (EAAs) in the fermented meals were substantially higher than the raw CPH meal. The additive effect of *R. stolonifer* during fermentation as single cell protein might have contributed to the observed increase because they are richer in EAAs particularly lysine and methionine. Data on amino acid profile of fermented CPH meal is limited hence, importance of this study. Donkoh et al., (1991) reported 10 to 15% levels of TEAA and 56% of TAA which was lower than that reported in the current findings. Interestingly, Ramos et al., (2008) reported that enzymatic treatment of cocoa bean husk reduced the negative effects of dietary-induced hypercholesterolemia in an animal model. The lysine/arginine ratio, a determinant of the cholesterolaemic and atherogenic effects of protein was observed to be low for CPH protein. In turn, the TEAA of the fermented CPH meal showed that *R. stolonifer* may both enhance the protein quantity and improve the quality which agrees with the findings of Kutlu et al., (2000), Muhammad and Oloyede (2009) and Dairo et al., (2017) that fungi species are a rich source of proteins and also contain all the essential amino acids.

Conclusion

Results from this study showed that the nutritive values of FCPH meal greatly improved as days of fermentation increased till the 14th day when fermented with *R. stolonifer*. Increase in crude protein, reduction in crude fibre and anti-nutrient content of the CPH meal fermented with *R. stolonifer*, could lead to improved palatability, better feed intake and utilization which would make the meal more suitable for use as alternative feed ingredient for animals especially monogastrics in regions which depend on imported feed ingredients where cocoa pod husk are predominant but left to waste on the farm. The use of the FCPH in animal diets in place of the import dependent ingredients could help to stem the cost of finished feeds for monogastric and rabbit production and increase meat consumption among the resource poor.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Variable DNA methylation in Ensete (*Ensete ventricosum*) clones associated with developmental stage revealed by Amplified Fragment Length Polymorphisms (AFLPs) with Methylation-Sensitive enzyme

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Ensete (*Ensete ventricosum*) is an important perennial species in Ethiopia. It is used for food and fiber. The mode of propagation is asexual and sexual. Asexual form of reproduction is by natural and anthropogenic induced sucker. This research aims to investigate and measure the methylation diversity of natural and anthropogenic induced sucker forming Ensete. Twenty-seven individuals from Hawassa University research collection were used to identify using Amplified Fragment Length Polymorphisms (AFLPs) and Methylation-Sensitive Amplification Length Polymorphism (MS-AFLP).technology. The average values of Nei's genetic diversity (*Hj*) were 0.189 and 0.110 for natural sucker forming and anthropogenic induced sucker forming, respectively by AFLPs analysis. With MS-AFLP analysis, this value reduced to 0.145 for natural sucker forming Ensete but increased to 0.172 for anthropogenic sucker inducing populations. The AFLP Shannon information index (*Ho*) was 0.313 and 0.162 for natural and anthropogenic sucker forming Ensete, respectively. However, this value was reduced to 0.238 for natural sucker forming and 0.252 for anthropogenic sucker formed by the MS-AFLP analysis. UPGMA tree, structure analysis and principal coordinate analysis (PCoA) showed the two populations clustered separately. AMOVA revealed 24% of the genetic differentiation (*Fst*) occurred among populations. Gene flow (*Nm*) was limited among all populations. This concludes that AFLP did not show differentiation among populations; however MS-AFLP clearly showed the differentiation of populations which is an indication of epigenetic diversity but limited to developmental stages of Ensete.

Key words: Methylation, *Ensete ventricosum*, sucker, Amplified Fragment Length Polymorphisms (AFLPs), Methylation-Sensitive Amplification Length Polymorphism (MS-AFLP).

INTRODUCTION

Ensete is the staple food crop in Ethiopia where, 20 million people depend on for food, feed, medicine, fiber and ornament (Pijls et al., 1995). Improving the different qualitative and quantitative traits of this crop is important

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to increase food security and diversity. For this, employing molecular breeding tools is an efficient approach.

The diversity of Ensete is confirmed by morphological and ethno botanical studies (Birmeta et al., 2002, 2004; Negash et al., 2002; Olango et al., 2015; Tesfaye and Lüdders, 2003; Tobiaw and Bekele, 2011; Yemataw et al., 2018). The mode of reproduction in Ensete is also different where wild Ensete has sexual propagation method by forming flower and seed, which is a unique resource for its possibility of retaining genetic recombination (Olango et al., 2015). However, cultivated Ensete population reproduces by sucker formation. This sucker formation is natural or anthropogenic induced, and most of the cultivated ensete comprised of natural sucker forming (ET) and anthropogenic sucker inducing (NE). The majority of cultivated Ensete populations which are used as source of food are NE type. However, cultivated Enset with limited use for food are ET type. Furthermore, ET and NE type of Ensete have contrasting propagation system. ET is propagated exclusively by vegetative propagation. However, NE type of Ensete is manipulated by anthropogenic activity to form multiple sucker formation. If the NE type is left till it finishes its life cycle, the plant will form a flower, the basis for sexual reproduction and seed formation.

Over the past decades, many researchers employed molecular markers such as Random Amplified Polymorphic DNA (RAPD) (Birmeta et al., 2002), Amplified Fragment Length Polymorphism (AFLP) (Negash et al., 2002), Inter Simple Sequence Repeats (ISSRs) (Tobiaw and Bekele, 2011); Single Sequence repeats (SSR) (Olango et al., 2015) and Single Nucleotides Polymorphism (SNP) (Yemataw et al., 2018) to study the genetic diversity of Ensete. Molecular markers were able to give us the genetic diversity, richness, and differentiation without the effect of environmental variation. However, above employed markers studies gave little information about the differentiation of natural and anthropogenic sucker forming of Ensete.

Epigenetic alters phenotypes of a living organism by changing the morphology and biological molecules (Broeck et al., 2018; Fournier-Level et al., 2011; Fray and Zhong, 2015; Fresnedo-Ramírez et al., 2017; Kaeppler et al., 2000; Wang et al., 2016). However, these changes are not the result of the change in DNA sequence (Dhar et al., 2019). This phenomenon challenged the traditional dogma of phenotypes controlled by DNA only. Thus currently it becomes clear that not only DNA but also epigenetic is affecting the variation and diversity of living organisms.

One of the most studied parts of the epigenetic effect is the change which happens because of DNA Methylation. Methylation-Sensitive Amplified Polymorphism (MSAP) is one the technique which is used to study DNA Methylation in the living organism (Fulneček and Kovařík, 2014; Labra et al., 2002a, b, 2004; Pérez-Figueroa, 2013). Reyna-Lopez is the first to use this technique in Fungi (Fulneček and Kovařík, 2014). However, after his result showing the different pattern of Methylation in clonally propagated fungi, the approach has been used by many researchers in the model and cultivated plants (Dowen et al., 2012; Fournier-Level et al., 2011; Kashino-Fujii et al., 2018; Suter and Widmer, 2013; Wang et al., 2016). Furthermore, the technique is widely applied not only to study diversity but also to understand the ecological and evolutionary aspect of the species (Broeck et al., 2018; Dowen et al., 2012). Currently, the technique is used to understand the population epigenetic diversity of living organisms. For example there is clear Methylation diversity of barley (Chwialkowska et al., 2019), Arabidopsis (Kawakatsu et al., 2016) rice (Wang et al., 2016), sorghum (Rosati et al., 2019), wheat (Shaked et al., 2001), almond (Fresnedo-Ramírez et al., 2017), grape (Fournier-Level et al., 2011), tomato (Fray and Zhong, 2015). This analysis clarified that epigenetic is important in population differentiation and influencing morphological changes. Implying epigenetic effect is an important factor for phenotypic variation. Many studies clarified DNA Methylation is the common phenomenon of a natural population which can be a potential force for the evolutionary process in living organisms. This indicates understanding the epigenetic effect is important for understanding the evolutionary and ecological explanation of population diversity. Thus, for proper management of Ensete breeding, it is necessary to evaluate the local genetic/epigenetic structure in these populations. Unfortunately, there is no information about the extent and partitioning of genetic and epigenetic diversity in Ensete populations on a local scale, as well as the genetic/epigenetic relationships between natural sucker forming (ET) and anthropogenic induced sucker formation (NE) populations of Ensete.

The major objective of this research is to provide preliminary data to determine if there is DNA Methylation differences between ET and NE Ensete trees. This study focused on dynamic differential global Methylation patterns between trees having different propagation systems. The specific objectives of the research are: (1) to check the genetic and epigenetic diversity of branching and non-branching type of Ensete, and (2) to analyze the population structure of the two types of Ensete genotypes based on their genetic and epigenetic diversity.

MATERIALS AND METHODS

Study species and sampling sites

Genomic DNA was collected from emerging sucker on dead leaf (EB), emerging bud (MB), emerging shoot (ML), shoot of young plants (D), and young shoot of matured mother plants of ET (M1, M2 , M3, HU), and shoot of matured NE plant (Ado, Ganticha, Koba, Abebe, and Wild) from the field established in Hawassa University (Figure 1). The samples consisted of two types of sucker

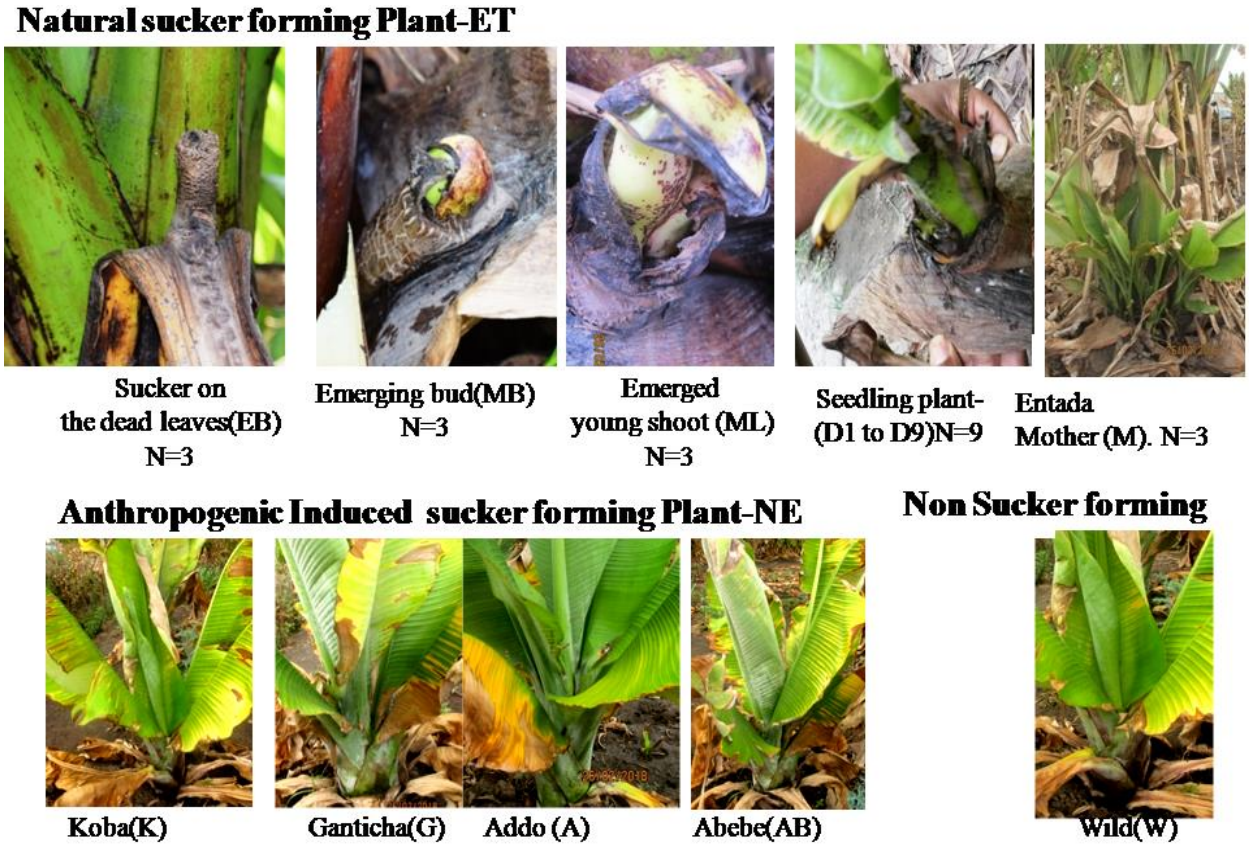


Figure 1. Phenotypic representation of the Ensete samples for the AFLP and MS-AFLP analysis at Hawassa University Research field.

formation: Mainly natural sucker forming-branching (ET) and anthropogenic induced sucker formation-non-branching (NE) form of Ensete (Figure 1). A total of 27 individuals' genomic DNA is extracted with the Qiagen DNeasy Plant Mini kit (Qiagen, Valencia, CA, USA).following the manufacture's protocol.

AFLP and MS-AFLP protocol

AFLP primer combinations using *Eco*RI+AAC (6-FAM), *Eco*RI+ AAG (6-FAM), and *Eco*RI +ACA (6-FAM) labeled primers with *Mse*I + CTC, *Mse*I + CAG, *Mse*I + CAC, *Mse*I + CTG, and *Mse*I + CTA unlabeled primers were selected from the literature (Trebbi et al., 2019). For MS-AFLP, the *Mse*I with *Msp*I and *Hpa*II isoschizomers (restricting CCGG) was replaced with different sensitivities to cytosine Methylation. The selective primers were *MspI*/*Hpa*II +TCAA, *MspI*/ *Hpa*II +TCTC, *MspI* / *Hpa*II +TCTT and *MspI*/ *Hpa*II +TCTG unlabeled primers. The primers were tested for polymorphism using two Ensete accessions (M1 and Ganticha). Following the polymorphism screening, fifteen and 9 primer combinations were selected for AFLP and MS-AFLP analysis, respectively (Table 1). Thus those polymorphic primers were used to screen all samples' genomic DNA for AFLP and MS-AFLP protocol to ensure reliable scoring of fragments. AFLP analysis was carried out using a modified protocol described by Blignaut et al. (2013), Schulz et al. (2013), Vos et al. (1995) and Vuylsteke et al. (2007). The detailed AFLP and MS-AFLP analysis were carried out with a volume of 40 µl, containing 1 µl of DNA sample (500 ng), 0.12 µl of enzyme EcoRI (Thermo Scientific TM), 0.1 µl MseI, and 0.2 µl HpaII/MspI (Thermo Scientific TM). The reaction is buffered with 4 µl Tango Restriction Buffer (Thermo Scientific TM) adjusted to the final volume of 40 with molecular grade water. The restricted DNA is incubated for 3 h at 37°C. The restricted fragment is ligated with 1 µl of T4 ligase (Invitrogen), 1 µl of ligation buffer, 1 µl of EcoRI adaptor (Thermo Scientific TM), 1 µl of MseI/HpaII/MspI adaptor (Thermo Scientific TM) and 14 µl of molecular grade water. These restriction-ligation mixtures were incubated at 37°C for 2 h and then denatured at 70°C for 15 min. The pre-selective amplification reactions were prepared in a final volume of 20 µl containing 5 µl of diluted the restricted-ligated DNA in to 1:9 dilution factor; 0.25 µl of polymerase (Invitrogen); 2 µl PCR buffer-$MgCl_2$ (10 mM); 0.4 µl dNTPs (10 mM); 0.6 µl $MgCl_2$ (50 mM) and 7.5 µl of water. The pre-selective PCR reactions were then amplified in a DNA thermo cycler programmed under the following conditions: 72°C for 2 min (1 cycle); 94°C for 30 s., 56°C for 1 min, 72°C for 2 min (30 cycles) and 72°C for 30 min (1 cycle). The pre-selective amplification products were then visualized on a 1.5% agarose gel. The selective amplification reactions were prepared in a final volume of 10 µl containing 2.5 µl of template DNA from pre-selective PCR, 0.625 µl of PrimerEcoRI-FAM+3 (10mM), primer MspI+3/MpaII+3/MseI+3 selective primer set (10 µM, Thermo Scientific TM), 12.5 µl of Taq polymerase (5 U/µl) adjusted to final volume of 10 with water (nuclease-free). The selective PCR reactions were then amplified in a DNA thermo cycler programmed as follows: 1 cycle at 95°C for 15 min, 95°C for 30 s, 66°C for 30 s and 72°C for 2 min. The annealing temperature was then lowered by 0.7°C per cycle during the first 12 cycles, and then 23 cycles were performed at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s,

Table 1. Sequences of adapters and primers used for AFLP and MS-AFLP analysis.

MS-AFLP primer and adaptor sequence (5' to 3')		AFLP primer and adaptor sequence (5' to 3')	
Oligo name	**Sequence (5' to 3')**	**Oligo Name**	**Sequence (5' to 3')**
HpaII/MspI Adaptor	GATCATGAGTCC	MseI-AdaptorF	GACGATGAGTCCTG
HpaII/MspI RAdaptor	CGAGCAGGACTCATGA	MseI-AdaptorF	TACTCAGGAC
EcoRI-FAdaptor	CTCGTAGACTGCGTACC	EcoRI-FAdaptor	CTCGTAGACTGCGTACC
EcoRI-RAdaptor	AATTGGTACGCAGTC	EcoRI-RAdaptor	AATTGGTACGCAGTC
HpaII/MspI-T	ATCATGAGTCCTGCTCGGT	Mse-A	GACGAT GAG TCC TGA GTA AC
EcoRI-A	GACTGCGTACCAATTCA	EcoRI-A	GACTGCGTACCAATTCA
*Eco*RI+AAC +(6-FAM)	GACTGCGTACCAATTCAAC	*Eco*RI+AAC +(6-FAM)	GACTGCGTACCAATTCAAC
*Eco*RI+AAG + (6-FAM)	GACTGCGTACCAATTCAAG	*Eco*RI+AAG + (6-FAM)	GACTGCGTACCAATTCAAG
EcoRI+ACA+ (6-FAM)	GACTGCGTACCAATTCACA	EcoRI+ACA+ (6-FAM)	GACTGCGTACCAATTCACA
HpaII/MspI-TCAA	ATCATGAGTCCTGCTCGGTCAA	*Mse*I+CTC,	GACGAT GAG TCC TGA GTA ACA CTC
HpaII/MspI-TCTC	ATCATGAGTCCTGCTCGGTCTC	*Mse*I+CAG,	GACGAT GAG TCC TGA GTA ACA CAG
HpaII/MspI-TCTT	ATCATGAGTCCTGCTCGGTCTT	*Mse*I+CAC,	GACGAT GAG TCC TGA GTA ACA CAC
HpaII/MspI-TCTA	ATCATGAGTCCTGCTCGGTCTA	*Mse*I+CTG,	GACGAT GAG TCC TGA GTA ACA CTG
HpaII/MspI-TCTG	ATCATGAGTCCTGCTCGGTCTG	MseI+CTA	GACGAT GAG TCC TGA GTA ACA CTA

then at 4°C forever. The amplification selective PCR products were diluted to 1:9 dilution factors and denatured with formamide. A 500-bp Liz standard marker was used to estimate the molecular size of the fragments. The mixture is analyzed using the NMBU University GENE Center facility for fragment analysis on an ABI3130XL.

Data analysis

To score AFLP and MS-AFLP fragments, the gene-mapper software was used. We manually set bin widths using the graphic interface with the minimum relative fluorescence units for band identification at 50 bp. These parameters resulted from bands coded as "1" or "0" for present and absent, respectively. Throughout we use "locus" to indicate a specific fragment size in the AFLP and MS-AFLP results. "Haplotypes" was use to indicate the binary variable positions (dominant genotypes) for each individual's collection of AFLP loci, and "epigenotype" to indicate the collection of binary variable positions of MS-AFLP loci. Both AFLP and MS-AFLP data were analyzed using GenALex version 6.5 and POPGENE version 1.31 software. The assumption of population structure was tested for both genetic and epigenetic loci by calculating standard population genetics statistics within and among populations using GenALex 6.5. GenALex was also used to estimate genetic and epigenetic differentiation using hierarchical analysis of molecular variance (AMOVA) to determine if there was structure associated with the population. This analysis assessed structured genetic or epigenetic differences by comparing variation in marker profiles (AFLP or MS-AFLP) among populations (Φ_{RT}) and within populations (Φ_{PR}). 9,999 permutations was used to estimate statistical significance and an initial alpha of 0.05.

The dendrogram was constructed based on Neil's genetic distance using the unweighted pair group method with arithmetic average (UPGMA). The partitioning of total genetic diversity (H_T) into within (H_S) and among (D_{ST}) accession components was examined using Nei1's genetic diversity statistics ($H_T = H_S + D_{ST}$; $G_{ST} = D_{ST}/H_T$).

It was initially assumed that each population is clonally propagated, thus little diversity and no epigenetic variation is expected. Using the resulting data, Bayesian clustering was performed using Structure v.2.3.4 to identify how many different groups are represented among the individuals. Structure estimates the number of groups (k) present among individuals and assigns individuals to each k using Bayesian modeling. Five populations (k=1-5) were tested which is three populations more than the maximum anticipated based on the sampling, with five independent runs at each k. We used both the log probability of observing the data (ln Pr (x|k)) method of Structure and Delta k (Earl, 2012; Evanno et al., 2005; Falush et al., 2003) which determines the number of populations that best fit the data. Clustering was performed with the admixture model, 50,000 burn-in steps, 100,000 post-burn-in steps, and correlated allele frequencies were allowed. Individuals were assigned to groups based on the highest q-value.

RESULTS

Phonotypical characterization of natural sucker forming and anthropogenic induced sucker forming Ensete established at Hawassa University research field

Under the normal growth conditions, there is a notable phenotypic difference observed between ET (the natural sucker forming) and Anthropogenic induced sucker forming (Ado, Ganticha, Koba, Wild type and Abebe) Ensete (Figure 1). The growth pattern of the two types of Ensete was evaluated in the established garden. The morphology show a difference pattern of growth which shows the emerging of sucker on dead leaves of the matured ET type of Ensete. However, this process is completely absent in the common cultivated Ensete types (Ado, Ganticha, Koba, and wild). These indicated that natural sucker formation is absent in the common group of Ensete while maintained in the Entada accessions. Observation of Ensete population for more than ten years (data not presented) confirmed three types of multiplication in the sampled genotypes. The first multiplication method is exclusive vegetative. This is observed only by ET and Abebe accessions. The second multiplication system is anthropogenic induced sucker

formation which is observed among cultivated Ensete like Ado, Ganticha and Koba. The third multiplication system is exclusively sexual by forming seed as represented by Wild (W) type Ensete.

Polymorphism of AFLP and MS-AFLP data

The AFLP yielded a total of 3745 bands ranging from 41 to 400 bands per primer combinations (Table 2a). All primers produced polymorphic bands for both populations (Table 3). The effective number of allele ranged from 1.78 to 1.927. This value varies among ET and NE populations. The number of privet bands for NE and ET is 101 and 2051, respectively (Table 3). Unbiased haploid genetic diversity for each population also varied (0.198 and. 0.146 for ET and NE, respectively).

The MS-AFLP analysis also showed significant polymorphism by each of the nine primer combinations. The total number of bands generated by the MS-AFLP was 2846 (Table 2b). The number of bands per primer combination ranged from 14 to 319. Polymorphic bands ranged from 14 to 226. The effective number of alleles ranged from 1.585 to 2.0. The number of privets bands was 835 and 479 for ET and NE, respectively. Unbiased haploid genetic diversity for each population also varied with a value of 0.151 and. 0.229 for ET and NE, respectively. This showed an increased number of privet bands for NE compared to the AFLP analysis.

Population differentiation

Total genetic diversity per primer combination ranged from 0.145 to 0.208 for AFLP analysis. However, the genetic diversity within (H_S) the population ranged from 0.135 to 0.191 indicated higher diversity within the population than among the population (D_{st}) which ranged from 0.011 to 0.017. However, the differentiation index (G_{ST}) ranged from 0.045 to 0.104. This value indicates lower differentiation among the population. However, for MS-AFLP, total genetic diversity (H_T) ranged from 0.13 to 0.265, while the genetic diversity within (H_S) the population ranged from 0.117 to 0.217. The D_{st} value ranged from 0.013 to 0.071, indicating low diversity. However, the differentiation index (G_{ST}) ranged from 0.09 to 0.339. The relative higher differentiation index value by the MS-AFLP analysis indicated the moderate differentiation of the population.

Analysis of molecular variance revealed no genetic differentiation among the population in the AFLP analysis but significantly higher differentiation among the population by the MS-AFLP analysis ((Φ=0.25, P = 0.001). The result of this analysis indicated that 24% of molecular variance was present among the two Ensete types, whereas 76% of the molecular variation was within the samples in the population (Table 4). This data clearly demonstrated that the presence of cytosine Methylation increased the differentiation of the population.

Cluster analysis

To further determine the genetic relationships among the two populations, UPGMA clustering was carried out using Nei's unbiased genetic distance matrix. The dendrogram failed to revealed inter-population relationships by AFLP analysis (data is not presented). In the MS-AFLP analysis two groups (A and B) were separated by the MS-AFLP analysis. Eighteen individuals were clustered in one group (A) and nine individuals clustered into another group (B). Further, sub-group A-I and A-II are composed of 9 individual from which exclusively consisted of samples of sucker on dead plant (MB, MB2 and MB3), emerged bud (MS1, MS2 and MS3), and emerged young shoot (ML1, ML2 and ML) type ET and daughter plants (D12, D13, D17, D20, D21, D22, D37, D38 and D39) of ET mother plant. And sub-group B-I is composed of four individual of cultivated Ensete of NE type (Ado (A), Ganticha (G), Koba (K), and Wild (W)), whereas sub-group B-II is composed of the mother plants of ET (M1, M2, M 3 and HU). The exception is one individual (Abebe (AB)) which is grouped as the outlier in B-II. This implies that the MS-AFLP analysis distance is greater than the AFLP analysis confirming an important role for DNA Methylation in the Ensete epigenome since the differences between two types of Ensete are enhanced.

Structure analysis

The AFLP procedure did not cluster the population significantly (data not presented). For the MS-AFLP procedure, the structure of ET and NE was analyzed with no apriori information, using the Structure software. The result showed a clear peak (ΔK = 19.13) at the K value of 2 (Figure 2b to d) based on likelihood plots of the models, the stability of grouping patterns across different runs, and germplasm information. Group 1 had eighteen individuals while group 2 is composed of nine individuals. Group 1 is composed of the young plant from Entada (D1 to D9), a sucker from dead leaves (EB), emerging bud (MB), and emerged young shoot (ML). While group two are composed of all the anthropogenic sucker inducing plants (Ado, Ganticha, Koba), the mother plant of Entada (M1, M2, M3, HU and Abebe), and wild type which reproduced by seed formation. The result indicated that 78.8% from the ET group are clustered in the same group while 21.3% are grouped in the second group. However, 99.65 of the NE are grouped in the second group. The average distance between the individuals within the cluster was 0.12 and 0.22, for cluster 1 and cluster 2, respectively. The mean F_{st} value for each cluster is 0.56 and 0.11, for cluster 1 and cluster 2, respectively. The

Table 2. Comparison of genetic differentiation for natural sucker forming (ET) and anthropogenic induced sucker forming (NE) by (a) AFLP and (b) MS-AFLP markers.

Primer set		NOL	PL	%PL	A_e	H_T	H_S	D_{ST}	G_{st}
a. AFLP									
EcoRI+AAC	**MseI+CTC**	**41**	38	**92.7**	**1.927±0.26**	0.199±0.29	**0.190±0.28**	0.013	**0.045**
EcoRI+AAC	MseI+CAG	181	148	81.8	1.818±0.39	**0.145±0.02**	**0.135±02**	**0.011**	0.073
EcoRI+AAC	MseI+CAC	277	227	81.9	1.819±0.38	0.151±0.02	**0.138±0.01**	0.013	0.083
EcoRI+AAC	**MseI+CTG**	**400**	**370**	**92.5**	1.925±0.26	**0.208±0.02**	**0.191±0.02**	**0.017**	0.081
EcoRI+AAC	MseI+CTA	224	198	88.4	1.884±0.32	0.147±0.16	0.137±0.01	**0.011**	0.072
EcoRI+AAG	MseI+CTC	152	136	89.5	1.898±0.31	0.145±0.19	0.1295±0.01	0.015	**0.104**
EcoRI+AAG	MseI+CAG	238	179	**79.8**	1.798±0.40	0.163±0.02	0.148±0.02	0.014	0.084
EcoRI+AAG	MseI+CAC	293	269	91.9	1.918±0.27	0.189±0.02	0.173±0.02	0.016	0.083
EcoRI+AAG	MseI+CTG	291	255	87.6	1.876±0.33	0.183±0.02	0.167±0.02	0.016	0.087
EcoRI+AAG	MseI+CTA	247	208	84.2	1.842±0.37	0.162±0.02	0.147±0.02	0.016	0.096
EcoRI+ACA	MseI+CTC	293	270	**92.2**	1.921±0.27	0.190±0.02	0.174±0.02	0.016	0.083
EcoRI+ACA	MseI+CAG	293	271	**92.5**	1.925±0.26	0.173±0.02	0.159±0.02	0.015	0.081
EcoRI+ACA	**MseI+CAC**	**42**	**33**	78.6	1.786±0.42	0.170±0.34	0.154±0.03	0.021	0.094
EcoRI+ACA	MseI+CTG	152	135	88.8	1.888±0.32	0.145±0.02	0.130±0.01	0.015	**0.102**
EcoRI+ACA	MseI+CTA	293	269	91.8	1.918±0.27	0.189±0.02	0.173±0.02	0.016	0.083
b. MS-AFLP									
EcoRI+AAC	HpaII/MspI-TCAA	231	187	81.9	1.809±0.39	**0.265±0.35**	0.175±0.02	**0.071**	**0.339**
EcoRI+AAC	HpaII/MspI-TCTC	59	53	89.8	1.898±0.30	**0.258±0.03**	**0.217±0.02**	0.042	0.162
EcoRI+AAC	HpaII/MspI-TCTT	171	100	**58.5**	**1.585±0.49**	0.140±0.23	**0.117±0.01**	0.023	0.164
EcoRI+AAC	HpaII/MspI-TCTA	**319**	**226**	70.8	1.708±0.45	0.193±0.03	0.161±0.02	0.029	0.166
EcoRI+AAC	HpaII/MspI-TCTG	170	118	69.4	1.694±0.46	0.158±0.02	0.14±0.02	0.017	0.113
EcoRI+AAG	HpaII/MspI-TCAA	22	**14**	63.4	1.636±0.49	**0.13±0.02**	0.118±0.02	**0.013**	**0.09**
EcoRI+AAG	HpaII/MspI-TCTC	235	196	83.4	1.834±0.37	0.184±0.02	0.156±0.02	0.025	0.149
EcoRI+AAG	HpaII/MspI-TCTT	**14**	14	**100**	**2.00±00.0**	0.189±0.02	0.171±0.02	0.026	0.094
EcoRI+AAG	HpaII/MspI-TCTA	202	180	89.1	1.891±0.31	0.211±0.02	0.186±0.02	0.023	0.118
EcoRI+AAG	HpaII/MspI-TCTG	231	172	74.5	1.745±0.44	0.257±0.04	0.169±0.02	0.071	**0.343**

NOL, Total number of loci per primer combination; PL, Number of polymorphic loci; PLP, percentage of polymorphic loci; Ae, Effective number of alleles per locus; H_T, Total gene diversity; H_S, Average gene diversity within accessions; D_{ST}, The genetic diversity among accessions; G_{ST}; $G_{ST} = H_T - H_S/H_T$, Genetic differentiation coefficient.

mean α value is 0.05, 0.04, 0.03 and 0.02 for K = 2, 3, 4 and 5, respectively, showing significant difference between each cluster.

Principal coordinate analysis

The genetic relationship among the studied populations was also visualized by performing PCoA based both on the AFLP and MS-AFLP data (Figure 3). For AFLP only, the first two components accounted for 14% (7.5 and 6.5%) of variation observed in the populations. The two estimated population intersected and shared a large part of the ellipse area. However, in MS-AFLP analysis only the first two components accounted for 34.3% (19.7 and 12.6%) of variation observed in the populations. Where the major and minor axes, which show the dispersion degree of the population, indicates the dispersion is higher even though some level of overlap among the populations. A similar pattern of clustering is revealed by the PCoa analysis and UPGMA dendrogram (Figure 2a and Figure 3).

DISCUSSION

This study offers an analysis of the genetic and epigenetic diversity and population structure of *Ensete ventricosum* in Ethiopia. Both MS-AFLP and AFLP have been proven to be valuable for the determination of genetic and epigenetic diversity among the collected genotypes in Hawassa University research field.

MS-AFLP and AFLP profiles were generated from Ensete genomic DNA extracted from plant tissue of Ado, Ganticha, Koba, Abebe, Wild type and Entada which represent the mother plants, a sucker from the dead

Table 3. Genetic diversity of 27 Ensete accessions representing natural sucker forming (ET) and anthropogenic induced sucker forming (NE).

Population	Techniques of analysis			
	AFLP		MS-AFLP	
	ET	NE	ET	NE
No. of bands	2937	987	1701	1345
No. of bands frequency ≥5%	2103	987	1182	1345
No. private bands	2051	101	835	479
No. of locally common bands (≤25%)	0	0	0	0
No. of locally common bands (≤50%)	0	0	0	0
Diversity(h)±SE	0.189±0.00	0.110±0.0	0.145±0.0	0.172±0.0
Unbiased diversity (uh)±SE	0.198±0.0	0.146±0.0	0.151±0.0	0.229±0.0
No. of different alleles (Na)	1.705	0.563	1.2	0.9
No. of effective alleles (Ne)	1.277	1.185	1.23	1.29
Shannon's information Index (I)	0.313	0.162	0.238	0.252
Polymorphic [%]	85.2	27.6	61.3	42.8
Nei genetic identity [%]	97.00		92.00	
Nei genetic distance	0.03		0.08	

ET vs NE, Population names; Np, number of polymorphic loci; PLP, percentage of polymorphic loci; Na, observed number of alleles per locus; Ne, effective number of alleles per locus; Hj, Nei's gene diversity index; I, Shannon information index.

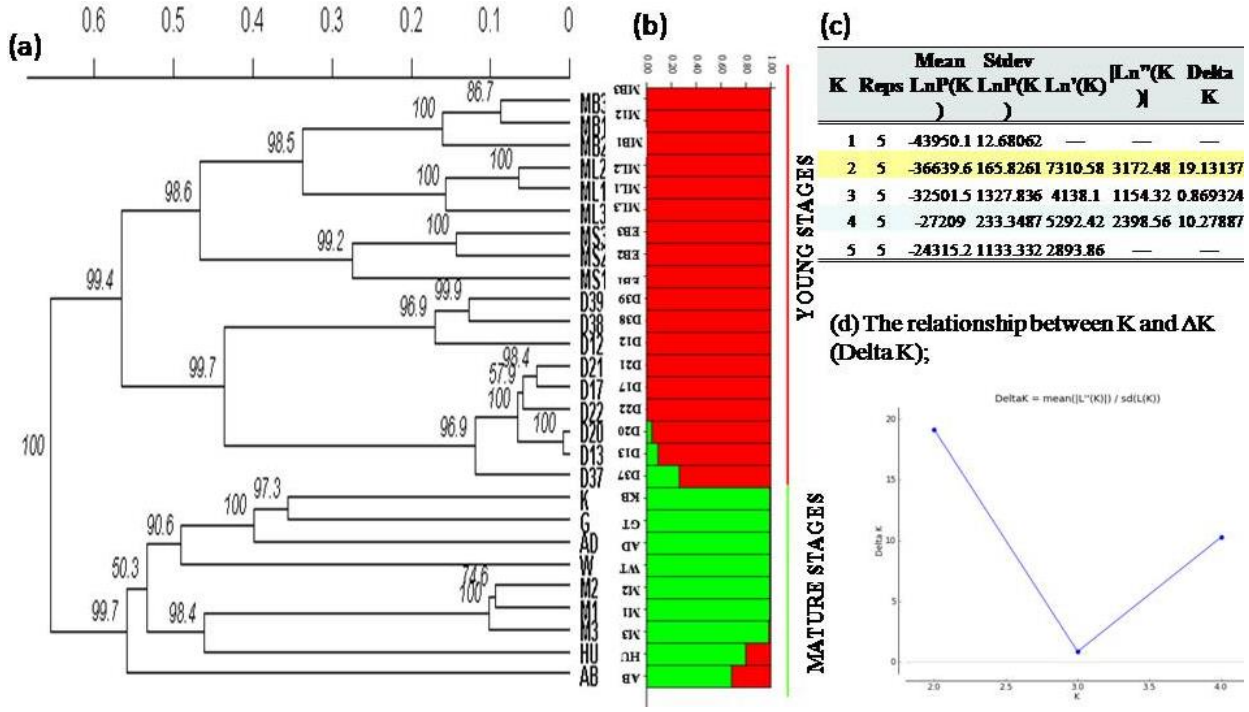
Table 4. Analysis of molecular variance (AMOVA) among and within populations of natural sucker forming (ET) and anthropogenic induced sucker forming (NE) of *Ensete* using AFLP and MS-AFLP markers.

Analysis	Source	Degree of freedom	Sum of square	Mean sum of square	Estimated variation	% variation	Φ	*P*(rand ≥ data)
AFLP	Among pops	1	19.746	19.746	0	0	-0.008	0.56
	Within pops	25	522.402	20.896	20.896	100		
	Total	26	542.148	40.642	20.896	100		
MS-AFLP	Among pops	1	733.1	733.1	74.05	24	0.25	0.001
	Within pops	25	5713	228.5	228.5	74		
	Total	26	6446	247.9	302.6	100		

leaves, emerging bud, emerged shoot and separated seedlings from the same Entada plant (Figure 1). The quality of AFLP and MS-AFLP was good; as a result, comparing the DNA profiles of the different tissues and plants was possible for comparing the genetic and epigenetic patterns in the selected plants.

Restriction of genomic DNA with *Eco*RI/*Hpa*II/MspI creates three groups of fragments (Vos et al., 1995; Vuylsteke et al., 2007). However, the target was to measure the fragments which are preferentially amplified by EcorI-/MspI/HpaII selective primers. This lowered the number of amplified fragments in MS-AFLP analysis than the AFLP analysis.

As a result, the following four classes of fragments originated: Un-methylated, hemi-methylated internally, hemi-methylated externally and fully methylated (Schulz et al., 2013). Conservation of the MseI site indicates the genetic similarity of the germplasm while variation indicates the genetic diversity which can be quantified using G_{ST}, Amova, Nei genetic diversity and structure software (Pérez-Figueroa, 2013). The fifteen primer combinations resulted in more than 3000 loci with higher polymorphic bands. However, the AFLP polymorphic loci were not able to differentiate the two populations into natural sucker forming (ET) and anthropogenic induced sucker forming (NE) groups. There are many polymorphic bands as a result of EcorI/MseI restriction analysis. A similar result has been reported by different author using different population AFLP (Negash et al., 2002). Even if the research was done using a different collection of Ensete from different agro ecology, there is moderate genetic diversity within the population than among the population of Ensete. Depending on their population determination little or no population differentiation has been exhibited. This indicates the clones are the result of cross-pollination.



K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	5	-43950.1	12.68062	—	—	—
2	5	-36639.6	165.8261	7310.58	3172.48	19.13137
3	5	-32501.5	1327.836	4138.1	1154.32	0.869324
4	5	-27209	233.3487	5292.42	2398.56	10.27887
5	5	-24315.2	1133.332	2893.86	—	—

Figure 2. Cluster analysis of (a) UPGMA dendrogram based on MS-AFLP profiles obtained from Sardinian Ensete ample. The Jaccard similarity index is indicated on the X axis. The sample names with their previously assigned (or not) genotype are reported on the Y axis. (b). Bar plot of estimated membership probability (Q) for K = 2 for the MS-AFLP data of Ensete sample names are indicated on the X axis. The estimated membership probability (Q) for K = 2 are represented on the Y axis. (c) Mean estimated log-normal (Ln) probability of the data in relation to the simulated number of clusters K. (d) Delta K in relation number of clusters K.

Yet, with nine pairs of primer combinations, the MS-AFLP analysis revealed more polymorphic bands in the population and significant differentiation of the two populations. This might be the result of fragments which are methylated with their internal cytosine and externals cytosine or fully Methylation as the result of the two schizomeric restriction enzymes (HpaII /MspI). This indicates that there is some level of epigenetic variation exhibited by the population. Furthermore, the small percentage of polymorphism (0.145 and 0.229 for ET and NE, respectively) found in the MS-AFLP analyses shed important information about the Methylation pattern and the presence of independent allele as a result of Methylation in the two populations (Michalakis and Excoffier, 1996). This indicates the presence of a different pattern of Methylation on the natural and anthropogenic induced sucker forming populations. Such a high level of diversity indicates the presence of high epigenetic diversity.

Changes in Methylation patterns occur by de novo Methylation which is catalyzed by Methyl transferase enzyme (Lyko, 2017). In this result, a different pattern of Methylation where the two populations exhibited differently was observed. The pattern of DNA Methylation is exhibited by the different number of unique fragments in the MS-AFLP analysis. For example, an increased number of privet bands (835) were exhibited in the ET population while less number (479) was found in the NE populations. This indicates that de-methylation of the NE population which might have happened due to the failure in maintaining the Methylation pattern through DNA replication. This might have created a different number of unique bands by MS-AFLP. The absence or disappearance of bands in the NE and ET population might be attributed to the status of the Methylation sites which need to be elucidated further.

The presence of more effectively amplified fragments by the AFLP showed a higher frequency in the ET populations. The high number of the fragment in the ET population might reveal the de-methylation of the genome sequence in the population while Methylation in the NE populations made it inaccessible for the digestion by EcoRI/MseI enzyme combinations. De-Methylation further is supported by the increased fragments number in the ET population than the NE population by the HpaII/MspI enzyme combinations that indicate the effect of the de-methylation events occurring during the different developmental stages of ET ensete.

The overall explanation of the data reflects the presence of Methylation and De-methylation in the NE and ET population which occur in Ensete population. This explanation can be supported by the fact that cytosine

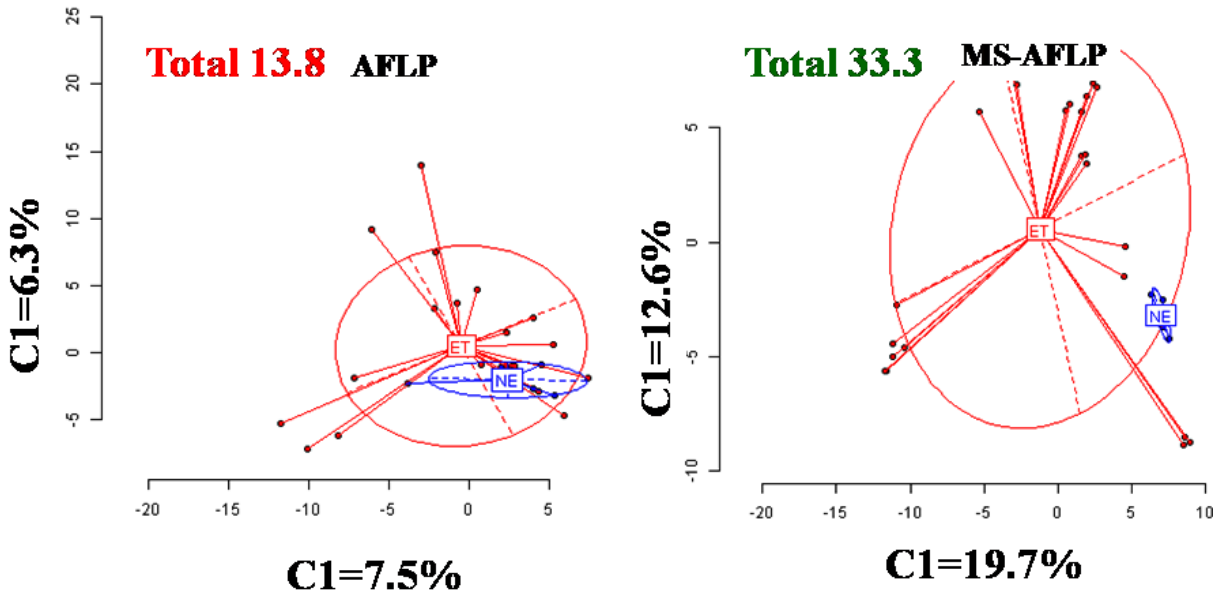


Figure 3. Representation of principal coordinate analysis (PCoA) for epigenetic differentiation among groups. Different colors represent different populations, called ET and NE. The Et and NE symbols indicate the position of the genetic centroids. Ellipses represent the average dispersion of those individual data points around their centre. The long axis of the ellipse shows the direction of maximum dispersion and the short axis, the direction of minimum dispersion.

Methylation is a well-known phenomenon in plants as it is observed by different plant species (Broeck et al., 2018; Dowen et al., 2012; Fresnedo-Ramírez et al., 2017). Methylation affects the gene expression which in turn affects plant adaptation and productivity (Kashino-Fujii et al., 2018). Thus, de-methylation might be one aspect of differentiation in the Methylation pattern of plants at a different stage of their development. This further characterizes that the genes or the Methylation pattern of Ensete are important to unravel the developmental pattern of Ensete.

The explanation behind the variability of the two forms of Ensete (ET vs NE) based on MS-AFLP results is an indication of the selection strategy of the germplasm for vegetative reproduction. The ET form is not able to form a flower but propagate by the sucker. Thus, the absence of recombination can reduce to generate variation in the population. Thus, ET relies on epigenetic which will be the only way for the plant to exist. This could explain why the different developmental stages are the target for epigenetic variability and creating population structure.

The key evidence for the above explanation is the DNA Methylation fingerprint that was distinctly different (Φ *ST* = 0.25,*p* = 0.001) among the two forms of Ensete. This uncovers a disjunction between the global DNA Methylation pattern and the developmental state of Ensete as revealed by the structure analysis. Therefore, the difference that was observed between the Methylation fingerprints of developmental stages and genotypes could reflect the developmental stage-related phenotypic plasticity.

To date, regulation of gene expression through DNA Methylation has been described for many clonally propagated plants like grape (Fournier-Level et al., 2011), almond (Fresnedo-Ramírez et al., 2017), but not for Ensete. The Methylation patterns described in those earlier studies were generally associated with ecological contexts, developmental stages, and genotypes. In this results, there is no geographical scope where all the population is established in the same environment for more than three years. But epigenetic analysis differed between the different populations where little genetic diversity is exhibited. There are few reports with big genetic variation and the epigenetic difference in plants. The current research supports the idea of change in Methylation involved in the developmental stage of the plant which includes a natural sucker forming Ensete. This concept could open new perspectives for a better understanding of Ensete developmental biology and mechanism of sucker formation. The epigenome analysis of branching and non-branching Ensete population along with different ecology is needed. Discovering the key genes involved in the change in the branching and non-branching patter mechanism, as well as their epigenetic variation, is necessary to clarify the pattern of epigenetic variation evidenced in this study.

Conclusion

Results indicate that the genetic diversity of *Ensete ventricosum* is high based on MS-AFLP analysis. Less

genetic differentiation occurred among the AFLP markers which are based on the genetic difference. This study highlights the presence of Epigenetic variation among the population which should be further elucidated. The information generated from this study will facilitate future works on the epigenetic relationship and different morphological and developmental phenomena in *E. ventricosum* which is one of the important crop plants in Ethiopia.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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NORHED project “Controlling diseases in sweet potato and Enset in South Sudan and Ethiopia”.

ABBREVIATIONS

NE, Anthropogenic sucker inducing; **ET,** Natural sucker inducing; **EB,** sucker on the dead leaves; **MB,** Emerging bud; **ML,** emerged young shoot; **D,** seedling plant 1 to 9; **M,** Entada mother; **K,** Koba; **G,** Ganticha; **A,** Addo; **AB,** Abebe; **W,** Wild.

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Development and validation of an analytical method for quantification of total flavonoids in *Alternanthera brasiliana* by ultraviolet-visible absorption spectrophotometry

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***Alternanthera brasiliana* is popularly known in Brazil as "penicillin" or "benzetacil" and is used in traditional medicine for the treatment of infections and for the healing of wounds. It is also used as an ornamental plant due to the characteristic purple coloration of its leaves when cultivated in shade. The objective of this study was to develop and validate an analytical methodology by Ultraviolet-Visible absorption spectroscopy to quantify total flavonoids in crude ethanolic extract of *A. brasiliana*. The parameters analyzed in validation were those indicated in resolution 166/2017 of ANVISA, as selectivity/specificity, linearity, accuracy, precision, robustness, limits of detection and quantification, and also by ICH Q2(R1) for analytical validation. The method developed was simple, fast, low cost, linear, selective, precise, accuracy and robust. All parameters analyzed were within the limits recommended by the Brazilian legislation. Thus, this methodology can be useful for quality control of the extract and vegetal derivatives of *A. brasiliana*.**

Keywords: Analytical validation, Amaranthaceae, UV-Vis, natural products, quality control.

INTRODUCTION

The Amaranthaceae family has about 170 genera and 2,000 species, occurring in Brazil 27 genera (6 endemics) and 157 species (74 endemics) (Marchioretto et al., 2010). About 20 genera and 94 species occur in the

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Brazilian Northeast, and 13 genera and 29 species in Pernambuco State (A Flora do Brasil, 2020). Several species of this family present in their composition biologically active compounds such as betalains, ecdysteroids, flavonoids, saponins and triterpenes (Ferreira and Dias, 2000).

Plants of *Alternanthera* genus are known to have antimicrobial and antiviral properties. In some species the inhibition of lymphocyte activity, hepatoprotective, analgesic, antifungal and antidiarrheal activity were reported. However, although the number of species of this genus is significant, the number of studies aiming to determine the chemical composition is still scarce (Delaporte et al., 2002; Ferreira et al., 2003).

Alternanthera brasiliana is used in folk medicine to treat infections and is popularly known as "terramycin", "penicillin" or "benzetacil" (Facundo et al., 2012; Caetano et al., 2002). In relation to its phytochemistry, several constituents have already been isolated or identified. Glycosylated flavonoids (Brochado et al., 2003), oxylipines (Trapp et al., 2015), alkaloids and triterpenoids (Anunciação, 2012) were isolated from the leaves. From the leaves and stalks, flavones, flavonols, steroids, betalains, betacyanins, betaxanthines, hodroxybenzoic acid derivatives and hydroxycinnamic acids has already been reported (Deladino et al., 2017).

Some studies conducted with *A. brasiliana* sought to validate their ethnopharmacological uses, such as the antimicrobial activity, proven for chloroform, ethyl acetate and methanolic fractions of their aerial parts (Silva et al., 2011). The analgesic effect of its crude ethanolic extract has also been reported, being its response more effective than the drugs used as standards (acetylsalicylic acid, dipyrone and indomethacin) (Souza et al., 1998). The anti-inflammatory effect was also studied and was mainly attributed to the glycosylated flavonoids present in its composition (Brochado et al., 2003). The wound healing effect of *A. brasilaina* has also been reported in both immunocompromised rats and aged rats, showing retraction of the wound halos larger than the standard drugs used (Enechi et al., 2013; Barua et al., 2009, 2012a, b).

Flavonoids are the main biologically active compounds present in *A. brasiliana*, so they were selected in this study to develop and validate analytical methodology for their quantification in samples of this species (Deladino et al., 2017). The importance of validating procedures for analytical safety and obtaining reliable results is known. Thus, for the development of an analytical method, adaptation or implementation of a known method involves an evaluation process that estimates its efficiency in the routine of the laboratory and this process is the validation (Brito et al., 2003). For validation of analytical methodologies some parameters must be analyzed such as, selectivity/specificity, linearity, robustness, precision, accuracy, limit of detection and limit of quantification and all of this is of fundamental importance in product quality control, being the validation part of the good manufacturing practices and control (Brasil, 2017). Therefore, the objective of this work was to perform an adaptation and validation of an analytical methodology for the quantification of total flavonoids in *A. brasiliana* by ultraviolet-visible (UV-Vis) absorption spectroscopy.

MATERIALS AND METHODS

Chemicals, glassware, solvents and equipment

All solvents used were analytical grade: sodium nitrite (Sigma-Aldrich®), sodium hydroxide (Alphatec®), aluminum chloride (Vetec®), methanol (MeOH, Synth®, AppliChem®). Phox® glassware was used. As standard for flavonoids, hydrated rutin (Sigma-Aldrich®), purity ≥ 94% was used. The equipment used was EVEN® analytical balance (FA-2204B model, Brazil); Cristófoli® ultrasonic bath; Ethik Technology® stove with air circulation (420-6TD model, Brazil); Solab® knife mill (SL-31 model, Brazil); EVEN® UV-Vis spectrophotometer (IL-592 model, Brazil); Nova Instruments® UV-Vis Spectrophotometer (NI-1600 model, Brazil).

Plant material and extraction process

The aerial parts of *A. brasiliana* (L.) KUNTZE growling wild were collected from Campus of Agricultural Sciences of Federal University of San Francisco Valley (UNIVASF). A botanist from Centro de Recuperação de Áreas Degradadas da Caatinga (CRAD) identified the sample by comparison with a voucher (number 19072) already deposited in Herbarium Vale do São Francisco (HVASF). The harvested material was oven dried with air circulation at 40°C (62.80% of humidity) and then pulverized in a knife mill (20 mesh) (SOLAB, SL31 model, Brazil). The dried and pulverized material was macerated with 95% ethanol in a stainless steel vessel. Three extractions were performed, replacing the solvent every 72 h. Then, the extractive solution was concentrated in a rotary evaporator under reduced pressure at 50°C, obtaining the *A. brasiliana* crude ethanolic extract (Ab-CEE) (13.14% yield) (Silva et al., 2014).

Experimental procedures and adaptation of the analytical method

The development of analytical method for quantification of total flavonoids by UV-Vis was made using the method proposed by Silva et al., (2014) for *A. brasiliana*, with the necessary adaptations. Initially an extract stock solution of 5 mg/ml was prepared using 10% methanol in distilled water. A dilution was performed to obtain a concentration of 1 mg/ml by adjusting the final volume with distilled water. The analyzes were performed in UV-Vis spectrophotometer (EVEN®, model IL-592), using quartz cuvettes with 1 cm of optical path.

The methodology used by Silva et al., (2014) as the starting point was as follows. The volumes of 1.5 ml of distilled water, 300 µl of the 1 mg/ml extract solution and 90 µl of 5% (w/v) sodium nitrite solution ($NaNO_2$) were initially added and waited 6 min. Then 180 µl of 10% (w/v) aluminum chloride ($AlCl_3$) was added and waited 5 min. Finally, 600 µl of 1 M sodium hydroxide (NaOH) was added and the final volume was adjusted to 3 ml with 330 µl of distilled water. The spectrophotometric reading was performed at 520 nm.

A UV-Vis scan of 300 to 600 nm was performed for the procedures with and without the complexing agent, in order to verify the bathochromic effect provided by the complexing agent, which

was the function of $AlCl_3$, and also with in order to verify the wavelength that the maximum absorption of the sample occurs. Thus, the procedure was performed as previously described for the procedure with complexation (PWC) and for the procedure without complexation (PNC), instead of adding 180 µl of $AlCl_3$, the same volume of distilled water was added. After the maximum absorption wavelength of the sample was verified, the methodology was developed and adapted to the chosen wavelength (370 nm).

Then, it was decided to check if there was a need to wait the methodology times (6 min after adding $NaNO_2$ and 5 min after adding $AlCl_3$), because in literature consulted it was found that the complexation with $AlCl_3$ can occur immediately (Sampaio et al., 2018a). In this way, the analyzes were performed with the times determined by the initial methodology and without the predetermined times, in which case a reagent added immediately after the previous one, preceded only by homogenization of the contents, and therefore, two types of procedure: the immediate procedure and the procedure with the times predetermined by the methodology. In addition, for each of these procedures, the influence of the reading time of the analysis at 0, 10, 20 and 30 min times was verified. It was also verified whether the volume of complexing agent could be relevant for the quantification of total flavonoids in the methodology. Thus, at the time of adding the complexing agent, different volumes of $AlCl_3$ were added (140, 160, 180 and 200 µl).

Validation of the analytical method

To validate the analytical method, the standards established by the Brazilian National Health Surveillance Agency (ANVISA) were used, in accordance with Resolution N° 166/2017, which defines what should be considered during the validation of analytical methods and non-chromatographic methods, such as UV-Vis spectrophotometry. The ICH Harmonised Tripartite Guideline for Validation of Analytical Procedures Q2(R1) was also consulted for validation. The following parameters were evaluated: selectivity, linearity (working range), precision (repeatability, intermediate precision and reproducibility), limit of detection (LOD), limit of quantification (LOQ), accuracy and robustness. All analyzes were performed in triplicate and the reliability of the parameters was verified by the relative standard deviation (RSD%) (Fernandes et al., 2015; Hollands et al., 2017). Only the procedure with complexation was validated considering that the procedure without complexation does not satisfactorily quantify the total flavonoids in *A. brasiliana* extract.

Selectivity

The selectivity of the method was demonstrated by the overlapping of the standard spectra used (the flavonoid rutin) and the Ab-CEE sample of the PWC, with the extract solution (1 mg/ml) and the rutin (0.25 mg/ml), obtained by the scanning curve in the range of 300 to 600 nm.

Linearity

The linearity (working range) of the extract sample was evaluated from the mean of the analyzes of three curves with five concentration levels (0.5, 0.75, 1.0, 1.5 and 2.0 mg/ml) for the PWC. The calibration curves were obtained from the mean absorbance as a function of concentration. For quantification of flavonoids in rutin equivalents, three calibration curves were obtained for rutin analytical standard in five concentration levels (0.05, 0.1, 0.25, 0.5 and 0.75 mg/ml) for the PWC. The results were statistically treated by linear regression, to determine the straight line equation and the coefficient of determination (r), with the minimum acceptable value being > 0.990 for the analytical standard rutin, and > 0.980 for the extract solution.

Precision

Precision was evaluated in terms of repeatability (intra-run precision) and intermediate precision (inter-run precision). Intra-run precision was obtained from three stock solutions at the concentration of 1 mg/ml, the analyzes being carried out in six-fold, by one analyst on the same day, giving a total of 18 determinations. The inter-run precision was performed in the same way, in six-fold of each of the three stock solutions, and the analyzes were done by two analysts on two different days, totaling 18 analyzes for each analyst. Also, the reproducibility test of the method was carried out in another laboratory, in this case, varying the entire physical infrastructure.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated (mg/ml) from the respective calibration curves for the PWC, according to their formulas, where SDa is the standard deviation of the intercept with the y axis, obtained from the average of the three linearity curves, and S is the slope of the line of the respective calibration curves. The LOD and LOQ can be calculated by Equations 1 and 2:

$$LOD = SDa \times 3/S \quad (1)$$

$$LOQ = SDa \times 10/S \quad (2)$$

Accuracy

Accuracy was assessed by the rate of recovery, from the addition of a known amount of rutin, a flavonoid present in Ab-CEE. In the analyzes for PWC, the same procedures were performed as previously described, but now adding at the same time with the sample 100 µl of analytical standard rutin (200 µg/ml). The result of the recovery was obtained by Equation 3:

$$R\ (\%) = TFC - CFE / CFP \times 100 \quad (3)$$

Where R is the percent recovery, TFC corresponds to total flavonoid concentration (rutin) added to Ab-CEE solution, CFE corresponds to concentration of rutin in Ab-CEE and CFP corresponds to rutin concentration.

Robustness

This parameter was performed by performing small changes in the wavelength of the analyzes (370 by 380 nm), as well as by modifying the label of the solvent (methanol) that was used to prepare the stock solution (exchange of the Synth® laboratory by AppliChem®).

Statistical analysis

All analyzes were performed in triplicate and the reliability of the parameters was verified by the relative standard deviation (RSD%). The results were analyzed statistically by analysis of variance (ANOVA), One-Way or Two-Way, when applicable, being considered statistically significant F calculated less than tabulated F ($p > 0.05$). The statistical treatment was obtained by the software

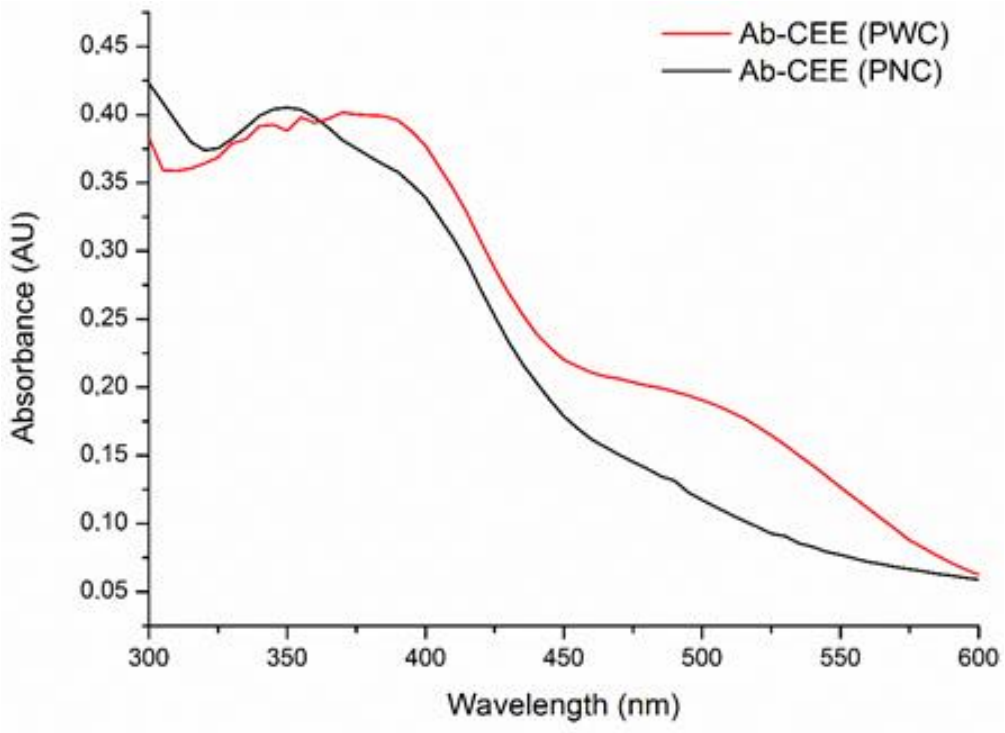

Figure 1. Superposition of scanning graphs of the procedure with complexation (PWC) and without complexation (PNC).

OriginPro® 8 (OriginLab).

RESULTS AND DISCUSSION

For the development and adaptation of the method proposed by Silva et al., (2014) was initially scanned from 300 to 600 nm for procedures with and without the complexing agent, in order to verify the bathochromic effect provided by $AlCl_3$, and to verify the wavelength where the maximum absorption of the sample occurs. Figure 1 shows the curves superposition of the solutions with complex agent and without complexation (1 mg/ml). Still in Figure 1, one can perceive the maximum absorption wavelength at 370 nm for the PWC sample.

Next it was verified whether there was a need to wait the predetermined times for the methodology between the addition of each reagent. From the analysis and application of specific statistical test (unpaired Student t test), the results obtained (in absorbance) were 0.421 ± 0.008 for the procedure with the predetermined times and 0.399 ± 0.009 for the procedure performed immediately. So that, it was possible to conclude that, for the study sample, it is necessary to wait the predetermined times, since the time of complexation interferes in the quantification.

The influence of the reading time of the analysis was verified, being this realized in times 0, 10, 20 and 30 min. The results obtained (in absorbance) were 0.421 ± 0.008 in time 0 min (T0) and 0.417 ± 0.006 in time 30 min (T30) for the procedure with the predetermined times. For the procedure performed immediately, the results were 0.399 ± 0.009 in T0 and 0.391 ± 0.009 in T30. The results showed that the analysis can be performed in both time 0 and 30 min times, since there is no statistically significant difference in T0 and T30 (unpaired Student t test). In this way, time 0 was selected to perform all the analyzes. Comparing the results in T0 for the procedure with and without the predetermined times, there is significant difference between the analysis, showing that it is necessary to wait for the times between the addition of the reagents, otherwise it will interfere with the quantification.

The last step in the adaptation of the methodology was to verify the $AlCl_3$ volume in the quantification, so different volumes of complexing agent (140, 160, 180 and 200 µl) were tested. The volumes of 140, 160 and 180 µl had no significant statistical difference. The volume of 200 µl made it impossible to read the analysis because it provided a cloudy solution, and its reading was inadequate. In this way, the volume of 140 µl was selected, being the smallest one used in the development in order to use the minimum of reagents. The changes that occurred from the predetermined methodology for the methodology adapted in this work was the reading wavelength of the analysis, which went from 520 to 370

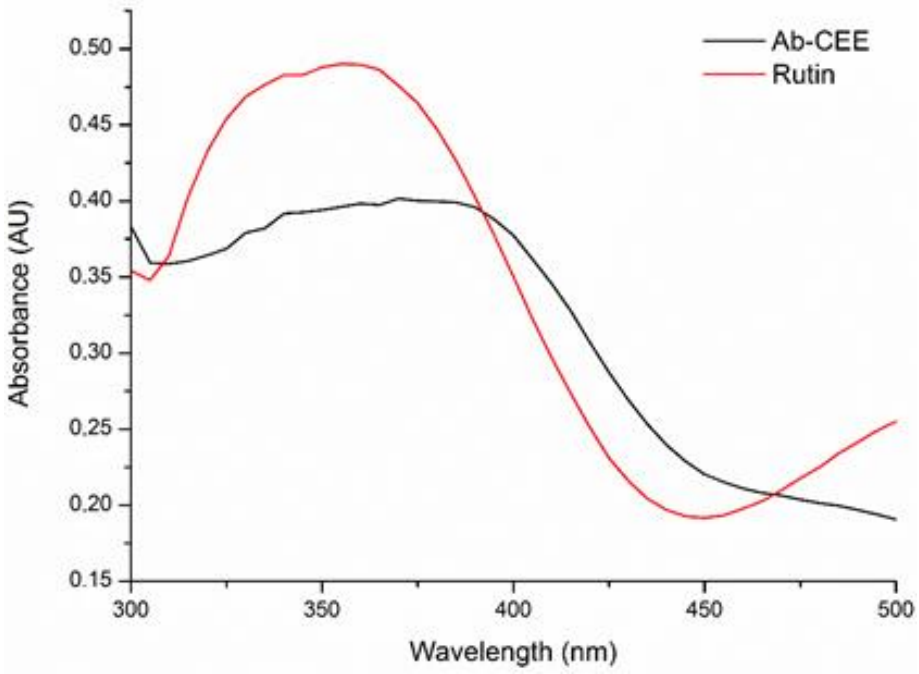

Figure 2. Selectivity of the method, constructed with the analytical standard rutin and Ab-CEE.

nm, and the volume of $AlCl_3$ added as a complexing agent dropped from 180 to 140 µl.

Validation of the analytical method

The selectivity was analyzed from the evaluation of different scanning spectra. The evaluation demonstrated the efficiency of the method for this assay, showing that other components do not interfere in the reading of the solutions, since they do not absorb in the region of the wavelength (370 nm) used for the quantitative analysis of the extract. It is possible to observe that there is similarity between the spectra of the analytical standard rutin and the extract, as can be observed in Figure 2.

Linearity corresponds to the ability of the method to provide a response directly proportional to concentration of analyte of interest present in the sample. The correlation coefficient (R^2) allows an estimate the quality of the obtained curve, so the closer to 1.0, the less the dispersion of the set of experimental points and the less the uncertainty of the estimated regression coefficients (Sampaio et al., 2018b). To verify the linearity, it was observed the linear equation and the correlation coefficient, which can be verified in Figure 3 (linearity of the Ab-CEE) and Figure 4 (linearity of the analytical standard rutin). The result of the regression obtained for R^2 was 0.99, proving that more than 99% of the method showed satisfactory linearity between the increase of the analyte concentration and the spectrophotometric response, in the concentration range chosen and the analysis of the mean residuals showed homoscedasticity. The working range of the method for Ab-CEE was determined to be 0.5 to 2 mg/ml. After calculating the total flavonoids, it was obtained as a result 0.3387 ± 0.0054 µg of rutin equivalents per mg of extract, which is equivalent to approximately 33.87% of total flavonoids in *A. brasiliana* crude ethanolic extract.

The LOD and LOQ were calculated from the equation line. LOD and LOQ values were 18.04 and 60.15 µg/ml, respectively. This shows that the method provides spectrophotometric responses with high sensitivity to detect and quantify flavonoids in extracts of *A. brasiliana* in very low concentrations (Hollands et al., 2017).

Precision represents the dispersion of results between independent and repeated assays of the same sample, similar samples or standards, under defined conditions (Padilha et al., 2017). For the validation, the precision was considered in three distinct levels: repetitiveness (intra-run), intermediate precision (inter-run) and reproducibility (inter-laboratory). For precision assays, the results of repeatability and intermediate precision (Table 1) showed RSD values below 5%, which is the maximum value recommended. In the repeatability, the value of the coefficient of variation (CV) was 0.008%. For the intermediate precision, the calculated F was lower than the table F ($p > 0.05$), that is, no significant statistical difference was observed when the same analyst evaluated the method on different days, and when different analysts evaluated on different days. In the

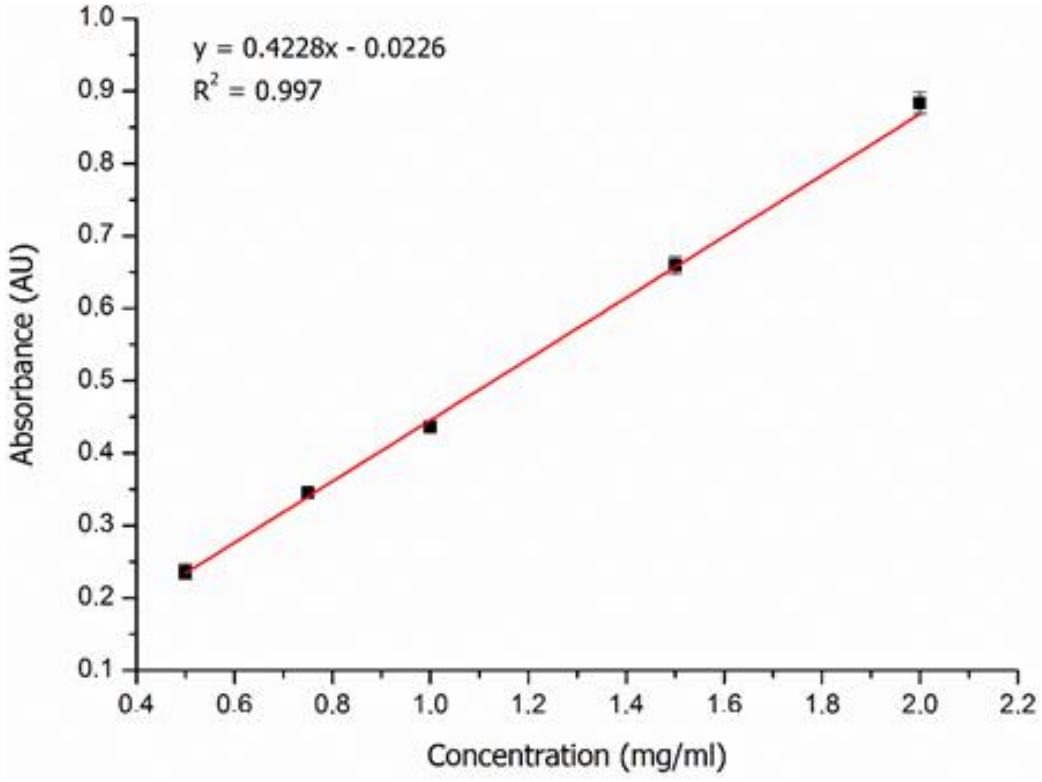

Figure 3. Linearity of the Ab-CEE.

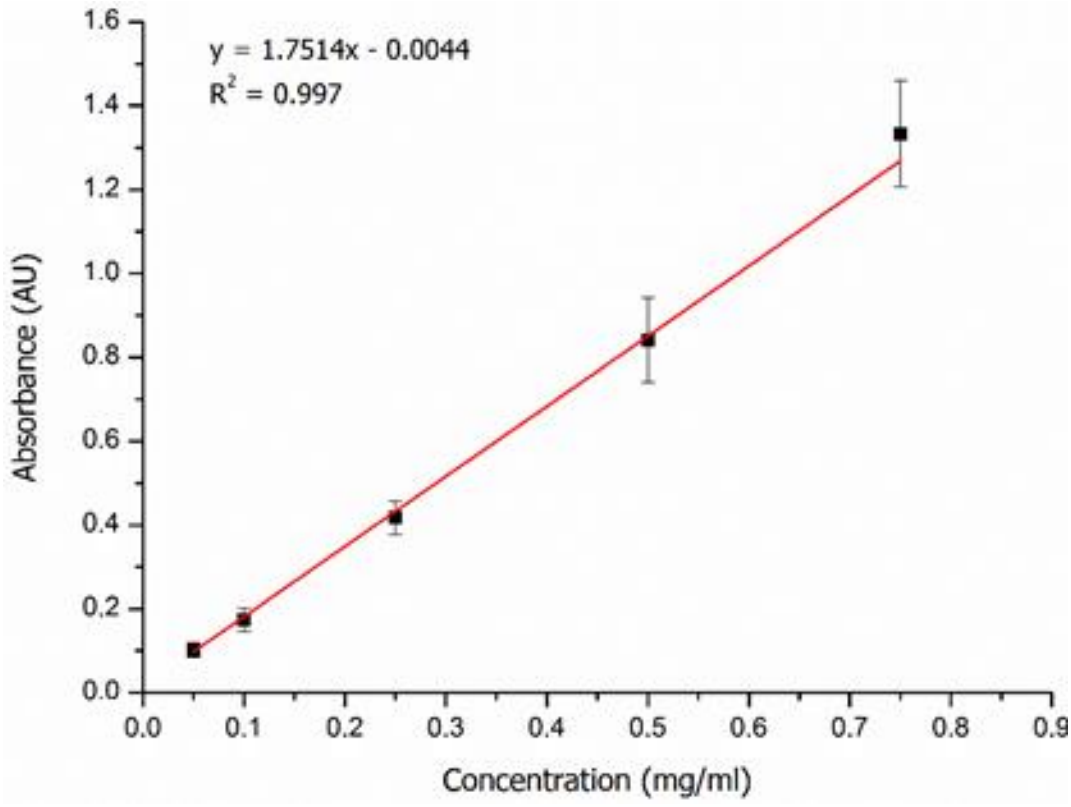

Figure 4. Linearity of the analytical standard rutin.

Table 1. Results obtained in intermediate precision analysis of the method.

Analist	Day 1 (mean of absorbance)	Day 2 (mean of absorbance)	F values
Analist 1	0.4366	0.4364	F *cal* 0.0956
Analist 2	0.4288	0.4322	F *tab* 0.3236

Table 2. Results obtained in reproducibility analysis of the method.

Parameter	Variable	Mean (absorbance) ± RSD%	F values
Spectrophotometer UV-Vis	EVEN® (IL-592 model, Brazil)	0.455 ± 0.093	F *cal* 0.4885
	Nova Instruments® (NI-1600 model, Brazil)	0.408 ± 0.003	F *tab* 0.6551

Table 3. Robustness test result for the evaluated method.

Parameter	Variable	Mean (absorbance) ± RSD%	F values
Wavelength	370 nm	0.446 ± 0.028	F *cal* 0.0082
	380 nm	0.417 ± 0.010	F *tab* 0.0645
Solvent brand	Synth®	0.442 ± 0.012	F *cal* 0.1180
	AppliChem®	0.487 ± 0.015	F *tab* 0.9882

reproducibility (Table 2), the calculated F was lower than the table F, that is, no statistical difference was observed, and the method was reproducible.

Regarding the parameter accuracy, the average recovery of the rutin was 93.46% ± 0.87 (CV = 1.03%), an acceptable value for natural products. These values show that the analytical method developed is sufficiently accurate. The recovery represents the degree of agreement between the individual results found in a given test and a reference value accepted as true (Fernandes et al., 2015).

Robustness is the measure of the method's ability to withstand small and deliberate variations in analytical parameters, such as the sample analysis wavelength, the pH of the solution used as the solvent or eluent, and the brand of the solvents (Brasil, 2017). The data obtained showed that the procedure was robust in terms of the analyzed parameters (difference in analysis wavelength and solvent brand used), since the calculated F values were lower than those of tabulated F (one-way ANOVA), as can be seen in Table 3.

Conclusions

This study aimed to adapt and validate an analytical method for the quantification of total flavonoids in *A. brasiliana* extract, a medicinal plant with strong pharmacological and technological potential. The method developed proved to be simple, fast, low cost, linear, selective, precise, accuracy and robust. Therefore, this methodology can be useful for quality control of the extract, plant drugs and herbal medicines obtained from *A. brasiliana* and to quantify the total flavonoids in this species.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Characterization of three full *Iris yellow spot virus* genes of a garlic-infecting isolate from Zimbabwe using next-generation sequencing technology

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Iris yellow spot virus **(IYSV) is an important pathogen of *Allium* species worldwide. It has a tripartite genome consisting of the large (L), medium (M) and small (S) RNA segments. Despite its worldwide distribution, very few complete gene and genome sequences are available in public databases. The aim of this study was to obtain full gene sequences of a garlic-infecting IYSV isolate by next-generation sequencing (NGS) for understanding its evolution. Total RNA was extracted from an IYSV-positive garlic leaf and sequenced on the Illumina HiSeq platform using paired-end chemistry 125 × 125 bp reads. The quality of raw reads was assessed using FastQC software before trimming with Trimmomatic version 0.36. The resultant paired-end sequences were used for both *de novo* and reference-based genome assembly. The resultant consensus gene sequences were analyzed using SIAS (for sequence identity and composition), ExPASy (for protein molecular weight) and ORF Finder (for open reading frame identification). Three full gene sequences, that is, nucleocapsid (N), nonstructural protein (NSs) and movement protein (NSm) were recovered. The N gene did not display any distinct clustering patterns based on geographical locations and was most identical to an onion-infecting isolate from Serbia (Accession KT272878). The NSs and NSm genes clustered closely with homologous sequences of IYSV isolates that were retrieved from GenBank and EMBL. This study lays the foundation for complete genome studies of IYSV in Zimbabwe.**

Key words: *Allium* species, emerging pathogen, reverse transcription polymerase chain reaction (RT-PCR), serology, tospovirus.

INTRODUCTION

Iris yellow spot virus, IYSV, is an important emerging pathogen of *Allium* species worldwide responsible for causing significant yield and quality losses in alliaceous and ornamental crops (Bag et al., 2015). It was first isolated and characterized in iris (*Iris holandica*) in The Netherlands (Cortês et al., 1998) and has now been confirmed to be present in over 40 countries worldwide (CABI, 2018). In Zimbabwe, IYSV was first detected infecting onions (*Allium cepa*) in 2014 (Karavina et al., 2016) and was subsequently reported infecting garlic (Allium sativum), leeks (Allium ampeloprasum) and shallots (A. cepa var. aggregatum) (Karavina and Gubba,

2017).

IYSV belongs to the genus *virus* in the family Tospoviridae. Like other tospoviruses, IYSV has quasi-spherical enveloped particles that are 80 to 120 nm in diameter (Pappu et al., 2009). It has a tripartite single-stranded RNA genome consisting of the large (L), medium (M) and small (S) segments. The L RNA encodes the RNA-dependent RNA polymerase (RdRp) in the viral complementary sense (Bag et al., 2010). The M RNA is ambisense and has two open reading frames (ORFs). The viral sense strand of the M RNA encodes the non-structural movement (NSm) protein, while the anti-viral sense strand encodes the Gn/Gc glycoproteins. The NSm protein is responsible for virus particle movement during systemic infection, while the two glycoproteins serve as virus attachment proteins (Bandla et al., 1998). The S RNA is also ambisense and encodes the non-structural (NSs) protein in the viral sense strand and the nucleocapsid (N) protein in the antiviral sense strand. The NSs protein is involved in suppressing RNA silencing, while the N protein encapsidates the RNA segments (Bag et al., 2015). In addition to these genes, both M and S RNAs contain intergenic regions (IGRs) capable of forming stable hairpin structures (King et al., 2012). IYSV is currently known to be transmitted by two thrips species, *Thrips tabaci* and *Frankliniella fusca* in a persistent and propagative mode (Srinivasan et al., 2012).

IYSV identification and characterization is carried out by employing biological, serological, morphological and molecular techniques (Bag et al., 2015). Biological characterization, also known as host indexing, involves the inoculation of indicator plant species that produce typical symptoms. However, it normally takes several days for symptoms to develop on inoculated hosts (Bag et al., 2012). Serological detection can lead to ambiguous results, especially amongst closely related viruses like IYSV and Tomato yellow ring virus (TYRV). Also, antibodies to IYSV must be raised if serological detection is to be successful. Morphological identification involves the use of electron microscopy, a technique that is technically challenging and not affordable in most research and academic institutions in developing countries. Being RNA viruses, reverse transcription-polymerase chain reaction (RT-PCR) can also be employed for IYSV identification (Walsh et al., 2001). However, primers specific for IYSV detection must be available/designed.

Next-Generation Sequencing (NGS) provides an unbiased, quick, cost- and labour-effective characterization procedure for plant viruses (Kreuze et al., 2009). Two assembly methods, namely, *de novo* assembly and reference-based mapping are used to recover the virus genome from the sequenced data. With NGS, full viral genomes can be recovered at once (Adams et al., 2009).

In public databases like NCBI and EMBL, except for the N gene, there are no more than five complete IYSV gene sequences available. This has negatively impacted evolutionary studies of this pathogen. In this study, NGS was used to characterize three full genes of a garlic-infecting IYSV isolate from Zimbabwe.

MATERIALS AND METHODS

Sources of materials used

IYSV was isolated from garlic plants collected at a horticultural farm in Chegutu, Zimbabwe, in 2015. Garlic plants that displayed necrotic, irregularly-shaped and grey-to-bleached lesions typical of IYSV infection were targeted during sample collection. Young leaves from symptomatic plants were collected in RNA*later*® solution (Thermo Fisher Scientific, USA) and transported to the University of KwaZulu-Natal (South Africa) for pathogen characterization.

Serological detection

Eight leaf samples were tested for IYSV in duplicate wells using a commercial kit supplied by Loewe® Biochemica GmbH (Sauerlach, Germany) following the manufacturer's instructions. Briefly, microtiter plates were coated with IYSV-specific antibodies. About 0.5 g of garlic leaf tissue showing disease symptoms were excised and ground in liquid nitrogen in a pestle and mortar. Macerated tissues were mixed with Conjugate Buffer at 1:20 dilution, and 0.2 ml mixture added to each microtiter well before overnight incubation. After four washes, an enzyme-labelled antibody-AP-conjugate was applied to the plate wells. In the final step, 0.2 ml of the Substrate Buffer Solution containing the dissolved substrate was applied to the microtiter plate. After 2 h of incubation, the reaction was visually assessed for yellow color development which is characteristic of a positive test.

RNA extraction and RT-PCR

Total RNA was extracted from the garlic leaves using the Quick RNA MiniPrep Kit (Zymo Research, USA) according to manufacturer's instructions. RT-PCR was performed using the RevertAid RT Reverse Transcription Kit (Thermo Fischer Scientific, USA) targeting the N gene. The resultant cDNA was used for PCR amplification using the KAPA2G Fast Hot Start ReadyMix Kit (KAPA Biosystems, USA) and N gene-specific primers (IYSV_2F: 5'-GGCGGTCCTCTCATCTTACTG-3' and IYSV-NCP2_R: 5'-GAAGTTCCAGGAGTGCATTTAGTC-3') (Lee et al., 2011) under the following cycling conditions: initial denaturation at 95°C for 2 min; 35 cycles of 94°C for 15 s (denaturation step), 57°C for 15 s (annealing step), and 72°C for 15 s (extension step), followed by a 5-min incubation period at 72°C. PCR products were analyzed by

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1.5% agarose gel electrophoresis. The amplicons were excised, purified using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA) and directly sequenced in both directions at Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). Sequences generated were blasted into MEGA 6.06 program.

Sample preparation, sequencing and data analysis for NGS

A sample that was IYSV-positive by both DAS-ELISA and RT-PCR was randomly selected and RNA extracted for NGS at the Agricultural Research Council's Biotechnology Platform (ARC-BTP) (Pretoria, South Africa). RNA quality and quantity were assessed using a nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). Total RNA samples were pre-treated with Ribo-Zero (NEB, UK) prior to library preparation.

NGS was performed on the Illumina HiSeq platform using paired`-end chemistry 125 × 125 bp reads. Subsequent sample demultiplexing was done using the CASAVA pipeline software (Illumina, USA). Read lengths of less than 25 nucleotides were trimmed and pair-end sequence libraries were generated. FastQC version 0.11.5 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess quality of the raw reads generated by NGS before and after trimming with the Trimmomatic v 0.36 with the following settings:

(ILLUMINACLIP: TrueSeq3-PE-2.fa:2:30:9:1:true LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15:MINLEN:36) (Bolger et al., 2014).

The pair-end sequences were subsequently used for both *de novo* assembly and reference-based mapping. *De novo* assembly was performed using the SPAdes Genome Assembler version 3.10.1 with the parameters: careful mode; only assembler; –k 21, 33, 55, 77, 99 (Bankevich et al., 2012), while referenced-based genome mapping was done using the BBMap Short Read Aligner version 37.28 (Bushnell, 2014) using IYSV genomes as references. All contigs generated by the *de novo* assembly method were subjected to BLAST using ncbi-blast 2.6.0+ (www.ncbi.nih.gov).

IYSV sequence analysis and phylogeny

All contigs that matched IYSV genomes were selected and aligned using the Clustal W embedded in MEGA 6.06 software (Tamura et al., 2013) to generate the consensus sequences of the N, NSs and NSm genes. The open reading frames (ORFs) on the IYSV genes were identified using ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder). Molecular weight (Mw) of proteins was determined using the online ExPASy bioinformatics tool (Gasteiger et al., 2005). Phylogenetic trees of the nucleotide sequences of the three IYSV ORFs were inferred by Maximum Likelihood method based on the best evolutionary models as determined by MEGA 6.06. The trees were rooted using the TSWV sequence as an outgroup. Bootstrap analyses were conducted using 1000 replicates. Details of isolates used in the phylogenetic analysis are shown in Table 3. Nucleotide and amino acid sequence compositions and sequence identities were calculated using SIAS program (www.imed.ucm.es/Tools/sias.html).

RESULTS

Serology and RT-PCR analyses

Of the eight samples that were tested, six were positive for IYSV by both DAS-ELISA and RT-PCR. All IYSV-positive samples turned yellow within 2 h of incubation after the final wash step. The 236-bp bands were visualized from samples that were IYSV-positive after electrophoresis on 1.5% agarose gel stained with SYBR Safe Gel stain (Life Technologies, USA).

RNA quality assessment and NGS data analysis

The concentration of the RNA sample that was sent for NGS was 194.38 ng/µL, with an absorbance A_{260}/A_{280} ratio of 1.95. The size of the NGS data generated was 3.3 GB and consisted of 6 921 806 raw reads. The average reads length after trimming was 119 bp (Table 1). A total of 107 102 contigs were generated by *de novo* analysis. Of these, 18 matched to the L (2), M (8) and S (8) RNA segments of the IYSV genome. From the reference-based mapping, a total of 671 reads were mapped to the IYSV genome (Table 1). Although the results obtained using both *de novo* assembly and reference-based mapping methods were consistent, full IYSV genome segments of the garlic Zimbabwe (garlic-Zim) isolate were not recovered. However, three genes (N and NSs found on the S RNA segment and the NSm present on the M RNA segment) were found to be complete after visual inspection and analysis on the ORF Finder. Consequently, these genes were considered for phylogenetic analyses. The nucleotide sequences of these genes were deposited in GenBank under the accession numbers shown in Table 2.

Sequence characteristics and phylogenetic analyses of the N, NSs and NSm genes were from the IYSV garlic-Zim isolate

The N gene of the IYSV garlic-Zim isolate was 822 nt long and coded for a protein with a molecular weight of 30.46 kDa. The NSs and NSm proteins had molecular weights of 50.11 and 34.73 kDa, respectively (Table 2).

The N gene of the garlic-Zim isolate had a sequence identity of 93.06% to the onion-infecting Serbian isolate (KT272878) at the nucleotide level, while at the protein level; it was most identical (95.25%) to the onion-infecting Sri Lankan isolate (GU901211). It shared the lowest nucleotide and protein sequence identity with the Iranian isolate (HQ148173). The NSs gene sequence of the garlic-Zim isolate was 91.66% identical to The Netherlands isolate (AF001387) at the nucleotide level. As for the NSm gene, it was most identical to the USA isolate (FJ361359) at both nucleotide and amino acid levels (Table 3).

Phylogenetic analysis of the N genes produced two distinct clusters (A and B; Figure 1). The N gene of the IYSV garlic-Zim isolate was in cluster A along with homologous sequences of isolates from Australia, Brazil, Egypt, India, Israel and Sri Lanka. Cluster B composed of N sequences of isolates from Iran, Japan, Serbia, The Netherlands and The UK (Figure 1).

The NSs gene of the garlic-Zim isolate clustered with the homologous sequences of isolates from India and

Table 1. Characteristics of the NGS data generated from total RNA infected by IYSV.

Characteristics	Statistics
Number of raw reads	6 921 806
Average read length	125 bp
Number of reads after trimming	5 758 205
Average reads length after trimming and adapter removal	119 bp
Number of contigs generated	107 102
Length of contigs	100-31303 bp
Contigs matching IYSV	18 (S: 2, M: 8, L: 8)
Number of reads mapped to L RNA	336
Number of reads mapped to M RNA	133
Number of reads mapped to S RNA	202

Table 2. Sequence characteristics of the N, NSs and NSm genes of the garlic-Zim isolate.

Segment	ORF polarity	Accession number	Protein coded	ORF length (nt)	Number of amino acids	Protein weight (kDa)
S	(-)	MF359019	N	822	273	30.46
	(+)	MF359021	NSs	1332	443	50.11
M	(+)	MF359020	NSm	983	311	34.73

Table 3. Percentage nucleotide (nt) and protein (aa) sequence identities between the IYSV N, NSs and NSm genes and other IYSV isolates.

Accession No.	Isolate	N		NSs		NSm	
		Nt	Aa	nt	aa	nt	aa
AF001387	-	41.36	17.14	91.66	82.02	-	-
AF067070	-	92.33	93.79	-	-	-	-
AF271219	-	91.11	92.7	-	-	-	-
KT272878	163-14	93.06	93.23	-	-	-	-
JQ973066	Washington	92.94	94.52	-	-	-	-
AB180919	SgOniD1	42.09	16.08	-	-	-	-
AM900393	-	41.22	17.62	-	-	-	-
AY345226	NSW-1	89.9	92.7	-	-	-	-
EU310295	IYSV-On-Vir	91.97	93.79	-	-	-	-
EU477515	New Zealand	92.7	93.23	-	-	-	-
EU586203	605-SRB	41.96	16.18	-	-	-	-
GU901211	-	92.57	95.25	-	-	-	-
HQ148173	5	41.05	15.82	-	-	-	-
KF171105	-	92.21	94.52	-	-	-	-
KT225547	IYSV-Egyptian	85.76	86.49	-	-	-	-
KJ868797	DOGR	-	-	91.51	83.59	-	-
FJ361359	-	-	-	-	-	97.11	99.67
AF213677	-	-	-	-	-	95.19	95.83
AF214014	-	-	-	-	-	89.63	94.55
KM035409	DOGR	-	-	-	-	87.07	89.42

The Netherlands (Figure 2A). As for the NSm gene, it also clustered with homologous sequences of IYSV isolates from Brazil (AF213677) and The USA (FJ361359) (Figure 2B).

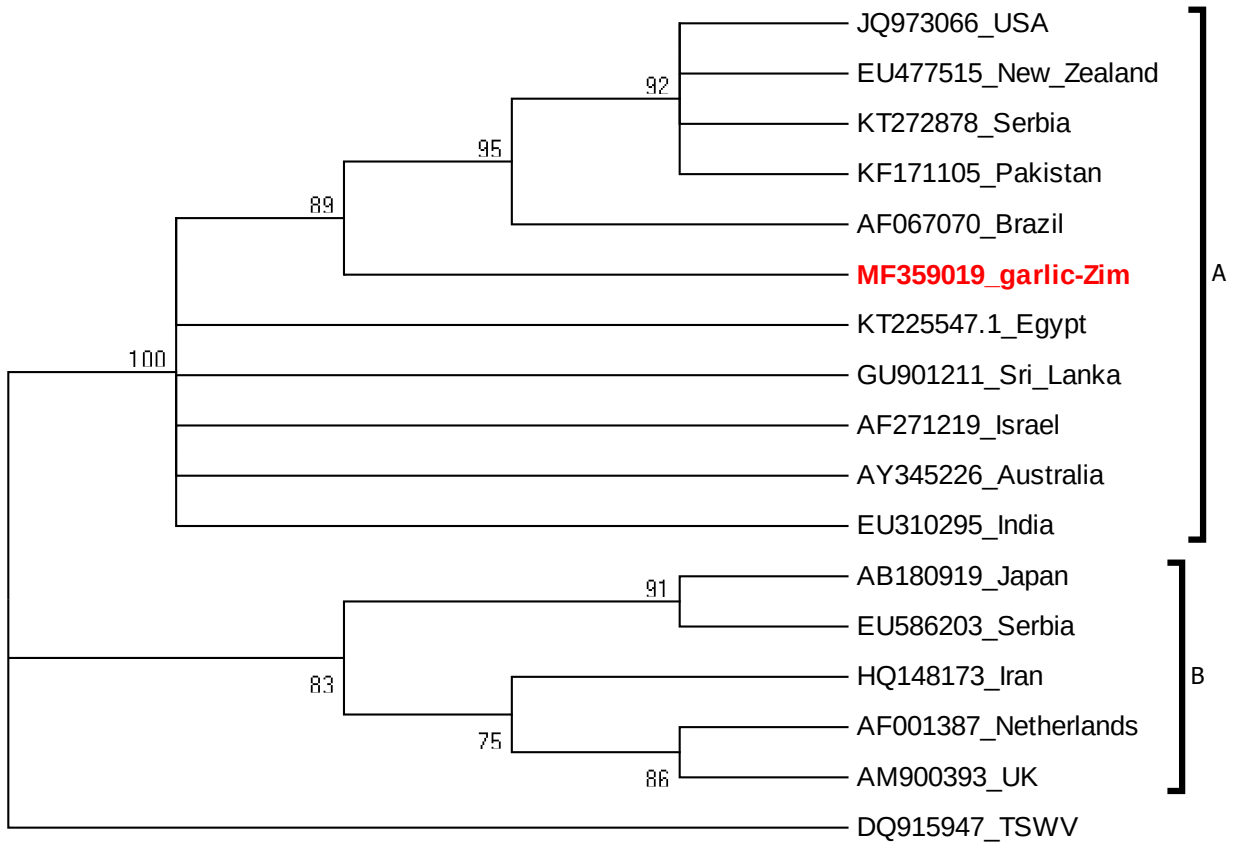

Figure 1. Phylogenetic analysis of the N gene of the garlic-Zim isolate by the Maximum Likelihood method based on the Tamura 3-parameter model.

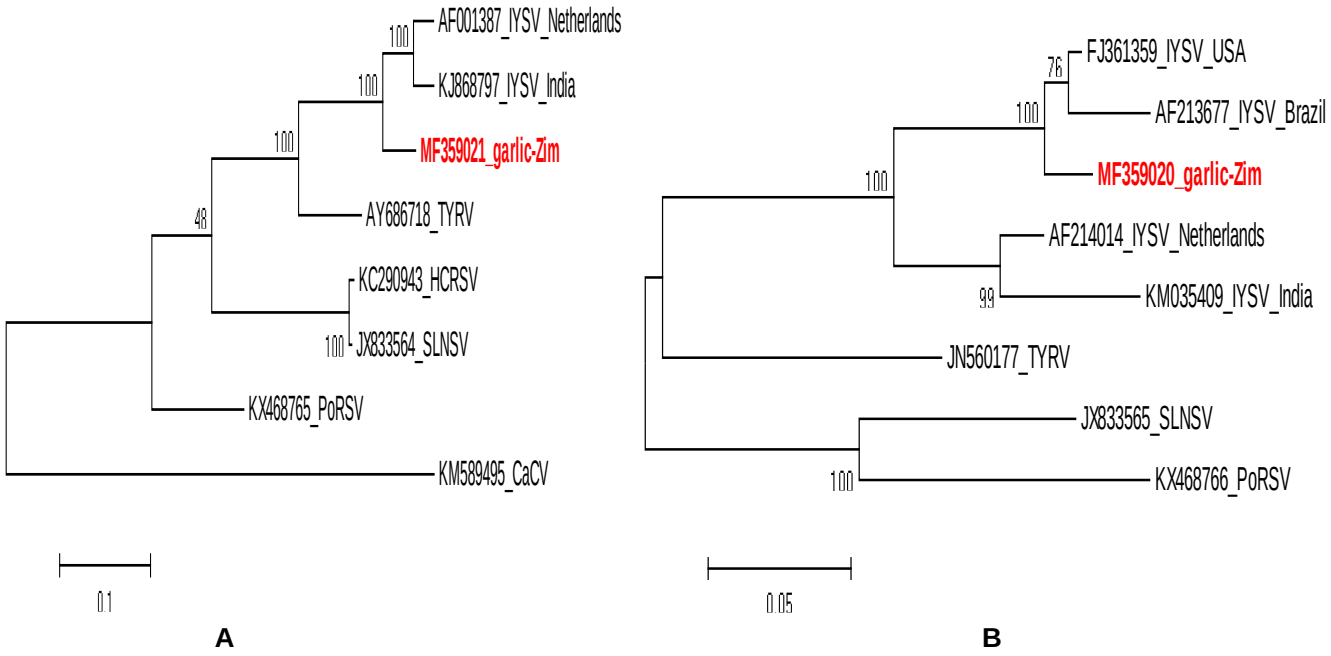

Figure 2. Molecular phylogenetic analyses of the NSs (A) and NSm (B) genes by Maximum Likelihood method based on the Tamura 3-parameter model.

DISCUSSION

The genomic organization of the garlic-Zim isolate is typical of tospoviruses in being tripartite (Pappu et al., 2009). Though no full genomic segments were recovered, NGS enabled the simultaneous recovery of the two full genes on the S RNA segment and one gene on the M segment. This is not possible with Sanger sequencing. This study lays the foundation for future studies on the full genome of IYSV in Zimbabwe.

Despite the global importance of IYSV, only a few full genome and gene sequences have been characterized (Gawande et al., 2015). This greatly compromises studies to understand pathogen evolution and management. The current study is first in Africa to characterize more than one full gene sequence of the same IYSV isolate. The only other full IYSV gene sequences from Africa deposited in public databases are from Egypt (Accessions KT225547.1 and KC161369.1).

Phylogenetic analysis of the N gene showed no specific clustering patterns based on geographical locations. This could suggest the possibilities of long-distance migration, recombination and reassortment events in IYSV. Such events are highly prevalent in tospoviruses (Margaria et al., 2015; Zhang et al., 2016). Being a pathogen of some internationally-traded ornamental plants (Bag et al., 2015; CABI, 2018), it is possible that IYSV and its vectors have been unintentionally distributed worldwide in live plant shipments. Also, the smuggling of live host plants across borders could have contributed to pathogen's worldwide distribution. The S segment is known to be substantially more prone to recombination than the M and L segments (Gawande et al., 2015). For the occurrence of either recombination or reassortment to be confirmed in the Gar-Zim isolate, full genomic segments must be recovered and analyzed.

In addition to DAS-ELSIA and serology, NGS was employed in further confirming the occurrence of IYSV in Zimbabwe. Knowledge of IYSV presence is crucial in epidemiological studies towards developing effective disease control strategies. When compared to serology and RT-PCR, NGS is a more rapid procedure and it also produces nucleic acid sequences for substantial parts of the viral genome. Another major advantage of NGS over DAS-ELISA and RT-PCR is that the latter approaches require reagents designed exclusively to detect their viral target and any variation in the virus genome may cause the assay to fail. NGS is non-targeted and requires no prior knowledge of the target. Therefore, it can detect existing strains, new variants and even new strains (Adams et al., 2009).

To maximize the chances of detecting IYSV in the sequenced data, two different methods (*de novo* assembly and reference-based mapping) were employed. *De novo* assembly recreates the original genome sequence through overlapping reads while reference-based mapping requires a previously assembled genome to be used as a reference. A major advantage of *de novo* assembly over reference-based mapping is that it gives the virome of the host(s) studied. It also detects other viruses not targeted by the study (Martin and Wang, 2011). In this study, *Garlic common latent virus*, *Garlic virus B*, *Garlic virus C* and *Shallot virus X* were detected (data not shown). There were no significant differences in the IYSV genes that were recovered by both *de novo* and reference-based mapping.

The fact that viruses other than IYSV were detected in the sample that was sent for NGS shows that mixed and multiple infections are common in nature. This implies that symptom expression cannot be conclusively relied upon for disease diagnosis.

Conclusion

The characterized IYSV genes of the garlic-Zim isolate are the foundation for future full genome studies of this important pathogen. Knowledge of the full genome is critical in understanding the evolutionary patterns of IYSV. Pathogen genomic information is also important in developing IYSV disease management strategies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Economic viability of the biogas produced on pig farms in Brazil for electric power generation

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Electric energy generated by clean and renewable sources, such as biogas, is a subject widely discussed and of global importance. Biogas, besides being an alternative to other fuels uses a raw material, which in many cases is considered disposable, worthless and harmful to the environment when not correctly disposed of. Several factors influence the design, operation and collection of the final product. This paper is a technical economic viability report about three biogas projects, which have already been published, and presents the main difficulties and advantages encountered during the whole process involving biogas production, as well as its economic viability when used to generate electrical energy. The analysis show that the use of biogas from wastes of pig farms is economically viable, since all biogas produced is nearly totally used for electrical power generation.

Key words: Carbon credits, biodigester, biomass, methane, biomethane, greenhouse gases.

INTRODUCTION

Biogas was discovered in 1667, however, only 100 years after Alessandro Volta noted the presence of methane in its composition, opening the possibility of its potential to produce heat, and leading to its widespread use in rural installations (Classen et al., 1999). In Brazil, the technology for producing biogas appeared in the 1970's and did not reach any importance initially. Nonetheless, over recent years, mainly due to the escalation in the price of other types of energy sources, such as those derived from oil, biogas regained its place as a viable alternative source of energy.

Different to other renewable energy sources, such as biodiesel and alcohol, biogas does not have the need for the cultivation of any type of culture, such as sugar cane, corn, beetroot etc. The primary material used in the production of biogas is detritus, agricultural waste, materials that in many cases would have no value or use (Barreira, 1993). Noteworthy here is that some countries in Europe use corn plantations as biomass for biogas production. However, Nigatu et al. (2012) conduct their study toward the potential use of the plant Eragrostis *Tef* for the production of biogas, focusing on the care that should be taken in plantations used specifically for the production of biogas, as these areas, when not planned correctly, can lead to a breakdown in food production in that region.

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On the other hand, as shown on Table 1, biogas is composed, in greater part, of methane (CH_4) and carbondioxide (CO_2), although in the composition of biogas there exist other gases but in less significant quantities.

Therefore, the most important component of biogas is methane, which is the main reason for opting for the exploration of this type of energy. According to La Farge (1979) “methane and carbon dioxide represent 60 to 80% and 20 to 40% of the total gas volume, respectively”. This concentration is affected by the type of biomass origins, which, in turn, interferes in the heating potential of the fuel. This represents the maximum energy content of the fuels and in fact this parameter alters with the elementary chemical composition (Barros et al., 2018).

Highlighted also, in agreement with Vanti et al. (2015), is that raw biogas has a strong odor, low heat potential and is highly corrosive, which means that it is not indicated for internal combustion engines, thus there arises the need for purification processes to be applied to the raw product in order to elevate its heat potential and remove components that are responsible for corrosion and the bad odor.

Different to other fuels used as heat sources, such as wood, coal, oils etc., methane when burned does not leave behind residues, and is of low impact environmentally. Table 2 is a list showing the approximate equivalent of other fuels when compared to biogas, where 1 m³ of biogas is used as a base (Sganzerla, 1983).

In this study, the biomass addressed comes from pig waste. Pig waste is often used for biogas production. One of the reasons for such use can be demonstrated through an experiment performed by Zagorskis et al. (2012). The aim of the experiment by Zagorskis was the analysis of biogas generated through the mixture of chicken excrement with leftover plant material at a ratio of 90:10%, as well as pig excrement and leftover plant material also at a ratio of 90:10%. The conclusion reached from the results of these experiments was that the mixture with pig excrement is better for the production of biogas, as the maximum concentration of methane was around 68%, which was approximately 50% greater than that of the chicken excrement and plant mixture.

Biomass is classified as being all and any biological input that can be decomposed through biological action, and as such “any type of organic material of animal or vegetable origin is considered biomass” (Sganzerla, 1983). Biomass is the most common fuel in nature and for this reason it has greatest ease of access and use, at least until the beginning of the 20th century, when petroleum was finally discovered (Rossilo-Calle, 2000). Currently, due to its use as gas for the production of energy, biomass has started to retake a relevant portion of the market simply because of the biomass digestion process, performed by bacteria, which results in the production of two main inputs, biogas and biofertilizer. Excrement from animals, abattoir waste, sugarcane bagasse, domestic waste and sewage are among the main composts that can be used as biomass.

Biofertilizers, produced by means of anaerobic digestion in biodigesters, are organic fertilizers rich in nutrients that can be used as substitutes for chemical fertilizers. As these are the result of the decomposition of organic animal or vegetable material, biofertilizer possesses in its composition live cells and several microorganisms. This type of fertilizer possesses a more liquid nature than solid, which therefore facilitates its use in the field (Medeiros and Lopes, 2006). In the study by Kocyigit et al. (2017), the authors reached the conclusion that the biofertilizer that results from anaerobic digestion of biomass has a lower rate of mineralization than other organic fertilizers. Mineralization in biology is the conversion of organic matter into inorganic matter.

The biodigester is a chamber into which the biomass is deposited, maintaining the appropriate proportions between solid and liquid mass, in order that the digestion process occurs as expected, liberating in this way the biogas and the biofertilizer as its final products. Currently, there exist various models of biodigester, depending on the type of biomass, final product, available materials, among other factors (Tiago Filho and Ferreira, 2004). According to these authors, the biodigester can act in continuous mode, where the installation possesses a collection mode, in which biogas is continuously collected with no interruption, and batch mode, when the process is maintained inside the biodigester for a given period until it is concluded and the biogas is removed at the end. The two most common models of biodigester are the Indian and Chinese models, where both are continuous biodigesters, as illustrated in the schematic drawing shown in Figure 1. The Indian model of biodigester is built with a bellflower design gasometer, and in this way the pressure inside the gasometer remains constant during the whole process. In Figure 1, a two-dimensional plan of the biodigester, in which the input and output entrances can be seen, thus allowing for a constant supply without the need to interrupt the process (Cervi et al., 2010). The Chinese model also possesses an uninterrupted mode of working. The differences are that, in the Chinese model, the biodigester is constructed in brick and is fixed, and, in this way the pressure inside the chamber is not constant as happens in the Indian model. In both models, the structures are supplied with biomass in solid concentration of around 8%, in such a way to avoid the occurrence of possible blockages in the input pipes (Deganutti et al., 2002).

In recent years, the world market has seen high price rises on fossil fuels and their derivatives, where both petroleum and natural gas are highlighted. These constant price changes, among other market factors, occur due to increases in demand and the absence of

Table 1. Biogas composition.

Type of gas	Biogas composition (%)
Methane (CH_4)	60 to 80
Carbon dioxide (CO_2)	20 to 40
Hydrogen sulfide (H_2S)	until 1.5
Nitrogen (N)	Trace
Hydrogen (H)	Trace

Source: Obtained from La Farge (1979).

Table 2. Comparison of biogas with other fuels.

Fuel	Corresponding value at 1 m³ of biogas
Alcohol	0.790 L
Gasoline	0.610 L
Diesel	0.550 L
Kerosene	0.570 L
Liquid gas	0.450 kg
Wood	1.538 kg
Electric energy	1.428 KWh

Source: Originally from Sganzerla (1983).

conditions for increasing the production of these fuels in a way that meets such demands, even though there has been growth in fossil fuel production over time. Petroleum started to be exploited around 1845, and its production went on to surpass 86 million barrels per day" according to the International Energy Agency (IEA) (2010), leading to a number of problems. The most important problem concerning the use of this type of fuel is based on the fact that it is not renewable, thus at some moment in the future, the production of petroleum will fall and the price, in virtue of increased demand, will rise even more, making it impractical. To avoid this problem in the future, investments on renewable energies are necessary, and according to Song et al. (2014), the anaerobic fermentation of agricultural wastes for production of biogas is a good alternative than the use of fossil fuels.

Faced with this, several countries see as alternatives the search for new sources of renewable energy, such as energy from hydroelectric, wind turbines, solar systems, biodiesel and biogas, which are all excellent alternative sources to the use of petroleum. The importance of these renewable energy sources is increasing, not only to substitute fossil fuels but also to protect the environment.

According to the Mines and Energy Ministry (2017), which pointed out in its newsletter "World Ranking of Energy and Socioeconomics", Brazil is one of the highest producers of renewable energy, although the exploitation of some sources is still very much in their initial stages. Nevertheless, with adequate investment, the power generation of the country in this area will be much higher than that currently presented. Unexplored sources, or those that are in their initial stages of exploration, represent a great potential in energy generation.

One of the biggest concerns that lead to the search of renewable sources is associated with the increase in the greenhouse effect, which is caused by the emission of polluting gases denominated GHG (Greenhouse gases). This group includes gases such as methane (CH_4) and carbon dioxide (CO_2), which are among the main aggravating components for this type of problem. If we take into consideration agricultural and livestock production, there exist other aggravating gases, such as carbon monoxide and nitrous oxide. According to the Intergovernmental Panel on Climate Change (IPCC) (1996), the agricultural sector is responsible for an increase of around 20% of global radiative forcing, which is an index used for analyzing possible impacts of greenhouse gases on the climate by means of studies related to its radiative forcing characteristics. According to Pertl et al. (2014), the greenhouse gas from digestion of organic wastes is less prejudicial to the climate than the use of renewable agricultural resources to the production of energy.

The use of biogas is not only an alternative to the use of natural gas or any other fuel in the generation of electric energy, but it also provides a reduction in pollution loads. In addition, organic material deposited in the biodigester, after being digested, ends up being used

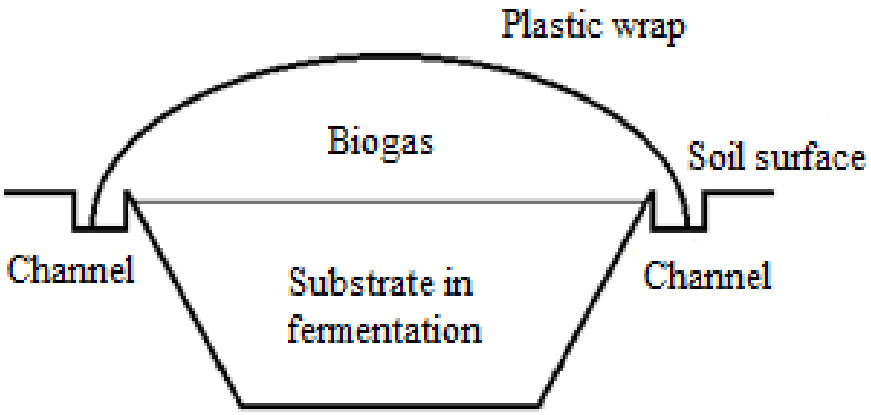

Figure 1. Cross-section of the tubular biodigester model.
Source: Originally from Cervi et al (2010).

Table 3. Yield of conversion technologies from chemical energy to electric.

Yield		
Gas turbine	**Steam turbine**	**Internal combustion engines**
25 to 40%	14 to 35%	Diesel Cycle: 35 to 45%

Source: Adapted from Antônio (2016).

as compost. This means the reuse of something that would be simply discarded can now be used for the generation of energy and fertilizer. This is very attractive for investment, in terms of the financial point of view (Pereira, 2005).

Currently, China is one of the fastest growing countries in terms of economy in the world, and the fact that it is the country that possesses the largest quantity of biodigesters is an indication that the generation system can, in the future, become responsible for a considerable percentage of electric energy production. In Brazil, the production of electricity using biodigesters has transformed rural properties into self-sustaining units, producing in this manner new incomes and jobs. It is in this way that “the use of these biodigesters collaborates with the maintenance of labor in the field and as way to reduce rural migration” (Sganzerla, 1983). In addition, the dependence on external networks of electric energy is no longer a difficulty in this sector.

In line with these prospects, this study proposes the investigation of the subject of electric energy generation using biogas as a primary source, with the aim of presenting basic concepts employing the main models of biodigester currently in use. The proposal here analyze if the electric generation units supplied with biogas are in fact an economically viable alternative, by presenting cases in which projects were developed for the installation and operation of this type of plant.

The use of biogas in the generation of electric energy

Burning biogas in combustion engines or boilers is one of the ways to produce electric energy. To decide the best technological option when selecting equipment for electric energy generation, one needs to consider the following: The power that will be generated, the fuel employed, the yield and the type of motor or turbine used. Another point that needs to be considered relates to global yield. Some conversion technologies are more efficient when thermal energy is used for cogeneration of electric energy. However, such technology will be economically viable when one needs to use the heat generated from burning biogas.

In order to demonstrate the yield from generators, the conversion efficiency of some technologies is emphasized in Table 3 for those most commonly used.

Legislation and free trade of energy

In Brazil, one option for increasing the attraction of this type of installation is the trade of electric energy within the free energy market. It allows the energy of small electric sources to be sold as stated by the decree number 5.163 of July 30th, 2004. In this decree, it is determined that there exist two types of trade: (1) The Regulated Contracting Environment (RCE), which is the

segment of the market, in which one performs the buying and selling operations of electric energy among selling and distribution agents, prior to bidding, except for those cases covered by law, as in specific rules and commercial procedures, and (2) The Free Contracting Environment (FCE), which is the segment of the market in which one realizes the buying and selling operations of electric energy, as the object of freely negotiated bilateral contracts, as set out in rules and procedures of specific trade.

The RCE is based on the regulated market, to which most consumers are associated. Within this setting, tariffs and distribution are regulated by the Brazilian government. However, FCE allows for direct negotiation with the energy producer, in a way that enables the consumer to decide to buy energy according to their needs. According to the Energy Trading Chamber (2016), the participation in the Free Energy Market in the buying and selling scenario in Brazil was approximately of 27% in 2016, and the tendency is for increased growth over the coming years.

Carbon market

Over recent decades, the carbon credit trading model has become viable in the carbon market. Carbon credits are a type of exchange currency for the emission of greenhouse gases. Following the commitment made under the Kyoto Protocol, goals were set to which developed and developing countries would adhere, in relation to the emission of greenhouse gases. However, as some countries are unable to adapt over the short term and as such forfeit the targets set out, there exists the possibility of buying these credits as a compensation for the emission of these gases.

Each equivalent ton of CO_2, which a country no longer emits into the atmosphere, is equal to 1 carbon credit. In Brazil, this can be negotiated by means of auctions organized by BM&FBOVESPA. In such events, brokers associated with the organization representing their clients can participate, including market traders from REC (Reduced Emissions Certificate) and from the European permissions market, financial multilateral organizations, global carbon market accredited by BM&FBOVESPA, carbon funds and government entities (Brazil, 2012).

Mitigating factors in the production of biogas

Biogas can be obtained from many types of raw materials. Some of them are more commonly used than others, due to efficiency in the production of gas, handling facility and availability. Some sources are brewery waste, abattoir waste, farm waste, animal excrement and grass. Highly fibrous materials, such as sugar cane, have a less efficient digestion, resulting in a lower biogas production when compared to other materials rich in starches, as is the case of grains and proteins, waste and blood from abattoirs. They present an elevated efficiency when it comes to biogas production (Prati, 2010).

Considering the two basic continuous models, in which the biodigester is continually receive biomass, without the need to terminate the digestion process, and the batch type biodigester, where the biogas is only removed at the end of the digestion process, the following systematization occurs. In the first case, in order to maximize the production of biogas, it is necessary that the concentration of dry mass is of 7 to 9%, which is a concentration considerably lower when compared with the second model, in which the concentration can reach 25% without incurring problems (Mazzuchi, 1980). The digestion process is also affected by the pH present in the biomass, where its ideal level is of pH 6.0 to 8.0. In cases where the pH reaches values below 6.0, the process starts to wane, to the point where it may even stop. If this occurs, it becomes necessary to perform a pH correction; also it is important to monitor these acidity levels (Comastri Filho, 1981).

Another item that should be assessed is temperature. In this case, the main precaution to be taken is with sharp variations, as some microorganisms responsible for the process are sensitive to these variations. Higher temperatures also produce better results. For temperatures around 35 to 37°C, the digestion process occurs at an accelerated rate, and in the other extreme, for temperatures below 15°C the process can arrive at a stop (Barreira, 1993). There should also be a level of attention paid to the presence of unwanted substances in the biodigester. In other words, care should be taken with substances that can, not only damage the digestion process but also present a risk to the installation itself. Excessive quantities of nutrients, strong disinfectants, and oil derivatives can all cause the loss, in greater part, of the bacteria involved in the process. For this reason, according to Comastri Filho (1981), the water used in the cleaning of equipment and installations is not adequate for use in biodigesters.

MATERIALS AND METHODS

Here, the objects of study dealt with herein are presented; these involve economic aspects and case studies.

Economic viability

The economic viability concerning the use of biogas to produce electric energy is directly linked to several different factors. Some of such factors can be the type of biomass to be used, the demand and/or selling of electric energy/fertilizer produced, active operation time of the electric generator, the initial investment, the costs with future maintenance, depreciation and labor. Therefore, one notes that the installation of an energy generator via biogas is not always

economically viable.

In order to assess whether a project is economically viable, it is common to use mathematical indicators, such as NPV (net present value), IRR (internal rate of return), BCR (benefit-cost ratio), SPB (simple payback), EPB (economic payback), as well as the use of MARR (minimum attractive rate of return), which is the minimum rate desired for the investment to become economically viable. When applied in a correct fashion, these calculations aid in the execution or rejection of a project, as well as for applying corrections along its course. In a simplified manner, NPV as determined through Equation 1, is the sum of all the revenues presently assigned to the project, subtracting the costs and taking into consideration interest rates and the duration of the undertaking.

$$NPV = -In + \Sigma_{j=0}^{n} R_j(1+i)^{-j} - \Sigma_{j=0}^{n} C_j(1+i)^{-j} \quad (1)$$

Where variable In is the initial investment, R_j is the current value of the revenue, C_j is the current value of costs, *I* is the interest rate, *j* is the period in which the costs or revenues occur and *n* is the duration of the project. A positive NPV means that the project presents a good result. However, negative values make the project impossible to execute. When positive, "the higher the obtained NPV value, the better will be the project performance" (Dossa, 2000). The IRR is a more complex technique than NPV; nevertheless it is still widely used. According to Gitman (2002), by using the same parameters, it is the same as the NPV calculation when it presents a null value, which is: NPV = 0, as calculated using Equation 2.

$$0 = -In + \Sigma_{j=0}^{n} \frac{R_j - C_j}{(1+IRR)^j} \quad (2)$$

To know whether a project will be accepted, or not, the following analysis can be used: if IRR is higher than the cost of capital, consider the project as viable and attractive. It should be discarded, if the opposite condition occurs. In Equation 3, the cost benefit, is the ratio of the current values of expected benefits and the current value of expected costs and for a project to be considered financially interesting, this value should be greater than 1 (Dossa, 2000).

$$BCR = \Sigma_{j=0}^{n} \frac{B_j(1+r)^j}{C_j(1+r)^j} \quad (3)$$

In Equation 3, B_i is the benefit of the project in monetary units in year *i*, C_i is the cost of the project in monetary units in year *i*, *I* is the time counter in years, *j* is the discount rate as a percentage, and *n* is the period representing the useful life of the investment in years. Finally, one has the capital recovery period, also known as *SimplePayback* (SPB), which represents, in years, the time necessary for the cash flow to equal itself to the initial investment and the economic payback (EPB), which considers a minimum attractive rate for calculating the recuperation period according to Casarotto Filho and Kopittke (2007).

Case study A

In Cervi et al. (2010), a project developed at a rural property with a diversified production, including poultry, coffee as well as pig, cattle and sheep farming, was presented. In this investigation, the decision was reached for the use of pig excrement, due to the fact that it holds great potential for generating biomass by means of this type of culture in that place. To carry out the project, the choice of a continuous operation biodigester was made, with a working capacity of 496 m³.

The floor where the biomass is deposited is made of bricks. And the top part is made of a plastic sheet covering, as illustrated in the schematic drawing of Figure 1.

Case study B

In the study developed by Lindemeyer (2008), emphasis is given to the economic viability analysis, based on the use of biogas as a source of electric energy at a pig farm in Santa Catarina, Brazil. The study was developed around the breeding of 2,500 animals. By means of the digestion of waste in the biodigester, an average of 158 m³ of biogas was produced per day. In the plant, there is a generator of 50 kVA that remains on 4 h per day, which consumes 80 m³ of biogas as fuel.

Case study C

The objective of the study presented by Antônio (2016) is found in the economic viability of the generation of electric energy through the use of the biogas produced at a pig farm in Minas Gerais, Brazil. According to the author, the goal of the study was to analyze the production capacity and use of biogas from pig farming, as a fuel for generating electric energy. In this project, information from 22 farms was collected, registering data such as the number of animals and the maximum consumption of electric energy. In this manner, the quantity of biogas produced by each farm, as well as the quantity of biogas necessary to supply the consumption of electric energy of the establishment was estimated.

In order to calculate the economic indicators (NPV, IRR, BCR, SPB, EPB and MARR), the cash flow for ten years was calculated by Antônio (2016), where the output values considered were the installation of the biodigester, generation equipment, connection to the electric network, labor, availability of electric energy, depreciation of the generation equipment, maintenance of the biodigester and the generator. The cash flow input corresponds to the savings generated by the autonomy ascertained by the electric energy. Also there is an important increase in the tenth year due to the residual value of the generation equipment, and the tariff used was US$ 0.0975 per KWh and a MARR (minimum attractive rate of return) of 8.75% per year.

Case selection

Considering that Brazil is a great producer of pigs, it was selected for the analysis of the use pig farms to produce biogas. Another reason for the choice is that pig manure is a good material to produce biogas with high methane concentration.

The Table 4 shows some differences and similarities, about the biodigester and biogas, of the three cases.

RESULTS

The results shown refer to the three case studies covered herein listing the collected and simulated values.

Case study A

The estimated production of biogas obtained at the end of the Project reached 670,760.5 m³/year. In the same study, 72.072 m³ of biogas/year were also estimated,

Table 4. Differences and similarities among the three cases.

Cases	Substrate type	Biodigester type	Biodigester working capacity (m^3)	Biogas consume per day (m^3)
Case A	Pig manure	Tubular model	496	197
Case B	Pig manure	Canadian model	900	158
Case C	Pig manure	Indian model	1,479 ~ 8,074	168 ~ 6,058

Source: Cervi at al. (2010), Lindemeyer (2008), and Antônio (2016).

Table 5. Simulation for the consumption of electric energy for use at 10.5 h/day.

Energy consumption (KWh)	Working period (h/day)	Benefit (US$/year)	Cost* (US$/year)	NPV (US$)	IRR (%)	BCR (index)	SBP (years)	EPB (years)
20	10.5	5,925.34	5,737.84	(-27,878.62)	-	0.05	-	-
25	10.5	7,406.67	5,737.84	(-16,787.47)	-9.10	0.43	-	-
30	10.5	8,888,01	5,737.84	(-5,696.32)	1.35	0.81	10.30	-
35	10.5	10,369.34	5,737.84	5,394.83	9.34	1.18	7.32	9.04
40	10.5	11,850.68	5,737.84	16,485.98	16.24	1.56	5.79	6.75

*In this cost the interest over the capital is not included. Source: Originally from Cervi at al. (2010).

considering the consumption of 72.072 m^3 of biogas/year and a working period of 3,276 h/year for the generators.

For a per day consumption of 17.1 KWh, adopted at the property, there was an annual financial return of US$ 5,066.16. This was calculated considering the electric energy tariff applied during 2008, the year in which the Project was developed. In that year, the tariff was US$ 0.0937 per KWh in the dry season (7 months) and of US$ 0.0858 per KWh in the wet season (5 months), both at off-peak h. As one notes from Table 5, the annual cost of the Project was calculated at US$ 5,737.84. That means, for this scenario, that the production and use of biogas only produces desirable results for higher end consumers than those addressed herein. Finally, a new study was performed that considered the employment of the generator for a greater working period. It was 10.5 h outside of the peak and 3 h at peak, resulting in 4,212 h of energy generation per year. In this second case, one notes that for the same consumption of 17.1 KWh, adopted by the property, as well as possible increases in consumption up to 40 KWh, the project becomes viable as noted from Table 6.

Case study B

The project made possible an annual production of 58,400 KWh of electric energy. As the consumption on the farm was 58,400 KWh and the KWh tariff at US$ 0.092, this led to a generated economy of approximately US$ 5,400. In the farm place, Lindemeyer (2008) performed an economic analysis and calculated for a 15 years period the items for the working life of the generator, NPV, IRR, and payback. After he recalculated the economic parameters for an alternative scenario, by considering that the generator would remain on 8 h per day, a use of 158 m^3 of biogas generated per day was calculated. To achieve this, he considered that the surplus KWh would be sold to the energy utility for US$ 0.076. This was the price paid by COPEL (The Energy Company of Parana), where it was possible to sell the surplus electric energy produced at that time. In both conditions, the income generated by the selling of carbon credits was also considered in the calculation of economic indicators. Table 7 provides the results for the economic viability of the two calculated conditions, involving 4 h in the first condition and 8 h in the second condition. Note that, operating 8 h per day, the result presented herein is higher than that for the operation occurring for only 4 h.

Case study C

By analyzing Table 8, which contains the economic indicators for some farms, one notes the best and worst results. This is in addition to allowing for possible motivation of some farms not producing a positive result.

As shown on Table 8, Farms 4, 5, 13 and 19 obtained a good performance in the analysis, and this is seen mainly due to the fact that they possess a positive NPV and high value. Another point is that the IRR presents a much higher value than MARR, which was only 8.75%. Taking into consideration the average working life of around 15

Table 6. Simulation for the average energy consumption for use at 13.5 h/day.

Energy consumption (KWh)	Working period (h/day)	Benefit (US$/year)	Cost* (US$/year)	NPV (US$)	IRR (%)	BCR (index)	SBP (years)	EPB (years)
17.1	13.5	11,290.31	6,567.74	6,076.64	9.79	1.21	7.20	8.85
20	13.5	13,205.05	6,567.74	20,412.75	18.52	1.70	5.41	6.22
25	13.5	16,506.3	6,567.74	45,130.17	31.79	2.54	3.95	4.32
30	13.5	19,807.57	6,567.74	69,847.59	44.04	3.39	3.21	3.44
35	13.5	23,108.83	6,567.74	94,565.01	55.82	4.23	2.77	2.92
40	13.5	26,410.09	6,567.74	119,282.43	67.37	5.07	2.48	2.59

Source: Originally from (Cervi et al., 2010).

Table 7. Comparison of economic indicator.

Parameter	1st condition - 4 h	2nd condition - 8 h
Initial investment	US$ 68,306.01	US$ 88,797.81
Payback	5.56 years	3.33 years
IRR	14%	24.71%
NPV	US$ 76,956.19	US$ 169,097.60
Generator operating	4 h per day	8 h per day

Source: Adapted from Lindemeyer (2008).

Table 8. Economic indicators for investment in the use of biogas for generating electric energy.

Farm	NPV (US$)	IRR (%)	SPB (years)	EPB (years)
4	264,852.23	21	4.3	5.62
5	33,156.24	11,99	6.49	9.27
13	791,893.14	25.11	3.72	4.7
16	(-106,318.76)	4.28	9.15	-
17	(-119,938.48)	-15.64	-	-
19	430,347.72	25.47	3.69	4.66

Source: Adapted from (Antônio, 2016).

Table 9. Comparison of the number of animals and electric energy consumption for farms 5 and 17.

Farm	Number of animals	Maximum consumption of electric energy (KWh/month)
5	9.604	42.120
17	9.800	6.226

Source: adapted from Antônio (2016).

years for the generation equipment, an EPB of less than six years is attractive for this type of investment. By the analysis of farms 5 and 17, and considering the data in Antônio (2016), some inferences are listed at Table 9.

As shown on Table 9, the number of animals is almost the same; however, the consumption of energy of farms 5 is almost 7 times higher than that of 17. This meant that the economic viability study for the use of biogas on farm

Table 10. Data for farm 16.

Farm	Number of animals	Maximum consumption of electric energy (kWh/month)	Estimation of biogas produced (m³/day)	Estimation of biogas necessary (m³/day)
16	52,430	79,102	9,437.40	1,618.00

Source: Adapted from Antônio (2016

Table 11. Economic viability comparison between the cases.

Case	Worst situation			Best situation		
	NPV (US$)	IRR (%)	SPB (years)	NPV (US$)	IRR (%)	SPB (years)
Case A	(-27,878.62)	-	-	119,282.43	67.37	2.48
Case B	76,956.19	14	5.56	169,097.60	24.71	3.33
Case C	(-119,938.48)	(-15.64)	-	430,347.72	25.47	3.69

Source: Cervi at al. (2010), Lindemeyer (2008) and Antônio (2016).

5 was positive, while for farm 17 presented a dire performance. The electric economy generated on farm 17 is very low in terms of the viability of using biogas for the generation of electricity only for supplying local demand. Should the case arise where the selling of excess electric energy is permitted, farm 17 would also become a viable option. Farm 16 demonstrated a low performance, as shown on Table 8, in the investment study for the use of biogas for the generation of electricity, which is due to the negative value of NPV and IRR that had a value below MARR. This occurred due to the high cost in the construction and maintenance of the biodigester, because as shown on Table 10, the biogas production estimate is much higher than the estimate necessary for farm 16 to become self-sufficient in electric energy. In this way, the savings will be lower than the cost. However, if the possibility to sell the excess electric energy arises, this farm will obtain a large financial return, as it has a high estimative to produce biogas.

Comparison

Observing Table 11, it is possible to see that in each case there is a situation in which the project has a worst and a good performance. This depends on the amount of biogas converted to electricity. The worst situations are when it uses low amount of biogas for electricity production while the best situations used practically all biogas produced.

DISCUSSION

The authors performed an analysis of distinct projects with the aim of investigating if in fact the use of biogas for generating electricity is a good investment. To gain a better understanding into the study, concepts were introduced as to how biogas is produced and how the calculation is performed for its economic indicators, which allows the knowledge if an investment is lucrative. Three cases A, B and C was also presented allowing the realization of the study and the reaching of its conclusions.

Case study A

An explanation for the result obtained in the first study can be reached if one takes into consideration the low use of the potential for biogas production, where only 10.74% of the total biogas produced is in fact being converted into electric energy. In this way, emphasis is also given to the point that to produce 17.1 KWh, only 43% of its nominal power output is being used. Noteworthy also is that, in the second case, the project becomes viable even for the partial use of the nominal power offered by the generator, as can be shown on Table 6, even when the annual cost increase is considered, due to higher utilization, which results in a new cost estimated at US$ 6,567.74.

Case study B

Based on observable data, the second condition, where it was considered that the generator would remain on for 8 h, thus consuming the total generated biogas, one would establish the condition in which near total financial return would be achieved. However, this would only be possible

if the energy utility were to buy the surplus energy produced.

Case study C

The analysis of the third case showed that some laws should be in place to induce the power distribution company to buy the surplus energy would be welcome. Farm 16 is an example of this, as it possesses a high potential for energy production. However, this cannot be fully exploited until it is possible to sell the surplus production to the energy utility.

Conclusion

In situations where the consumption of energy in the form of electricity is considerably less than the capacity of biogas production, as is the situation on small rural properties, the installation of a biogas plant becomes invaluable due to high costs of its installation and maintenance.

However, under balanced conditions, when faced with other alternative sources of energy, biogas is economically viable, as it represents a great investment potential with a guaranteed return, in those cases of high efficiency. In other words, the almost complete conversion of the biogas produced in the installation into electric energy can be achieved. In those cases the surplus should be sold. Nevertheless, there is still the use and/or selling of the biofertilizer generated, and finally the participation in the carbon credits market when such credits are sold through the appropriate entities. All three cases analyzed show that the use of biogas from wastes of pig farm is economically viable, since the biogas is nearly totally used for electrical power generation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Review

Scientific applications and prospects of nanomaterials: A multidisciplinary review

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Nanotechnology is the science of objects <100 nm in size. Research into the development and application of nanomaterials takes a material science and engineering-based approach to nanotechnology. Nanomaterials often possess interesting optical, electronic and mechanical properties. The capacity to construct large, intricate structures with nanometer precision is rapidly increasing and consists several top-down reductive approaches and bottom-up additive approaches to satisfy its applicability in several fields of science. The physicochemical property of nano-functional materials and structural flexibility, promotes its vast application in chemistry and chemical engineering; physics and electronics; biochemistry and medical science, exploration and mining; computer science and engineering. A large pool of information was accessed via several reputable published books and articles, with the sole aim to contribute to the establishment of a stronger theoretical basis for the growing application of nanomaterials in several field of science. It has been established that nanomaterials and advancement in nanotechnology holds great potential in solving several global problems, and if properly harnessed with the right synergy between disciplines or fields in science, would increase the quality of life on Earth.

Key words: Application, disciplines, nanotechnology, nanomaterials, properties, science.

INTRODUCTION

Scientific research in the development of new materials with functional properties for nanotechnology has received global attention and hundreds of products such as sunscreens, electrical gadgets, cosmetics, textiles, and sports equipment; are all based on scientific advancements that have been made. Nanotechnology has found applications in the field of medicine especially in drug delivery, biosensors, and other biomedical applications. Nanomaterials are also being developed for use in environmental applications, e.g. remediation of different environmental compartments via clean-up of environmental pollutants (Lyddy, 2009).

Several applications that were motivated by advances in nanotechnology exist across several disciplines. This is

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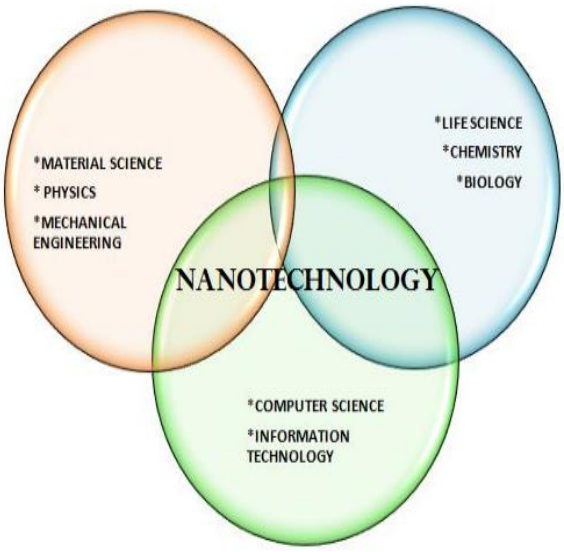

Figure 1. Relationship between Nanotech-nology and several Disciplines

evident in Schummer (2004), where the author sampled and carried out visualization research of 600 publications published in journals considered to be nanotechnology inclined in 2002 and 2003, using the journal subject categories. Also, the work of Grodal and Thoma (2008) identified migration of concepts between nanotechnology and biotechnology. The authors found that the ‘‘nano-biotechnology’’ keyword activity is growing to a greater extent than that of either of the parent nanotechnology or of the biotechnology.

The improvement of the quality of materials via re-arrangement or refining their nano-structures is a form of nanotechnology regarded as incremental nanotechnology. When the size reduction of materials leads to changes in physicochemical properties that provide problem solving potentials and new economic opportunities, then such applications are considered to be examples of what has been termed evolutionary nanotechnology (Jones, 2004). The Venn diagram (Figure 1) gives a pictorial explanation to the relationship that exists in several fields of science and nanotechnology.

In recent times, nanoparticles, nanomembrane and nanopowder have found application in detection and removal of chemical and biological substances such heavy metals (e.g. cadmium, copper, lead, mercury, nickel, zinc), minerals and nutrients (e.g. phosphate, ammonia, nitrate and nitrite), cyanide, trace organics, algae (e.g. cyanobacterial toxins) viruses, bacteria, parasites and pharmaceuticals (e.g. antibiotics). There are four classes of nanoscale materials potentially evaluated as functional materials for water treatment applications; they are metallic nanoparticles, carbon-based nanomaterials, zeolites and dendrimers. Carbon nanotubes and fibers have also shown positive results. Nanomaterials provide better results than other techniques used in water treatment because of its high surface area (surface/volume ratio). Nanoparticles are used in a wide range of applications including pharmaceuticals, cosmetics, medical devices, food ware, clothing and water purification, among other uses, due to their antimicrobial properties (Dhermendra et al., 2008).

Generally, nanotechnology has presented many essential applications in many aspects of oil and gas operations and broad application prospects in oil-field exploration. Various collections of nano-sized materials such as metallic nanoparticles, metal oxide nanoparticles, carbon nanotubes, and magnetic nanoparticles have been widely used in various types of oil and gas operations.

THE CHEMISTRY AND REMEDIAL APPLICATIONS OF NANOMATERIALS

Wastewater treatment processes are designed to achieve improvements in the quality of wastewater. The various treatment processes may reduce: (i) suspended solids, (ii) biodegradable materials, (iii) pathogenic bacteria, (iv) nitrates and phosphates, etc. Wastewater treatment is classified into three types: (a) Primary, (b) Secondary, and (c) Tertiary treatments (Abdel-Raouf et al., 2012). Based on the type of treatment and stages involved in purification, hence, nanomaterials are selected for the effective removal of pollutants and germs from the water systems. Compared to their counterparts in bulk states, carefully synthesized nanomaterials have the merits of better adjustable electronic properties, better tunable optical properties, and higher reactivity (Deniz et al., 2015).

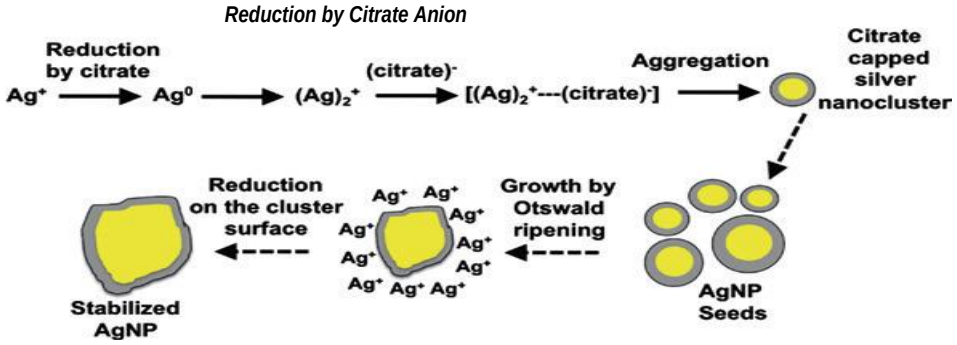

Figure 2. Representation of the nucleation and growth mechanisms for AgNP (Pillai and Kamat, 2004).

Most of the remediation technologies available today are not economical as they involve high costs of chemical consumption (cleaning agents, anti-scaling agents, biocides, etc.), high energy costs, high maintenance costs, low efficiency (≤50%), high costs of concentrate handling; especially when dealing with heavily polluted water e.g. agricultural waste waters, textile waste waters, etc. Furthermore, they are time consuming, particularly the pump-and-treat methods (Sharma and Bhattacharya, 2017). Thus, the capability to remove toxic compounds from surface and sub-surface and other environments are very difficult to access *in situ*, and doing so rapidly, efficiently and within reasonable costs is the ultimate goal and subject to more research. Secondly, the importance of water for domestic and industrial applications cannot be over-emphasized, however the devastating consequences of polluted water is also alarming and attracts global attention.

There are reports of synthetic route which have been used for the production of nanoparticles, they include chemical methods, photochemical methods, electrochemical methods, etc. Nanoparticles can be synthesized using the top-down or bottom-up approach. Research articles have reported different methods for silver nanoparticles (AgNP) synthesis. Most versatile bottom-up approaches include, chemical reduction, photochemical and electrochemical methods. The mechanisms involved in the particle nucleation were discussed as a key to predicting the outcome of any synthetic method. The end use or application of the nanomaterial determines the choice of the synthetic route, and this is not an easy decision as the product size and shape depends on the metal precursor, capping election, temperature of the reaction, amongst other factors (Pacioni et al., 2015). Several nanoparticles have been prepared using the chemical reduction method. The metal salts were reduced using either sodium borohydride, hydrazine or formaldehyde. The metal ions in aqueous solution are expected to produce stable, colloidal particles with appreciable size if treated with strong reducing agent and subsequently with a weaker reducing agent (Landage et al., 2014; Tinwala et al., 2014). Mechanism for the reduction of silver ion to silver nanoparticles (AgNP) using citrate as a reducing agent is described in Figure 2.

Water pollution is a world-wide environmental problem and nanotechnology is an efficient tool which can provide solution to the global menace. Silver nanoparticles among other metal-nanoparticle, are known to exhibit microbial toxicity, with strong biocidal effect yet nontoxic to the human body at low concentration (Harikumar et al., 2010). The antimicrobial potential of biosynthesized Ag nanoparticles for the treatment of water have been reported in literature (Figure 3). Silver materials have been synthesized using *Escherichia coli* and *Klebsiella* species and the *Carica papaya* plant extract. The product was characterized using UV-Visible spectroscopy, scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDS). The silver nanoparticles were adsorbed on granular activated carbon and used as a bacterial filter for treating contaminated water. The enzymatic reaction involved in the synthesis of nanoparticles may be the nitrate reductase provided by the microbe. This enzyme induced by nitrate ions reduces silver, nickel and iron ions to metallic state with zero oxidation number. The possible mechanism that may involve in the reduction of the metal ions is the electron shuttle enzymatic metal reduction process, which was proposed for gold nanoparticles (Harikumar et al., 2010).

Pesticide removal in aqueous solution

Several studies have been carried out to explain the interaction of pesticides in environmental media and the ability of pesticides to get adsorbed on adsorbent has been established (Adeola, 2018; Ololade et al., 2018). Thus, silver oxide nanoparticles loaded or embedded in chitosan beads have been synthesized and applied to remove pesticides from water (Rahmanifar and Dehaghi, 2013). A simple approach was adopted to prepare AgO

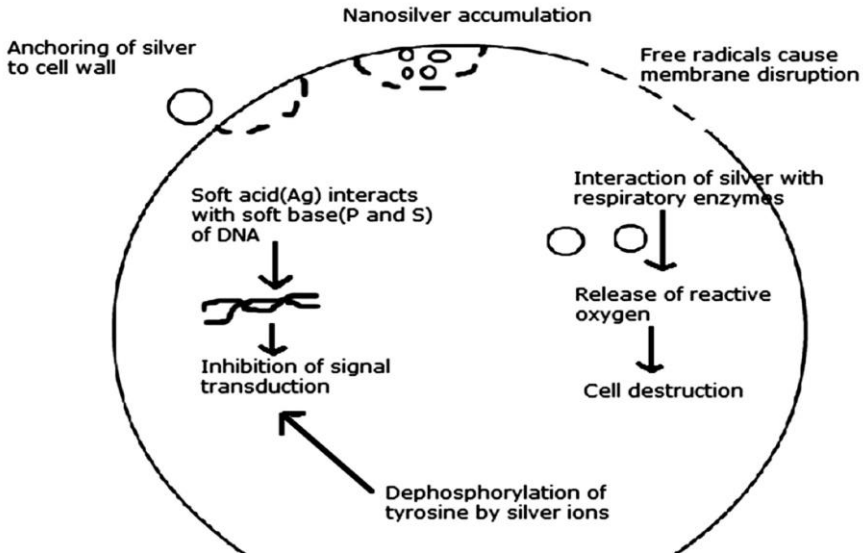

Figure 3. Schematic diagram of antimicrobial activity of AgNP (Prabhu and Poulose, 2012).

nanoparticles on the surface of chitosan. The chitosan-AgO nanoparticles (CS-AgONPs) composite was characterized by infrared spectroscopy (FTIR), X-ray diffraction (XRD), and SEM. The CS-AgONPs composite beads were optimized to remove maximum permethrin (pesticide), while varying parameters such as the amount of sorbent, agitating time, initial concentration of pesticide, and pH parameters. The optimum conditions, room temperature, pH 7, and the CS-AgONPs, the removal efficiency was 99% pesticides of permethrin solution (0.1 mg L^{-1}) which was determined by using UV spectrophotometer at a wavelength of 272 nm. While comparing experimental data with the pure chitosan, the percent removal efficiency of CS-AgONPs beads has been enhanced by 49%. The CS-AgONPs composite beads possess high adsorption capacity as an adsorbent which has potential as a new nano-scale, eco-friendly strategy for pesticide pollution remediation (Altaher, 2012; Zhu et al., 2012).

Dye removal via adsorption

Nickel nanoparticles have also been synthesized, characterized and applied to decolourize dye effluent in aqueous solution (Kale and Kane, 2016). C. I. Reactive Blue 21 was taken as a reference dye and polyvinyl pyrrolidone (PVP) as a stabilizer to prevent agglomeration of nanoparticles. Experimental parameters such as pH, dye initial concentration, nanoparticle concentration, alkali addition, salt addition and contact time was studied for dye decolourization or degradation. To ascertain the attachment of metabolites of dye on the nanoparticles, FT-IR analysis was done. About 98% colour removal efficiency was recorded with concurrent reduction in chemical oxygen demand (COD) (Nateghi et al., 2012).

Abou-Gamra and Ahmed (2015) carried out a similar research involving a successful route for synthesis titania nanoparticles by controlled sol-gel progress. Chitosan as bio-template was used as stabilizer in the synthesis to increase the surface area and create a defined particle and enhanced pore structure. The crystalline behavior and the nanostructure of the synthesized nanoparticles were elucidated using X-ray diffraction (XRD) and transmission electron microscope (TEM). Result obtained depicts that a transition in sample crystallography from anatase to completely amorphous nanoparticles upon adsorption of malachite green dye indicates a strong adherence of the dye which led to a breakdown in the crystalline morphology of titania sample (Li et al., 2013).

The remediation of dye in that aqueous system has been studied over wide range of dye concentrations and dosage of catalyst sample was varied (Shu et al., 2015; Olaremu and Adeola, 2018). Adsorption isotherms was studied using Freundlich, Langmuir, Temkin and Dubinin models to ascertain the mechanism of adsorption and calculate the maximum adsorption capacity and correlation coefficients (Deniz et al., 2015; Ololade et al., 2018). The kinetics of adsorption process is well investigated using different models as pseudo first order, pseudo second order, Elovich, Morris and Weber. The adsorption isotherm indicates the adsorption capacity of 6.3 $mg \cdot g^{-1}$ TiO_2. The value of enthalpy change ($\Delta H°$) for malachite green dye adsorption is 19 kJ/mol, which indicates that the removal process is endothermic. The adsorption process follows pseudo-second order rate equation and the negative values of standard free energy

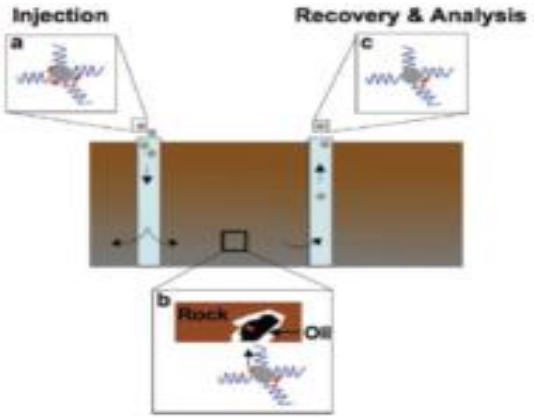

Figure 4. Hydrocarbon detection by nano-reporter (Jacob et al., 2011).

(ΔG°) suggest that the adsorption process is spontaneous (Ghaedi, 2012; Abou-Gamra and Ahmed, 2015).

GEOPHYSICAL AND GEOLOGICAL APPLICATIONS OF NANOMATERIALS

The unique physical and chemical properties of nanomaterials have led to their application in geophysical exploration hydrocarbon, reservoir characterization, drilling, cementing, production and stimulation, enhanced oil recovery (EOR), refining and processing (Munawar et al., 2017). This review article presents brief discussion on the most recent development of nanomaterials and their roles in new or enhanced applications in the exploration and mining industries.

Hydrocarbon exploration and reservoir characterization

One of the most essential yet expensive and high-risk activities in oil and gas industry is hydrocarbon exploration. The objective of this process is simply to find hydrocarbon accumulations beneath the earth's surface. However, it often presents many unique challenges such as the unexpected hazard, which may greatly increase the total cost of production. Many conventional sensing methodologies, with exception of seismic techniques, can only provide little information about reservoir as they can only penetrate a few inches from the wellbore. Current sensing technologies are still unable to obtain high-resolution reservoir imaging and lacking on the ability to penetrate deeply into reservoir to get key information about reservoir characteristics. Furthermore, many sensing techniques like conventional electrical sensors are often unable to provide reliable information at certain extreme reservoir conditions. Despite the advancement of recent state-of-the-art exploration techniques such as 3D and 4D seismic surveys, new, simple, low-cost, non-damaging and sensitive sensing technologies that can accurately locate hydrocarbon accumulation are still desired. The integration between new reservoir mapping and computational strategies is also needed to attain better discovery, sizing, and characterization of reservoirs (Kong and Ohadi, 2010).

Implementation of nanotechnology in accurate prediction of hydrocarbon accumulations and characterization of hydrocarbon reservoirs has been extensively studied. Owing to the virtue of their size-dependent optical, magnetic, chemical and electrical properties, nanoparticles can be used as nanosensor as they would easily migrate through pores of the surrounding geological structures and collect information about the reservoir characteristics. A new sensing technology based on nanoparticles also enables one to probe rock properties in deeper reservoir regions and to obtain data about the complex interaction between reservoir rock and fluids or distribution of immiscible fluids. Polyvinyl alcohol functionalized with oxidized carbon black effectively act as hydrophobic compound in variety of oil field types and releases the compound when rock contains the hydrocarbon, which helps in detection of *in-situ* hydrocarbons in the reservoir as shown in Figure 4 (Jacob et al., 2011).

Drilling and completion

Drilling is one of the most crucial processes in creating access to hydrocarbon reservoir rocks for producing the crude oil and natural gas. The well is created by drilling a hole of 5 to 50 inches in diameter into the earth with a drilling rig that rotates a drill string with a drill bit. Nanoparticles are used in drilling and completion, such as, clay stabilization, enhanced viscosity of drilling fluids, and fluid loss control, sloughing (wall collapse) control, stability of well bore, torque and drag friction, hydraulic fracturing and cementing, etc. In broad-spectrum, several types of additives (commonly polymers) are used to enhance the properties and performances of drilling fluids such as in thermal stability, salty resistance, filter cake generation, rheological and filtration properties. Conversely, the use of several types of nanoparticles has also been reported in drilling fluids formulation recently. For example, several studies have revealed that the presence of nanoparticles in drilling fluid has contributed to the formation of effective, dense, thinner and impermeable cake as sealing for micro-cracks during drilling operation (Cheraghian et al., 2013; Yao et al., 2014).

Production and stimulation

One of the greatest challenges in the current oil and gas productions is the recovery from unconventional

resources such as heavy and extra heavy oil, shale gas and liquid, tight gas and oil, coal bed methane (CBM), and bitumen hydrocarbons due to the nature of their physical and chemical properties as well as their geological difficulties. Recently, the development of nanotechnology has enabled one to effectively and efficiently harvest hydrocarbon from unconventional resources. For example, several types of nanocatalysts such as nano-sized transition metals and metal oxide nanoparticles have been used in aquathermolysis process for the improvement of heavy and extra heavy oil production (Khalil et al., 2015; Chen et al., 2009). In aquathermolysis process, it is believed that the significant improvement of oil production is mainly due to the reduction of oil viscosity as a result of degradation of large hydrocarbon molecules such as resin and asphaltene. It is reported that there is a great possibility that some chemical reactions such as hydrocracking, hydrodesulphurization (HDS), hydrodenitrogenation (HDN), and hydrogenation can occur during the process. In addition, it is also believed that one of the main reasons for the degradation of these large molecules is due to the cleavage of CaX (X = S, N, O) bonds (Maity et al., 2010).

Cementing

It has been widely known that wellbore failures and well integrity issues due to cementing and/or cementing stability issues are considered as one of the major problems in oil and gas exploration and production. Typically, these problems may occur during preproduction such as in drilling operation and during production processes (Teodoriu et al., 2013). During drilling operation, several cementing problems such as casing centralization (incomplete cementing), formation damage due drilling operations and cementing, inadequate cement-formation or cement-casing bond, cement shrinkage, incomplete cement placements, filtration of cement slurry, contamination of cement by drilling or formation fluids, and fracture formation with cement can seriously affect well integrity.

The development of smart cement materials based on nano-sized materials as additives with desired specific properties that solve or minimize many practical issues in the field has widely been reported in literatures. For example, several types of metal oxide nanoparticles such as nanosilica (Lin et al., 2008; Jo et al., 2007; Qing et al., 2007), TiO_2 (Nazari and Riahi, 2010a, 2011b), Fe_2O_3 (Li et al., 2004; Khoshakhlagh et al., 2012), Al_2O_3 (Nazari and Riahi, 2011, 2012), ZrO_3 (Nazari and Riahi, 2010a, 2011b), CuO (Nazari and Riahi, 2011), ZnO_2 (Nazari and Azimzadegan, 2012), and several other types of magnetic nanoparticles (Blyszko et al., 2008) have been used as additives in cement modification. These metal oxide nanoparticles are added mostly to improve several cements and concrete properties such as strength, resistance to water penetration, accelerate hydration reaction, control calcium leaching, to provide self-cleaning properties, and many more.

Refining and fuel production

Unlike in the upstream and midstream sectors, nanotechnology and nanomaterials have been used for over a decade in downstream sector of oil and gas industry, mainly in refining and processing process. One of the most common applications of nanomaterials in the oil refining and petrochemical industry is in the utilization of nanoparticle-based catalysts (Wei et al., 2007). Over the last several years, the advancement in nanotechnology has contributed substantially in the development of more effective and efficient refining and processing processes in converting crude hydrocarbon into useful products such as liquefied petroleum gas (LPG), gasoline, kerosene, jet fuel, diesel, and other valuable chemical feedstock. Nanotechnology has allowed researchers to develop catalysts that can substantially increase refining capacity and speed, improve the efficiency of hydrocarbon conversion, reduce or even eliminate catalyst poisoning issue, and provide better refining efficiency for extra heavy and sour crude oils (Okunev et al., 2015).

Lately, the application of nanomaterials in conversion and upgrading process of heavy crude oil and its derivatives have attracted many attentions since nanocatalysts provide a large surface area for the appropriate catalytic reactions. Over the last few years, different types of nanocatalysts such as metal oxide nanoparticles have been used in hydroprocessing of crude oil due to their good asphaltenes adsorption/ oxidation, and their high oxygen storage/release capacity. For example, Montoya et al., (2016) recently investigated the effect of NiO and PdO supported on fumed silica nanoparticles catalysts in catalytic thermal cracking of n-C7 asphaltenes. Based on their results, it is found that the presence of NiO or PdO was able to show a better catalytic activity than fume silica support alone.

Physicochemical properties of nanomaterials

Size and surface effect

As the unconventional oil and gas resources are developed further, more and more conventional chemicals cannot satisfy the reservoir injection requirements. Nanomaterials can not only improve the injection effectively, but also present peculiar penetrating capacity, especially in unconventional oil and gas resources. It is the small-size effect that increases the diffusion rate of chemicals in reservoirs greatly and injects the nano-fluid

into the target areas in the reservoirs, to enhance the recovery factor significantly (Ayatollahi and Zerafat, 2012).

Nano-particles also have strong surface effect. The specific surface area of nanoparticles is large, so the bond strength of chemical bonds between nanoparticles and other media (e.g., mineral surface and metal salt) is increased. The surface of all nanomaterials (oxide of silicon, vanadium, molybdenum, and tungsten) is enriched with active modification sites (e.g., end oxygen and bridge oxygen), which provide the basis for stabilization modification and improvement at the surface of nanomaterials. Only the nanochemicals whose surface is modified present the special properties of wettability alteration, micro-particle migration inhibition, nanofiltration and shear thickening so that they can satisfy the actual requirement at each stage of oilfield development (Liu et al., 2016)

Nanometer photocatalytic property

Nanometer photocatalytic agent has the redox ability under ultraviolet radiation, so nanometer photocatalytic technology is used to purify contaminants. This technology is especially suitable for the purification of organic matter, and it is of great potential in purifying deeply the oilfield sewage (Xu et al., 2010). Nanometer photocatalytic agent is usually acted by TiO_2, whose photocatalytic reaction happens only after being excited by ultraviolet light (wave length less than 385 nm) (Li and Xu, 2010).

Shear thickening property

Existing water plugging and profile control materials are mainly acted by gel, volume expansion particle and polymer microsphere. These materials cannot be deformed by themselves and their physical and chemical properties do not vary with the ambient conditions. The shear thickening property of nanomaterials provides a technical solution to deal with this situation. Shear thickening fluid (STF) consists of shear thickening liquid, shear thickening gel, etc. Bender and Wagner (1996) described the shear thickening mechanism of this type of nanomaterial.

Nano-corrosion and wear resistance

In the sector of oil drilling engineering, the surface and down hole tools suffer complex environments of wear, corrosion, high temperature, high pressure, high H_2S and high CO_2 content, which result in tool damage and corrosion, cost increase and production decline and increase negative impact (e.g., operation hazard and environment pollution). For example, the key vulnerable parts (e.g., drilling bit, expansion cone, plunger, rotator and polished rod) may be improved by using high-performance nanocoating. The new high-hardness wear-resistance nanocoating is different from the traditional wear-resistance coating (e.g., single-phase nanocrystalline). The new nanocoating mainly performs periodic modulation on microstructures by using two-phase ceramics to form multilayer nanomembrane structures of coherent and epitaxial growth, so that the vulnerable parts are characterized by high hardness and wear resistance (Liu et al., 2016).

Particle migration inhibition

In the process of oilfield development, mineral micro-particles migrate at different levels, decreasing the permeability of porous media and damaging the reservoirs. Some solutions can be developed by using nanomaterials or emulsion. The nano-fluids containing nano-particles (magnesia, silica, and alumina) have lower oil-water interfacial tension and strong adsorption tendency. The research results by Al-Malki et al. (2016) showed that if drilling fluid is added with sepiolite nano-particles, its rheological stability is kept and filtration resistance and clay swelling inhibition are improved.

NANOTECHNOLOGICAL ADVANCEMENT IN PHYSICS AND ELECTRONICS

Nanomaterials are materials which possess single unit and size in at least one dimension to the order 10^{-9} m. (Figure 6) Usually, 1 to 100 nm is the usual definition of nanoscale (Buzea et al., 2007). Nanomaterials research takes a materials science and engineering-based approach to nanotechnology, leveraging advances in materials synthesis, micro-fabrication and other developmental research.

Nanomaterials often possess both interesting optical and electronic, or mechanical properties (Hubler and Osuagwu, 2010). Nanomaterials and nanophysics focus on designing, fabricating and controlling materials and its components on the nanoscale dimension.

Nanotechnology can be used to develop new optic and electronic components and new materials for use in communications technology, sensor technology or catalysis. Nanophysics focus on the special electronic and optical characteristics of nanomaterials such that there are numerous possibilities for development of nanotools and nanodevices.

Sources of nanomaterials

Engineered

Nanomaterials have been deliberately engineered and

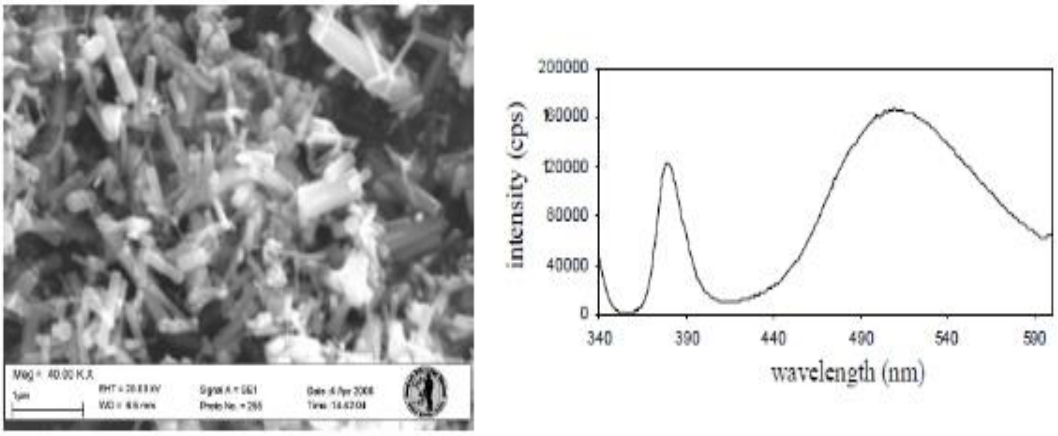

Figure 5. SEM image and photoluminescence spectral of ZnO nanopowders (Pereira et al., 2006).

manufactured in the laboratory placing premium on certain properties over the others (US NIOSH, 2013). The practice of engineering on the nanoscale is called nanoengineering.

Incidental

Nanomaterials have overtime been produced incidentally as a byproduct of mechanical or industrial processes (Sahu and Casciano, 2009).

Natural

Biological systems are characterized by both natural and functional nanomaterials (Figure 9). The structure of foraminifera (mainly chalk) and viruses (protein, capsid), the wax crystals covering a lotus or nasturtium leaf, spider and spider-mite silk (Novel Natural Nanomaterial Spins Off from Spider-Mite Genome Sequencing, 2015).

Application of nanophysics

Semiconductor

Zinc Oxide (ZnO) is a wide bandgap semiconductor and it has been the subject of considerable research due to its potential applications in the areas of photonics, electronics and sensors. Nano-ZnO offers several advantages over existing biosensing platforms, most notably a large surface area for greater bio-functionalization and an inherent photoluminescence (PL) signal, which consist of two emission peaks. The first peak is in the UV region, due to near band edge emission while the other is in the visible (green) region, due to oxygen vacancies caused by crystalline defects (Jason et al., 2006). Two specific semiconducting nanocrystals of interest are titanium dioxide (TiO_2) and zinc oxide (ZnO), however, utilizing TiO_2 as an optical sensing material may be difficult. Concerns regards it inability to optically detect a real-time binding event due to the single broad visible emission band unlike Nano-ZnO which is a wide band gap material with a high exciton binding energy (60 meV) that contains an inherent photoluminescence (PL) signal consisting of two emission peaks. One peak is in the UV, due to near band edge emission and the other is in the visible (green) region, due to oxygen vacancies caused by crystalline defects (Figure 5) (Jason et al., 2006).

In a bid to overcoming some of the current sensing platform limitations, efforts are focused on semiconducting nanocrystalline materials. Large surface area, mechanical and thermal stability, and inherent photoluminescence signal (Lei and Zhang, 2001; Banerjee et al., 2004) make them promising materials for an optically responsive sensing platform. Nano-ZnO has recently been demonstrated as a gas sensor by utilizing changes in its electrical resistivity (Guo et al., 2000; Zhiyong et al., 2004; Zhiyong and Jia, 2005).

Semiconductor nanocrystals (NCs) are made from a variety of different compounds. They are referred to as II-VI, III-V or IV-VI semiconductor nanocrystals based on the periodic table groups into which these elements are formed. For example, silicon and germanium are group IV, GaN, GaP, GaAs, InP and InAs are groups III-V, while those of ZnO, ZnS, CdS, CdSe and CdTe are groups II-VI semiconductors (Sagadevan, 2013). These novel properties of semiconductor nanomaterials have attracted significant attention in research and applications in emerging technologies such as nanoelectronics, nanophotonics, energy conversion, miniaturized sensors and imaging devices, solar cells, detectors, etc.

Nanoelectronics

Nanoelectronics refer to the use of nanotech in

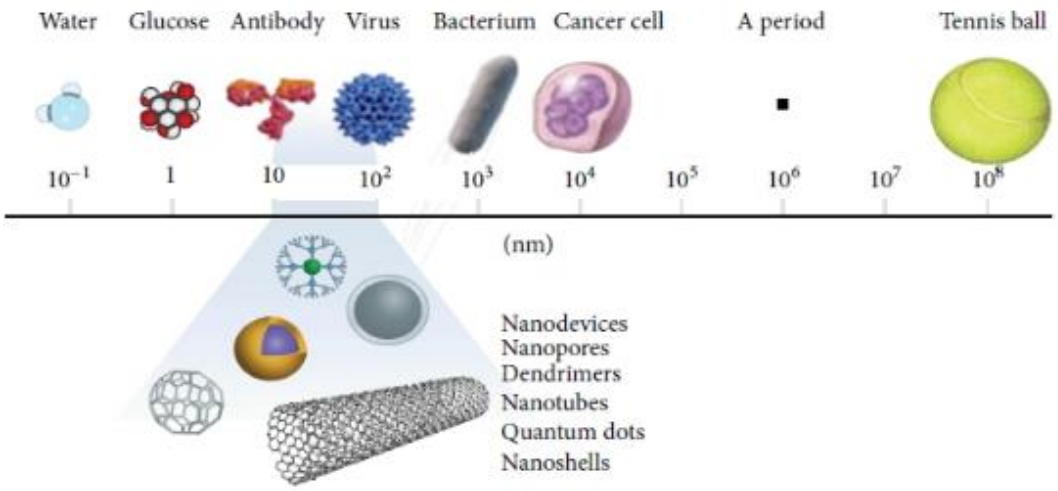

Figure 6. A size comparison of nanoparticles with other larger-sized materials (Amin et al., 2014).

fabricating electronic components. Examples include: hybrid molecular/semiconductor electronics, one dimension nanotubes (nanowires) (e.g. Silicon nanowires/ Carbon nanotubes), electron Transistor or advanced molecular electronics (e.g. New silicon CMOS). Nanoelectronics are sometimes considered as disruptive technology because they are significantly different from traditional electronic components. For example the electron transistors, which involve transistor operation based on a single electron: besides being small and allowing more transistors to be packed into a single chip, the uniform and symmetrical structure of nanowires and/or nanotubes allows a higher electron mobility, a higher dielectric constant (faster frequency), and a symmetrical electron/hole characteristic (Goicoechea et al., 2007).

Nanoelectronic device includes computer processors (nanomaterials such as nanowires or small molecules in place of traditional CMOS components, field effect transistors now operational using both the semiconducting carbon nanotubes (Postma et al., 2001), memory storage which uses a carbon nanotube based memory (called Nano-RAM) and the Hewlett-Packard has proposed the use of memristor as a replacement of Flash memory). Optoelectronic devices are replacing the traditional analog electronic devices due the enormity of their bandwidth, Displays (Silicon nanowires and carbon nanotubes), Quantum computing (which rely heavily on the understanding and application of the quantum nature/behavior of atomic charge carriers), etc.

Material science and engineering

Material engineering process dates to the proper understanding of surface physics such that engineered materials are deliberately made by humans to possess certain required characteristics for specific functionality. Most current nanomaterials could be organized into four types: Carbon Based Materials, Metal Based Materials, Dendrimers, and Composites. One outstanding application of nanotechnology in material engineering involves providing affordable solutions to water/ wastewater treatments by the use of nanoparticles/fibers for the removal of pollutants from water/wastewater (Abdo, 2016). This process does not rely on large infrastructures or centralized systems (Amin et al., 2014). Developments in nanoscale research have made it possible to invent economically feasible and environmentally stable treatment technologies and one of such suggested that nanotechnology can adequately address many of the water quality issues by using different types of nanoparticles and/or nanofibers (Savage and Diallo, 2005).

Carbon nanotubes (CNT) are one of an illuminative example for the potential of nanotechnology. The tensile strength of high carbon steel is around 1.2 GPa but the tensile strength of carbon nanotubes (CNT) is 63 GPa. While the longer carbon nanotubes will increase inter-tube contact areas and therefore yield higher tensile strength, decreasing the CNT diameter may also increase the yarn tensile strength (Mottal et al., 2005; Zhang et al., 2007a, b; Liu et al., 2008). Also they are known to be one of the strongest materials by nanotechnology so far. Figure 7 shows some examples of different type of nanomaterials based on nano-engineered dimension.

NANOTECHNOLOGICAL CONTRIBUTIONS TO COMPUTER SCIENCE

Major advancements in computer science began with miniaturization. The idea of miniaturization started in the

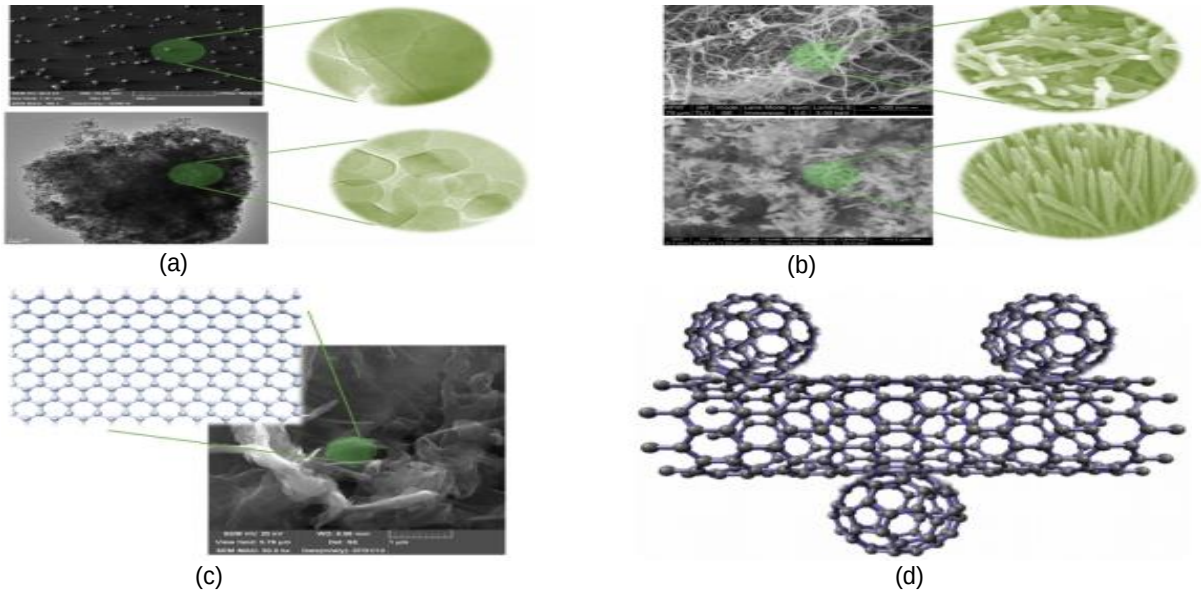

Figure 7. (a) Typical scanning electron microscopy (SEM) image of 0-D nanostructured materials; silver nanoparticles (upper image) and titania nanoparticles (lower image) with transmission electron microscopy (TEM) of each (right side). (b) Typical scanning electron microscopy (SEM) image of 1-D nanostructured materials: carbon nanotubes (upper image) and zinc oxide nanorods (lower image) with enlarged part of each image. (c) Typical scanning electron microscopy (SEM) image of graphene nanosheet and its structure as an example of 2-D nanostructured materials. (d) Carbon nanobud formed of carbon nanotube and fullerene as an example of 3-D structure (Abdo, 2016).

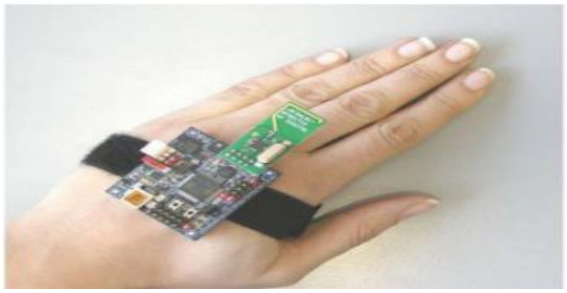

Figure 8. iNEMO board placed on hand (Brigante et al., 2011).

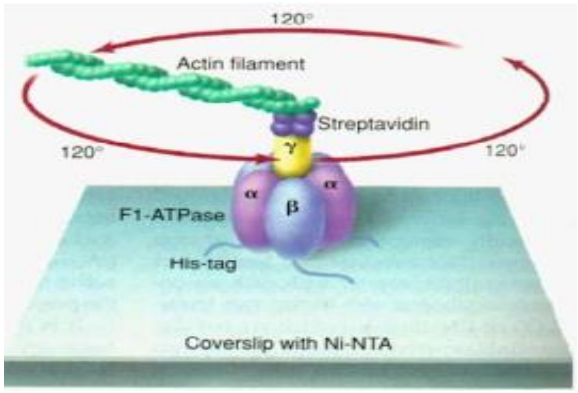

Figure 9. The molecular motor protein F1-ATPase (A naturally occurring nanomachine) (Nikalje, 2015).

early 1980s and it is regarded as the foundation of nanotechnology (Mamalis, 2007). The work of Kostoff et al. (2007) defined nanotechnology as the development and use of techniques to study physical phenomena and construct structures in the physical size range of 1 to less than 100 nm, as well as the incorporation of these structures into applications. Several researchers have worked on the area of nanotechnology and its applications (Zahn, 2001; Chen and Bruce, 2012). Porter and Youtie (2009) carried out extensive research to emphasize that nanotechnology is interdisciplinary. The result showed that nanotechnology cuts across almost all disciplines, most especially computer science. Nanotechnology is made possible through technological advances made in several disciplines (Mamalis, 2007).

The techniques used for nanotechnology applications are the energy beam processes, based on the principle that the energy carried on a beam can remove material by melting, vaporization or ablation (Chen and Bruce, 2012).

The most significant application of nanotechnology is in the production of microchips. Example of this is in Brigante et al., (2011), where a wearable system for real-time human motion capture called iNEMO was developed. The developed system makes use of nanotechnology to embed several MEMS sensors on its board (Figure 8).

The developed system, due to its performance, size, and weight can be easily embedded in a tracksuit for total

body motion reconstruction. Also, the production cost for the iNEMO system when compared with other systems that performs the same function reduced by a factor of about eight. This was made possible by advances in nanotechnology.

Nanotechnology has immeasurably improved and revolutionized information technology. Early research on the application of nanotechnology in the Computer Science field such as Heath et al. (1998), developed a defect fault tolerant computers using nanotechnology. The developed system called “Teramac”, incorporates a very high communication bandwidth that enables it to easily route around defects, and also paved way for future nano metre scale computer paradigm. The work concluded that future nanoscale computers may consist of extremely large configuration memories that are programmed to perform specific tasks.

In recent time, nanoscale transistors that are faster, more powerful, and energy-efficient are being developed; soon our computers’ entire memory may be stored on a single tiny chip (Kumar et al., 2014). Computer scientists believe that nanotechnology will eventually bring them closer to the goal of creating computer systems that can simulate and emulate the brain's abilities for sensation, perception, action, interaction and cognition (Berger, 2010). Nanotechnology has witnessed four generations till date (Ullah, 2012), and the fourth generation of nanotechnology basically deals with the manufacturing and development of nano computers.

APPLICATIONS OF NANOTECHNOLOGY TO BIOCHEMISTRY AND MEDICAL SCIENCES

Nanotechnology is a leading scientific technique that offers sensing technologies and miniature devices to diagnose disease accurately and within time. There is wide range of applications of nanotechnology in the field of drug delivery and furthermore, to simplify the oral absorption of proteins and peptides nano carriers are modified with specific ligands (Veiseh et al., 2015). Nanotechnology holds a promising future in the field of Biochemistry both in Clinical Biochemistry and in Food and Nutrition Biochemistry. Diseases like diabetes, cancer, Parkinson’s disease, Alzheimer’s disease, cardiovascular diseases and multiple sclerosis as well as different kinds of serious inflammatory or infectious diseases (e.g. HIV) constitute a high number of serious and complex illnesses which are posing a major problem for the mankind. Nano-medicine is an application of nanotechnology which works in the field of health and medicine. Nano-medicine makes use of nano materials and nano electronic biosensors.

Clinical biochemistry

Drug delivery

In nanotechnology nano particles are used for site specific drug delivery. In this technique, the required drug dose is used and side-effects are lowered significantly as the active agent is deposited in the morbid region only. This highly selective approach can reduce costs and pain to the patients. Thus variety of nano particles such as dendrimers, and nano porous materials find application. Micelles obtained from block co-polymers, are used for drug encapsulation. They transport small drug molecules to the desired location. Similarly, nano electromechanical systems are utilized for the active release of drugs. Iron nano particles or gold shells are finding important application in the cancer treatment. A targeted medicine reduces the drug consumption and treatment expenses, making the treatment of patients cost effective. Nano medicines used for drug delivery are made up of nano scale particles or molecules which can improve drug bioavailability. For maximizing bioavailability both at specific places in the body and over a period of time, molecular targeting is done by nano engineered devices such as nano robots (Cavalcanti et al., 2008). The molecules are targeted and delivering of drugs is done with cell precision.

Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease and affects one in every 100 persons above the age of 65 years. PD is a disease of the central nervous system; neuro inflammatory responses are involved and lead to severe difficulties with body motions. The present day therapies aim to improve the functional capacity of the patient for as long as possible but cannot modify the progression of the neurodegenerative process (Ravichandran, 2009a, b)

The aim of the applied nanotechnology is regeneration and neuro protection of the central nervous system (CNS) and will significantly benefit from basic nanotechnology research conducted in parallel with advances in neurophysiology, neuropathology and cell biology. The efforts are taken to develop novel technologies that directly or indirectly help in providing neuro protection and/or a permissive environment and active signaling cues for guided axon growth. In order to minimize the peripheral side-effects of conventional forms of Parkinson's disease therapy, research is focused on the design, biometric simulation and optimization of an intracranial nano-enabled scaffold device (NESD) for the site-specific delivery of dopamine to the brain, as a strategy. Peptides and peptidic nano particles are newer tools for various CNS diseases. Nanotechnology will play a key role in developing new diagnostic and therapeutic tools. Nanotechnology could provide devices to limit and reverse neuro pathological disease states, to support and promote functional regeneration of damaged neurons, to provide neuro protection and to facilitate the delivery of drugs and small

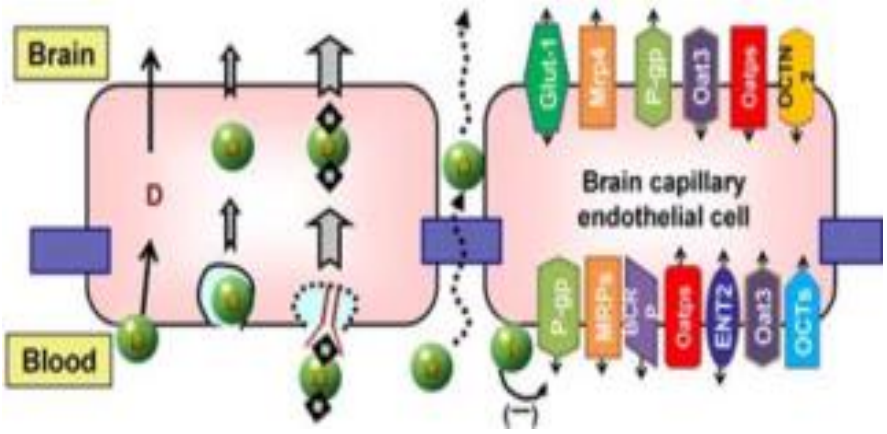

Figure 10. Delivery of nano medicine to CNS through BBB (Nilkaje, 2015).

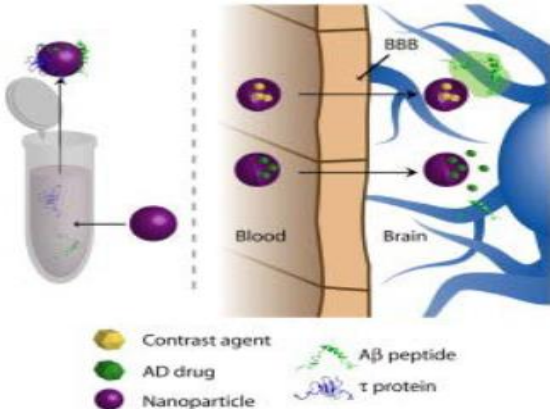

Figure 11. Use of nano particles in Alzheimer's disease (Nilkaje, 2015).

molecules across the blood-brain barrier.

Nikalje (2015) reported that for the delivery of CNS therapeutics, various nano carriers such as dendrimers, nano gels, nano emulsions, liposomes, polymeric nano particles, solid lipid nano particles, and nano suspensions have been studied (Figure 10). Transportation of these nano medicines has been effected across various *in vitro* and *in vivo* BBB models by endocytosis and/or transcytosis, and early preclinical success for the management of CNS conditions such as, Alzheimer's disease, brain tumors, HIV encephalopathy and acute ischemic stroke has become possible. Future development of CNS nano medicines needs to focus on increasing their drug-trafficking performance and specificity for brain tissue using novel targeting moieties.

Alzheimer's disease

Worldwide, more than 35 million people are affected by Alzheimer's disease (AD), which is the most common form of dementia. Nano technology finds significant applications in neurology. These approaches are based on the, early AD diagnosis and treatment is made possible by designing and engineering of a plethora of nano particulate entities with high specificity for brain capillary endothelial cells. Nano particles (NPs) have high affinity for the circulating amyloid-β (Aβ) forms and therefore may induce "sink effect" and improve the AD condition. *In vitro* diagnostics for AD has advanced due to ultrasensitive NP-based bio-barcodes and immune sensors, as well as scanning tunneling microscopy procedures capable of detecting Aβ1-40 and Aβ1-42. The recent research on use of nanoparticles in the treatment of Alzheimer's disease is as shown in Figure 11 (Davide et al., 2011).

Tuberculosis treatment

Tuberculosis (TB) is the deadly infectious disease. The long duration of the treatment and the pill burden can hamper patient lifestyle and result in the development of multi-drug resistant (MDR) strains. Tuberculosis in children constitutes a major problem. There is commercial non availability of the first-line drugs in pediatric form. Novel antibiotics can be designed to overcome drug resistance, cut short the duration of the treatment course and to reduce drug interactions with antiretroviral therapies. A nanotechnology is one of the most promising approaches for the development of more effective and compliant medicines. The advancements in nano based drug delivery systems for encapsulation and release of anti-TB drugs can lead to development of a more effective and affordable TB pharmacotherapy.

Food and nutrition biochemistry

Food technology is regarded as one of the industry sectors where nanotechnology will play an important role

in the future (The Eleventh ASEAN Food Conference, 2009). It is commonly distinguished between two forms of nanofood applications: food additives (nano inside) and food packaging (nano outside). Nanoscale food additives may for example be used to influence product shelf life, texture, flavor, nutrient composition, or even detect food pathogens and provide functions as food quality indicators. In the context of food packaging, nano-technologies are mainly considered to be of use to increase product shelf life, indicate spoilt ingredients, or generally increase product quality, e.g., by preventing gas flow across product packaging (Nickols-Richardson and Piehowski, 2008).

For food applications, nanotechnology can be applied by two different approaches, either “bottom up” or “top down” (Ravichandran, 2009a, b). The top down approach is achieved basically by means of a physical processing of the food materials, such as grinding and milling. For example, dry-milling technology can be used to obtain wheat flour of fine size that has a high water-binding capacity (Degant and Schwechten, 2002). This technology has been used to improve antioxidant activity in green tea powder. As the powder size of green tea is reduced to 1000 nm by dry milling, the high ratio of nutrient digestion and absorption resulted in an increase in the activity of an oxygen-eliminating enzyme (Shibata, 2002).

By contrast, self-assembly and self-organization are concepts derived from biology that have inspired a bottom-up food nanotechnology. The organization of casein micelles or starch and the folding of globular proteins and protein aggregates are examples of self-assembly structures that create stable entities. Self-organization on the nanometer scale can be achieved by setting a balance between the different noncovalent forces (Dickinson and Van, 2003). The electron microscope and, more recently, the development of tools such as probe microscopes have provided unparalleled opportunities for understanding heterogeneous food structure at the submolecular level (Chaudhry et al., 2008). This has provided new solutions to previously intractable problems in food science and offers new approaches to the rational selection of raw materials, or the processing of such materials to enhance the quality of food products.

CONCLUSION AND FUTURE PROSPECTS

In the light of exponential increase in world population and the growing rate of environmental pollution from domestic and industrial indiscriminate release of chemicals to the environment, the development and application of nano-remediation strategy is needful and must be further explored. Several metal and metal oxides nanoparticles have biocidal activities on harmful microorganisms and biodegradative properties on chemicals such as dyes, pesticides and other pollutants. The “easy to handle, easy to recover” attributes of nanomaterials especially magnetic nanomaterials, provide a vast research potential for how nanotechnology would provide an easier, cheaper and effective means of saving our environment from imminent collapse due indiscriminate release of harmful substances that can lead to disease outbreak, global warning as a result of ozone depletion, poisoning from polluted water, agricultural products, etc.

With the rapid development of nanotechnology, it is predicted that new crucial technologies will arise successively in the future. The future of nanotechnology in computer science and even other fields of science is hinged on the development new systems capable of promoting and enhancing scientific revolution and evolution in world of research, development and technology. In the field of computer science, new quantum computers are being designed, which will allow all electronic systems like computers, storage devices, mobile phones, power, sensors, and artificial intelligent systems to fit onto a micro-chip. Miniaturization which is a concept of reduction in size, for convenience and ease of transportation, yet effective is the future of computer and other electronic gadgets. Such systems cannot be developed except suitable nano-functional materials are developed.

Generally, nanotechnology has presented many essential applications in many aspects of oil and gas operations and broad application prospects in oilfield exploration. Various collections of nano-sized materials such as metallic nanoparticles, metal oxide nanoparticles, carbon nanotubes, and magnetic nanoparticles have been widely used in various types of oil and gas operations. In the future, the oilfield development technologies have to be equipped with “objective orientation” and “complex function”, and nanomaterials provide the technical feasibility for it (Liu et al., 2016). For example, nano-molecular deposition film can be used to decrease the pressure and increase the injection rate of low-permeability oil reservoirs. Intelligent nano-fluid can be used for water plugging and profile control. Nano-particles can be used to improve drilling fluid behavior. Nano-catalyst and nano-filter membrane can be used for *in-situ* oil reservoir stimulation and late water treatment. Nano-coating can be used for engineering anti-corrosion.

In the future, nano medicine will benefit molecular nanotechnology. The medical area of nano science application has many projected benefits and is potentially valuable for all human races. With the help of nano medicine early detection and prevention, improved diagnosis, proper treatment and follow-up of diseases is possible. Certain nano scale particles are used as tags and labels, sensors capable of monitoring biological processes quickly (Figure 9), testing has become and will become more sensitive and more flexible. Gene sequencing will be more efficient with the invention and

possible optimization of nano devices like gold nano particles, which if tagged with short segments of DNA can be used for detection of genetic sequence in a sample. With the help of nanotechnology, damaged tissue can be reproduced or repaired. The invention of nano-engineered artificially stimulated cells can be used in tissue engineering, which might revolutionize the transplantation of organs or artificial implants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparison of embryo developmental rates in Nguni, Bonsmara and Boran beef cattle breeds following *in vitro* fertilization and artificial insemination

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The aim of this study was to compare the embryonic developmental rates in the Southern African cattle breeds. To do this, cryopreserved semen straws from Nguni, Bonsmara, and Boran bulls were thawed at 38°C and evaluated for sperm motility characteristics using Sperm Class Analyser (SCA). The fertilizing ability of frozen/thawed sperm was evaluated by performing artificial insemination (AI) and *in vitro* fertilization (IVF). For AI, superovulated cows were inseminated with frozen/thawed semen and then further evaluated for embryo development. For IVF, oocytes from the respective cows were retrieved using ovum pickup, and then matured. Following maturation, oocytes were co-incubated with semen for 6 h. In the Nguni breeds, the IVF method of embryo production was mildly superior to the *in vivo* method at the morula stage while the Bonsmara breed revealed the opposite effect at both the morula and blastocyst stages. In the Boran breed, the IVF method was highly superior with the *in vivo* method at the 8-cell stage while the opposite effect was observed at the blastocyst stage of embryonic development. This study suggests that the Boran breed is less susceptible to loss of embryonic development as compared to the Nguni and Bonsmara breeds.

Key words: Nguni, Bonsmara, Boran, embryo, beef breeds, motility, artificial insemination, *in vitro* fertilization.

INTRODUCTION

The success of the Southern African regional beef industries depends on its indigenous cattle beef breeds for their sustainability and competitiveness in the global stage (Morgan et al., 1991; Mckenna et al., 2002; Scholtz and Theunissen, 2010). Among the Southern African regional indigenous cattle beef breeds, the Nguni, Bonsmara (a composite breed) and Boran have great meat quality attributes, such as, good carcass qualities

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with carcass marbling and meat tenderness which are favourable to consumers (Scholtz, 1988; Strydom et al., 2008; Scholtz and Theunissen, 2010). All these breeds possess survival traits that are suitable for local conditions including tolerance to diseases and harsh environmental conditions. In addition, they possess superior growth and reproductive performance and are outstanding beef breeds (Strydom et al., 2008).

Despite their superior qualities, instances of costly reproductive failures occur as a result of a number of factors that interfere with conception or cause the loss of foetuses in cycling females (Mokantla et al., 2004). Artificial insemination (AI) has often been used in our local beef industries to increase production using fresh or frozen/thawed semen (Ferraz et al., 2012). The *in vivo* fertilizing capacity of frozen/thawed semen has always been suspect because it is influenced not only by semen quality and fertilizing ability but also by the respective recipient cow fertility related issues (Dalton, 2011). Hence, the efficiency of *in vivo* fertilization has become a pressing issue worth investigating in our local beef breeds.

Moreover, the investigation of the *in vitro* fertilization sufficiency using frozen/thawed semen has revealed variable outcomes in embryo formation and development, due to poor semen quality (Sudano et al., 2013). It has been previously demonstrated that in frozen/thawed semen about 50% of sperm cells are rendered immotile following thawing and the fertilizing ability of such semen is significantly lower leading to decreased fertilization rates (Watson, 2000). However, advances in semen cryopreservation technologies, such as, the use of percoll gradient and centrifugation, promises to improve post-thaw semen qualities and insemination rates as shown by improved fertilizing rates during AI and IVF (Oliveira et al., 2012). Others have argued that reactive oxygen species are higher in such semen resulting in poor 2-4 cell and 8-cells embryonic developmental stages and poor blastocyst inner cell mass formations (Oliveira et al., 2012; Arias et al., 2017).

As a result of these observations, this study was conducted to compare and investigate the embryonic developmental rates in our Southern African Nguni, Bonsmara and Boran beef breeds using *in vivo* fertilization and IVF as an attempt to address some of the reproductive failures in these breeds. In addition, this study was conducted to establish any relationships or interactions between the semen qualities, embryo production method (AI or IVF) and embryo production rates in these local beef cattle breeds.

MATERIALS AND METHODS

Animals

All animals used in this study are aged between 24 and 36 months and are kept at a quarantine area with access to water at all times. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Animals under the guidelines of the Agricultural Research Council, Animal Production Institute Animal Ethics Committee (APIEC16/029). Bulls were fed on eragrostis at ad lib. A total of sixty (20 Nguni; 20 Bonsmara; 20 Boran) non-pregnant, healthy cycling cows were used as donors and evenly distributed for two embryo production methods namely, ovum pick-up and embryo flushing. The donor cows were feeding on 7 to 10 kg Lucerne, *ad lib* Eragrostis, 1.5 kg of Afgri® embryo concentrate per day.

Evaluation of sperm motility characteristics

A total of 15 bulls (5 Nguni; 5 Bonsmara; 5 Boran) cryopreserved bull semen straws were thawed in warm (38°C) sterile water. Percoll gradient subjected semen was generously supplied by Embryo Plus (Embryo Plus, Brits, South Africa). This percoll gradient and centrifugation were used to separate live and motile sperm cells from the immotile and dead sperm cells and thus enrich the live and highly motile sperm population (Oliveira et al., 2012). To analyse for semen quality, a drop of about 10 µl semen was placed on a microscopic slide and covered with a microscopic cover slip. Thereafter, motility characteristics such as total motility, progressive motility, non-progressive motility, rapid motility, medium motility, slow and static motility were evaluated using a sperm class analyser (SCA).

AI, superovulation and embryo flushing

A total of 30 donor cows (10 Nguni; 10 Bonsmara; 10 Boran) were superovulated according to the method described by Pontes et al. (2009) with slight modifications on dosage. Briefly, a controlled internal drug release (CIDR®) (1.9 g/ml, Pfizer (Pty) ltd., Sandton, Republic of South Africa, RSA) was placed into the vagina of each cow of the three different breeds accompanied by intramuscular (i.m) injection of estradiol benzoate (1 g/ml Pfizer (Pty) ltd., Republic of South Africa) on day 0. An i.m of Cloprostenol Sodium (263 µg/ml, Estrumate®, Isando, RSA) was administered (i.m) to the cows after CIDR® removal on day 8 of oestrous synchronization followed by i.m injection of half the original dosage of estradiol benzoate on day 9. Heat was observed with the aid of heat mount detectors on day 9 (Kamar®, RSA). Day 0 was repeated by inserting a new CIDR three days after heat observation. On day 4, two injections of Follicle Stimulating Hormone (FSH), Folltropin-V® (20 mg, Armidale, Australia) were administered at 12 h intervals initiated for four days on a decreasing dosage, plus two injections of estrumate 12 h apart on the last two days of Folltropin®. Then cows were inseminated twice (12 and 24 h) after detection of standing oestrous with frozen/thawed semen from Nguni, Bonsmara and Boran bulls. Thereafter, embryo recovery was performed seven days after AI, whereby an epidural anaesthesia (lignocaine) was performed with a standard non-surgical technique to flush the uterine horns using a three way folley catheter. Retrieved embryos from the breeds studied were transferred into an embryo filter containing holding medium and evaluated using a stereo-microscope (Olympus SZ40, Olympus, Japan). Embryos were evaluated for embryo development (2-4 cells, 8-cell, Morula, Blastocyst).

Ovum pickup

Ovum pick up was performed as described by Petyim et al. (2000) with a minor modification. A total of 30 normal cycling donor cows (10 Nguni; 10 Bonsmara; 10 Boran) were restrained in a crush pen then given an epidural injection (Lignocaine) on the head of the tail. Thereafter, the rectum was emptied and the vulva was cleaned

Table 1. Motility characteristics of Bonsmara, Boran, Nguni following thawing (Means±SE).

Breed	Motility characteristics						
	Total motility	Progres	Non-prog	Rapid	Medium	Slow	Static
Bonsm	80.7±6.9^{b}	10.7±7.8^{b}	70.0±6.2^{ab}	10.2±7.4^{b}	8.3±4.5^{a}	62.2±8.7^{a}	19.3±7.0^{ab}
Nguni	75.1±4.2^{c}	13.6±5.0^{b}	61.5±5.5^{bc}	7.9±2.1^{b}	9.7±1.5^{a}	56.9±3.7^{a}	25.4±4.2^{a}
Boran	93.2±3.6^{a}	39.7±3.4^{a}	53.5±6.8^{c}	36.1±5.9^{a}	17.6±3.2^{a}	39.4±5.3^{b}	6.8±3.5^{c}

^{abc}Within the same column, numbers with different superscript differs significantly ($p<0.05$).

Figure 1. Stained embryos following *in vitro* or *in vivo* fertilization showing the 2-4 cells stage with the male and female pronuclei (PN) and the blastocyst stage of embryonic development.

thoroughly with 70% alcohol. Following cleaning, the transducer was advanced into the external of the cervix. Thereafter, ovaries were held through the rectum and positioned over the transducer face so that the targeted follicle is transacted by the built in puncture line on the ultrasound monitor, which represented the projected needle path. When the targeted follicles were stabilized on the puncture line, the needle was inserted in the guide and advanced through the vaginal wall and into the follicle antrum. Follicular fluid was then aspirated using continuous negative pressure (about 95 mmHg) then transferred into the laboratory for oocytes searching under the stereo microscope.

IVF and Embryo production

In vitro maturation, fertilization and oocyte culture were done using procedures as described (Huang et al., 2001; Nazem et al., 2016) with slight modification on the use of bovine follicular fluid and hormonal concentrations. Briefly, the cumulus oocytes complexes (COCs) were matured for 24 h in TCM-199 (Gibco, Grand Island, NY) consisting of 10% FBS, 10% follicular fluid, 10 µg/ml Leutenizing Hormone, 1 µg/ml prostaglandin E_2 and 1 µg/ml FSH under humidified atmosphere of 5% CO_2 at 38.5°C. Following maturation, oocytes were fertilized in 100 µl drops of frozen-thawed percoll gradient subjected semen for 18 h at 38.5°C. Thereafter, oocytes were cultured in synthetic oviductal fluid (SOF) supplemented with bovine serum albumin (BSA) and incubated at 38.5°C in 5% CO_2 for seven days.

Embryo staining

Fixing and staining were done based on methods described (Hossaini et al., 2007; Nazem et al., 2016). Briefly, seven days following culture, a sample of embryos were fixed in 4% paraformaldehyde for 48 h then later stained in 50 µg/mL of Hoechst 33342. Thereafter, embryos were placed individually on a microscope glass slide and covered with a coverslip and then evaluation under fluorescence microscope (Olympus-BX51TF).

Statistical analysis

Cleavage and blastocyst formation data were analysed by ANOVA. Significant differences of the means were measured at (5% level). Means of the cleavage rates were separated using fishers protected least significant different (LSD) test. This test was only run if there was significance difference following the ANOVA analysis.

RESULTS AND DISCUSSION

Percoll treatment of frozen/thawed semen revealed an improved total motility greater than 70% in all three local breeds. The Boran breed had a significantly ($P<0.05$) higher total sperm motility (93.2±3.6) compared to Bonsmara (80.7±6.9) and Nguni (75.1±4.2) breeds. Furthermore, Boran had a significant ($P<0.05$) higher percentage of progressive (39.7±3.4) and rapid (36.1±5.9) motility as compared to other breeds (Table 1).

Despite some donor cows being non-responsive to flushing, the overall oocyte recovery was impressive in all breeds. Hoechst 33342 staining of developing embryo revealed clear developmental stages into the 2-4 cells to blastocyst stages of embryonic development (Figure 1). The comparison of the *in vivo* and *in vitro* embryo

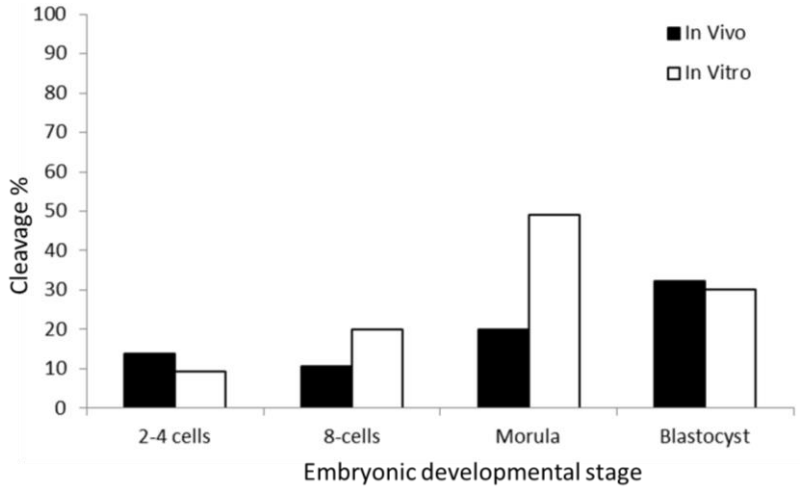

Figure 2. Embryo development in Nguni cows following the *in vivo* and *in vitro* methods of embryo production ($p<0.05$).

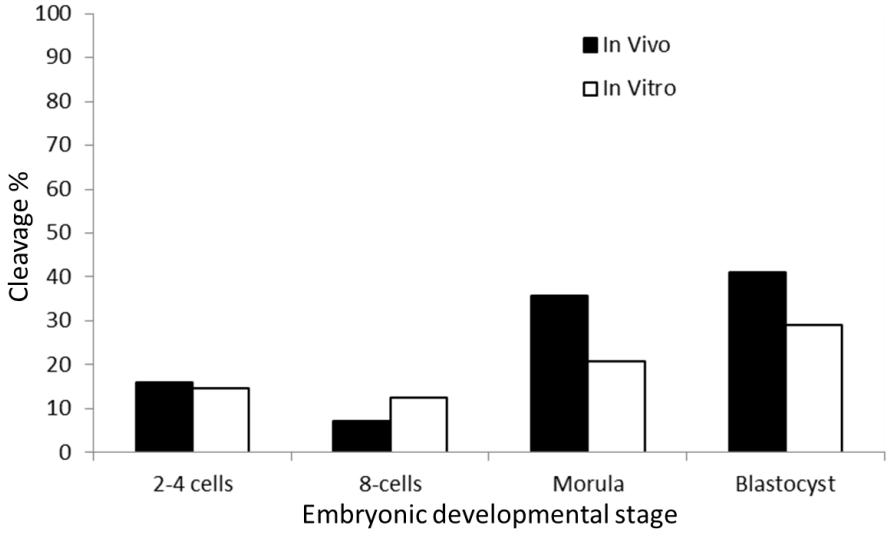

Figure 3. Embryo development in Bonsmara cows following the *in vivo* and *in vitro* methods of embryo production ($p<0.05$).

production methods revealed the *in vivo* method of embryonic development was superior in the Nguni breed at the morula stage of embryonic development than the *in vitro* method of embryo production (Figure 2). Also, for the Bonsmara breed, the *in vivo* embryo production method was superior at both the morula and blastocyst stages of embryonic development (Figure 3). In the Boran breed, the *in vitro* method of embryo production was significantly superior to the *in vivo* embryo production method at the 8-cell stage of development; however the *in vivo* method became superior at the blastocyst stage of development (Figure 4). Interestingly, only the Boran breed revealed a linear progression of embryonic development when the using the *in vivo* embryonic development method (Figure 4). The higher sperm motility observed in the Boran breed cannot account for this positive interaction between the embryo production method and the breed, since it was not observed in the *in vitro* method of embryo production (Table 1 and Figure 4).

A comparison of the breed effect on the embryonic developmental stages in embryos produced by either the *in vivo* or *in vitro* method revealed that the number of 2-4 cells of the embryonic development was very low with no significant deference among all the breeds (Figure 5). At the 8-cells stage, the number of produced 8-cells of

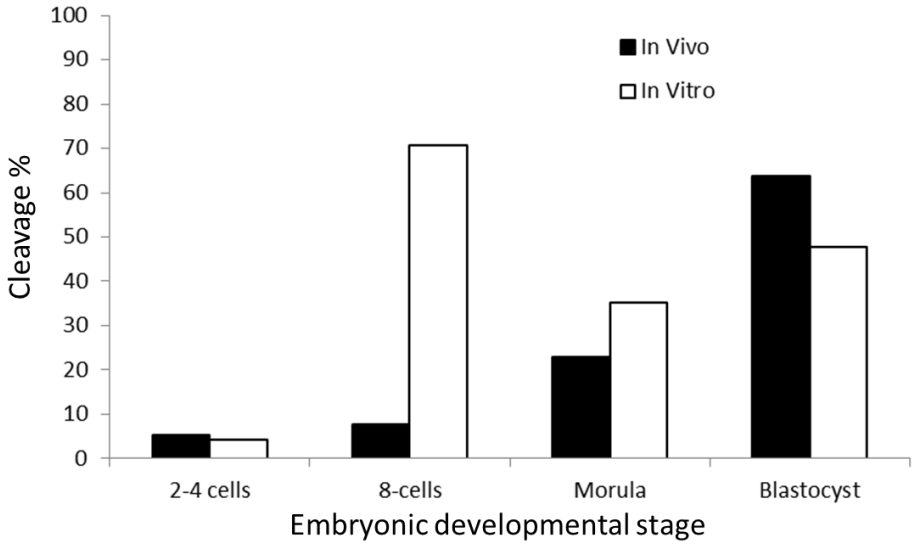

Figure 4. Embryo development in Boran cows following the *in vivo* and *in vitro* methods of embryo production ($p<0.05$).

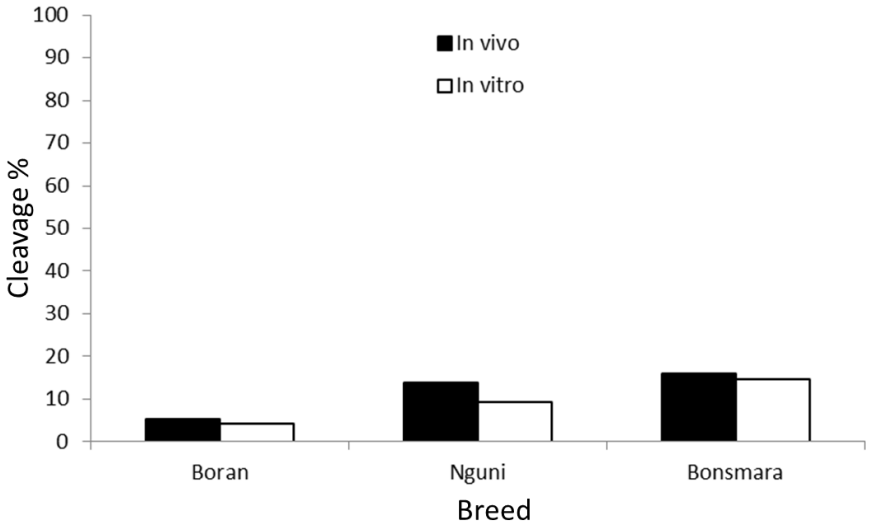

Figure 5. Comparison of embryo production at the 2-4 cells stage of embryonic development in the Nguni, Boran and Bonsmara breeds under both the *in vivo* and *in vitro* methods of embryo production ($p<0.05$).

embryonic developmental stage, only embryos produced in the Boran breed revealed a highly significant number albeit under those embryos produced using the *in vitro* method of embryo production (Figure 6). At the morula stage of embryonic development, the Boran and Nguni breeds revealed higher number of developing embryos produced under the *in vitro* method, while the *in vivo* method produced higher number of morula stage embryos in the Bonsmara breeds (Figure 7). For the blastocyst stage of embryonic development, only the Boran breed showed significantly high number of blastocysts produced under both the *in vivo* and *in vitro* methods of embryo production as compared to the Nguni and Bonsmara breeds (Figure 8).

This study suggests that reproductive failures observed in the Nguni breed are likely to occur at the 2-4 cell stage of embryonic development when the *in vivo* embryo production method is used. However, both the Bonsmara and Boran breeds are likely to progress to the blastocyst stage of embryonic development when the *in vivo* method of embryo production were used. Ironically, when the *in vitro* method of embryo production was used, production

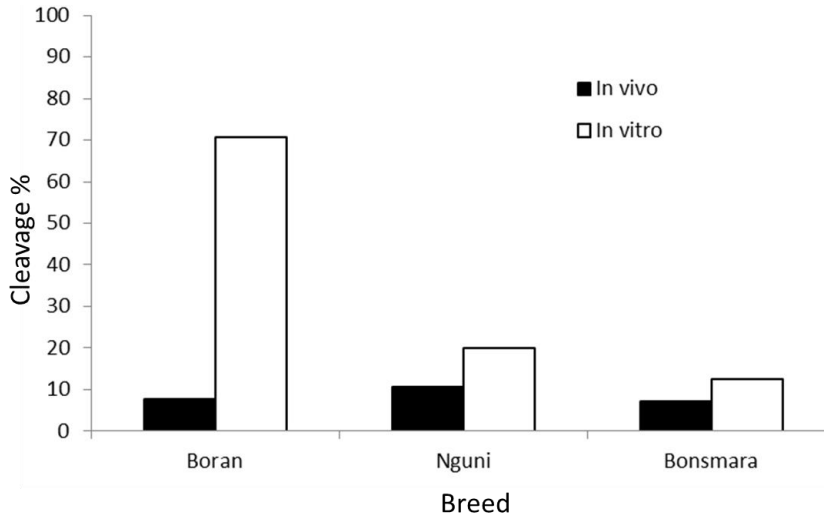

Figure 6. Comparison of embryo production at the 8-cells stage of embryonic development in the Nguni, Boran and Bonsmara breeds under both the *in vivo* and *in vitro* methods of embryo production, least significant difference (LSD) =2.112, ($p<0.05$).

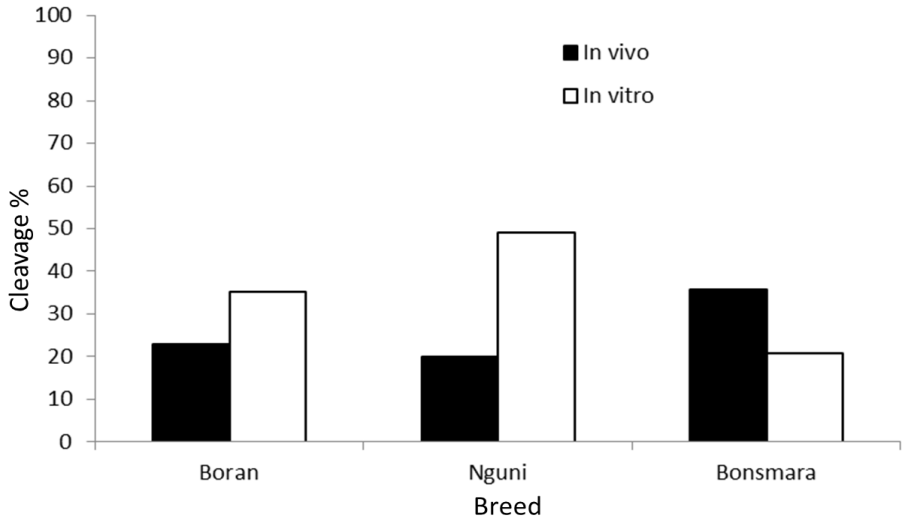

Figure 7. Comparison of embryo production at the Morula stage of embryonic development in the Nguni, Boran and Bonsmara breeds under both the *in vivo* and *in vitro* methods of embryo production ($p<0.05$).

failures are likely to occur at all stages of embryonic development in all the breeds.

This is the first study comparing embryos produced through flushing during *in vivo* and *in vitro* fertilization in our local Nguni, Boran, and Bonsmara beef breeds. In this current study, Boran breed presented a high oocyte recovery (18.2±1.7) compared to Nguni (13±0.8) and Bonsmara (11.2±1.6) accompanied by high blastocysts produced *in vivo*. Moreover, the Boran breed obtained a higher number of 8-cell embryos during the *in vitro* production which seem to deteriorate at blastocyst stage while the opposite is true *in vivo*. This could suggest that the Boran breed is likely to have minimal reproductive failures as compared to Bonsmara and Nguni breeds. Overall, the development of embryos produced *in vitro* was low across all breeds compared to their *in vivo* counterparts. This study is comparable with the findings of Machatkova et al., (2008) and Lonergan and Fair (2014) who indicated that the development of embryos produced *in vitro* from oocytes of selected donors and

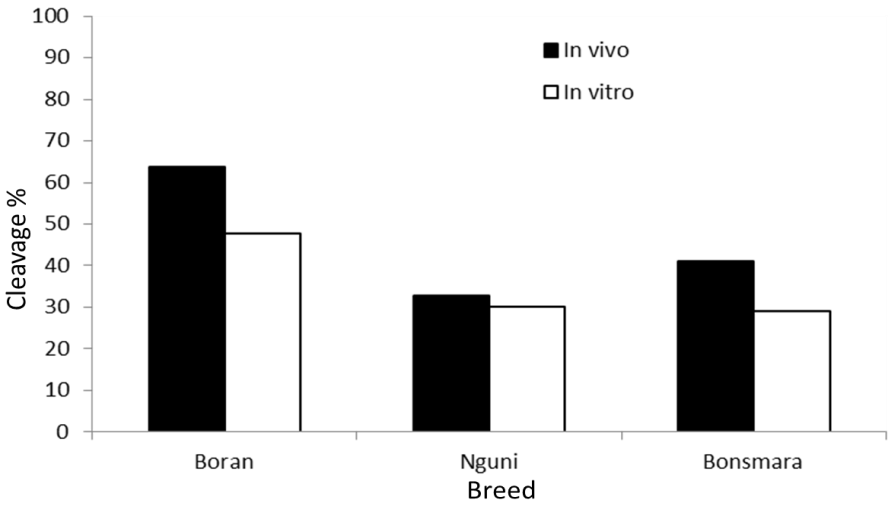

Figure 8. Comparison of embryo production at the blastocyst stage of embryonic development in the Nguni, Boran and Bonsmara breeds under both the *in vivo* and *in vitro* methods of embryo production, least significant difference (LSD) =1.953, ($p<0.05$).

survival of these embryos after cryopreservation was low compared with embryos produced *in vivo* from superovulated donors.

Furthermore, the study showed that semen from individual bulls may differ in their ability to fertilize oocytes to blastocyst stages after *in vitro* and *in vivo* fertilization methods. These findings are in agreement with *in vitro* fertilization method where semen from different bulls has been used with varying capabilities to fertilize oocytes (Abdel Dayem et al., 2009; Nagy et al., 2015). Theoretically, the higher the sperm progressive motility is accompanied by higher rates of fertilization *in vitro* as well as *in vivo*. Therefore, as previously indicated, production failures could occur since male and breed effect result in variable *in vivo* and *in vitro* outcomes in farm animals as previously discussed by others (Mahmoud et al., 2013).

In conclusion, the Boran breed seems to be superior in embryonic development following *in vivo* and *in vitro* production methods. This observation indicates that the Boran breed is less likely to have reproductive failures as compared to Nguni and Bonsmara breeds but is not immune from these failures. Also, there appears to be a relationship between the sperm motility rate and fertilization rate on *in vitro* and *in vivo* embryo development in beef cattle, as demonstrated by the Boran breed. Without excluding the failures due to female reproductive factors however, reproductive failures that occur when semen from individual bulls is used during *in vivo* and *in vitro* embryo production remains an area of greater interest among researchers. In addition, we recommend that further studies on the follicular waves of these three breeds should be studied to support the oocyte recovery rates and progressive embryonic development which might help address the observed differences.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Genetic diversity assessment of yams (*Dioscorea* spp.) from Ethiopia using inter simple sequence repeat (ISSR) markers

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The genetic diversity and relationships of 70 accessions of yam belonging to *Dioscorea cayenensis/Dioscorea rotundata* complex (55), *Dioscorea bulbifera* (13) and *Dioscorea alata* (2, as a reference) were assessed using six inter simple sequence repeat (ISSR) primers. DNA was extracted from a bulk of two plants per accession using a modified cetyl trimethyl ammonium bromide (CTAB) method. Six ISSR primers amplified 77 fragments with 75 (97.40%) polymorphism at genus level. The genetic diversity, estimated by gene diversity and Shannon's index were 0.36 and 0.53, respectively, revealing a high level of genetic variation at genus level. At species level, 75 bands were amplified for *D. cayenensis/D. rotundata* complex, out of which 71 were polymorphic accounting for 92.2% polymorphism. Gene diversity and Shannon's index for *D. cayenensis/D. rotundata* complex were 0.33 and 0.49, respectively. In the case of *D. bulbifera*, a total of 64 bands were scored, out of which 55 were found to be polymorphic which resulted in 71.4% polymorphism. Gene diversity and Shannon's index for this species were 0.24 and 0.47, respectively. Genetic diversity analysis of *D. cayenensis/D. rotundata* complex accessions showed that Gedeo was the most diverse among populations and South among groups. Analysis of molecular variance (AMOVA) indicated the presence of higher proportion of variation within species (63.9%) than among species (36.1%). AMOVA for *D. cayenensis/D. rotundata* complex also showed higher within population variation (53.6) than among populations (46.4). In addition, cluster analysis for relationship between *D. cayenensis/D. rotundata* complex accessions showed grouping of some of the accessions according to their population but it failed to produce clear species boundary between *D. cayenensis/D. rotundata* complex. The results suggest that there is a high level of genetic diversity in Ethiopia yams to be exploited for future improvement (breeding) of the crop.

Key words: *Dioscorea bulbifera*, *Dioscorea cayenensis*/*Dioscorea rotundata* complex, genetic diversity, yam.

INTRODUCTION

Yams are one of tuber crops which belong to the genus *Dioscorea* in the family *Dioscoreaceae.* The family is believed to be among the earliest angiosperms and probably originated from Southeast Asia (Coursey, 1967;

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Wilkin, 1998). Yams (*Dioscorea* L. spp.) are the fourth ranked and most important tuber crops in economic terms next to potatoes (*Solanum tuberosum* L.), cassava (*Manihot esculenta* Crantz) and sweet potatoes (*Ipomoea batatas* (L.) Poir.) (Mignouna et al., 2005). Yams (*Dioscorea*spp.) are one of the most important native tuber crops of Ethiopia (EIB, 2009) and the country ranks 10th in the world in terms of their production (FAOSTAT, 2013). The cultivated species of yam, *Dioscorea bulbifera, Dioscorea abyssinica* and *Dioscorea schimperiana* are native to Ethiopia (Westphal, 1975).

The advent of the polymerase chain reaction (PCR) was a breakthrough for molecular marker techniques and made possible many fingerprinting methods. Among all markers, RAPD, inter simple sequence repeat (ISSR), amplified fragment length polymorphism (AFLP) and most recently genotyping by sequencing (GBS) markers are the most widely applied probably because they do not require the knowledge of genome sequences and the protocols used are relatively simple, rapid and cost effective (Srivastava et al., 2004; Vijayanet al., 2005; Elshireet al., 2011).

Different types of DNA molecular assays have been applied in yam in different countries (Arnau et al., 2017; Zhou et al., 2008; Nascimento et al., 2013; Muluneh et al, 2007; Wendawek et al., 2013a, b; Atnafua, 2014). However, only few studies: AFLP (Muluneh et al., 2007; Wendawek et al., 2013b) and SSR (Atnafua, 2014) have been conducted on yams of Ethiopia using molecular markers. Few studies were also conducted based on morphological and agronomic traits (Muluneh et al., 2008; Tewodros 2013). Previous researchers on yams of Ethiopia suggested the need for further studies with inclusion of areas which were not included in their studies. For instance, accessions from Ilu Ababora were not included in any of the previous studies. Moreover, *D. bulbifera* in Southwestern Ethiopia were not studied using any molecular markers. Besides, there is no previous report regarding the use of ISSR markers for assessment of genetic diversity of yams (*Dioscorea* spp.) from Ethiopia. Hence, the present study aimed at assessing the level and pattern of inter- and intra-species genetic diversity and relationships among yams (*Dioscorea* spp.) accessions of Ethiopia using ISSR markers. This will be of great importance to supplement actions to be taken towards improvement and conservation of the crop.

MATERIALS AND METHODS

Collection of plant material

A total of 70 accessions (55 *D. cayenensis/D. rotundata* complex; 13 *D. bulbifera*; 2 *D. alata*) belonging to 7 populations (3 from Oromia and 4 from Southern Nations, Nationalities and Peoples Regional State, SNNPRS) were obtained from Ethiopian Institute of Biodiversity (EIB). For DNA extraction, five fresh and young leaf samples were randomly selected from each accession and silica gel dried in a zip lock bag.

DNA extraction, primer screening and PCR optimization

The ISSR marker assay was conducted at Plant Genetics Research Laboratory of the Department of Microbial Cellular and Molecular Biology, Addis Ababa University, Addis Ababa, Ethiopia. Genomic DNA was extracted following the CTAB protocol (Borsch et al., 2003). A total of 15 ISSR primers were screened for their ability to generate clear, reproducible, polymorphic and high resolution bands, in four (two from *D. cayenensis/D. rotundata* complex; two from *D. bulbifera*) randomly selected accessions. Out of 15 candidate ISSR primers, six good ones with clear, reproducible and polymorphic bands (ISSR-811, ISSR-818, ISSR-844, ISSR- 848, ISSR-873 and ISSR-880) were selected and used for analysis of genetic diversity of *Dioscorea* spp. (Table 1). Various combinations (at different concentrations) of PCR components were tested to find out optimum concentrations of the PCR reaction components and the one with clear band was used.

PCR amplification and agarose gel electrophoresis

PCR amplification was carried out in a 25 µl reaction mixture containing 16.7 µl sterile deionized H_2O, 1 µl dNTPs (25mM each), 2.5 µl Taqbuffer (10X reaction buffer S), 2 µl $MgCl_2$ (25mM), 0.4 µl primer (20 pmol/µl), 0.4 µl Taqpolymerase (5 unit/µl) and 2 µl diluted template DNA. Polymerase chain reactions were conducted in Biometra T3 Thermocycler withthe followingamplification program: a preheating and initial denaturation at 94°C, for 4 min, then 15 s for 40 cyclesat 94°C, 1 min primer annealing at primer annealing temperature (varies based on primers used), primer extension at 72°C for 1.30 min and final extension at 72°C for 7 min. The PCR products (10 µL for each sample) together with 1 µL 6X loading dye were loaded on 1.67% agarose gel with 100 bp ladder as a size standard (on the first lane of the gel) and subjected to electrophoresis in BIORAD min-sub® (from Biometra® cell GT at 80V standard power pack P25) for about 2 h and visualized under UV light using gel documentation system (Biosens SC750), stained by immersing in 1% ethidium bromide solution (50 µl ethidium bromide diluted with 450ml H_2O) and destained with distilled H_2O. The image of both stained and destained was photographed with BiosensSC750.

Statistical analysis

Amplified products were scored as present (1) or absent (0) to form a binary matrix. For analysis, scored data were subjected to different software like POPGENE (Yeh et al., 1999) to calculate genetic diversity as: number of polymorphic loci (NPL), percent polymorphic loci (PPL), Nei's gene diversity (H) and Shannon's information index (I). Areliquin (Excoffier et al., 2006) was used to compute AMOVA, while NTSYS- pc (Rohlf, 2000) and Free Tree (Pavlicek et al., 1999) were used to generate UPGMA and NJ tree, respectively. PAST & STATISTICA (Hammer et al., 2001; Statistica Soft, Inc.2001) were also used to generate two dimensional (2D) and three dimensional (3D) plots.

RESULTS AND DISCUSSION

Banding patterns of the ISSR primers

Six ISSR primers namely 811, 818, 844, 848, 873 and 880, were selected based on the presence of well defined, informative and good resolution bands. A total of 77 bands with a size ranging from 200 to 3000 bp and an

Table 1. List of primers, primer motif, annealing temperature, repeat motives and amplification quality used for optimization and screening.

ISSR-primers	Primer motif	T(°C)	Amplification quality	Repeat motif
809	(AG)8G	48	No amplification	Di-nucleotide
810	(GA)8T	45	Not reproducible, not polymorphic	Di-nucleotide
811	(GA)8C	48	Polymorphic, reproducible	Di-nucleotide
812	(GA)8A	45	Not reproducible, not polymorphic	Di-nucleotide
815	(CT)8G	48	Not reproducible, not polymorphic	Di-nucleotide
818	(CA)8G	48	Polymorphic, reproducible	Di-nucleotide
824	(TC)8G	48	Not reproducible, Not polymorphic	Di-nucleotide
844	(CT)8RC	48	Polymorphic, reproducible	Di-nucleotide
848	(CA)8NG	48	Polymorphic, reproducible	Di-nucleotide
866	(CTC)6	55	Not reproducible, not polymorphic	Tri-nucleotide
873	(GACA)4	45	Polymorphic, reproducible	Tetra-nucleotide
876	(GAYA)4	45	No amplification	Tetra-nucleotide
880	(GGAGA)3	45	Polymorphic, reproducible	Penta-nucleotide
881	(GGGTG)3	48	Not reproducible, not polymorphic	Penta-nucleotide

N = (A, T,G,C); R = (A, G); Y = (C, T).

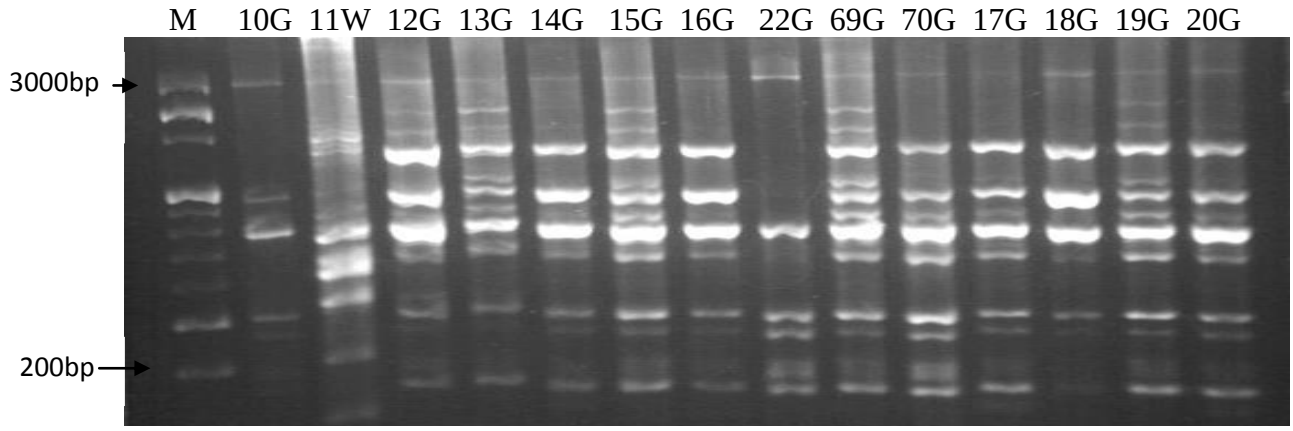

Figure 1. ISSR fingerprint generated for some *Dioscorea* spp. accessions using ISSR-Primer 848. Key: G= Guinea yam (*D. cayenensis/D.rotundat0061* complex); W= winged yam (*D. alata*).

average of 13 bands per primer were obtained with six ISSR primers on 70 accession of *Dioscorea* spp. The highest number of scorable bands (17) was generated by penta-nucleotide ISSR-primer 880, whereas di-nucleotide ISSR-primers 844 and 811 generated the least number of scorable bands (11). The remaining ISSR-primers 818, 848 and 873 generated 13, 13 and 12 bands, respectively. Out of the six ISSR primers used, gel electrophoresis pattern obtained using primer ISSR-848 is illustrated in Figure 1.

For *D. cayenensis/D. rotundata* complex, a total of 75 bands were generated with number of bands produced by each primer ranging from 10 to 16, with the average bands per primer being 12. The highest number of bands was again amplified by primer 880, while primer 844 amplified the lowest number of bands (Table 2).

In *D. bulbifera* accessions, a total of 64 bands with 7 to 13 bands for each primer and with an average of 11 bands per primer resulted from the six ISSR primers used. Primer 880 and 818 produced the highest number of bands, while the lowest number of bands was amplified by primer 811 (Table 2).

Species specific ISSR bands in *D. cayenensis/D. rotundata* complex and *D. bulbifera*

Out of the six ISSR primers used, five ISSR primers showed species specific bands (Table 2). A total of 13 bands specific to *D. cayenensis / D. rotundata*complex

Table 2. List of ISSR primers used and their banding pattern in *D. cayenensis/D. rotundata* complex and *D. bulbifera.*

Primers	Repeat motif	No. of scorable bands			No of species specific bands	
		G	A	G and A	G	A
811	$(GA)_8C$	11	7	11	4	0
818	$(CA)_8G$	13	13	13	0	0
844	$(CT)_8RC$	10	8	11	3	1
848	$(CA)_8NG$	13	12	13	1	0
873	$(GACA)_4$	12	11	12	1	0
880	$(GGAGA)_3$	16	13	17	4	1
Total		75	64	77	13	2
Average		12	11	13	2.16	0.33

G = Guinea yam (*D. cayenensis*/*D. rotundata* complex), A = Aerial yam (*D. bulbifera*), G and A = combination of Guinea and aerial yam, N = (A, T,G,C); R = (A, G).

were generated. Primers 811 and 880 showed the highest number of specific bands to this species (four bands). Only two bands specific to *D. bulbifera* were amplified by six primers. Two bands specific to *D. bulbifera* were generated by primers 844 and 880. ISSR primer 818 generated no specific bands for both species whereas primers 811, 848 and 873 generated specific bands for only *D. cayenensis/D. rotundata*complex (Table 2).

Application of ISSR markers in *Dioscorea* species genetic diversity assessment

Six ISSR primers amplified 77 fragments with 75 (97.40%) polymorphism at genus level. The genetic diversity, estimated by Gene diversity and Shannon's index were 0.36 and 0.53, respectively, revealing a high level of genetic variation at genus level. At species level, 75 bands were amplified for *D. cayenensis/D. rotundata* complex, out of which 71 were polymorphic accounting for 92.2% polymorphism. Gene diversity and Shannon's index for *D. cayenensis/D. rotundata* complex were 0.33 and 0.49, respectively. Comparable results have been reported by Wendawek et al. (2013b) who used AFLP fingerprinting to evaluate and characterize 43 individuals belonging to different populations of wild and cultivated guinea yam (*D. cayenensis/D. rotundata* complex) using three-primer combination and detected 78% polymorphism.

Bressan et al. (2014) also evaluated 21 local varieties of *D. cayenensis* and two *D. rotundata* accessions using 7 isozyme loci and 24 morphological markers, and reported the existence of high genetic variability with 100% polymorphism using isozyme marker. Dansi et al. (2000) and Mignougna et al. (2002) also studied genetic diversity of *D. cayenensis/D. rotundata* complex using isozyme markers in 7 and 6 isozyme systems, respectively, and reported the existence of high diversity (polymorphism in all analyzed isozyme systems) which is in agreement with the present study.

In the case of *D. bulbifera*, a total of 64 bands were scored, out of which 55 were polymorphic which resulted in 71.4% polymorphism. Gene diversity and Shannon's index for this species were 0.24 and 0.47, respectively. Despite its smaller sample size (13), *D. bulbifera* accessions still showed high genetic diversity but lower than *D. cayenensis/D. rotundata* complex. This shows that small populations or individuals are not always associated with a lack or low level of genetic variation (Yingjuan and Ting, 2009). Likewise, Tewodros (2013) studied the level of genetic diversity within *D. bulbifera* accessions collected from South and Southwestern Ethiopia based on key agronomic traits and reported the existence of high diversity in the region. Silva et al. (2016) also evaluated genetic diversity among 42 *D. bulbifera* accessions from Brazil using microsatellite markers and found high genetic diversity.

Both *D. cayenensis/D. rotundata* complex and *D. bulbifera* showed high genetic diversity, 92.2 and 71.4% at the species level, respectively. Shannon's information index of both species (0.49 for *D. cayenensis/D. rotundata* complex; 0.47 for *D. bulbifera*) was also higher than the average values for widespread species (0.202) as suggested by Hamrick and Godt (1989).

Among the two species, *D. cayenensis/D.rotundata*complex was more diverse, this might be due to its larger sample size, being a combination of various populations and for representing two species complex. Genetic diversity analysis of *D. cayenensis/D. rotundata* complex populations showed that Gedeo was the most diverse, while East Wellega was the least diverse. Gedeo areas are known for their traditional agro-forestry system in which they grow a variety of crop plants including tuber crops (Wubalem, 2014), which might be the reason for the highest diversity

Table 3. Analysis of molecular variance (AMOVA) at the genus level for *Dioscorea*.

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation indices	*P*-value*
Among species	1	98.767	6.400	36.128		0.001
Within species	66	596.896	11.317	63.871		0.001
Total	67	695.663	17.718		0.361	

d.f = Degree of freedom, *significance tests after 1023 permutations.

Table 4. Analysis of molecular variance (AMOVA) for Guinea yam (*D. cayenensis/D. rotundata* complex).

Source ofvariation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation indices	*P*-value*
Among populations	6	219.969	1.96	46.444		P< 0.001
Within populations	40	212.121	7.24	53.555		P< 0.001
Total	46	432.090	11.591		0.464	

d.f = Degree of freedom, *significance tests after 1023 permutations.

of yams in the area. In the case of East Wellega, it is geographically a bit isolated with no/little incoming genes or tubers as a seed. Therefore, it is more likely that the genetic diversity in this area might be lower due to presence of lower or no tuber exchange with other populations.

Partitioning of genetic diversity in yams (*Dioscorea* spp.)

AMOVA revealed the presence of higher proportion of variation within species (63.9%) than among species (36.1%) (Table 3).This might be due to the presence of several shared bands between these species, which indicates that they might have close evolutionary relationship and/or admixture on farmer's field might have facilitated gene flow (pollen flow). It has been reported that *D. cayenensis/D. rotundata* complex and *D.* bulbifera are most likely cross compatible due to their similar ploidy level (2n= 40, 60, 80) (Coursey, 1967; Asiedu, 1997). The potential for gene exchange has long been recognized even between taxa with large differences in chromosome numbers (Stebbins, 1971).

AMOVA for *D. cayenensis/D. rotundata* complex also showed moderately higher within population variation (53.6) than among populations (46.4) (Table 4). Similarly, Similarly, Loko et al. (2016) used microsatellite marker to study genetic diversity and relationship of guinea yam germplasm of Benin and found 96% of variation within population and 4% among population. Muluneh et al. (2007) also assessed genetic diversity of yam (*Dioscorea* spp.) germplasms from Ethiopia and their relatedness to the main *Dioscorea* spp. by AFLP markers and found 81% of the total genetic variation being attributed to within populations and only 19% to among populations. Wendawek et al. (2013a) also evaluated genetic diversity and population structure of guinea yams and their wild relatives in South and South West Ethiopia using microsatellite marker, reporting the same pattern. This could be due to gene flow through seed materials exchange among local farmers.

Genetic relationships within and among species

Cluster analysis showed grouping of most of the accessions according to their species. Mignouna et al. (2005) also used RAPD and double stringency PCR (DS-PCR) and reported similar result. In addition, cluster analysis for relationship between *D. cayenensis/D. rotundata* complex accessions showed grouping of some of the accessions according to their population. Accessions from East Wellega clearly formed their own cluster, accessions from Semen Omo were grouped together with those of Hadiya-Kembata, while accessions from Gedeo were clustered together with those of Jimma (Figure 2). Both of the two (Figure 3a) and three dimensional PCO plots (Figure 3b) showed the same pattern. Similarity between Semen Omo and Hadiya-Kembata population is expected due to geographical proximity of those areas. However, genetic similarity was also present between Gedeo and Jimma accessions in spite of their geographical distance. Hence, this study showed that there is no strong correlation between geographic distance and genetic diversity. This could be explained in terms of movement of the people carrying tubers and distribution of cultivars over great distance as clones in the course of human movement.

ISSR data failed to produce any clear boundary between different types of Guinea yams that showed domestication characteristics of different species (wild,

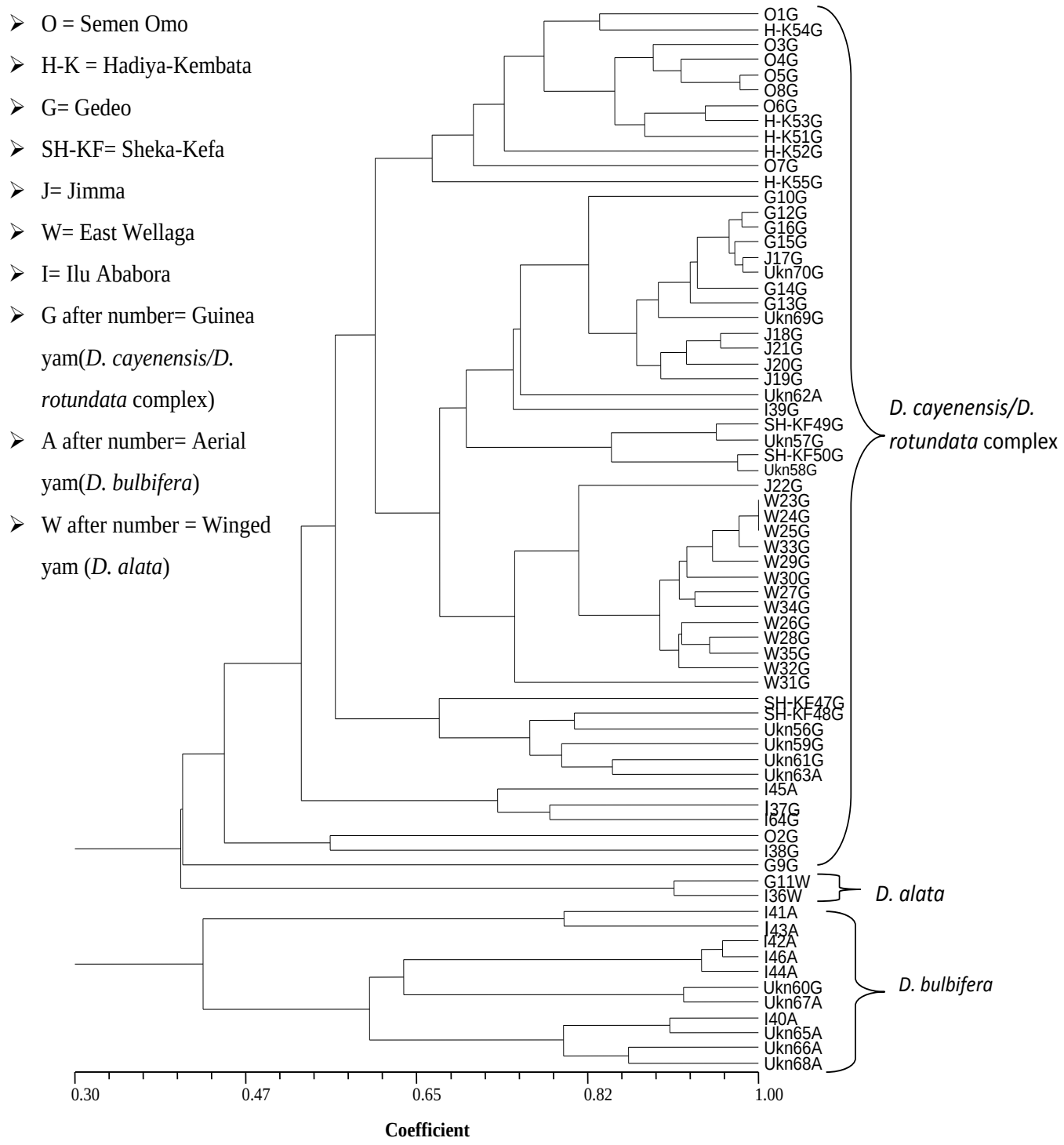

Figure 2. UPGMA dendrogram based on Jaccard's similarity coefficient among 55 *D. cayenensis/D. rotundata*complex, 13 *D. bulbifera* and 2 *D. alata* accessions.

cultivated and intermediate) based on their tuber flesh colour. Similarly, Wendawek et al. (2013b) used AFLP genetic finger printing to evaluate and characterize 43 individual plants belonging to different populations of wild and cultivated guinea yams and reported that ordination and cluster analysis did not produce any clear boundary between either the guinea yam accessions or between them and their wild relatives. The finding of the present

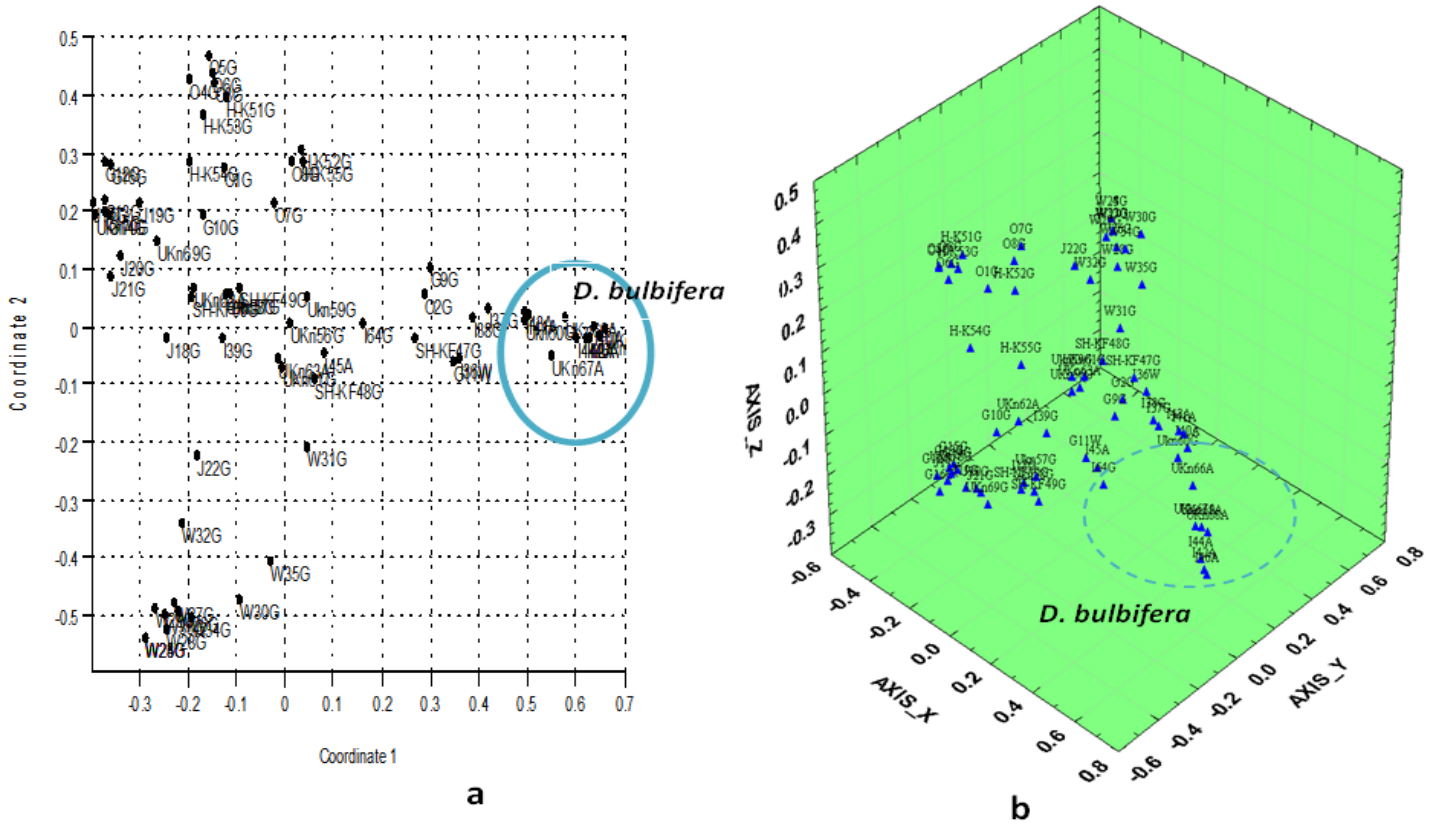

Figure 3. Two dimensional (a) and three-dimensional (b) plot obtained from principal coordinate analysis of 70 (55 *Dioscorea cayenensis/D. rotundata* complexes; 13 *D. bulbifera*; 2 *D.alata*) accessions using Jaccard's similarity coefficient.

study supports the reports of Miege and Sebsebe (1997), which indicates that they are species complex with many intermediates.

Conclusion

Both *D. cayenensis/D. rotundata* complex and *D. bulbifera* showed high genetic variation. In *D. cayenensis/D. rotundata* complex, the highest genetic diversity was found within Gedeo population, which indicates that this population can be considered as a source of diverse individuals in future improvement of the crop. On the contrary, East Wellega population, which showed the least diversity, needs special attention for conservation. Variation within species seemed to be greater than that of among species. Similarly, AMOVA analysis of *D. cayenensis/D.* rotundata complex populations showed higher within population variation than among population variation which indicates existence of high level of gene flow. Cluster and PCO analyses showed clustering of most of the accessions to their respective species and in some cases, to their geographic origin. However, they failed to differentiate between different guinea yam (*D. cayenensis/D. rotundata* complex) types, which support the idea that they are species complex.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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