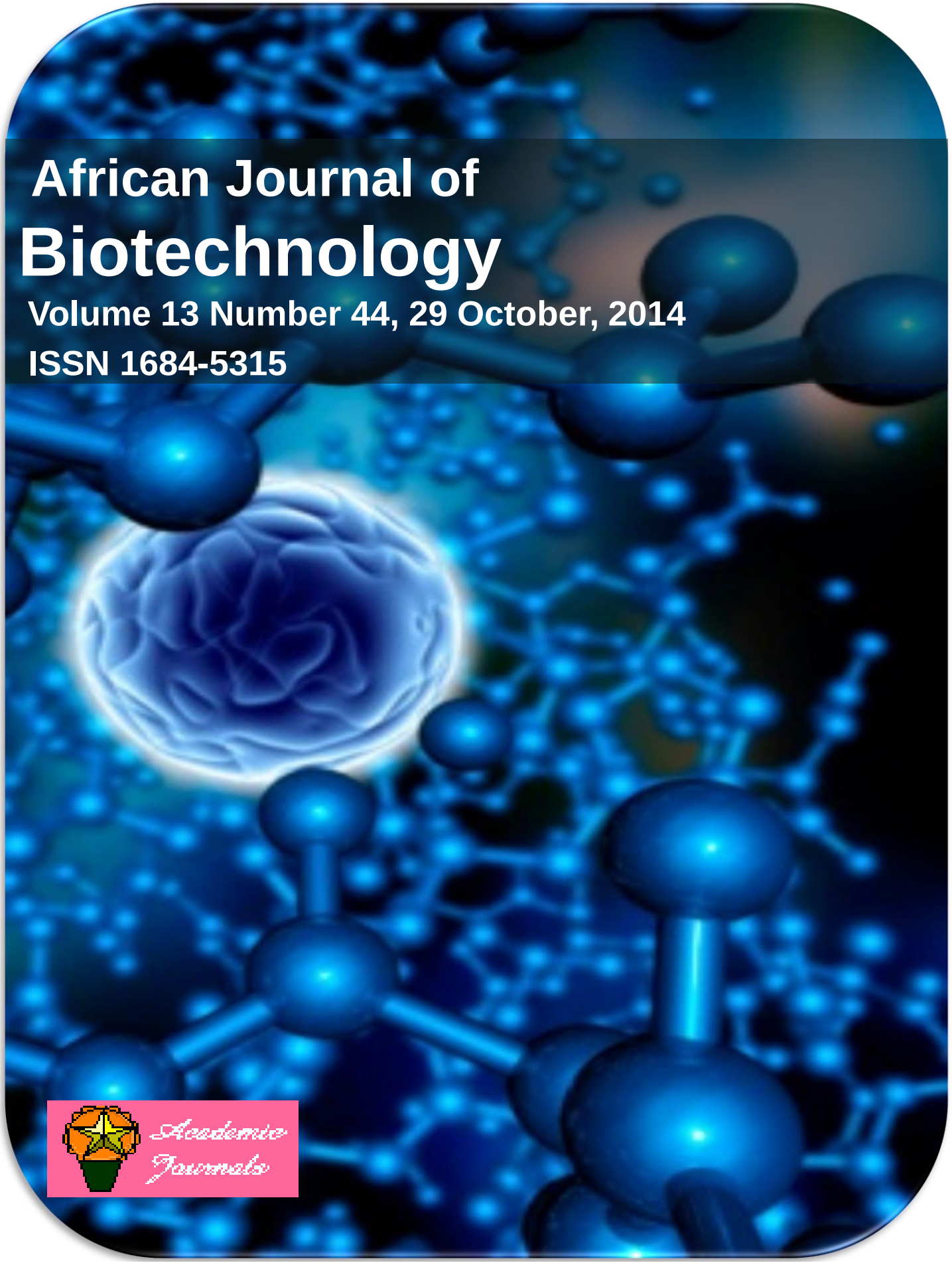

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Full Length Research Paper

Potentials of non-edible *Abrus precatorius* seed oil towards biodiesel production

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Abrus precatorius **seed oil is not edible and possesses lethal toxicological properties; hence, alternative use as low cost feedstock for biodiesel production was investigated. The n-hexane extracted oil was characterized for its chemical and physical properties, and subsequently transesterified using 1% sodium hydroxide at 60°C to produce biodiesel. The biodiesel produced had acceptable quality following characterization of its fuel properties. The relative density was found to be 0.889 with kinematic viscosity of 3.34 mm^2/s within limits of Thailand biodiesel fuel standard. The acid value was 0.281 mg KOH/g, iodine value 52.43 mgI_2/g, peroxide value 3.45 mEq/kg, saponification value 227.8 mg KOH/g and 2.87% free fatty acid content. The flash point and the cetane number were 137°C and 58.3, respectively while the heat of combustion was 38.28 MJ/Kg. The low temperature operability properties of *A. precatorius* seed biodiesel determined by parameters such as cloud point, pour point and cold-filter plugging point were - 2, 1 and - 4°C, respectively. The sulfated ash value and refractive index were found to be 0.09% and 1.457, respectively. GC analysis of the fatty acid methyl esters profile revealed 50.86 and 49.1%, saturated and unsaturated fatty acid methyl esters (FAME), respectively. A percentage FAME yield of 86.1%; with higher content of methyl palmitoleate (31.94%) and a lowest value for methyldecanoate (1.27%) was obtained. Although a n-hexane oil yield was low (2.52% w/w), the results show that Abrus seed oil derived biodiesel has commercially acceptable fuel properties and may be suitable as fuel for internal combustion engine.**

Key words: Biodiesel, *Abrus precatorius*, oil, non-edible oil, fatty acid methyl ester.

INTRODUCTION

The use of vegetable oils as fuels in internal combustion engines dates back to over a century ago, when in 1912 Rudolf Diesel successfully tested peanut oil as fuel for his engine (Knothe et al., 2005). During a demonstration at

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the World's Fair, he predicted that, the use of vegetable oils for engine fuels although might seem insignificant then, but such oils may become, in the course of time, as important as petroleum and the coal tar products of the present time. His words have taken on added significance today. In the 1930s and 1940s, vegetable oils were used as diesel fuels from time to time but usually only in emergency situations. When petroleum-derived oils became available, the vegetable oils were displaced as fuels for engines (Knothe et al., 2005). The continued and increasing use of petroleum will intensify local air pollution and magnify the global warming problems caused by CO_2 emission rate above which the atmosphere can naturally contend with. Recently, the intermittent global energy crisis often prompted by the unpredictable crude oil economy and environmental issues stemming from downstream petroleum processing and burning of fossil fuels has spawned research efforts towards alternative energy. Consequently, increasing demand for bioenergy has generated a strong interest in the bioconversion of agrowastes including vegetable oils into fuels. Around one-tenth of global primary energy use is based on bioenergy sources, of which about 10% are produced from modern bioenergy in form of power, heat and fuel. Currently, biodiesel is considered a promising alternative fuel due to its renewability, better gas emission, CO_2 neutrality, non-toxic and also biodegradability (Hossain et al., 2010); excellent lubricity properties and is typically low in sulfur content, thus meeting the needs of the EPA and new generation fuels. Biodiesel (B100) is a fuel comprised of mono-alkyl esters of long-chain fatty acids derived from vegetable oils or animal fats. It is a fuel designed as a blend stock for use in blending with petroleum diesel fuel and also considered to be an alternative for petroleum-based diesel fuel. Despite possessing attractive prospects, the traditional biodiesel production still faces some bottlenecks as follows: the limited supply of lipid feedstock which generally relies on geographical and seasonal conditions, and the chemical trans-esterification which is energy consumption intensive and needs further waste treatment processes (Ibeto et al., 2011). Considerable researches have been done on vegetable oils as diesel fuel; including palm oil, soybean oil, sunflower oil, coconut oil, rapeseed oil and tung oil. Animal fats, although mentioned frequently, have not been studied to the same extent as vegetable oils (Ma and Hanna, 1999). The most common technique for producing biodiesel is trans-esterification, which refers to a catalyzed chemical reaction of triacylglycerides of vegetable oil with alcohols. However, the vegetable oil and alcohol must be substantially anhydrous and have low free fatty acid content because the presence of water or free fatty acid or both promote soap formation. Generally, trans-esterification is used to produce biodiesel from vegetable oil or animal fat containing low free fatty acid through a reaction involving alcohol and an alkaline catalyst (Ma et al., 1998; Gerpen et al., 2004; Prateepchaikul et al., 2007). When biodiesel is produced from high FFA oils by trans-esterification, the high FFA content in the oils reacts with the metallic alkoxide to produce soap (saponification) (Brown et al., 2003, Gerpen et al., 2004). In addition, if oil contains high moisture content, saponification and hydrolysis occur. These reactions cause a lower yield and washing of biodiesel very difficult. These problems can be solved via either one of four methods: enzymatic-catalyzed trans-esterification, acid-catalyzed trans-esterification, a super-critical carbon dioxide technique or a two-stage process (Ma and Hanna, 1999).

As with bioalcohol production from food based raw materials, use of edible feedstock for biodiesel production has attracted tremendous debate prompting research and development efforts to seek means of augmenting oil yield from food based feedstocks as well as identifying non-edible seed oils with potentials for biodiesel production. The production of biodiesel from edible and non-edible oil has progressively affected food uses , price, production and availability (Rashid et al., 2008). Vegetable oil seeds that do not compete with traditional food crops are needed to meet existing energy demands (Xu and Hanna, 2009). In order to achieve production cost reduction and make biodiesel more competitive with petroleum diesel, low cost feedstocks, such as non-edible oils, waste vegetable oils could be used as raw materials (Xiaohu and Geg, 2009). To reduce the impact on land resources and reserve edible feedstocks for consumption, an increase in biodiesel production will require the use of raw materials that are unconnected with food, especially non-edible oils such as the toxic *Abrus precatorious* seed oil. *A. precatorius* belongs to the family Fabaceae, and it is a creeping or climbing woody vine with pinnately compound leave (Figure 1) (Pokharkar et al., 2011). They are found in tropical climates of Nigeria, India, Thailand, Sri Lanka, the Philippine Islands, South China and West Indies (Hart, 1963).

The alkaloids of the seeds are abrine, hypaphorine, choline and precatorine. Proximate analysis of *A. precatorius* seeds revealed the presence of moisture (5.06%), oil yield (2.5%), crude protein (39.20%), crude fibre (9.08%), ash (5.38%) and total carbohydrate (42.42%) (Abu et al., 2011). *A. precatorius* seeds have been found to contain alkaloids, steroids, lectine, flavonoids and anthocyanins (Attal et al., 2010).

As part of our research and development efforts to explore edible and non-edible seed oils in Nigeria for biodiesel production, the potentials of the toxic *A. pretarious* red seed oil as feedstock for biodiesel were investigated in this study.

MATERIALS AND METHODS

Collection and processing of the samples

A. precatorius red seeds were collected and cleaned by washing with distilled water. The seeds were dried and ground into fine

Figure 1. *Abrus precatorious* plant.

coarse particles using commercial grinder.

Extraction of oil from *A. precatorius* red seeds

Dried and washed *A. precatorius* red seeds (6500 g) were crushed, using commercial grinder and fed to a Soxhlet extractor fitted with a 2-L round bottomed flask (Rashid et al., 2008). The extraction with n-hexane was executed on a water bath for 6 h. The solvent was removed under vacuum, using a rotary evaporator. The amount of oil extracted was determined as a ratio of weight of extract to the total weight of Abrus seeds used.

Determination of percentage free fatty acids (FFA) of the oil

Two grams of well-mixed *A. precatorius* sample was accurately weighed to conical flask into which 10 ml of neutralized 95% ethanol and phenolphthalein were added. This was then titrated with 0.1 M NaOH, shaking constantly until a pink colour persisted for 30 s (AOAC, 1984). The %FFA was determined using equation:

%FFA = [(Volume of NaOH)(Molarity of NaOH) / Weight of oil]*2.82

Where, 2.82 is a conversion factor for oleic acid.

Biodiesel production by two step acid-base trans-esterification

Acid pretreatment

The crude *A. precatorius* seed oil was heated to 50°C while stirring continuously using magnetic stirrer to homogenize the oil. The reaction was conducted in a 500 ml three necked round-bottomed flask attached with a reflux condenser and thermometer, and placed in a water bath with a temperature control. A concentrated sulfuric acid (2% based on oil weight) in 0.60 w/w methanol was heated to 50°C and added to the reaction flask containing pre-heated oil (Zullaikah et al., 2005; Bala, 2005). This mixture was stirred for 2 h. The reaction product mixture was then poured into a separating funnel and allowed to settle for 2 h. The top layer comprised unreacted methanol, whereas the middle layer was oil and fatty acid methylester (FAME) (small amount obtained by conversion of free fatty acids to esters), and water at the bottom layer (Zullaikah et al., 2005).

Alkaline trans-esterification

The acid pre-treated A*. precatorius* seed oil with low percentage free fatty acid was heated to 60°C in a four necked flask and stirred at 800 rpm with mechanical stirrer in a water bath. The catalyst sodium hydroxide (NaOH) 0.5% based on oil weight was dissolved in the required amount of methanol (ratio was methanol: oil = 6:1) and added to the pre-treated oil (Freedman et al., 1984). The reaction was conducted for 120 min. The resulting product was poured into a separating funnel and stood for 2 h. Two phases were distinct; biodiesel on top and the glycerol at the bottom. The two phases were separated and the excess methanol in biodiesel was recovered by using a rotary evaporator. The biodiesel was then washed thoroughly by using hot de-ionized water to wash out impurities like soap and other residues. Finally, the biodiesel was heated to 100°C for 1 h in oven to remove the moisture. Based on the initial amount of pre-treated oil, the biodiesel yield was then evaluated (Meher et al., 2006).

Analysis of physico-chemical properties of the Abrus oil extract and its biodiesel

Physicochemical properties were determined by using standard test methods. These standard values were calculated and compared with European organization (EN 14214). Kinematic viscosity (at 40°C) was determined using viscometer (Oswald U-tube) (AOAC, 1975), refractive index (at room temperature) was determined with Abbe refractometer (Alamu et al., 2008). The relative density (at 15°C) was determined using the relative density bottle (AOAC, 1975). The flash point was determined by the method of ASTMD93 using the Pensky-Martens closed cup tester. Other parameters such as ash content, cold filter plugging point, Pour point and cloud point were determined following the method described by the Association of Official Analytical Chemists (AOAC) (1984). Iodine, peroxide, acid and saponification values were determined as described by Hamilton and Hamilton (1992) while the heat of combustion was determined using bomb calorimeter. The cetane number (CN) of the biodiesel was calculated using empirical

Table 1. Percentage yield and physicochemical properties of *Abrus precatorious* seed oil.

Parameter	Amount
Oil yield (% w/w)	2.52
Relative density	0.92
Kinematic viscosity (mm^2/s)	23.4
Iodine value (mgI_2/g)	57.34
Peroxide value (mEq/Kg)	4.1
Acid value (mg KOH/g)	5.74
Saponification value (mg KOH/g)	227.8
Free fatty acid (%)	2.87

formula provided by Mohibbe et al. (2005).

Determination of fatty acid profile of biodiesel

The fatty acid composition of *A. precatorius* seed biodiesel was determined using agilent 6890 series gas chromatography (GC) equipped with flame ionization detector and capillary column (30 m×0.25 mm×0.25 mm). A quantity, 2 g of oil was weighed out in a small beaker and the exact weight was recorded. The sample was dissolved in 50 ml of chloroform and transferred into 100 ml volumetric flask and diluted to the mark. The most of the chloroform at room temperature was evaporated. One milliliter of inter-esterification reagent (20%v/v benzene and 55% v/v methanol) was added, sealed and heated at 100□C in water bath for 30 min. After inter-esterification, the extract of the methyl esters was mixed with hexane and water in a proportion of 1:1:1 and shook vigorously for 2 min. About half of the top layer (hexane layer) was transferred to the small test tube for injection. The detector temperature was programmed at 240□C with flow rate of 0.8 ml/min. The injector temperature was set at 240□C. Hydrogen was used as the carrier gas. The identification of the peaks characteristic and composition of *A. precatorius* seed biodiesel was achieved by retention times by means of comparing them with authentic standards analyzed under the same conditions (Knothe, 2005).

RESULTS

Percentage yield and physicochemical properties of *Abrus precatorious* seed oil

Table 1 shows that the yields of n-hexane oil extract of *A. precatorius* seed were very poor. The physical characterizations of the oil extract presented in Table 1 show that the oil extract has a lower relative density and lower kinematic viscosity. The Table also shows that the chemical characterization of *A. precatorius* seed oil has a lower iodine value, peroxide value, acid value with high saponification value and free fatty acid.

Biodiesel physicochemical and fuel properties

Table 2 shows the physical characterization of *A. precatorius* seed biodiesel in comparison with petro-diesel. The biodiesel from *A. precatorius* seed oil has higher density, kinematic viscosity, flash point, cloud point, pour point, cold filter plugging point, cetane number, ash contents and lower heat of combustion and refractive index than petro-diesel. *A. precatorius* seed biodiesel has lower acid value, iodine value and higher peroxide value than petro diesel.

Fatty acid profile of Abrus seed oil biodiesel

The result of fatty acid profile of *A. precatorius* seed oil biodiesel as shown in Table 3 reveals that the biodiesel contains methyl decanoate (1.27%), methyl undecanote (10.49%), methyl dodecanoate (6%), methyl eicosa-dienoate (17.16%), methyl tetradecanoate (4.59%), methyl palmitoleate (31.94%), methyl heptadecanoate (4.84%), methyl stearate (14.22%), methyl pentacosylate (3.7%) and methyl messilate (5.75%). The saturated FAME in *A. precatorius* seed oil biodiesel was 50.86% while unsaturated was 49.1%.

DISCUSSION

The oil yield content of n-hexane extract of *A. precatorius* seed was found to be 2.52%. This result is the same as the oil yield of petroleum ether extract of *A. precatorius* seed reported by Abu et al. (2011). However, the oil content suggests that *A. precatorius* seeds have low oil yield when compared to linseed (33.33%), soybean (18.35%) (Gunstone, 1999) and palm oil kernel (44.6%) (Akbar et al*.,* 2009); although, the oil yield of *A. precatorius* seed can be improved by using other extraction methods.

The physical characterization of the oils showed that the relative density of *A. precatorius* seed oil was 0.92. The result is similar to the oil of neem (0.918) (Sekhar et al*.,* 2009), coconut (0.91) (Alamu et al., 2010), *J. curcas* (0.901) (Belewu et al., 2010), shea butter (0.902) (Asuquo and Anusiem, 2010) and fluted pumpkin (0.908) (Ibeto et al., 2011). The relative density of *A. precatorius* seed oil is higher than the relative density specified by fuel standard. Therefore, using the oil as a biodiesel results in the delivery of a slightly greater mass of fuel which may influence engine output power since fuel injection equipment operates on a volume metering system.

The kinematic viscosity of *A. precatorius* seed oil was 23.4 mm^2/s which is above the limits of Thailand ASTM (1.9-8.0 mm^2/s) fuel standard. This value was found to be lower when compared to that of neem oil (44.00 mm^2/s) (Sekhar et al., 2009) and coconut oil (43.30 mm^2/s) (Alamu et al., 2010), but higher than that of *Jatropha curcas* oil (17.00 mm^2/s) (Wilson, 2010). Viscosity is a measure of the internal fluid friction or resistance of oil to flow, which tends to oppose any dynamic change in the fluid motion. Its value affects the atomization of fuel upon injection into the combustion chamber thus resulting to the formation of engine deposits. The high viscosities of

Table 2. Biodiesel physicochemical and fuel properties.

Parameter	Amount	Standard limits	Petrodiesel
FAME yield (%)	86.1 ± 0.01	-	-
Relative density	0.889	0.86 - 0.9[a]	0.84
Kinematic viscosity @ 40°C (mm^2/s)	3.34 ± 0.01	1.9 - 6[a], 1.9 - 8[bc]	2.98
Flash point (°C)	137 ± 0.02	Min. 130[d]	74
Heat of combustion (MJ/Kg)	38.29 ± 0.15	Min. 35[c]	42.85
Refractive index	1.457 ± 0.02	Max. 1.479[a]	1.483
Cloud point (°C)	- 2 ± 0.55	-3 to 12[d]	-16
Pour point (°C)	1 ± 0.23	-15 to 10[e]	-12
Cold filter plugging (°C)	- 4 ± 0.1	-20 to 5[f]	-18
Ash content (%)	0.088 ± 0.05	Max. 0.02[d]	0.02
Cetane number	58.3 ± 0.06	Min. 51[f]	49
Acid value (mg KOH/g)	0.281 ± 0.06	Max. 0.8[d]	0.35
Iodine value (mgI_2/g)	52.43 ± 0.2	Max. 120[f]	3.05
Peroxide value (mEq/Kg)	3.45 ± 0.17	-	0.2

Superscripts represent test methods; [a]ASTM D445, [b]Thailand ASTM, [c]EN14213, [d]ASTMD6751, [f]EN14214.

Table 3. Fatty acid profile of Abrus seed oil biodiesel.

Fatty acid methyl esters (FAME)	Amount (%) carbon molecules
Saturated FAME	50.86
Unsaturated FAME	49.1
Methyl decanoate	1.27 C_{10}
Methyl undecanoate	10.49 C_{11}
Methyl dodecanoate	6 C_{12}
Methyl eicosadienoate	17.16 $C_{20:2}$
Methyl tetradecanoate	4.59 C_{14}
Methyl palmitoleate	31.94 $C_{16:1}$
Methyl heptadecanoate	4.84 C_{17}
Methyl stearate	14.22 C_{18}
Methyl pentacosylate	3.7 C_{25}
Methyl messilate	5.75 C_{30}

vegetable oils are reduced through the process of trans-esterification (Alamu et al., 2008). Therefore, *A. precatorius* seed oil cannot be used as a fuel in engine due to high kinematic viscosity of it which would cause poor atomization of the oil in the combustion chamber thus resulting to the formation of engine deposits.

The results of the chemical characterizations of the oil extract showed that *A. precatorius* seed oil has iodine value of 57.43 mgI_2/g which classifies the oil as a non-drying oil. Non-drying oils have iodine value less than 100 (Asuquo et al., 2012). This value was lower than those of corn seed oil (103), sunflower oil (110), castor oil (83) and rubber seed oil (134.51) (Asuquo et al., 2012). Iodine value is the measure of unsaturation of fats and oil. The higher iodine value of fats and oil indicates high unsaturation and susceptibility to rancidity. This implies that the oil cannot be preserved for a long period of time. Iodine value is also used to measure the chemical stability property of oil against oxidation, and the higher the iodine value, the higher the number of double bond. *A. precatorius* seed oil has low iodine value compared to other non-drying oil such as castor oil (83) due to long chain fatty acid and higher saturated fatty acid. The low iodine value of *A. precatorius* seed oil is vital due to the fact that heating highly saturated fatty acids results in poor polymerization of glycerides which could reduce the formation of deposits in engines. It also reduces the rancidity in the oil, and makes it possible to preserve the oil and its biodiesel for long period of time.

The 4.1 mEq/kg peroxide value of *A. precatorius* seed oil showed the oxidative stabilities of the seed oil. The higher the peroxide values of oil, the greater the development of rancidity. This low peroxide value of *A. precatorius* seed oil must have resulted from proper handling of the oil during extraction, and also regulated heat treatment of the oil during extraction since heat favours oxidation of fatty acids thereby increasing the formation of peroxides (Oluba et al*.,* 2008). The low peroxide value of *A. precatorius* seed oil showed that the oil if exposed without addition or treatment with antioxi-dants will be stable over a long period of time and protected against rancidity and peroxidation.

The acid value of *A. precatorius* seed oil was shown to be 5.74 mg KOH/g. This acid value was higher than those of soybean oil (2.67 mg $KOHg^{-1}$), rape seed oil (2.88 mg $KOHg^{-1}$) (Jordanov et al*.,* 2007), palm oil (3.8 mg $KOHg^{-1}$) (Christian, 2006), but lower than that of *Cucurbita luffa* (36.47 mg KOH/g), *Brachystegia eurycoma* (27.08 mg KOH/g) (Ibeto et al*.,* 2011). The high acid value of *A. precatorius* seed oil indicates that the oil is non-edible, but may be useful for the production

of soaps, paints and biodiesel. Acid value measures the presence of corrosive free fatty acids and oxidation products. This is actually an important variable in considering the quality of oil because the lower the free fatty acid, the better the quality of oil with respect to its consumption (Balley, 1982). The percentage free fatty concentration of *A. precatorius* seed oils (2.87) is higher than the maximum limit of 2.0% (Codex Alimentairus Commission, 1993). Vegetable oils containing high free fatty acids have significant effects on the trans-esterification with methanol using alkaline catalyst. It interferes with the separation of fatty acid ester and glycerols (Ma and Hanna, 1999). The high acid value of *A. precatorius* seed oil suggests high levels of hydrolytic and lipolytic activities in the oils. Thus, this indicates that *A. precatorius* seed oil would be better converted to biodiesel using the two stage processes of esterification and trans-esterification to reduce the formation of soap.

The saponification value of *A. precatorius* seed oil was shown to be 227.8 mg KOH/g. This is higher than those of common seeds such as *J. carcus* (202.40 mg KOH/g), linseed oil (195 mg KOH/g) (Singh and Siroj, 2009) and fluted pumpkin (151.48 mg KOH/g), but lower than that of coconut (257 mg KOH/g) (Kyari, 2008). The higher saponification value of *A. precatorius* seed oil indicates the presence of high percentage of free fatty acids in the oil, and therefore implies the possible tendency to soap formation and difficulties in separation of products if utilized for biodiesel production. This would also suggest that using the oils for biodiesel production would lead to very low yields in the methyl esters.

The biodiesel yield (86.1%) of *A. precatorius* seed oil obtained is low when compared to conventional canola methyl ester (93.5%) (Leung and Guo, 2006), rape seed methyl ester (94%) (EI-Diwani et al*.,* 2009), but higher when compared to *J. carcus* seed methyl ester (80.2%) (Adebayo et al., 2011) and soybean ethyl esters (66.8%) (Hossain et al*.,* 2010). The biodiesel yield of the oil was low compared to that which was specified in EN14214. This low value of *A. precatorius* seed biodiesel could be due to the formation of soap which was so prominent during the conversion process as a result of high saponification and acid values of the oil.

The properties of the triacyglycerol and the biodiesel fuel are determined by the amounts of each fatty acid that is present in the molecules. Chain length and number of double bonds determine the physical characteristics of both fatty acid alkyl ester and triacyglycerol (Mittelbach and Remschmidt, 2004). Trans-esterification does not alter the fatty acid composition of the feedstocks and this composition plays an important role in some critical parameters of the biodiesel such as cetane number and cold flow properties (Ramos et al., 2009). There are three main types of fatty acids that are present in triacyglycerols; namely, the saturated (Cn:0), monounsaturated (Cn:1) and polyunsaturated with two or three double bonds (Cn:2,3). Various vegetable oils are potential feedstocks for the production of a fatty acid methyl ester or biodiesel, but the quality of the fuel will be affected by the oil composition. Vegetable oils that are rich in polyunsaturated fatty acid such as linoleic and linolenic acids as found in soybean and sunflower oil tend to give methyl ester fuels with poor oxidation stability (Gunstone, 2004). Vegetable oil with low degree of unsaturation tends to have high freezing point. This oil has poor flow characteristic and may become solid (for example, palm oil) at low temperatures though they may perform satisfactorily in hot climates (Gunstone, 2004). The predominant fatty acid alkyl ester of *A. precatorius* seed biodiesel consists of monounsaturated fatty acid methyl ester (31.94%), polyunsaturated fatty acid methyl ester (17.16%) and saturated fatty acid methyl ester (50.86%). According to the European standard, the concentration of linolenic acid and acid containing four double bonds in FAMEs should not exceed the limit of 12 and 1%, respectively. *A. precatorius* seed biodiesel does not contain linolenic acid and fatty acid containing three or four double bonds. The long chain and higher saturated fatty acids obtained showed that *A. precatorius* seed biodiesel has higher heat of combustion, high cetane number while high concentration of methyl eicosadienoate ($C_{20:2}$,17.16%) showed that *A. precatorius* seed biodiesel has a low cold temperature properties and low viscosity. It has been reported by Rodrigues et al. (2006) that more than one unsaturation in the carbon chain lowers both the crystallization temperature and the viscosity by hindering molecular packing.

The relative density obtained for the *A. precatorius* seed biodiesel (0.889) was in agreement with the specified value reported (ASTMD445), which range from 0.860 to 0.90 for biodiesel. The trans-esterification of *A. precatorius* seed oil to biodiesel reduced the density from 0.922 to 0.889 g/cm^3. Density is a very important property of biodiesel because fuel injection equipment operates on a volume metering system; hence a higher density for biodiesel results in the delivery of a slightly greater mass of fuel. Thus, changes in the fuel density will influence engine output power due to different mass of fuel injected. The relative density of *A. preatorius* seed biodiesel is higher than palm oil methyl ester (0.878) (Jansri and Prateepchaikul, 2011), but lower than castor oil biodiesel (0.917 g/cm^3) (Encinar et al., 2010).

The kinematic viscosity of *A. precatorius* seed biodiesel (3.34mm^2/s) was found to be within the limits of U.S.A ASTM D6751 (1.9 - 6.0 mm^2/s) fuel standard, and Thailand ASTM (1.9-8.0 mm^2/s) biodiesel fuel standard. The production of biodiesel from *A. precatorius* seed oil reduced the viscosity of the oil from 23.4 to 3.34 mm^2/s. Viscosity of biodiesel depends on the structural composition of the parent or virgin oil used in biodiesel production. Viscosity increases with the number of CH_2 moieties in the fatty ester chain. For example, methyl esters of lauric, myristic, palmitic and stearic acids have kinematic viscosities of 2.43, 3.30, 4.38, and 5.85 mm^2/s,

respectively (Knothe and Steidley, 2005). It also decreases with an increasing degree of unsaturation as evidenced by comparison of the methyl esters of stearic (5.85 mm^2/s), oleic (4.51 mm^2/s), linoleic (3.65 mm^2/s) and linolenic (3.14 mm^2/s) (Knothe, 2008). The higher viscosity of *A. precatorius* seed biodiesel than petro diesel is due to long chain length of fatty acid alkyl ester of *A. precatorius* seed biodiesel ($C_{16:1}$), (C_{18}), ($C_{20:2}$), (C_{30}) and higher saturated fatty acid alkyl ester (50.86%).

The flash points of *A. precatorius* seed oil biodiesel (137°C) was found to be above the minimum value (120°C) of the EN 14214 biodiesel fuel standard and the minimum value (130°C) of the ASTMD6751 biodiesel fuel standard. Flash point of a fuel is the temperature at which it ignites when exposed to a flame. Therefore, flash point is an important parameter to be considered in the handling, storage and safety of a biodiesel. The flash point of *A. precatorius* seed oil biodiesel was comparable to that of jatropha biodiesel (135°C) but lower than that of palm kernel oil biodiesel (167°C), (Alamu et al., 2008). This value was however, higher than that of neem seed oil biodiesel (120°C) and more importantly, extremely higher than that of petro diesel (74°C). The relatively higher flash point value of *A. precatorius* seed oil biodiesel showed that it does not contain methanol contaminants which would have lowered the flash point and it is of prime importance for storage and transport-tation of the fuel.

The heats of combustion of *A. precatorius* seed biodiesels (38.28 MJ/Kg) was comparable to those of soybean oil biodiesel (38.1 MJ/Kg) (Rashid et al., 2008), but greater than neem seed oil biodiesel (35.2 MJ/Kg) (Sekhar et al., 2009). The heat of combustion is important parameter for estimating fuel consumption; the greater the heat of combustion, the lesser the fuel consumption (Knothe et al., 2006). The heat of combustion or heating value is not specified in the biodiesel standards ASTM D6751 and EN14214. However, a European standard for using biodiesel as heating oil, EN 14213, specifies a minimum heating value of 35 MJ/kg. The heat of combustion increases with an increasing chain length and decreases with an increasing unsaturation. For instance, methyl stearate possesses greater energy content (40.07 MJ/kg) than methyl laurate (37.97 MJ/kg) (Knothe, 2008). Therefore, the energy content of fatty acid alkyl esters is directly proportional to chain length since longer-chain fatty acid alkyl esters contain more carbons but a similar number of oxygen atom. The heat of combustion of *Abrus* seed biodiesel was higher than the minimum value for heating biodiesel. The high heat of combustion of *A. precatorius* seed biodiesel is due to long chain length of fatty acid alkyl ester of *A. precatorius* seed biodiesel ($C_{16:1}$), (C_{18}), ($C_{20:2}$), (C_{30}) and higher saturated fatty acid alkyl ester (50.86%), underscoring the importance of Abrus seed oil-derived biodiesel as a useful petrol diesel supplement. The lower values of heats of combustion of *A. precatorius* seed biodiesel, and conventional biodiesels in general, when compared to that of petro diesel might be due to higher oxygen content and lower carbon-to-hydrogen ratio in the former than in the later.

The refractive index of *A. precatorius* seed biodiesel was 1.4567 which meets the value of ASTMD6751 of 1.479 maximum (ASTM International, 2002). It was lower than the refractive index of petro diesel (1.4831). The refractive index which is the ratio of the velocity of light in vacuum to the velocity of light in a medium is an indi-cation of the level of saturation of the biodiesel (Oderinde et al., 2009). As chain length of fatty acid and degree of unsaturation increases, the refractive index increases. Refractive index is widely used in quality control to check for the purity and adulteration of fatty materials (Hoffmann, 1986). The lower value of refractive index of *A. precatorius* seed biodiesel than petro-diesel indicates higher purity and saturation of the biodiesel than petrodiesel.

The low temperature operability properties of *A. precatorius* seed biodiesel determined by parameters such as cloud point (CP), pour point (PP) and cold-filter plugging point (CFPP) were 2, 1 and -4°C, respectively. The cloud point of *A. precatorius* seed biodiesel is within specified range of ASTMD6751 (-3 to 12°C) biodiesel fuel standard, but higher than petro-diesel (-16°C). The pour point (PP) of *A. precatorius* seed biodiesel was within specified range of ASTMD97 (-15 to 10°C) biodiesel fuel standard, but higher than petro diesel (-12°C) while cold-filter plugging point (CFPP) is within EN 14214 (-20 to 5°C) international biodiesel fuel standards, but higher than petrodiesel (-18°C). The CP is the temperature at which the first solids become visible when cooling a diesel fuel; the PP is the temperature at which the fuel ceases to flow while cold filter plugging point is the temperature at which the diesel fuel blocks the filter device as a result of the formation of crystal agglo-merates. These parameters are related to the cold engine start, and should be sufficiently low because when the biodiesel freezes, the engine will not start (Encinar et al., 2005). An increase in chain length of saturated fatty acid methyl ester and decrease in degree of unsaturation of fatty acid result in a corresponding increase in melting point (Lee et al., 1995). For instance, compounds of similar chain length but increasing levels of unsaturation display lower melting point as evidenced by the melting point of C18:0 (methylstearate, melting point 39°C), C18:1 (methyloleate, melting point -20°C), C18:2 (methyl linoleate, melting point -35°C), and C18:3 (methyl linolenate, melting point -52°C) (Lee et al., 1995). The cloud point, pour point and cold filter plugging point of *A. precatorius* seed biodiesel (49.1% unsaturated fatty acid methyl ester and 50.86% saturated) are lower than palm oil methylester (49.9% saturated and 47.7% unsaturated) (CP = 13°C,PP=16°C) (Dunn, 2005), karanja oil methylester (68.23% unsaturated and 26.04%saturated) (CP = 3.4°C, PP = 6°C) (Bobade and Khyade, 2012),

tallow oil methyl ester (55.3%unsaturated and 39.6% saturated) (CP = 17°C,PP =15°C,CFPP = 9°C) (Dunn, 2005). The lower cold temperature operability properties of *Abrus precatorius* seed biodiesel are due to high concentration of polyunsaturated fatty acid ($C_{20:2}$ =17.16%wt) of fatty acid alkyl ester of *A. precatorius* seed biodiesel. It has been reported by Rodrigues et al. (2006) that more than one unsaturation in the carbon chain lowers the cold temperature operability properties by hindering molecular packing.

The value of sulfated ash obtained for *A. precatorius* seed biodiesel 0.09% is slightly higher compared to the standard specified 0.02% max (ASTMD6751). Ash content describes the amount of inorganic contaminants, such as abrasive solids and catalyst residues and the concentration of soluble metal soaps contained in a fuel sample. It has higher ash contents than jatropha seed biodiesel [0.06%] (Adebayo et al., 2011). However, the value was higher than that obtained for diesel fuel 0.02%. The slightly higher ash contents of *A. precatorius* seed biodiesel was due to the contamination with metals from water used in purification of the biodiesel and the use of crude oil.

A. precatorius seed oil biodiesels had cetane number of 58.30 which is well above the minimum value of the ASTM D6751 (40 minimum) and EN 14214 (51 minimum) international biodiesel fuel standards. Cetane number (CN) is widely used as diesel fuel quality parameter related to the ignition delay time and combustion quality. High cetane numbers help to ensure good cold start properties and minimize the formation of white smoke. The CN of an individual compound depends upon the structure of the compound. The CN increases with an increasing chain length (that is, methyl esters of lauric (CN 67), palmitic (CN 86) and stearic (CN 101) acids), and also increases with increasing saturation of fatty acid (that is, methyl esters of stearic (101), oleic (59), linoleic (38), and linolenic (23) acids) (Knothe et al., 2006). The cetane number of *A. precatorius* seed oil biodiesel was found to be higher than some conventional biodiesels such as soybean oil biodiesel (49) (Ramos et al*.,* 2009) and sunflower oil biodiesel (55) (Rashid et al., 2009). Thus, the higher cetane number of *A. precatorius* seed oil biodiesel when compared to petro-diesel (49) and other conventional biodiesel is due to long chain length of fatty acid alkyl ester of *A. precatorius* seed biodiesel ($C_{16:1}$), (C_{18}),($C_{20:2}$), (C_{30}) and higher saturated fatty acid alkyl ester (50.86%).

The chemical characteristics of *A. precatorius* seed biodiesel showed that the acid value of *A. precatorius* seed oil biodiesel was 0.281 mg KOH/g which is within the limits of the ASTM D6751 (0.8 mg KOH/g maximum) and EN 14214 (0.5 mg KOH/g maximum) biodiesel fuel standards. However, the acid value of *A. precatorius* seed oil biodiesel is lower than that of sunflower oil biodiesel (0.40 mg KOH/g) and *Jatropha* oil biodiesel (0.4 mg KOH/g maximum) (Wilson, 2010). The lower acid value of *A. precatorius* biodiesel when compared to other conventional biodiesels and petro-diesel (0.350 mg KOH/g) indicates that the fuel has low levels of free fatty acids in the biodiesel, which also suggests low levels of hydrolytic and lipolytic activities in the biodiesel.

A. precatorius seed biodiesel has iodine value (52.43 mgI_2/g) which is lower than the standard iodine value for biodiesel of 120 by Europe's EN 14214 specification. Iodine number is a measure of total unsaturation within a mixture of fatty acid material. The limitation of unsaturation of fatty acid is vital due to the fact that heating highly unsaturated fatty acids results in polymerization of glycerides which could lead to the formation of deposits (Mittelbach, 1994). The iodine value of *A. precatorius* seed biodiesel is lower than *Jatropha* oil biodiesel (101 mgI_2/g) (Tamalanpundi et al., 2008). The lower iodine value of *A. precatorius* seed biodiesel will allow it to be used as alternative fuel for diesel engine that leaves very small carbon deposits on the injector and in combustion chamber thus improving life of components and increasing inter service period.

The peroxide value of *A. precatorius* seed oil biodiesel was 3.45 mEq/kg. It is higher than petro-diesel (0.20 mEq/kg). The peroxide value measures the presence and amount of unstable hydroperoxide which is a portion of deteriorated biodiesel formed when oxygen from the air react with fatty esters. The peroxide value of the biodiesel can be reduced by adding of antioxidants (Frankel, 2005). The low peroxide value of *A. precatorius* seed oil biodiesels indicates little peroxidative rancidity of the biodiesels as a result of proper handling.

Conclusion

In this present studies, the physiochemical characterization of *A. precatorius* seed oil and its biodiesel properties have highlighted the potentials of the oil and its biodiesel as a very good resource. Due to high availability of the seed, ability of the seed to grow easily as well as the poisonous nature of the seed, it is considered good resource for industrial process. The oil yield of the seed which is quite small compared to other high oil yielding seeds could be modified genetically and the yield can also be improved by using other extraction methods. *A. precatorius* seed oil has high saponification value showing that it is also good for soap production. *A. precatorius* seed biodiesel is suitable when compared to conventional biodiesels and petro-diesel. Considering the percentage biodiesel yield in this study, the fatty acid methyl esters (FAME) confer on the biodiesel improved fuel qualities. Some of the fuel properties investigated had shown, to a reasonable extent, that quality biodiesel can be produced from *A. precatorius* seed. Hence, improving *A. precatorius* seed cultivation more than its present state via mechanization and genetic engineering can, therefore, facilitate its incorporation as an additional feedstock for biodiesel.

Conflict of Interests

The author(s) have not declared any conflict of interest.

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Review

Wheat stem rust in South Africa: Current status and future research directions

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Plant diseases are among the major causes of crop yield loss and food insecurity. In South Africa, stem rust caused by *Puccinia graminis* Pers. f. sp. *tritici*. Eriks. & E. Henn. (*Pgt*) is an important disease of wheat. Records of stem rust occurrence in South Africa date back to the late 1720's, when it was first discovered in the south-western wheat growing areas of the Western Cape. Although growing stem rust resistant cultivars is one of the most economic and environmentally feasible strategies, one of the challenges in host-plant resistance is that *Pgt* frequently acquires new virulence to overcome resistance genes in existing cultivars. There is strong evidence that the pathogen continues to evolve through mutation and genetic recombination. The appearance of stem rust race Ug99 (TTKSK, North American race notation) in East Africa in 1999 and subsequent epidemics in Kenya and Ethiopia was accompanied by the occurrence of four Ug99 variants (TTKSF, TTKSP, PTKST and TTKSF+) in South Africa. These have resulted in a renewed interest in understanding the status of *Pgt* races and stem rust resistance in the world as well as the need for a new host-plant resistance strategy. The current review summarises up-to-date literature on the prevalence of stem rust races in South Africa, and also draws attention to the resistance genes and strategies currently deployed to combat this disease. The aim of the review was to provide perspectives on research milestones and guide future research programs for reducing losses incurred by stem rust of wheat in South Africa.

Key words: Durable resistance; Pathogen variability; *Puccinia graminis* f. sp. *tritici.*

INTRODUCTION

Wheat is one of the most important grain crops in South Africa with an estimated annual production of 1.8 to 2 million tons between 2013 and 2014 (South African Grain Information Service 2014 - http://www.sagis.org.za/Flatpages/Oesskattingdekbrief.htm). Compared to the global wheat market, South Africa is

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a minor player producing less than 0.4% of the world's total. South Africa is a net importer of wheat, since wheat prices are trading at import parity levels; it is more economical to import wheat products than to produce them locally (Louw et al., 2013). According to the Department of Agriculture, Forestry and Fisheries (DAFF) wheat market value chain profile (2011/2012), South Africa is the largest producer of wheat in the Southern African Development Community (SADC) region and the fourth largest producer in the African continent. Approximately 27% of the total cultivated area is under irrigation with the remaining being dry land cultivation (SAGIS 2014 - http://www.sagis.org.za/Flatpages/Oesskattingdekbrief.htm).

Spring wheat cultivars are suited to the winter rainfall areas, while the winter wheat and intermediate types are suited to the summer rainfall areas of South Africa (Jordaan, 2002). The wheat growing areas are also differentially suitable for the development of stem, stripe and leaf rust diseases of wheat caused by *Puccinia graminis* Pers. f. sp. *tritici* (*Pgt*), *Puccinia striiformis* f. sp. *tritici* (*Pst*) and *Puccinia triticina* Eriks (*Pt*), respectively (Pretorius et al., 2007).

This review focuses specifically on stem rust in South Africa. Special emphasis will be placed on tracking the evolution and spread of the Ug99 races in South Africa and neighbouring countries, while providing a general description of the pathogen, the damage it causes and mitigation strategies.

STEM RUST PATHOGEN: MORPHOLOGY, REPRODUCTION AND THE MODE OF DISPERSAL

Puccinia graminis has an intricate life cycle that includes both sexual and asexual reproduction cycles. The fungus is heteroecious with uredinial (asexual) and telial stages occurring on wheat and other species of *Poaceae*, while its pycnial (sexual) and aecial stages occur on alternative hosts including *Berberis* spp. and *Mahonia* spp. (Campbell and Long, 2001). *Berberis* plants have not yet been found in South Africa, thus it is believed that sexual reproduction does not occur in the country. Hence, this section of the review describes the pathogen life cycle from a perspective of what commonly occurs in South Africa.

The asexual stage on wheat host begins with the production of urediniospores (Roelfs, 1985). Owing to the absence of the alternative host, *Pgt* can persist in the asexual or uredinial stage on wheat throughout the year, moving from winter wheat crops to the spring and intermediate wheat crops. Uredinia are produced after a successful infection of a grass host by urediniospores. These occur most frequently on the leaf sheaths of a wheat plant, but can also occur on true stem tissues, leaves, glumes and awns (Singh et al., 2011). The urediniospores produced during the crop season are oval and red-orange in colour varying from 15-20 by 40-60 µm in size and are easily shaken off a plant (Knott 1989; Singh et al., 2006, 2011). Pathogen infection appears as elongated blister-like pustules with the uredinia being about 3 by 10 mm in size. The urediniospores are dispersed by wind and water, and can spread over vast distances. In addition to natural dispersal mechanisms, accidental transfer by means of farm implements, contaminated clothing or goods may also contribute to the spread of spores (Singh et al., 2008). The rate of *Pgt* infection is heavily influenced by cultivar susceptibility, the virulence of the rust race as well as favourable environmental conditions such as temperature and humidity.

EFFECTS OF STEM RUST ON GRAIN YIELD AND END-USE QUALITY OF WHEAT

Wheat rusts are one of the major biotic stress factors leading to serious economic losses in South Africa and the world (Singh et al., 2006; Pretorius et al., 2007; Sharma et al., 2013). *Pgt* is highly aggressive making it the centre of attention of wheat breeders, geneticists, pathologists and farmers. An apparently healthy looking crop three weeks prior to harvesting can be severely devastated and destroyed by the explosive build-up of stem rust if sufficient inoculum arrives from a heavily infected wheat crop from a nearby field (Leonard and Szabo, 2005; Singh et al., 2006). When wheat stems are severely infected, nutrient flow to the developing heads is interrupted, resulting in shrivelled grain. Shrivelled grain usually results to lower test weight, flour yield and increased grain protein content. In addition, stems weakened by rust infection are prone to lodging, causing yield loss (Knott, 1989). Studies from around the world show stem rust incurs an average grain yield loss of 35% (Pretorius, 1983). This loss can increase to a total loss depending on the growth stage of the host when infection starts (Roelfs, 1985; Leonard and Szabo, 2005).

STEM RUST RACES IN SOUTH AFRICA

Effective management of stem rust requires a co-ordinated effort, including race monitoring, collection and characterisation of sources of resistance, and resistance breeding (Boshoff et al., 2000). The phenotyping of the stem rust pathogen in South Africa was initiated in the 1920s. This resulted in standard races 34 and 21 being the first to be identified in 1922 and 1929, respectively, and since then have been the most prevalent races (Pretorius et al., 2007). However, from 1960 to 1980 there was renewed interest in *Pgt*, leading to more regular surveys, the establishment of an improved differential set to characterize races, and a structured approach towards identifying effective sources of resistance

(Pretorius et al., 2007). In 1980, the Agricultural Research Council-Small Grain Institute (ARC-SGI) in Bethlehem, South Africa, initiated annual rust surveys which coincided with the mandatory inclusion of stem rust resistance genes in all newly released cultivars (Pretorius et al., 2007). The surveys were conducted to assist wheat breeding programmes with information on effective resistance genes.

The annual stem rust surveys were extended in 2013 to include the genetic characterization of all field isolates using simple sequence repeat markers (SSRs). However, due to the very high genetic similarity between the members of the Ug99 lineage, SSR analysis cannot distin-guish between them. For that reason, Dr. LJ Szabo at the USDA-ARS, St Paul, Minnesota, USA developed single nucleotide polymorphism (SNP) markers based on TaqMan technology (Szabo, unpublished results). A combined SNP and SSR analysis are thus currently employed to identify all the field isolates from the survey. Preliminary results indicated that combined with the phenotypical data, a very accurate assessment of the diversity of the local *Pgt* population can be made.

In South Africa, the first documented epidemic of wheat stem rust occurred in the South-western wheat growing areas of the Western Cape province in 1726 (Pretorius et al., 2007). As wheat production expanded in the country, epidemics became a recurring phenomenon and turned out to be particularly devastating in the winter-rainfall production regions of the Western and Eastern Cape, as well as in the summer-rainfall regions of the Free State. The last epidemic occurred in 1985 on *Sr24*-derived wheat cultivars in the Albertinia area of the Western Cape (Boshoff et al., 2000). Although the *Sr24* stem rust resistance gene was effective against most races of *Pgt* and was used in commercial wheat cultivars worldwide (Jin et al., 2008), detection of the races 2SA100 and 2SA101 (Agricultural Research Council of South Africa race notation) that were virulent to *Sr24* substantially increased the vulnerability of South African wheat cultivars (Le Roux and Rijkenberg, 1987). Over 30 *Pgt* races of wheat and triticale have been characterised in South Africa since the early 1980s (Table 1). *Pgt* continues to acquire virulence against resistance genes through genetic recombination, mutation and new introductions from other countries. Growing a single cultivar over a large area also contributes to the develop-ment of new virulent races. For example, SST44 carrying *Sr24* was widely used in the 1980s and the prevalence of race 2SA100, which is virulent to *Sr24*, increased drama-tically (Le Roux, 1985). The timely detection of stem rust races with new virulence is hence important to resistance breeding as information on the virulence profiles of new races would enable wheat geneticists and breeders to utilise effective resistance genes in their breeding programs.

The *Sr31* resistance gene that was transferred from rye to wheat was once one of the most effective and durable resistance genes. Durable resistance is described as a mechanism conferring effective resistance to a cultivar for long period of time during its widespread cultivation in a favourable environment for a disease (Johnson, 1984). Nevertheless, the first *Pgt* race with virulence to *Sr31* was reported in 1999 in Uganda, East Africa (Pretorius et al., 2000). The race was later designated as TTKS by Wanyera et al. (2006) using the North American nomenclature system. When a fifth set of differential lines was added, this race was re-named TTKSK (Jin et al., 2008, 2009). During 2003 and 2004, the majority of Kenyan cultivars and a large portion of CIMMYT wheat germplasm with gene *Sr31* planted in Kenya were susceptible to stem rust (Wanyera et al., 2006). Since the discovery of TTKSK, seven other variants of the Ug99 lineage have been detected in different parts of Africa (Singh et al., 2011). The evolution and occurrence of Ug99 variants have further broken down most of widely deployed stem rust resistance genes existing in commercial cultivars (Haile and Röder, 2013). Also, the introduction of new cultivars could contribute to the evolution of new stem rust races.

In total, four of the eight Ug99 race group members (TTKSF, TTKSP, PTKST and TTKSF+) were found in South Africa, as well as in several other countries in Southern Africa (Mukoyi et al., 2011). The first South African member of the Ug99 lineage was TTKSF (2SA88). A genetic study indicated strong genetic resemblance to TTKSK (Visser et al., 2009), except for avirulence towards *Sr31* (Pretorius et al., 2007). This proved that TTKSF did not develop from the South African race 3SA43 as was previously suggested (Pretorius et al., 2007). This was the first genetic study confirming that the South African *Pgt* population was an introduction from another location.

The second South African Ug99 group member TTKSP (2SA106) with virulence to *Sr24* was detected in 2007 in the Western Cape, South Africa (Terefe et al., 2010). TTKSP is phenotypically similar to PTKST except for avirulence to *Sr31* and similar to TTKSF except for virulence to *Sr24* (Visser et al., 2011). The *Sr24* gene is one of the genes that were initially identified as conferring resistance to Ug99 (Jin et al., 2008). Virulence of TTKSP to *Sr24* was of particular concern in South Africa as more than 20% of the commercial cultivars and elite germplasm had *Sr24* as a major resistance gene (Le Roux and Rijkenberg, 1987).

In November 2009, the third Ug99 race PTKST (2SA107) was detected at two locations (Cedara and Greytown) in Kwa-Zulu Natal Province. PTKST is virulent to both *Sr24* and *Sr31* (Pretorius et al., 2010), but unlike TTKSK, it is avirulent to *Sr21* (Terefe et al., 2010). Races with similar virulence phenotypes as 2SA107 were previously known in Kenya and Ethiopia which suggest that PTKST, just like TTKSF might have entered South Africa through an introduction from one of the East African countries (Terefe et al., 2010). The final South African

Table 1. Avirulence/virulence patterns of the stem rust races identified in South Africa.

Race	Avirulence/virulence genes
2SA2	*Sr5, Sr6, Sr9b, Sr9e, Sr17, Sr24, Sr30, Sr36/ Sr7b, Sr9g*
2SA4	*Sr8b, Sr9g, Sr21, Sr24, Sr27, Sr31, Sr36/Sr5, Sr6, Sr7b, Sr8a, Sr9b, Sr9e, Sr11, Sr17, Sr23, Sr30, Sr37, Sr44, SrGt*
2SA6	*Sr9e, Sr24, Sr36/Sr5, Sr6, Sr7b, Sr9b, Sr9g, Sr17, Sr30*
2SA10	*Sr6, Sr7b, Sr9b, Sr9e, Sr17, Sr24, Sr30, Sr36/Sr5, Sr9g*
2SA18	*Sr7b, Sr9e, Sr24, Sr30, Sr36/Sr5, Sr6, Sr9g, Sr17*
2SA20	*Sr9e, Sr24, Sr30/Sr5, Sr6, Sr7b, Sr9b, Sr9g, Sr17, Sr36*
2SA32	*Sr5, Sr6, Sr7b, Sr9b, Sr9g, Sr17, Sr24, Sr30*
2SA33	*Sr7b, Sr9e, Sr9g, Sr17, Sr24, Sr30/Sr5, Sr6, Sr9b, Sr36*
2SA36	*Sr9e, Sr9g, Sr23, Sr24, Sr27, Sr30, Sr37, Sr44, SrGt/Sr5, Sr6, Sr9b, Sr11, Sr17, Sr36*
2SA39	*Sr5, Sr9b, Sr9e, Sr17, Sr24, Sr30/Sr6, Sr7b, Sr9g*
2SA43	*Sr24, Sr36/Sr5, Sr6, Sr7b, Sr9b, Sr9e, Sr9g, Sr17, Sr30*
2SA45	*Sr9e, Sr9g, Sr24, Sr36/Sr5, Sr6, Sr7b, Sr9b, Sr17, Sr30*
2SA48	*Sr9e, Sr9g, Sr24, Sr30/Sr5, Sr6, Sr7b, Sr9b, Sr17, Sr36*
2SA49	*Sr9e, Sr9g, Sr24/Sr5, Sr6, Sr7b, Sr9b, Sr17, Sr30, Sr36*
2SA51	*Sr9b, Sr9e, Sr9g, Sr17, Sr24, Sr30, Sr36/Sr5, Sr6, Sr7b*
2SA52	*Sr9e, Sr24, Sr30, Sr36/Sr5, Sr6, Sr7b, Sr9b, Sr9g, Sr17*
2SA53	*Sr24/Sr5, Sr6, Sr7b, Sr9e, Sr9g, Sr17, Sr30, Sr36*
2SA54	*Sr7b, Sr9e, Sr9g, Sr24, Sr36/Sr5, Sr6, Sr9b, Sr17, Sr30*
2SA55	*Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr9g, Sr17, Sr21, Sr24, Sr27, Sr30, Sr31, Sr36, Sr38, SrEm, SrKw, SrSatu, SrTmp/Sr8a, Sr9a, Sr9d, Sr10, Sr11, Sr44, SrMcN*
2SA88	*Sr13, Sr15, Sr21, Sr22, Sr24, Sr25, Sr26, Sr27, Sr29, Sr31, Sr32, Sr33, Sr35, Sr36, Sr39, Sr43, SrAgi, SrEm, SrKw, SrSatu/Sr5, Sr6, Sr7a, Sr7b, Sr8a, Sr8b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9f, Sr9g, Sr10, Sr11, Sr12, Sr14, Sr16, Sr17, Sr19, Sr20, Sr23, Sr30, Sr34, Sr38, SrLc*
2SA88+	*Sr24, Sr27, Sr31, SrKw, SrSatu, SrTmp/Sr6, Sr7b, Sr8a, Sr8b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9g, Sr10, Sr11, Sr17, Sr21, Sr30, Sr88, Web, SrMcN*
2SA99	*Sr5, Sr6, Sr9b, Sr9e, Sr21, Sr24, Sr27, Sr31, Sr36, Sr38, SrKw, SrSatu/Sr7b, Sr8a, Sr9g, Sr11, Sr17, Sr30*
2SA100	*Sr8b, Sr9e, Sr9g, Sr21, Sr27, Sr30, Sr36, Sr37, Sr44, SrGt/Sr5, Sr6, Sr7b, Sr8a, Sr9b, Sr11, Sr17, Sr23, Sr24*
2SA101	*Sr9e, Sr30, Sr36/Sr5 Sr6, Sr7, Sr9b, Sr9g, Sr17, Sr24*
2SA102	*Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr11, Sr17, Sr21, Sr23, Sr24, Sr30, Sr31, Sr36, Sr37, SrGt/Sr8a, Sr9g, Sr27, Sr30, Sr44*
2SA103	*Sr5, Sr6, Sr9b, Sr9e, Sr9g, Sr11, Sr17, Sr23, Sr24, Sr36, Sr37, SrGt/Sr27, Sr30, Sr44*
2SA104	*Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr17, Sr21, Sr24, Sr30, Sr31, Sr36, Sr38, SrEm, SrSatu, SrTmp/Sr8a, Sr9a, Sr9d, Sr9g, Sr10, Sr11, Sr27, Sr44, SrKw, SrMcN*
2SA105	*Sr5, Sr6, Sr7b, Sr8b, Sr9b, Sr9e, Sr17, Sr21, Sr24, Sr30, Sr31, Sr36, Sr38, SrEm, SrTmp/Sr8a, Sr9a, Sr9d, Sr9g, Sr10, Sr11, Sr27, Sr44, SrKw, SrSatu, SrMcN*
2SA106	*Sr21, Sr27, Sr31, Sr36, Sr44, SrEm, SrKw, SrSatu, SrTmp/Sr5, Sr6, Sr7b, Sr8a, Sr8b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9g, Sr10, Sr11, Sr17, Sr24, Sr30, Sr38, SrMcN*
2SA107	*Sr13, Sr14, Sr21, Sr22, Sr25, Sr26, Sr27, Sr29, Sr32, Sr33, Sr35, Sr36, Sr37, Sr39, Sr42, Sr43, SrSt 44, SrEm, SrTmp, SrSatu/SrSr5, Sr6, Sr7b, Sr8a, Sr8b, Sr9a, Sr9b, Sr9d, Sr9e, Sr9g, Sr10, Sr11, Sr16, Sr17, Sr24, Sr30, Sr31, Sr34, Sr38, Sr41, SrMcN*

member of the Ug99 lineage is 2SA88+ (TTKSF+) that was identified in December 2010 at Afrikaskop in the Eastern Free State (Pretorius et al., 2012). It is thought to represent a local adaptation from TTKSF with virulence on cv. Matlabas. The similarity of *Pgt* races in Zimbabwe, South Africa and Mozambique thus supports cross boundary intro-ductions of races (Mukoyi et al., 2011). As such, all stem rust introductions and new races that evolved locally could have a substantial impact on resistance breeding strategies in all neighbouring countries.

GENES DEPLOYED FOR STEM RUST RESISTANCE IN SOUTH AFRICA

The rapid adaptation and distribution of Ug99 and its variants incited a renewed interest leading to a robust search and deployment of new resistance genes effective against stem rust races. The gene deployment strategy is important and should be considered in all breeding programs around the world. This strategy has mostly been advocated in recent years by the Borlaug Global Rust Initiative (http://www.globalrust.org/), a group of rust pathologists, breeders, geneticists and policy makers. For gene deployment, there are more than 57 different stem rust resistance genes catalogued and identified to date (McIntosh et al*.,* 2011; Haile and Röder, 2013). Most of these resistance genes and gene complexes were sourced from relatives of wheat (McIntosh et al*.,* 2003; Jin et al*.,* 2007; Liu et al., 2014) and near-isogenic lines carrying individual *Sr* genes are available in several wheat backgrounds. These resistance genes are spread throughout the three wheat genomes (A, B and D). Multiple resistance alleles are known to exist at the *Sr7*, *Sr8*, and *Sr9* loci. Of these multi- resistance loci, the *Sr9* locus carries a large number of alleles (McIntosh et al., 2003; Tsilo et al., 2007; Rouse et al., 2014). Additionally, closely linked stem rust genes with resistance to Ug99 races have also been reported at this locus (Hiebert et al., 2010; Zurn et al., 2014). Recently, a wheat stem rust resistance gene *SrWeb* subsequently designated as *Sr9h*, an allele at the *Sr9* locus has been identified from cultivar Webster, also present in Ug99 resistant cultivar Gabo 56 (Rouse et al., 2014).

In general, host-plant resistance against *Pgt* is grouped into two broad categories: seedling or major resistance genes (effective from seedling stage onwards) and adult plant or minor resistance genes (effective at the later or adult stage of the wheat plant) respectively. Apart from the *Sr2*, *Sr55*, *Sr56, Sr57* and *Sr58* genes, about 53 of the designated *Sr* genes are single-locus major genes conferring resistance at all stages of plant development. In many cases this resistance is short lived due to the emergence of new virulent races (Singh et al*.,* 2008; Jin et al*.,* 2009; Njau et al*.,* 2010). In South Africa, most wheat cultivars have not been intensively characterised for stem rust resistance. Wheat cultivars released between the late 90's to early 2000, however, had major genes for resistance to locally prevalent *Pgt* races which became defeated with time as more virulent races were reported (Pretorius et al*.,* 2007). The only race specific resistance genes that are known to be deployed include *Sr9e*, *Sr24*, *Sr27, Sr36* and *Sr39* found in different cultivars (Le Roux and Rijkenberg, 1987; Smith and Le Roux, 1992). Amongst those still effective is *Sr39* which was transferred from the wild relative *Aegilops speltoides* L. (Kerber and Dyck, 1990). Nevertheless, this gene has not been used extensively in wheat breeding worldwide because of the negative effects associated with *A. speltoides* chromatin; however, Niu et al*.* (2011) and Mago et al*.* (2009) have developed a set of recombinants with reduced *Ae. speltoides* fragments addressing the problem of linkage drag associated with the *Sr39* gene and the seed source is made available. Rouse et al. (2014) demonstrated that a race specific gene *Sr9h* in cultivar Gabo 56 confers resistance to *Pgt* race TTKSK. A robust allelism test determined that the *Sr9h* resistance gene is a new allele of *Sr9*, adding to the six alleles already present at this locus.

The adult plant resistance (APR) gene that was deployed in South Africa is *Sr2* (Pretorius and Brown, 1999), a recessive gene that provides a slow rusting response at adult plant stage and has been used worldwide (Singh et al*.,* 2006). *Sr2* was originally selected by McFadden in the 1920s from Yaraslav emmer wheat (Dubin and Brennan, 2009) and was mapped on chromosome 3BS of wheat close to the *csSr2* marker (Mago et al., 2011). Resistance given by this gene is unique in that it is pleiotropic, conferring resistance to other diseases including leaf rust, stripe rust and powdery mildew (McIntosh et al*.,* 1995). One of the first cultivars confirmed to carry *Sr2* called Palmiet, was released in 1985 in South Africa. It contributed significantly to the reduction of stem rust levels until its withdrawal from production in 1999 (Pretorius et al*.,* 2012).

It was recently shown that approximately 50% of the historic South African wheat cultivars carried the *Sr2* gene (Ntshakaza, unpublished data). The frequency of *Sr2* in recent commercial cultivars, however, appears to be low based on the use of the *Sr2* marker *csSr2* with only three cultivars PAN 3377, Inia and Steenbras having the gene (Pretorius et al*.,* 2012). The loss of *Sr2* in commercial cultivars could be attributed to the fact that breeding for durable stem rust resistance may have not been the main focus of breeders (J Smith and SC Smith, unpublished data). Hence, many commercial cultivars are susceptible to *Pgt* races 2SA102 and 2SA103. Pretorius et al. (2012) also showed that very few recent cultivars have resistance to all South African *Pgt* races as 88% of entries tested susceptible at seedling stage to at least one of the tested races. The status of stem rust resistance in commercial cultivars will probably change in the near future, as current and future breeding efforts aim to re-introduce *Sr2* in combination with other effective stem rust resistance genes back into commercial cultivars (Table 2). Nevertheless, the *Sr2* gene does not provide sufficient level of protection when deployed singly; it interacts with other genes that express intermediate levels of resistance to condition acceptable levels (Bansal et al*.,* 2014). Ayliffe et al. (2013) have previously demonstrated additive resistance when the *Sr2* resistance gene was combined with *Sr33* in Chinese Spring wheat seedlings. Identification of other APR genes such as *Sr55* (*Lr67/Yr46/Pm46*) (McIntosh et al., 2012), *Sr56* (Bansal et al*.,* 2014), *Sr57* (*Lr34/Yr18/Pm38*) (Lagudah et al*.,* 2006) and *Sr58* (*Lr46/Yr29/Pm39*)

Table 2. Effective and ineffective stem rust resistance genes against common races in South Africa as detected in the fields during evaluations.

***Sr* genes**	
Effective	**Ineffective**
Sr2, Sr13, Sr14, Sr22, Sr25, Sr26, Sr29, Sr32, Sr33, Sr35, Sr36, Sr37, Sr39, Sr42, Sr43	*Sr5, Sr6, Sr7a, Sr8a, Sr9a, Sr9b, Sr9d, Sr9e, Sr9f, Sr9g, Sr10, Sr11, Sr12, Sr15, Sr16, Sr17, Sr18, Sr19, Sr20, Sr21, Sr23, Sr24, Sr27, Sr30, Sr31, Sr34, Sr38, Sr41, Srwld-1*

(McIntosh et al., 2012) has aided breeding for durable stem rust resistance in wheat, and efforts to characterise more of these APR genes should be considered.

DEVELOPMENT OF STEM RUST RESISTANT GERMPLASM IN SOUTH AFRICA: CURRENT AND FUTURE DIRECTIONS

Despite the occurrence of stem rust in South Africa, information on the genetic basis of stem rust resistance in leading South African wheat cultivars and breeding lines is limited. Understanding the status of stem rust in South Africa and neighbouring countries (Lesotho, Zimbabwe and Botswana) should provide useful and detailed information on which genes were already used, so that new sources of resistance could be introduced and deployed strategically. More importantly, breeders should have this information to target specific genes as they routinely recycle germplasm and breeding lines in their breeding programs.

As a result of renewed interest on host resistance, different research groups in South Africa comprised of farmers, plant pathologists, geneticists and breeders together with international collaborators are exploring ways to combat stem rust disease by searching for new sources of durable resistance. As confirmed in the past, the conventional methods of phenotyping wheat to search and pyramid genes into a single genotype, have proven to be difficult and time consuming; hence, it may not be feasible for a regular breeding program to apply all necessary rust races in selecting lines with multiple rust resistance genes. Even so, multi-race tests are commonly performed in the glasshouse on seedlings and important adult plant resistance may not be recognised. It is also possible that multiple-race selection does not identify which genes are effective; hence a more advanced technique, such as DNA markers, is needed to identify and diagnose each effective resistance gene.

Marker-assisted selection (MAS) has been proven to be a powerful tool to facilitate the development of cultivars with multiple resistance genes or gene pyramids (Dubcovsky, 2004; Bernardo et al., 2013; Haile and Röder, 2013). Molecular markers linked to effective stem rust resistance genes can be used to predict the presence of specific genes with high accuracy without the need for disease evaluation, thus helping with the transfer of several genes into adapted material (Tsilo et al., 2008, 2009; Pumphrey, 2012; Bernardo et al*.,* 2013). Several laboratories, worldwide, have made concerted efforts to develop and identify molecular markers that are linked to effective race-specific and non-race specific stem rust resistance genes against *Pgt* and with reference to the Ug99 race group (Table 3). Nevertheless, some of the markers linked to effective resistance genes are not suitable for routine applications of MAS in breeding programs. Efforts of optimising DNA markers and developing the most diagnostic markers are on-going processes in many laboratories. With the availability of next-generation sequencing platforms, more diagnostic resistance gene markers will be made available for high-throughput screenings and application of MAS in breeding for stem rust resistance. Cloning of the identified resistance genes is extremely important, not only can it lead to developing excellent markers but can also assist in detecting important resistant gene families. To date, only a few stem rust resistance genes have been cloned, namely *Sr33* (Periyannan et al., 2013) and *Sr35* (Saintenac et al*.,* 2013), making it easier to apply resistance gene-derived markers. Moreover, fixing of these incorporated resistance genes into suitable genetic backgrounds and subsequent development of inbred lines can still take a considerable number of years before a new cultivar is ready for field trials. The doubled haploid (DH) technique can shorten the inbreeding process to a single cycle, without compromising the integrity and the agronomical quality of the lines (Wessels and Botes, 2014).

At the Agricultural Research Council - Small Grain Institute, MAS is routinely used to pyramid stem rust resistance genes into adapted germplasm in order to deploy a durable rust resistance strategy as proposed by the Borlaug Global Rust Initiative. The targeted genes include *Sr2* (3BS), *Sr25* (located on 7Ae#1 of *Thinopyrum ponticum* that was translocated onto 7DL chromosome), *Sr26* (located on 6Ae#1 of *T. ponticum* that was translocated onto 6AL chromosome) and *Sr39* (from chromosome 2BS of *Aegilops speltoides*). These genes are introgressed into the widely used South African bread wheat cultivars as a means to increase broad spectrum resistance. The genes used in this pyramiding scheme have been engineered to reduce the likelihood of linkage drag and originated from sources

Table 2. Chromosomal location, description, linked DNA markers and references for wheat stem rust resistance genes effective against *Puccinia graminis* f. sp. *tritici* races with special reference to Ug99 resistance in Africa.

Sr gene	Chromosomal location	General description	Linked DNA marker	Original source	References
Major genes (race-specific resistance)					
SrWeb (*Sr9h*) *and SrWLR*	2BL	✓ Allelism tests demonstrated that *SrWeb* from cultivar Webster is *Sr9h* adding to six alleles already present at this locus. ✓ Effective against Ug99 race TTKSK.	*GWM47* *IWA6121* *XwPt-3132* *XwPt-8460* *IWA6122* *IWA7620* *IWA8295* *IWA8362*	*Triticum aestivum*	Hiebert et al., 2010; Zurn et al., 2014; Rouse et al., 2014
Sr13	6AL	✓ Confers moderate resistance to TTKS. ✓ Frequent gene in durum varieties.	*BARC104* *WMC580* *DUPW167* *CK207347* *CD926040* *BE403950* *dupw167* *AFSr13* *gwm427*	*Triticum turgidum*	Simons et al., 2011; Periyannan et al., 2014
Sr22	7AL	✓ Confers resistance to Ug99 and other important races. ✓ The region surrounding this gene was modified to minimize effects of yield penalty and delay in heading date.	*WMC633* *CFA2123* *CFA2019* *BARC121* *IH81-BM* *IA81-AG* *cssu22*	*Triticum monococcum*	Khan et al., 2005; Olson et al., 2010; Periyannan et al., 2011
Sr24	1BS	✓ Confers resistance to TTKS but not its variants. ✓ Useful due to its linkage to *Lr24*.	*SCS73719* *Sr24#12*	*Agropyron elongatum*	Mago et al., 2005; Olson et al., 2010
Sr25	7DL, 7AL	✓ Confers a high level of resistance only in some genetic backgrounds. ✓ Sources with EMS-induced mutation on yellow pigment gene are available.	*BF145935*	*Thinopyrum ponticum*	Knott 1980; Liu et al., 2010
Sr26	6AL	✓ Confers resistance to Ug99 and other races. ✓ Sources with short chromatin fragment on chromosome 6AL are available. ✓ Multiplexing two primer sets to detect co-dominancy.	*Sr26#43* *BE51879*	*Thinopyrum ponticum*	Mago et al., 2005; Dundas et al., 2007; Liu et al., 2010
Sr27	3A	✓ Effective against Ug99. ✓ Has not been used in wheat improvement.	-	*Secale cereale*	Singh et al., 2011

Table 3. Contd

Sr28	2BL	✓ Confers APR for most known races. ✓ Seedling stage resistance for races BCCBC, TTKSK and TTKST. ✓	*WMC322* *WPT7004* *wpt7004*	*Triticum aestivum*	Rouse et al., 2012
Sr32	2A, 2B, 2D	✓ Effective against Ug99 lineage. ✓ Several recombinant translocation stocks with reduced *Aegilops speltoides* chromatin harbouring *Sr32* are available. ✓	*csSr32#1* *cssu22* *csSr32#2*	*Aegilops speltoides*	Mago et al., 2013
Sr33	1DS	✓ Confers only moderate levels of resistance. ✓ Cloned gene ✓	*BE405778* *BE499711*	*Aegilops tauschii*	Sambasivam et al., 2008; Periyannan et al., 2013
Sr35	3AL	✓ Effective against race TTKSK (Ug99) and its variants (TTKST and TTTSK. ✓ Cloned gene ✓	*AK332451* *AK331487* *GWM480* *GWM271* *WMC169* *WMC559* *BARC51* *CFA2193* *CFA2170* *CFA2076* *BE423242* *BF485004* *AK3358187* *BE405552*	*Triticum monococcum*	Zhang et al., 2010; Saintenac et al., 2013
Sr36	2BS	✓ Confers a high level of resistance to Ug99 and some of its variants. ✓	*GWM319* *WMC477* *STM773-2*	*Triticum timopheevi*	Bariana et al., 2001; Tsilo et al., 2008
Sr37	4BL	✓ Effective gene against Ug99. ✓ Recombinants with smaller alien segments were produced using the *ph1b* system, however, inheritance of resistance in these recombinants has been unstable to date.	-	*Triticum timopheevii*	Zhang et al., 2012
Sr39	2B	✓ Moderately to highly resistant to Ug99 in seedling tests. ✓ Multiple stocks with reduced *Aegilops speltoides* chromatin segments harbouring *Sr39* on chromosome 2BS are available to prevent effects due to linkage drag.	*Sr39#22r* *Sr39#50s* *WMC471* *RWGS27* *RWGS28* *RWGS29*	*Aegilops speltoides*	Mago et al., 2009; Niu et al., 2011

Table 3. Contd

Sr40	2BS	✓ Confers resistance to Ug99. ✓ Four recombinants with reduced *Triticum timopheevii* chromatin are available to prevent effects of linkage drag.	*Sr39#22r* *Xwmc344* *Xwmc474* *Xgwm374* *Xwmx474* *Xwmc661*	*Triticum timopheevii* ssp. *armeniacum*	Wu et al., 2009
Sr42	6DS	✓ Confers resistance to TTKSK and variants TTKST and TTKSK. ✓ Mapped to the same position on 6DS as *SrtMp* and *SrCad* indicating that they are the same genes.	*BARC183* *GPW5182* *FSD_RSA*	*Triticum aestivum*	Ghazvini et al., 2012
Sr43	7D	✓ Confers resistance to TTKSK, TTKST, TTTSK. ✓ Recently, two putative translocation lines with reduced alien chromatin have been developed.	*Xcfa2040*	*Aegilops elongatum*	Niu et al., 2014
Sr44	7DS	✓ Moderately to highly resistant to Ug99 in seedling tests.	*BF145935*	*Thinopyrum intermedium*	Liu et al., 2010
Sr45	1DS	✓ A locus more proximal to *Sr33*. ✓ Confers moderate levels of resistance.	*ccsu45*	*Aegilops tauschii*	Periyannan et al., 2014
Adult plant resistance genes (race-non-specific resistance)					
Sr2	3BS	✓ Conferred durable resistance against all virulent races of *Pgt* worldwide for more than 50 years combined with other genes. ✓ Deployed in many wheat cultivars worldwide. ✓ Pseudo-black chaff (morphological marker).	*GWM533* *GWM389* *BARC133* *csSr2-SNP* *3B042G11* *3B028F08* *STM559TGAG*	*Triticum turgidum*	McNeil et al., 2008; Mago et al., 2011
Sr55 (*Lr67/Yr46/Pm46*)	4DL	✓ Confers adult plant resistance to stem rust and powdery mildew	*Xgwm165* *Xgwm192* *Xcfd71* *Xbarc98* *Xcfd23*	*Triticum aestivum*	Herrera-Foessel et al., 2011
Sr56	5BL	✓ Confers adult plant resistance to stem rust	*sun209* *sun320*	*Triticum aestivum*	Bansal et al., 2014
Sr57 (*Lr34/Yr18/Pm38)*	7DS	✓ Confers adult plant resistance to stem rust ✓ Under appropriate conditions it may confer resistance in seedlings to certain rust races	*csLV34*	*Triticum aestivum*	Lagudah et al., 2006
Sr58 (*Lr46/Yr29/Pm39)*	1BL	✓ Confers adult plant resistance to stem rust	*Xwmc44* *Xgw259* *Xbarc80* *Xgwm259*	*Triticum aestivum*	William et al., 2003

The most useful diagnostic markers are underlined.

developed by the late Dr. Douglas R. Knott from the University of Saskatchewan (sources of *Sr25* with EMS-induced mutation on yellow pigment gene) (Knott 1980), Dr. Ian Dundas from the University of Adelaide (reduced fragments of *Sr26*) (Dundas et al., 2004) and Dr. Steven S. Xu from the United State department of Agriculture - Agricultural Research Service (reduced fragment of *Sr39*) (Niu et al., 2011). Several of other diagnostic/most useful markers (Table 3) are used to select resistance genes. The Plant Breeding Laboratory of the University of Stellenbosch has also clearly demonstrated and reaffirmed the importance of a breeding scheme where conventional breeding strategies are integrated with MAS and DH techniques in order to speed up cultivar development. In a study by Wessels and Botes (2014) from University of Stellenbosch, several rust resistance genes were successfully incorporated into a population of inbred lines within two seasons. The study has also shown the importance of the incorporation of locally available resistance genes into a genetic background that is suitable for South African agroclimatic conditions. Other pre-breeding and breeding efforts at the University of Free State and two South African private breeding companies are underway to increase durable stem rust resistance using a variety of sources and resistance genes. With advancements in technology, adoption of high throughput MAS by all breeding programs will make it easier to continuously integrate new sources of resistance into the existing gene pool, thereby sustaining the stem rust management strategy.

CONCLUSION AND RECOMMENDATIONS

Norman Borlaug, the Nobel laureate, who is widely considered as the father of wheat improvement in the 1960s, once noted that 'rust never sleeps'. Although farmers controlled the stem rust fungus with chemicals including fungicides, the approach is not a long term solution and can raise costs on economy and environment. Host plant resistance, and more impor-tantly, the combination of several effective stem rust resistant genes remain the most feasible, economic and environmentally safe approach. For this reason, it is clear that South African breeders and pathologists, together with the international community, need to continuously replace susceptible cultivars with ones with durable stem rust resistance by introgressing new resistance genes.

Race characterisation using both phenotypic and genotypic data has improved our understanding of the dispersal mechanisms and evolution of *Pgt* across regions. The deployment of resistance genes in South Africa has also progressed from one gene per cultivar to multiple genes to offer a broad spectrum and durable resistance to stem rust. In this way, the chances of a pathogen defeating virulence to multiple genes are very low. This may also lead to lower levels of inoculum; and may reduce the spread of virulent races to other wheat producing areas outside of South Africa.

Efforts for effective implementation of integrated disease management practices and gene stewardship in the SADC region, as well as international collaborative initiatives with farmers, breeders and scientists should be adopted to decrease the chance of new races evolving and/or getting introduced. Emphasis should be placed on the promotion of preventive approaches such as releasing cultivars with durable resistance or multiple resistance genes, rapid seed multiplication systems, intensive surveillance approaches, institutional coordination and contingency planning for effective emergency response capability. There is a need to rally for more research funds to be allocated for the sustained and improved productivity of wheat at local, regional and international basis. This includes an intensive study and understanding of both the host and pathogen genetics and variability. A recent study by Pardey et al. (2013) concludes that farmers should also play a pro-active role in the research programs, and understand that maintaining yield and growth rates necessary to meet anticipated future demands will require a sustained effort to plant wheat varieties that are resistant to existing races of rust. Pardey et al. (2013) undoubtedly shows that this will necessitate an investment strategy that supports sustained research programs geared towards identifying and addressing evolving stem rust threats across the globe.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Review

Methane and nitrous oxide emission from livestock manure

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Methane (CH_4) is a greenhouse gas which contributes significantly to global warming and a significant proportion of atmospheric methane is produced by livestock. Livestock contribute 18% of global greenhouse gas (GHG) emissions. Apart from enteric emission, decomposition of livestock manure under anaerobic conditions is also a source of methane. The later condition arises in confined management system. There is difficulty in disposing off the excreta and wastes produced on a large scale; they are stored in large pits which provide suitable environment for CH_4 production. Another green house gas is nitrous oxide (N_2O), released during the nitrification-denitrification of nitrogen contained in livestock waste. Cattle and feedlots are responsible for 26% of N_2O emissions from anthropogenic sources. Being greenhouse gases, their large scale emission is detrimental to the environmental safety. So, different strategies are emerging to either subside their emission from faeces and animal wastes or to use them effectively for energy saving purposes.

Key words: Global warming, manure, methane, nitrous oxide.

INTRODUCTION

Livestock contribute to climate change through the emissions of greenhouse gases such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) which cause global warming. The global warming potential of N_2O and CH_4 are 310 times and 23 times the global warming potential of CO_2, respectively. They together contribute 18% of global greenhouse gas (GHG) emissions (Steinfeld et al., 2006). Apart from enteric emission, decomposition of livestock manure under anaerobic conditions is another important source of methane. Normally the organic matter in manure is hydrolyzed and converted to volatile fatty acids. Only when the manure is stored for a long time especially in confined management system without fast disposing off, does the multiplication of the methane producers result in substantial release of methane. Nitrous oxide emission is associated with manure management and the application and disposition of manure as fertilizer. Considering the detrimental effect of the above gases to environmental safety, different strategies are emerging to either subside their emission from faeces and animal wastes or to use them effectively for energy production purposes.

LIVESTOCK MANURE DISPOSAL

The manure disposal systems vary with variation in the

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Table 1. Methane emissions (MT) in terms of CO_2 equivalent from manure management (Source; Environment Protection Agency, USA).

Country	Year			
	1990	2000	2005	2020
India	18.83	80.52	23.20	27.48
China	15.70	19.76	80.91	28.32
France	13.79	13.30	13.25	13.29
Germany	27.10	23.27	19.63	16.65
USA	31.19	38.08	39.18	43.83
World total	222.52	225.38	234.57	269.47

species of livestock, size of herd, climate, type of animal management system, storage period of manure. Manure management systems can be classified into dry and liquid/slurry manure management systems. Dry systems include solid storage, dry feedlots, deep pit stacks and daily spreading of manures. Liquid systems use water to facilitate manure handling and manure is stored in concrete tanks and lagoons. The cattle manure is generally categorized into liquid stable manure, solid stable manure and meadow manure. The stable manure is produced by animals in confinement rearing system. Stable manure is also produced in grazing system at the night time in shelters and also by the dairy animals during milking. The liquid stable manure is stored in the manure tanks outside the animal houses which provide an anaerobic environment. Mostly aerobic condition occurs in meadow manure, produced by the animals in grazing. Sheep are grazing animals and spend the cold winter months inside animal houses only, where they produce solid manure. Goats also produce solid manure. Pig manure is generally of liquid or semi solid type. Poultry birds are generally kept in cage system and produce solid manure except laying hens.

Liquid systems create the ideal anaerobic environment for methane production whereas warm climate make the condition more conducive. In solid system methane production is very less though an increased production has been noticed with rainfall. N_2O production from manure is an initial aerobic and then an anaerobic process. Dry, aerobic management systems may provide an environment more conducive for N_2O production though the relationship between degree of aeration and N_2O production form manure has not been established (Jun et al., 2000).

METHANE AND NITROUS OXIDE EMISSIONS FROM LIVESTOCK

The contribution of GHG emission from enteric fermentation and manure management is almost in the ratio of 9:1. In the industrial model of livestock production under which a large number of animals are housed in confinement, the faeces and animal wastes are stored in massive lagoons that create a suitable anaerobic pool for CH_4 production. Methane contributes to 15% of enhanced greenhouse effect whereas agriculture and associated sectors are responsible for 50% of the anthropogenic methane emissions (Bhatia et al., 2004). The annual global emission of CH_4 was reported to be 535 MT (Houghton et al., 1996). In India the methane's share in total GHG emissions was 30% in 1985 which declined to 27% in 2008 due to relatively higher CO_2 emissions from the fossil fuels. But there was a rise in the absolute value from 18.85 Tg in 1985 to 20.56 Tg in 2008 (Garg et al., 2011). The methane emission figures from manure management in several countries and world total have been suggested by Environmental protection agency of USA (Table 1).

Nitrous oxide contributed to 5% of enhanced greenhouse effect. Agriculture and associated sectors were responsible for 70% of the anthropogenic emissions of N_2O (Bhatia et al., 2004) whereas cattle and feedlots were responsible for 26% of N_2O emissions from anthropogenic sources (IPCC, 2001). Kroeze et al. (1999) reported annual global emissions of 17.7 MT of N_2O. The reports of Mirzaei and Hari Venkatesh (2012) suggested 75% contribution of livestock sector to agricultural N_2O emissions that equates to 2.2 billion tonnes of CO_2 equivalent. IPCC (1996) has reported the global N excretion in the range of 60-100 kg $year^{-1}$ for dairy cattle, 40-70kg $year^{-1}$ for non-dairy cattle, 12-20 kg $year^{-1}$ for sheep and 16-20 kg $year^{-1}$ for swine, respectively. As per the report of INDITE (1994) in UK the NH^{+}_{4}-N stored in livestock wastes is 250 kt and when applied to lands accounted for more than 12% of the total N_2O-N emissions from all terrestrial sources. Sneath et al. (1997) reported N_2O emissions of 800 mg from UK livestock buildings. MAFF (1989) estimated a total flux of 2 kt of N_2O-N $year^{-1}$ from excreta (dung and urine) by grazing animals on 5 Mha grazing land in the UK. The uncertainty in livestock methane emission data is due to the lack of information about emission factors for the various sources. Singhal and Mohini (2004) estimated total methane emission on the basis of 'methane per kg feed intake' from different categories of animals in different

agro-climatic conditions fed on different types of feeds. Prusty et al. (2014) reported that methane production by buffalo per day could be predicted most reliably (R^2 = 0.82) from the NDF, NFC and CP intake through fodders. Methane emission factors of 45 kg $hd^{-1}year^{-1}$ for dairy cattle manure and 3 kg CH_4 hd^{-1} $year^{-1}$ for beef cattle manure has been recommended by Pattey et al. (2005) in the North-America under cool conditions.

Global warming potential of methane and nitrous oxide

The contribution of methane is less than 2% of all the factors leading to global warming. The global warming potential is 21-23 times (UNFCC, 1995; IPCC, 2001) more than carbon dioxide. N_2O emissions contribute to depletion of ozone in the stratosphere, as in stratosphere nitrous oxide is converted to nitric oxide gas which is hazardous at sea-level. A significant increase of atmospheric N_2O concentration at a rate of 0.22 ± 0.02% per year has been reported (Battle et al., 1996). High atmospheric life of N_2O (166 ± 16 years) along with its 310 times (Tomlinson et al., 2013) global warming potential raised huge concern for the emission of N_2O.

Methane generation from livestock manure

Manure from livestock consists of a proportion of organic volatile solids which are fats, carbohydrates, proteins and other nutrients that act as source of food and energy for the growth and reproduction of anaerobic bacteria. The acid formers group of bacteria break down the volatile solid in manures to a series of fatty acids in the acid forming stage and in the next stage highly specialized methane formers convert the acids to methane gas and carbon dioxide. The methane formers are pH (6.8-7.4) sensitive, strict anaerobes and functions best at 95°F (Monteny et al., 2001). These conditions often occur when large numbers of animals are managed in a confined area (for example, dairy farms, beef feedlots and swine and poultry farms) where manure is stored in large piles or disposed of in lagoons. The main factors affecting methane emission from livestock manure are the amount of manure that is produced and the portion of the manure that decomposes anaerobically. The methane production is represented as methane conversion factor (MCF) in which the actual methane production is expressed as the ratio between the actual and the ultimate methane production, the later occurs with very long storage time.

Nitrous oxide generation from livestock manure

Nitrous oxide is produced during the nitrification-denitrification of nitrogen contained in livestock waste (Monteny et al., 2001). Initial nitrification step is important to provide the essential substrate (NO^-_3) for the microbial denitrification processes. There are several other ways of producing N_2O, as nitrifier-denitrification coupled chemical paths are also important. Its production requires an initial aerobic reaction and then an anaerobic process. Nitrous oxide is formed from N compounds of feeds or excreta during processes where oxygen is consumed. Dry, aerobic management systems may provide an environment more conducive for N_2O production. The majority of nitrogen in manure is in ammonia (NH_3) form. Nitrification occurs aerobically and converts this ammonia into nitrate. Denitrification occurs anaerobically, and nitrous oxide is one of the intermediate reaction products. Biochemical oxygen demand (BOD) and nitrogen concentration affect N_2O generation. Pereira et al (2012) observed a significant increase in the NH_3, CO_2 and CH_4 production from dairy cattle excreta with a change in storage temperature from 5 to 35°C.

$$NH_4 \xrightarrow{+\ \text{Nitrosomonas spp}} NO_2^- \xrightarrow{-\ \text{Nitrobacter spp}} NO_3^-$$

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

IPCC (1996) guidelines for measurement of methane and nitrous oxide emission from manure

The methane emission is expressed as kg CH_4 $year^{-1}$. The annual emission factor multiplied with total population from animal category, gives the assumed total methane emissions from the animal population. The annual emission factor is decided from the daily volatile solids (kg) excreted, maximum methane producing capacity (m^3 kg^{-1}) of volatile solid (VS) for manure, methane conversion factor for manure management system in particular climate region, fraction of manure handled using manure system of a particular animal population. The climate categories for emission factor as per IPCC recommendations are ‘cool’, ‘temperate’ and ‘warm’. Methane conversion factor for cool climate is 1% for solid waste system and 39% for liquid/ slurry waste system and deep litter (cattle, sheep) system. The volatile solids from manure of an animal category will vary with composition of diet and other factors such as straw addition. In conventional solid and slurry system the volatile solid produced is 87.3 and 84.3% of the cattle manure DM whereas it is 79.6 and 80.7% of the pig manure DM, respectively (Steineck et al., 1999). Below is given the composition of VS as suggested by Sommer et al. (2002) (Table 2).

The most important parameters for estimation of nitrous oxide are derivation of nitrogen excretion that is generally expressed as kg N $year^{-1}$. According to the guidelines, cattle, pigs and poultry only account for the nitrous oxide emissions and other animals like sheep, goat, camels, which do not account for manure management under wet

Table 2. Composition of volatile solids from animal slurry.

Parameter	Nutrient	Cattle slurry (%)	Pig slurry (%)
Degradable VS	Fat	9	10
	Protein	8	30
	Carbohydrate	21	25
Degradation resistant VS	Carbohydrate	52	35

VS, Volatile solids.

system are eliminated from the category of animals producing N_2O. Population data is same as used for estimation of methane from enteric fermentation and manure management. Nitrogen excretion values those are used for estimating nitrogen excretion animal^{-1} are as follows, dairy cattle - 60, non-dairy cattle - 40, pigs - 16 and poultry - 60 kg animal^{-1} year^{-1}. Nitrogen excretion (anaerobic lagoon/ liquid system and any other system) is derived as percentage of N_2 excretion from total N_2 excretion from animals. According to IPCC (1996) guidelines the percentage of N_2 excretion in anaerobic lagoon from non-dairy cattle manure is zero and for dairy cattle is 6 whereas in liquid system it is 4 for dairy cattle manure. In anaerobic lagoon, liquid system and other systems of storage the percentage of N_2 excretion were 1, 2 and 52 for poultry manure and 1, 38 and 0 for pig manure, respectively. Nitrous oxide emission per animal is determined by multiplying the nitrogen excretion using emission factors, which is the N_2O- N per kg excreted N. IPCC default emission factor for Asia is as follows: anaerobic lagoons and liquid systems-0.001 and others systems-0.005. Total emission is determined by multiplying the number of animals in each category with the emission factor. Emissions from all categories are aggregated and total emission expressed as Gg nitrous oxide year^{-1}.

FACTORS AFFECTING NITROUS OXIDE AND METHANE EMISSION

Housing system

Housing plays a more important role on GHG emissions in non-ruminants production systems since most of the emission in those systems comes from the manure. As described by the International Atomic Energy Agency (IAEA, 2008), the types of housing systems in Asia needs different strategies for manure treatment. Greater CH_4 emissions were reported from farmyard manure followed by liquid slurry and deep litter manure (Külling et al., 2003). Hristov et al. (2012) investigated the effect of manure management on barn floor on NH_3, CH_4, N_2O, and CO_2 emissions and found that CH_4 emissions were considerably lower for the flush manure systems (37 mg m^{-2} h^{-1}) than gravity-flow system (1,216 mg m^{-2} h^{-1}) on barn floor. Methane emissions from manure were much greater from dairy barns where manure is stored for prolonged periods of time compared with barns where manure is removed daily. Philippe et al. (2007) reported that fattening swine reared on deep litter released nearly 20% more GHG than those on slatted floors (0.54 Vs 1.11 g pig^{-1} day^{-1} for N_2O, and 16.3 Vs 16.0 g pig^{-1}day^{-1}for CH_4, respectively).

Deep litter system of pig housing had a great potential for N_2O production, mainly caused by poor O_2 availability in the compacted deep litter (Groenestein and Van Faasse,1996). Amon et al. (2001) observed similar N_2O emissions from the tying stall with manure managed in slurry based (609.6 mg N_2O livestock unit^{-1} day$^{-1)}$ or straw based system (619.2 mg N_2O livestock unit^{-1} day^{-1}).

Species and individual variation

Cattle slurry produced less N_2O than pig slurry and poultry manure whereas methane production depends on the organic matter content. The rate of organic matter production was highest from poultry manure followed by pig slurry and cattle manure (Corre et al., 1997). The Department of Animal Husbandry, Dairying and Fisheries (2010) of India estimated the amount of excreta year^{-1} in million tonnes to be 22.93 by sheep and goat, 8.26 by pig, 14.18 by poultry and 427.12 by cattle and buffalo. Methane production increased with the organic matter (volatile solids) content of the excreta. Poultry manure, pig slurry and cattle manure produced methane in decreasing order per kg of manure. But the large figure for CH_4 production from cattle is due their more volume of faeces excretion. Similar observations of higher methane production from pig (356 L kg^{-1} VS) and sow (275 L kg^{-1} VS) manure compared to dairy cattle manure (148 L kg^{-1} VS) has been reported by Moller et al. (2004). Bala (2013) reported non linearity in the methane emissions in relation to the manure mass in case of horses. He observed an emission of 1.40 and 9.31 g methane day^{-1} from 10 and 20 kg manure, respectively using fermentation chamber. Sharma (2014) suggested that selection of individual animals based on residual feed intake (RFI)

would be helpful to reduce enteric methane emissions. Animals with low RFI produced less methane while maintaining the productivity and thus contributing less GHG to the environment. Further research need to be done regarding relation of RFI with emission of gases from livestock manure.

Feed

Lodman et al. (1993) observed higher ($p< 0.05$) methane production from the manure of cattle fed a high grain diet compared to that of the cattle fed a forage det. Jarvis et al. (1995) observed an increased methane emission from grass and clover fed dairy cows and heifers. Hindrichsen et al. (2005) observed effect of feeding different concentrate diets based on oat hull, soybean hull, apple pulp, *Jerusalem artichoke*, molasses and wheat on the methane emissions from slurries of their origin. The slurry originating from molasses diet showed maximum methane emission at 14 weeks of storage though the proportion of methane produced from slurry compared to total emission (enteric and slurry) did not vary with treatments. Hindrichsen et al. (2006) observed 6.6% manure methane emission of the total methane in dairy cattle, fed on forages only, compared to 13% when fed on forage and concentrate in 1: 1 ratio. But Aguerre et al (2010) and Yohaness (2010) observed that increasing the grass and concentrate ration from 47: 53 to 68: 32 had no effect on manure methane emission. Doreau et al. (2011) observed higher manure methane production in hay and corn silage based diet compared to corn grain diet whereas the reverse was observed for N_2O and CO_2 emissions.

The amount of N excretion in dairy cows depends closely on the feed intake. By improving the protein quality of the diet according to the actual requirements, the gain of protein by the animal can be increased and the N excretion may be reduced. Nitrogen excretion of fattening animals increases with the live weight because the protein requirements for maintenance depend on the live weight. Dietary crude protein reduction reduced both CH_4 and N_2O emissions from stored manure (Atakora et al., 2011). Külling et al. (2001) reported decreased N_2O emissions from storage manure of dairy cows fed low-protein diets, but the total GHG emissions were not affected as there was an increased CH_4 emission from the low protein manure. Velthof et al. (2005) observed large decrease in the NH_3 and CH_4 emissions during manure storage and N_2O emission from soilby by decreasing the protein content of swine diets whereas reverse effect on N_2O emissions was reported in swine (Philippe et al., 2006) and dairy cattle (Arriaga et al., 2010) on lowering the dietary protein. Shifting N losses from urine to faeces is expected to reduce N_2O emissions from manure applied soil due to the lower concentration of NH_4^+ in manure. The N excretion of pigs can also be changed by feeding with bacterial fermentable substances such as cellulose, hemicellulose and pectin. Because these substances are degraded in the hindgut of the pigs by microbes, nitrogen is needed for bacterial growth, therefore the N excreted by the urine is reduced.

Stage of animal

N saving effect is more pronounced in producing animals than in growing animals. Milk yield of the cows and CP content of milk affected the N excretion inversely (Colmenero and Broderick, 2006). Variations in methane emissions were caused by difference in milk yield and feed intake (Amon et al., 2001).

Phase feeding

Phase feeding is an effective mitigation practice for GHGs. Reducing dietary protein concentration during the production cycle to better meet the requirements of the animal, significantly lowered the N excretion (Vasconcelos et al., 2007) and consequently losses from the pen surface. A two phase feeding reduced N excretion whereas further reduction was observed using four-phase feeding (Joachim and Heinz-Jürgen, 2001).

Management system

Dry systems include solid storage, dry feedlots, deep pit stacks, and daily spreading of the manure whereas liquid management systems often use water to facilitate manure handling. Liquid systems create the ideal anaerobic environment for methane production. The largest combined N_2O-CH_4 emissions in CO_2 equivalent were observed from the slurry storage, followed by the stockpile and lastly the passively aerated compost. This ranking was governed by CH_4 emissions in relation to the degree of aerobic conditions within the manure. The CO_2 equivalent emissions from the stockpiled manure was 1.46 times higher than from the compost for dairy and beef types of cattle manure (Pattey et al., 2005). Storage treatments with proper aeration and moisture management reduced CH_4 generation from poultry manure (Li and Xin, 2010). Ventilated belt removal of laying hen manure reduced CH_4 emissions compared to deep-pit storage (Fabbri et al., 2007). Amon et al. (2001) reported lower N_2O losses from an actively turned composting pile of solid cattle manure than from an undisturbed anaerobically stored pile.

Season of year

In practice, with substrate and microorganisms being

abundantly available, temperature and storage time mainly determine the amount of CH_4 produced. In Danish cattle the methane release from fresh dung pats in the field began immediately and ceased after 10-18 days and the total methane emissions varied from 37 to 170 ml kg^{-1} dung pat during late summer and spring, respectively (Holter,1997). Husted (2004) observed a significant increase in methane emission rate for pig and cattle slurries with an increase in storage temperature, with the peak emission observed at 35-45°C from pig slurry. Similar increase of two fold methane production with increase of storage temperature from 10 to 20°C has been reported by Masse et al. (2008).

In summer, the anaerobically stacked farmyard manure emitted about 4.5 times more greenhouse gasses than the aerobically composted farmyard manure. Due to the lack of oxygen supply in the winter compost, N_2O and CH_4 emissions were higher than from the summer compost (Amon et al., 2001). Ellis et al. (2001) observed that in uncovered yard N_2O emission rates were 3.3 µg N m^{-2} h^{-1} in winter and spring and 6.5µg N m^{-2} h^{-1} in summer. Pereira et al (2012) noticed that cumulative N_2O emissions were not significantly different between temperatures, although numerically slightly higher at 35°C compared to 5, 15 and 25°C.

Abatement strategy

Better manure management practice is the foremost strategy to reduce GHG emissions from manure whereas recovery techniques under which the recovered methane can be used for energy generation/ flaring is an attractive alternative. The flaring process decreases up to 95% of harmful atmospheric effect of methane. In developing countries, like in India there is rarely any provision of storing liquid manure. Instead the manure is used as fuel for households for cooking and preparation of compost to fertilize the aerable lands, otherwise they are thrown in open area. These activities give rise to very little amount of methane from the manure. If livestock manure is kept under aerobic condition by turning the manure regularly, methane emission from manure management can be reduced. The livestock excreta are spread on agricultural lands as manures which make the anaerobic condition unavailable for methanogenic bacteria to degrade the organic matter. Presence of inhibiting compounds (e.g., ammonium) also determines CH_4 production during storage and composting processes.

Abatement of N_2O should be considered as part of an integrated approach to improve the efficiency of N cycling in animal production systems. Current technologies could deliver up to 50% reduction in N_2O emissions from an animal housing system but only up to 15% from a grazing system (De Klein and Eckard, 2008). In animal housings if the air is centrally exhausted, the NH_3 may be stripped with sulfuric acid (Joachim and Heinz-Jürgen, 2001) for the production of ammonium sulfate fertilizer (efficiency up to 96%). When excreta have been applied to soil, nitrification inhibitors (for example, nitrapyrin) may conserve the applied NH_4-N as NH_4^+ and reduce N_2O emissions. Avoiding grazing at moist conditions might be helpful in mitigating N_2O emissions from urine patches in pastures (Van Groenigen et al., 2006). Increasing the hippuric acid concentration through dietary manipulation has been reported to be helpful for mitigating N_2O emissions. It is assumed that benzoic acid which is a breakdown product of hippuric acid has inhibitory effects on the denitrification pathway (Van Groenigen et al., 2006). Mechanically ventilated structures provide opportunity to treat emitted GHG through filtration and scrubbing. Another interesting mitigation technology for animal housing is use of titanium dioxide (TiO_2) paint on the interior walls. Industrial uses of TiO_2 stimulated its photocatalytic properties by UV light and lead to oxidation of NH_3 and N_2O (Allen et al., 2005).

Studies by Costa et al. (2012) in swine houses showed that GHG mitigation with TiO_2 paint hold promise for future GHG reduction strategy from manures. Capturing the gases produced using impermeable membranes, such as oil layers and sealed plastic covers, can reduce NH_3, N_2O, and CH_4 emissions. VanderZaag et al. (2008) suggested use of a vegetable oil layer as manure storage cover, which was very effective, but not practical because of degradability, generation of foul odors and difficulty in preventing the oil film from becoming mixed with the manure.

CONCLUSION

Methane and nitrous oxide are produced from livestock via two sources, enteric fermentation and manure management. There is rising concern over their increased production due to their hazardous effect on the environment. Feeding diets with balanced CP and fibre would optimize the release of N_2O and CH_4. The liquid piling of manures should be avoided. Instead aerated compost of manure would be helpful for decreasing methane emission and also increase the fertility of soil. Use of manure as fuel for households for cooking is another energy remunerating alternative. Other abatement strategies such as use of ammonium compounds during composting decrease methane emissions. When excreta have been applied to soil, nitrification inhibitors (for example, nitrapyrin) may conserve the applied NH_4-N as NH_4^+ and reduce N_2O emissions. Hippuric acid has inhibitory effects on the denitrification pathway. Mechanically ventilated structures, use of titanium dioxide (TiO_2) paint on the interior walls also decrease N_2O generation. The nutritional, management and other amendment strategies could be exploited for reducing the release of CH_4 and N_2O and simultaneously converting the released gases in to a source of useful energy.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Biocontrol efficacy of *Wickerhamomyces anomalus* in moist maize storage

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White maize cultivar 'Kasai' was harvested and stored moist, sealed in airtight plastic barrels, with and without inoculation of the biocontrol yeast, *Wickerhamomyces anomalus.* Microbes were enumerated at harvest and after 2, 5 and 8 months of storage. Moist storage of maize yielded a type of fermentation, in which high levels of lactic acid bacteria (LAB) naturally present on maize (10^8 cfu/g), were maintained throughout storage. Activity of LAB in both control and inoculated maize likely contributed to the decline in *Enterobacteriaceae* to < 10 cfu/g after 2 months storage, a good outcome for food and feed hygiene. The biocontrol yeast, *W. anomalus*, appeared to have died out in the inoculated treatment after 2 months; nevertheless, a positive effect was seen in significantly reduced mould counts to < 100 cfu/g compared with the uninoculated maize. Reducing moulds during storage minimises the risk for mycotoxin production. Storage for 8 months with the biocontrol yeast did not appear to affect nutritional parameters of the maize, such as dry matter, crude protein and total amino acids. Compared with the uninoculated control, inoculated maize had significantly better contents of three amino acids (alanine, aspartic acid and glycine), but poorer contents of six amino acids (arginine, lysine, ornithine, proline, serine and tyrosine). The absence of nutritional improvement in inoculated maize could be due to the poor survival of the biocontrol yeast, and altered formulation practices may address this.

Key words: Feed hygiene, microorganisms, storage, maize, *Wickerhamomyces anomalus*, biocontrol.

INTRODUCTION

Maize is one of the most widely cultivated cereal crops in the world. In Sub-Saharan Africa, it is a staple food for both humans and animals. Unfortunately, this cereal belongs to the top two crops that are susceptible to fungal infection. During their growth, fungi may produce mycotoxins, not only in field conditions but also during storage. Mycotoxins are secondary metabolites produced by fungi, typically, species of *Aspergillus*, *Fusarium* or *Penicillium*. When inhaled, ingested, or absorbed through the skin, mycotoxins may cause adverse health effects for exposed humans and animals, such as lowered performance and sickness, and, at high concentrations, even death (Wagacha and Muthomi, 2008).

Thus, mould infection and mycotoxin production

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in grain are a health concern that negatively affect food and feed production, leading to environmental and socioeconomic consequences. A pre-requisite for the transition to more sustainable crop production in the future is the development of environmentally-friendly, efficient and cost-effective agricultural technology that minimises the growth of mould and spoilage of cereal crops during storage in developing countries. Innovative biocontrol methods may be part of this strategy, with the goal of protecting both animal and human health.

Yeasts, in general, are microorganisms which are highly resistant and adaptable to environmental change. *Wickerhamomyces anomalus* (formerly *Hansenula anomala*, *Pichia anomala*) J121 is able to grow within a wide range of temperatures (3-37ºC) and pH values (2-12), at low water activity (0.92 [NaCl] and 0.85 [glycerol]), as well as in anaerobic conditions. It can also assimilate a substantial number of different carbon and nitrogen sources (Fredlund et al., 2002). These characteristics bode well for the application of such a yeast in the context of maize storage on-farm.

Previous studies have shown that *W. anomalus* has beneficial protective effects on both food and feed, such as mould-inhibition. Production of ethyl acetate appears to be the mechanism of inhibition of mould growth, whereas the mechanism by which *W. anomalus* inhibits growth of *Enterobacteriaceae* is not fully understood (Olstorpe and Passoth, 2011). Yeasts can contribute to the value of the feed, because they contain substantial amounts of vitamins, minerals and proteins (Olstorpe et al., 2010). In addition, the degradation of inositol hexaphosphate (phytate) in cereal grain by yeast phosphatases releases phosphorus as well as chelated essential trace minerals (Sandberg and Andlid, 2002; Olstorpe et al., 2009).

The normal spectra of microbial populations present in traditionally cropped maize during air-dried storage in Cameroon have been investigated (Leong et al., 2012). *Meyerozyma guilliermondii* was the dominant yeast during extended storage (5 months), whilst *W. anomalus* was not found to be naturally present**.**

The aim of this study was to evaluate the efficacy of *W. anomalus* as a biocontrol yeast of moist maize grain post-harvest and during long-term airtight storage in Cameroon. A secondary aim was to monitor the feed hygiene of moist maize grain in such a storage system (yielding a type of fermentation), by enumeration and identification of different microbial groups.

MATERIALS AND METHODS

Yeast isolate

The yeast *W. anomalus* J121 (CBS 100487) used during the study was originally isolated from stored grain in Sweden. The yeast is stored in the fungal collection of the Department of Microbiology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, in glycerol stocks at -70°C.

Experimental design

The experiment was conducted at a farm in Nforya-Bamenda, Cameroon, at 1239 m above sea level, approximately latitude 6° north, and longitude 10° east. The white maize cultivar 'Kasai' was harvested at the beginning of August. Immediately after harvest, husks were removed and kernels shelled from the cobs by hand. Kernels were divided into two treatments: 39 kg of *W. anomalus* inoculated maize ('inoculated'), and 39 kg of non-inoculated maize containing only the natural microbiota ('control'). Both inoculated and control maize were further divided into triplicate portions, each comprising 13 kg of maize packed in black 20 L airtight plastic drums with removable lids. The control grain was harvested and packed first (that is, before the inoculated maize), to minimise the risk of cross-contamination between treatments. After packing, the drums were closed to generate airtight conditions. Samples were collected from each replicate at harvest (post-inoculation), and after 2, 5 and 8 months of storage.

Maize inoculation

To simulate commercial production of yeast inoculum, *W. anomalus* J121 was cultured in pilot-scale on molasses, ammonia, phosphorus, magnesium and vitamins by Jästbolaget (Rotebro, Sweden), dried with emulsifier on a fluidized bed, and vacuum-packed into 50 g portion sachets. Prior to inoculation, yeast was rehydrated with tap water. The yeast suspension was mixed by hand into the maize, calculated to inoculate 1×10^5 cells/g moist maize.

Analytical methods

The moisture content (MC) of the maize was determined by drying samples at 103°C for 16 h. Grain water activity (a_w) was analysed using an AquaLab CX-2 (Decagon Devices Inc., Pullman, WA, USA) at 22°C.

Analysis of amino acids

The amino acid content of triplicate samples collected at harvest and after 8 months storage from both treatments was analysed by Eurofins AB, Lidköping, Sweden. Amino acid measurements were performed according to standard SS-EN ISO 13903:2005, except tryptophan that was analysed according to EU standard (EU Dir 2000/45/EG part C).

Quantification of microorganisms

Samples (20 g) from each triplicate were diluted with 180 ml sterile peptone water (Bacteriological peptone 2 g l^{-1}; Merck, KGaA., Darmstadt, Germany), supplemented with 0.15 g l^{-1} Tween 80 (Merck, OHG., Schuchardt, Hohenbrunn, Germany), and homogenised for 120 s at normal speed in a Stomacher 400 Laboratory blender (Seward Medical, London, UK). Serial dilutions were performed in peptone water and spread onto various solid culture media. The LAB were quantified on de Man Rogosa Sharp (MRS) agar (Merck, KGaA., Darmstadt, Germany) supplemented with 100 µg/ml Delvocide (active compound, natamycin; Gist-Brocades B.V., Ma Delft , The Netherlands) to inhibit fungal growth. Plates were incubated anaerobically using a GasPack system (Becton Dickinson; Sparks, Md., USA) at 30°C for 48 h. Yeasts were enumerated on yeast peptone dextrose agar (YPD) at 25°C for 2-3 days, supplemented with 100 µg/ml chloramphenicol (Sigma-Aldrich Inc., St. Louis, MO) to inhibit bacterial growth. Moulds were

quantified on YPD plates with a supplement of 100 µg/ml chloramphenicol and 10 µg/ml cycloheximide (Sigma-Aldrich Inc., St Louis, USA) to prevent growth of bacteria and yeasts, respectively, and the plates were incubated at 25°C for 3-4 days. *Enterobacteriaceae* were enumerated on Violet Red Bile Agar (Oxoid Ltd., Basingstoke, Hampshire, UK) by the pour plate method and incubated at 37°C for 24 h. Fungal and bacterial counts were expressed as Log_{10} mean (n=3) cfu/g feed.

DNA extraction, amplification and microbial identification

A total of 10 colonies of LAB and 10 colonies of yeast were randomly selected from each triplicate in both treatments at each sampling occasion. Colony PCR was performed for LAB isolates by selecting pure colonies as template. The 2 µl DNA template from yeast was extracted by boiling a single yeast colony in 50 µl nuclease-free water (Leong et al., 2012). PCR fingerprint patterns were generated by using the PuRe Taq ready-to-go PCR beads (GE Healthcare- Life Sciences, Uppsala Sweden) mixed with primer, DNA and water according to the instructions in the supplier's manual. Genotypic differentiation was studied with repetitive-DNA-element PCR fingerprinting, using the microsatellite primer GTG (5'-GTGGTGGTGGTG GTG-3'). The rRNA gene amplification and PCR conditions for yeast and LAB fingerprints were performed as described by Olstorpe et al. (2008). The PCR fingerprints were visualised with electrophoresis on a 1% agarose gel in 0.5× Tris-borate-EDTA. The yeast and LAB fingerprints were analysed with GelCompar II version 4.5 software (Applied Maths, Kortrijk, Belgium). Strains representing each unique profile were identified by amplifying the 16S rRNA gene in LAB (primers 16Ss / 16Sr) or D1 / D2 region of the 26S rRNA in yeasts (primers NL1 / NL4). Amplicons were sequenced at Macrogen, Korea, and isolates were identified by sequence comparison against the Genbank database. Representative mould isolates were identified by a combination of morphological and molecular techniques. The morphological data and the DNA sequences were combined for polyphasic identification using Genbank and the CBS database as previously described (Leong et al., 2012).

Strain conservation of LAB and yeast

Yeast and LAB that were identified by sequence comparison were conserved and stored at the culture collection of the Department of Microbiology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. LAB were inoculated in 9 ml MRS-broth (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 30°C overnight. Thereafter the tubes were centrifuged at 1000 *g* for 10 min and the supernatant discarded. The pellet was resuspended in 1 ml cryomedia (K_2HPO_4, 0,82 g/l; KH_2PO_4, 0.18 g/l; Na-citrate-dihydrate 0.67 g/l; $MgSO_4.7H_2O$, 0.25 g/l; 172 ml glycerol), and stored at –70°C. Yeast were inoculated in 2 ml yeast extract-peptone-D-glucose (YPD) broth (yeast extract, 10 g/l, bacteriological peptone, 20 g/l and D-glucose, 20 g/l) and incubated on a shaking board (130 rpm) at 30°C overnight. 1 ml of the suspension was mixed with an equal volume of glycerol and frozen at –70°C (Olstorpe et al., 2008).

Statistical analysis

The data generated from the nutritional analyses of the grain and the microbial counts were non-normally distributed; hence, a Wilcoxon ranking test suited for such non-parametric data was selected as the statistical analysis method. The effect of *W. anomalus* inoculation on nutritional values after storage was assessed using pairwise differences (harvest minus 'after storage') for each replicate as the input data. To estimate the effect of inoculation on microbial counts, the input data comprised counts obtained after 2, 5 and 8 months of storage. Mean values were considered significantly different at the 5% level.

RESULTS

Nutritional evaluation of the stored grain

After storage, the dry matter, crude protein, crude fibre, crude fats, and total amino acid contents did not differ significantly between the control maize and inoculated maize ($P>0.05$; Table 1). Of these parameters, dry matter, crude fibre and total amino acids had decreased from harvest levels to equivalent extents in both control and inoculated treatments. Inoculation with *W. anomalus* had significantly positive effects on the final contents of starch, and of three amino acids – alanine, aspartic acid and glycine compared with the control ($P<0.05$).

Starch and aspartic acid had decreased in the control samples during storage, whereas these were increased above harvest levels in the inoculated treatment. Alanine was increased after fermentation storage in both treatments, but to a greater extent in the inoculated samples. Glycine decreased in the control during storage, whereas it was maintained at harvest levels in the inoculated treatment. However, five amino acids decreased significantly more in inoculated than in control maize ($P<0.05$), namely, arginine, lysine, proline, serine and tyrosine; and ornithine was increased in the control maize but decreased in the inoculated maize after storage.

Microbial quantification

At harvest, microbial counts did not differ significantly between the control maize and the inoculated maize (Table 2). Yeast counts in both treatments increased up to 5 months, and then decreased markedly in the 8 month samples. Yeast counts increased more and maintained higher levels in the inoculated samples than in control maize, which by 8 months had reduced to levels below the detection limit. These overall elevated yeast counts in inoculated maize during storage were significantly higher than in control maize ($P<0.001$). Counts of LAB were fairly similar between treatments ($P=0.02$), maintaining levels similar to those present at harvest. Counts of *Enterobacteriaceae* decreased in both treatments after 2 months, and remained around the detection limit (log_{10} 1 cfu/g) for the remainder of storage; thus, statistical comparison of inoculated and control samples during storage was not meaningful. Mould counts showed a similar pattern: decreasing within the first 2 months, and then remaining around those levels until end of storage. Counts in inoculated maize decreased to levels at or below the limit of detection after 2 months and remained at those levels, whereas the

Table 1. Nutritional values and amino acid composition in non-inoculated control (C) and in *Wickerhamomyces anomalus* inoculated maize (I).

Variable	August		April		P-value
	C	I	C	I	
Dry matter (%)	50.67	50.00	29.67	29.33	0.90
Crude protein	105.67	105.67	105.00	105.33	0.47
Ash	16.13	16.10	16.00	16.00	0.78
Crude fibre	25.67	26.00	21.33	22.67	0.18
Crude fats	67.00	67.00	66.00	65.00	0.075
Starch	701.00	701.33	697.00	711.00	0.0014
Alanine	7.90	7.90	8.03	8.53	0.0015
Arginine	3.37	3.37	2.07	1.77	0.033
Aspartic acid	5.77	5.77	5.13	6.03	<0.001
Cysteine	2.43	2.47	2.33	2.37	0.74
Glutamic acid	19.07	19.13	15.60	15.43	0.079
Glycine	3.67	3.67	3.57	3.73	0.040
Histidine*	2.83	2.77	2.07	1.83	0.22
Hydroxyproline	< 0.1	< 0.1	< 0.1	< 0.1	1
Isoleucine*	3.83	3.87	3.60	3.73	0.19
Leucine*	12.53	12.53	11.53	11.67	0.15
Lysine*	2.83	2.83	2.27	1.73	0.002
Methionine*	2.07	2.10	2.00	2.10	0.26
Ornithine	0.33	0.33	0.53	0.23	0.014
Phenylalanine*	4.87	4.83	4.40	4.27	0.39
Proline	9.53	9.50	9.33	8.97	0.015
Serine	4.63	4.60	3.53	2.87	0.005
Threonine*	3.47	3.40	3.37	3.03	0.053
Tyrosine	3.87	3.83	2.97	2.40	0.002
Valine*	5.07	5.17	4.87	5.10	0.16
Total amino acids	97.97	98.07	87.20	85.80	0.060

Samples were taken for analysis at harvest (Aug) and after 8 months (Apr) storage. Data are given as g amino acid per kg dry matter (g/kg dm). The mean of three replicates is presented, and P-values compare the control and inoculated treatments, based on pairwise differences (harvest – after storage) for each replicate (Wilcoxon ranking test).The measuring tolerance given by the contract laboratory (Eurofins AB. Lidköping. Sweden) is 8% for each amino acid. *Essential amino acid.

Table 2. Colony forming units (cfu) of different microbial groups present at harvest (Aug), after 2 (Oct), 5 (Jan) and 8 (Apr) months storage in non-inoculated control (C) and *Wickerhamomyces anomalus* inoculated (I) moist maize.

Storage time	Treatment	Microbes			
		Yeast	Lactic acid bacteria	*Enterobacteriaceae*	Moulds
August	C	4.63±0.09	8.01±0.61	6.43±0.13	6.96±0.05
	I	4.69±0.23	8.04±0.59	6.24±0.27	6.92±0.03
October	C	5.84±0.30	8.28±0.45	< 1.00[b]	5.14±2.40
	I	7.01±0.29	8.86±0.37	< 1.00	1.80±0.17
January	C	6.79±0.45	8.48±0.49	1.33±1.09	4.40±0.69
	I	7.46±0.30	8.82±0.05	< 1.00	< 2.00[a]
April	C	< 2.00[a]	8.60±0.47	< 1.00	5.14±1.24
	I	6.41±0.37	8.91±0.17	< 1.00	< 2.00

[a]All three replicates below detection level (100 cfu/g grain) $\log_{10}$ 2.0. [b]All three replicates below detection level (10 cfu/g grain) $\log_{10}$ 1.0. Values are presented as mean (n=3) Log_{10} cfu/g grain ± std deviation, in which counts below the detection limit were assigned half the value.

Table 3. Yeast species present at harvest (Aug) and after 2 (Oct), 5 (Jan) and 8 (Apr) months storage in *Wickerhamomyces anomalus* inoculated (I) and uninoculated control (C) moist maize. Isolates were identified by D1/D2 large subunit rRNA gene sequencing, and relative abundance is given as the number out of 30 identified isolates.

Yeast species/sampling point	Treatment							
	I				C			
	Aug	Oct	Jan	Apr	Aug	Oct	Jan	Apr
Candida quercitrusa	6	0	0	0	27	0	0	0
Dekkera bruxellensis	0	0	0	0	0	0	8	0
Pichia galeiformis	0	0	0	0	0	1	1	0
Pichia kudriavzevii	1	28	23	10	0	22	16	0
Pichia manshurica	0	0	0	0	0	3	4	0
Pichia membranifaciens	0	2	6	20	0	0	0	0
Rhodotorula minuta	0	0	0	0	1	0	0	0
Rhodotorula mucilaginosa	0	0	0	0	2	0	0	0
Wickerhamomyces anomalus	23	0	1	0	0	0	0	0

Table 4. Bacterial species present at harvest (Aug) and after 2 (Oct), 5 (Jan) and 8 (Apr) months storage in *Wickerhamomyces anomalus* inoculated (I) and uninoculated control (C) moist maize. Isolates were identified by 16S rRNA gene sequencing and relative abundance is given as the number out of 30 identified isolates.

Bacterial sp./sampling point	Treatment							
	I				C			
	Aug	Oct	Jan	Apr	Aug	Oct	Jan	Apr
Acinetobacter ursingii	0	5	0	1	0	2	1	1
Enterococcus faecium	3	0	0	5	1	1	1	1
Lactobacillus brevis	0	0	1	0	0	1	4	0
Lactobacillus casei	0	1	0	6	0	0	0	8
Lactobacillus pentosus	0	0	8	0	0	1	0	2
Lactobacillus plantarum	12	15	11	12	12	11	12	13
Lactobacillus sp.	0	1	8	6	0	0	1	1
Leuconostoc citreum	3	1	0	0	3	0	0	0
Leuconostoc pseudomesenteroides	8	1	0	0	12	0	0	0
Leuconostoc sp.	0	0	0	0	2	0	0	0
Moraxellaceae bacterium	2	5	2	0	0	1	7	5
Unidentified bacteria	2	1	0	0	0	3	4	0

decrease in counts in control maize was less substantial; hence, overall counts during storage were significantly lower in inoculated maize than control maize ($P<0.001$).

Identified microbial species

Yeast

Candida quercitrusa was the dominant yeast isolated from the control maize at harvest, whereas, as expected, *W. anomalus* was dominant in the inoculated maize (Table 3). At 2 and 5 months, the dominant yeast species was *Pichia kudriavzevii* in both treatments. After 8 months, *Pichia membranifaciens* was the dominant yeast in the inoculated maize; yeasts were not isolated from the the control maize.

Lactic acid bacteria

Changes in LAB populations during storage were fairly similar in control and inoculated maize (Table 4). *Leuconostoc* spp. and *Lactobacillus plantarum* were common at harvest; the latter, together with other species of *Lactobacillus*, continued to be frequently isolated throughout the storage period.

Moulds

Mould species isolated at harvest included *Arthrinium arundinis*, *Aspergillus melleus*, *Bionectria ochroleuca*,

and *Pleosporales* sp. These species were not subsequently isolated during storage, in keeping with the overall decrease in mould counts (Table 2). *Paecilomyces variotii* was the dominant species isolated from maize during storage.

DISCUSSION

Previous studies have reported clear positive effects of *W. anomalus* inoculation, either as fresh yeast or freeze-dried preparations, on the amino acid and protein contents of moist stored grain, for example, in crimped barley stored in Swedish farm conditions for 5 months (Olstorpe, 2008; Olstorpe et al., 2010). Such effects were not observed in this first trial of moist (fermented) maize in Cameroon; rather, storage fermentation of maize with *W. anomalus* was not detrimental to the nutritional content, neither was it overtly positive. The total amino acids content did not differ significantly between the control and inoculated maize after storage, in that both treatments showed a decrease compared with levels at harvest. In inoculated samples, the contents of three amino acids were improved or maintained at harvest levels, but contents of six other amino acids, including the essential amino acid, lysine, decreased more than in control maize. The absence of clear improvements during this maize storage trial, compared with previous reports from other grains, may be due to the loss of the biocontrol yeast *W. anomalus* after 2 months' storage, with the population shifting towards *P. kudriavzevii* at 5 months, and *P. membranifaciens* at the end of storage. These latter species may not be associated with improved protein production for moist stored grain. Furthermore, yeast populations in inoculated maize were at their peak after 5 months of storage (log 7.46 cfu/g grain) and decreased to log 5.08 cfu/g at 8 months of storage. Protein evaluation was conducted at the end of the study when yeast numbers were lower, and no *W. anomalus* was present at that time.

In this study, the most notable finding in the use of *W. anomalus* for biopreservation of moist grain is the significant reduction in mould growth when the control and inoculated treatments were compared up to 8 months of storage. Note that the greatest reduction in mould counts was observed from harvest to 2 months' storage, that is, after the initial inoculation with *W. anomalus.* Thus, the greater mould reduction in inoculated *c.f.* control maize could well be attributed to this biocontrol yeast, despite it apparently having died off in the 2 months and subsequent samples. The reduction in mould growth for inoculated maize has important implications for maize spoilage in the tropics, as the prevalence of moulds in stored grains is of public health significance due to risks for mycotoxin production (Wagacha and Muthomi, 2008). The main mould isolated during storage was the non-toxigenic species, *Paecilomyces variotii*, which is favoured by warm, dry conditions, with an optimal temperature of 35-40°C (Pitt and Hocking, 2009), and, thus, is a potential human pathogen in immunocompromised hosts. It is also thought to grow well under low oxygen tensions, which may be created during airtight storage of moist maize. This mould was eliminated in the inoculated maize after 8 months' storage, which is a favourable outcome. Other studies have demonstrated the ability of *W. anomalus* to reduce mould growth through production of ethyl acetate during storage of crimped moist cereal grains and cereal by-products (Olstorpe and Passoth, 2011): for instance, in barley (Passoth et al., 2006; Olstorpe, 2008; Olstorpe et al., 2010), and wheat and oats (Petersson and Schnürer, 1998).

A further benefit of fermentation storage of moist maize grain demonstrated in this study was the reduction in *Enterobacteriaceae* to minimum detectable levels; this effect was observed in both control and inoculated grain, and is beneficial for grain hygiene and for reducing the risk of gastro-enteritis from pathogenic species that might be present. The constant levels of LAB observed in both treatments throughout storage and their production of organic acids and other anti-bacterial compounds may have contributed to the deleterious effect on *Enterobacteriaceae* (Lindgren and Dobrogosz, 1990). Similar reductions in *Enterobacteriaceae* during moist grain fermentations have been previously reported, particular with the addition of biocontrol yeast (Olstorpe et al., 2010; Olstorpe et al., 2012).

Conclusions

Moist storage (fermentation) of maize was sufficient and effective in reducing *Enterobacteriaceae*, a key parameter when monitoring grain hygiene; however, reducing the presence and growth of moulds during storage, thus minimising the risk for mycotoxin pro-duction, required inoculation with the biocontrol yeast, *W. anomalus.* The biocontrol yeast did not clearly improve the protein or amino acid contents of the stored maize in this trial, unlike previous trials with other grains. This could be attributed to its poor survival after 2 months; thus, formulation and preparation of the biocontrol ino-culum are avenues of further study, in order to optimise its survival and efficacy during moist maize storage in Cameroon.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Concentrations of iodine and some environmental goitrogens in two selected water bodies - Adada and Akoru in Nsukka, Enugu State, Nigeria

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This study was designed to investigate the distribution of iodine and goitrogens in two selected rivers: Adada and Akoru in Enugu Sate, Nigeria. Iodine concentrations were found to be 0.109±0.001 and 0.108±0.001 ppm, respectively in both water bodies. The evaluated environmental goitrogens were as follows in Adada and Akoru Rivers: Calcium 109.333±16.0 and 74.667±7.67 ppm; sulphate 33.400±7.40 and 65.000±2.60 ppm; chloride 183.000±13.20 and 257.7±14.30 ppm; potassium 0.733±0.02 and 0.733±0.02 ppm; nitrate 6.000±1.00 and 6.000±1.00 ppm; copper 3.455±0.46 and 2.792±1.35 ppm; phosphate 3.067±1.30 and 0.267±0.07 ppm; manganese 0.692±0.20 and 0.513±0.08 ppm; iron 0.476±0.05 and 0.181±0.01 ppm; magnesium 0.085±0.02 and 0.085±0.02 ppm, respectively. High values of these goitrogens in both rivers could be attributed to the high salt level of the two water bodies. Considering the iodine and goitrogen ratio in both Adada and Akoru rivers, the two water bodies would be a good source of iodine for the population and fresh water bodies that are dependent on them.

Key words: Adada River, Akoru River, iodine, goitrogens, hardness, acidity, pH.

INTRODUCTION

Iodine is an essential trace mineral in human nutrition obtained mainly from diet and water (Anderson et al., 2005). Iodine is irregularly distributed over the earth's crust and in some areas the surface soil becomes progressively poorer in iodine through accelerated deforestation, soil erosion and leaching processes (EGVM, 2002; Singh, 2004; Ujowundu et al., 2011). Concentration of iodine in unpolluted water in various parts of the world has been found to be generally less than 3 µg/L while drinking water has been shown to contain an iodine level of less than 15 µg/L, except in few cases where much higher levels are reported (Banerjee, 1985). The element is an essential part of the thyroid hormones Triodothronine (T3) and tetraiodothyronine (T4), which in turn are necessary for human growth and development (Banerjee, 1985). Goiter is not one of the manifestations of iodine deficiency and

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the wide spectrum of manifestations is now commonly termed Iodine Deficiency Disorders (Anderson et al*.,* 2005). These disorders include endemic cretinism, infant mortality, infertility, miscarriage, mental retardation neuromuscular defect or dwarfism (Anderson et al., 2005; Venturi and Venturi, 2009a; Underwood, 1977; Delange, 2000).

Goitrogens on the other hand are substances, which impair iodine uptake by the thyroid or impair iodine incurporation into thyroxine (JECDA, 1989; Venturi, 2001; Stoddard et al., 2008). It has been shown that there are some connections between the geogenic origin of water and the incidence of goiter (Dissanayake et al., 1999). Poor quality of drinking water has been discovered for years to cause goiter (Eskin et al., 1995; Venturi and Venturi, 2009b; Gaitan, 1983).

In Australia, New Zealand and several European countries, iodine deficiency is a significant public health problem. It is more common in third-world nations. Public health initiatives to lower the risk of cardiovascular disease have resulted in lower discretionary salt use at the table and with a trend towards consuming more processed foods. The non-iodized salt used in these foods also means that people are less likely to obtain iodine from adding salt during cooking (Anderson et al., 2005; Kulshreshtha, 1998; Venturi and Venturi, 2009a; Henniker, 1949; JECFA, 1989).

In areas where there is little iodine in the diet, typically remote inland areas and semi-arid equatorial climates where no marine foods are eaten, iodine deficiency gives rise to hypothyroidism, symptoms of which are extreme fatigue, goiter, mental slowing, depression, weight gain and low basal body temperatures (Lawrence, 2001). Iodine deficiency is the leading cause of preventable mental retardation, a result which occurs primarily when babies or small children are rendered hypothyriodic by a lack of the element (Lawrence, 2001; Spitzwag et al., 2000; Smyth, 2003). Breast strongly and actively concentrates iodine into breast-milk for the benefit of the developing infant but when iodine levels are low, it may develop a goiter-like hyperplasia, sometimes manifesting as fibrocystic breast disease (Lowe, 2006; Rasmussen et al., 2002). Studies indicate that iodine deficiency, either dietary or pharmacologic is a risk factor for breast cancer and can lead to dysplasia and increased incidence of malignancy in animal models, while iodine treatment can reverse dysplasia (Maekawa et al., 2006; Wu et al., 2002; Patrick, 2008; Lowe, 2006; Anderson et al., 2005).

MATERIALS AND METHODS

Water analysis

Two water samples were collected from two different water sources: Adada and Akoru Rivers both in Nsukka of Enugu State, Nigeria. An immediate analysis of these water samples were carried out in the laboratory owned by the Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka.

Chemicals (reagents)

The chemicals used were calcium working reagent (BDH), 6 N sulphuric acid (Sigma), 0.1% of gum ghatti solution (May & Baker), 4 N sodium hydroxide (NaOH) solution (BDH), conditioning reagent (Sigma), barium chloride solution (Cartivalue), vanadate molybdate solution (Merck), phenoldisulphoric acid (BDH), concentrated solution of ammonia (Merck), acetate buffer (Sigma), 2.5% of hydroquinone (BDH), 0.1% of α, α-dipyridyl solution (BDH), alcoholic mercuric thiocyanide (Marck), vanadate citrate mixture (Sigma), phenolphthalein indicator (Lab. Tech. Chemicals), sodium diethyldithiocarbonate (BDH), carbon tetrachloride (BDH), citric acid buffer (BDH), potassium phosphate solution (Sigma), Luico-crystal violet indicator (May & Baker), concentrated solution of Sulphuric acid (May & Baker), sodium pyriodate solution (BDH), sodium cobalt-nitrite solution (BDH), 70% ethanol solution (Sigma), choline hydrochloride solution (BDH), sodium ferricyanide solution (May & Baker), zinc uranyl acetate solution (Sigma), 1% acetic acid (May & Baker) and 10% potassium ferri-cyanide solution (May & Baker).

Methodology

The water collected from Adada River was tagged sample A while that from Akoru River was tagged sample B. 25 ml of the water samples (A and B) were measured into triplicates, and then 2 ml of the prepared buffer solution was added into each of the triplicate. 0.2 g of indicator powder was also added and then finally the resulting solution was titrated with the standard ethylenediaminetetraacetic acid (EDTA) solution until the reddish stench colour disappears. The formula applied to get the hardness of the two water samples is given as:

Hardness (EDTA) at mg $CaCO_3$/l = T* B * 1000/ml test portion

T = ml of the EDTA standard solution
B = mg of $CaCO_3$ equivalent of 1 ml of the EDTA standard solution.

Water sample analysis

Eleven different tests were conducted from the water samples collected which includes:

Determination of iodine (I_2)

Five milliliter (5 ml) of the water samples (A and B) were respectively collected in triplicates and 1 ml of citric acid buffer was added in each of the triplicates. Then, 0.5 ml of potassium persuphate and 0.1 ml of Luico-crystal violet indicator were added, respectively. Then, 3.9 ml of distilled water was added and mixed thoroughly and the absorbance reading was taken at a wavelength of 620 nm against the reagents blank.

Determination of calcium (Ca^{2+})

Five milliliter (5 ml) of the two water samples (A and B) were respectively taken in triplicates, that is, in three different test tubes, sample A was added to 0.5 ml while in another three different test tubes, sample B was added in the same volume. Also, 5 ml of distilled water was taken in another test tube tagged the BLANK.

Then, 3 ml of calcium working reagent was added into each of the triplicates containing sample A and B and also in the blank. Finally, the absorbance of each of the triplicates was taken at a wavelength of 612 nm against the blank.

Determination of magnesium ions (Mg^{2+})

Another triplicate was also prepared. In each of the triplicate test tubes (A and B), 5 ml of the water samples A and B were added, respectively. Also, in each triplicates, 2 ml of 0.6 N sulphuric acid was added. 1 ml of concentrated sulphuric acid was also added into 52.73 ml of distilled water to make a solution. 2 ml of the solution prepared was added into each of the triplicate samples (A and B). Then, 0.1% of gum ghatti was added into each of the triplicates and also in the blank. Also, 0.5 ml of 0.1% Titan yellow solution was added in each triplicate as well as in the blank. Then, 2 ml of 4 N NaOH was added in each triplicate and also in the blank. Finally, absorbance of the triplicates was taken at a wavelength of 520 nm against the blank.

Determination of sulphate (SO_4^{-2})

Five milliliter (5 ml) of the water samples (A and B) were respectively added in each of the triplicates A and B. Then 1 ml of conditioning reagent was added in each triplicate and also to the blank and shaken thoroughly. Then 0.1 g of barium chloride was added in each triplicate and also to the blank and was shaken for 1 min. The absorbance of these triplicates including the blank was taken at the wavelength of 420 nm.

Determination of phosphate (PO_4^{-3})

Five milliliter (5 ml) of the water samples (A and B) were respectively taken into triplicates including a blank. 2.5 ml of vanadate molybdate reagent was added into each triplicate and then 2.5 ml of distilled water was also added. Then the absorbance was taken at the wavelength of 470 nm.

Determination of nitrate (NO_3)

Five milliliter (5 ml) of the water samples (A and B) were respectively added into triplicate beakers and heated to dryness. Then 0.2 ml of phenoldisuphuric and reagent (PDA) was added, followed by 1 ml of concentrated ammonia. Then these triplicate beakers were heated for a little period of time (30 s) and were diluted to 5 ml, that is, after adding 0.2 ml of PDA and 1 ml of conc. NH_3, 3.8 ml of distilled water was added in each of the triplicate (A and B) to make up to 5 ml. Then, the absorbance was taken at a wavelength of 410 nm.

Determination of iron (Fe^{2+})

Five milliliter (5 ml) of the water samples (A and B) were respectively taken in triplicates. Then 1.5 ml of acetate buffer was added in each triplicate, followed by 1 ml of 2.5% hydroquinone. Then, 1 ml of 0.1% of α, α-dipyridyl reagent was added in each triplicate and then the pH of these triplicates were adjusted between 4.50 to 5.00 (the pH of the sample A triplicate was 4.70 and that of the B triplicate was 4.75). Then each triplicate was made up to 10 ml with distilled water and the absorbance taken at a wavelength of520 nm.

Determination of chloride (Cl)

Five milliliters (5 ml) of the water samples (A and B) were respectively taken in triplicates and in each triplicate 1 ml of ferric alum solution was added, followed by the addition of 1 ml of alcoholic mercuric thiocyanide in each triplicate as well. The mixtures were shaken thoroughly and allowed to stand for 10 min, and then the absorbance at a wavelength of 470 nm was taken.

Determination of copper (Cu^{2+})

Five milliliters (5 ml) of the water samples (A and B) was respectively collected and arranged in triplicate, then 1 ml of vanadate citrate mixture was added in each triplicate followed by the addition of an indicator-phenolphthalein (2 drops). Then, a solution of ammonia was also added (drop by drop) until the solution of each of the triplicates changed to pink. Also, 0.1 ml of 1% sodium diethyldithiocarbonate was added followed by the addition of 5 ml of carbon tetrachloride (CCl_4) into each of the triplicates. It was thereafter mixed vigorously and centrifuge for 5 min. Finally, the absorbance of the lower layer was taken at the wavelength of 440 nm against CCl_4.

Determination of potassium (K^+)

Five milliliters (5 ml) of the water samples A and B were respectively collected in triplicates; 2 ml of sodium cobalt-nitrite reagent was added, mixed vigorously and allowed to stand for 5 min. After the standing period, the triplicates were centrifuged for 15 min. After spinning, the supernatants were discarded and the residues were collected. To the residues, 2 ml of 70% ethanol was added, mixed vigorously and centrifuged for another 5 min. After spinning, the supernatants were also discarded and the residues collected were washed two times with 2 ml of 70% ethanol and centrifuged, respectively. To the residues, 2 ml of distilled water was added and boiled for 10 min. Then, 1 ml of choline hydrochloride was added, followed by addition of 1 ml of 2% sodium ferricyanide and also the addition of 2 ml of distilled water in each of the triplicates. Finally, the absorbance was taken at 620 nm against the blank.

Determination of sodium (Na^+)

Five milliliters (5 ml) of the water samples A and B were respectively collected in triplicates and 5 ml of zinc uranyl acetate reagent was added in each of the triplicates, mixed vigorously and allowed to stand for 5 min. After the standing period, the triplicates were centrifuged for another 5 min and 2 ml of the supernatants (of each triplicates) gotten after the spinning were transferred into another triplicate test tubes; 8 ml of 1% of acetic acid and 0.4 ml of 10% potassium ferricyanide were added into the new collected test tubes, respectively and then mixed. The absorbance reading was recorded at a wavelength of 480 nm against the reagent's blank.

Determination of hardness

Five milliliters (5 ml) of distilled water and 2 ml of buffer were added into the two test tubes. 1 ml of erichrome blathyle indicator was also added and the reading taken at 520 nm.

Determination of pH

The pH of both samples was determined using standard methods.

Table 1. Concentrations of minerals in Adada and Akoru Rivers.

Parameter	Adada River (Mean±SD)	Akoru River (Mean±SD)
Iron (µg/ml)	0.476±0.05	0.181±0.01
Phosphate (Mg/100 ml)	3.067±1.30	0.267±0.07
Calcium (Mg/100 ml)	109.333±16.0	74.667±7.67
Magnesium (µg/ml)	0.085±0.02	0.085±0.02
Copper (µg/ml)	3.455±0.46	2.792±1.35
Manganese (µg/ml)	0.692±0.20	0.513±0.08
Nitrate (µg/ml)	6.000±1.00	3.000±2.00
Sulphate (Mg/100 ml)	33.400±7.40	65.000±2.60
Chloride (Mg/L)	183.000±13.20	257.7±14.30
Potassium (Mg/100 ml)	0.733±0.02	0.703±0.01
Iodine (µg/ml)	0.109±0.001	0.108±0.0

Determination of acidity

Five milliliters (5 ml) of the natural samples of the two rivers were respectively added into a different test tubes and 1 ml of phenophthaline was added to each and was titrated with 0.02 M sodium hydroxide until turned pink.

RESULTS AND DISCUSSION

Iodine/goitrogen balance in water and food is an index for predicting the bioavailability of iodine to the population dependent on it as a source of iodine. However, there is no standard value at which iodine and goitrogens must be present in water or food to make iodine more available. It greatly depends on iodine goitrogen balance expressed as iodine goitrogen ratios (Anderson et al., 2005). The pH of both samples was determined and that of Akoru River was 6.61 while that of Adada River was 6.15.

Table 1 shows the levels of iodine and environmental goitrogens (calcium, sulphate, chloride, potassium nitrate, copper, phosphate, manganese, iron and magnesium) in Adada and Akoru fresh water bodies in Uzo-Uwani Local Government Area of Enugu State, Nigeria. Iodine concentration was found to be 0.109±0.001 ppm and 0.108±0.001 ppm, respectively in both water bodies. The evaluated environmental goitrogens were as follows in Adada and Akoru Rivers: Calcium 109.333±16.0 and 74.667±7.67 ppm; sulphate 33.400±7.40 and 65.000±2.60 ppm; chloride 183.000±13.20 and 257.7±14.30 ppm; potassium 0.733±0.02 and 0.733±0.02 ppm; nitrate 6.000±1.00 and 6.000±1.00 ppm; copper 3.455±0.46 and 2.792±1.35 ppm; phosphate 3.067±1.30 and 0.267±0.07 ppm; manganese 0.692±0.20 ppm and 0.513±0.08 ppm; iron 0.476±0.05 and 0.181±0.01 ppm; magnesium 0.085±0.02 and 0.085±0.02 ppm, respectively. High values of these goitrogens in both rivers could be attributed to the high salt level of the two water bodies. For the proper utilization of iodine for thyroid hormone synthesis some mineral are required at the right concentration and proportion (Ujowundu et al., 2010).

The mineral nutrients are interrelated and balanced against each other in human physiology. They cannot be considered as a single element with circumscribed functions. For instance, sodium, calcium, magnesium and phosphorus serve individual and collective purposes in the body fluid regulation. Inadequate mineral intake generally produces deficiency symptoms which include anaemia, impaired healing of wounds, delayed blood clotting severe diarrhea and chronic renal failure. Selenium and iodine ingestion have to be regulated as deficiency can lead to extreme fatigue, endemic goiter, cretinism and recurrent miscarriages (Ujuwundu et al., 2012). The results of this research correlated with the work of Enechi et al. (2013). Some inorganic mineral nutrients have been reported to be antagonistic and interfere with iodine metabolism (Ujuwundu et al., 2012; Walsh, 2003). Iron (Fe) deficiency lowers the thyroid peroxidase activity - a heme-containing enzyme that catalyzes the initial steps in thyroid hormone synthesis. High calcium (Ca) diets or hard water high in Ca, may increase the need for additional iodine (Jooste et al., 1999). Mineral nutrient deficiencies such as zinc, copper, iron, also contribute to inability to use iodine well and this may lead to the development of Goiter (Osman and Fatah, 1981).

Mineral malnutrition can have a negative impact on thyroid function but in the presence of adequate iodine supplies, it is less common for such factors to cause problems (Gartan, 1988). High levels of minerals above the recommended daily allowance (RDA) have also been shown to be goitrogenic (Osman and Fatah, 1981).

The evaluated cations and anions are within the standards for drinking and cooking water (Lawrence, 2001). Considering the iodine-goitrogen ratio, Akoru water body would be a better source of iodine than Adada water body for aquatic and human population.

Conclusion

In conclusion, the high values of cations and anions in both Adada and Akoru water bodies support the fact that they are salt water bodies which was further substantiated by the high calcium concentration.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Conditioners and significance of t-RFLP profile of the assemblage of prokaryotic microorganisms in crude oil polluted soils

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Following an increased interest in management practices designed to reduce posed phytotoxicity during phytoremediation experiments, crude oil polluted soil and variants from conditioners-aided phytoremediation experiment were subjected to terminal restriction fragment polymorphism (t-RFLP) to evalute the biodiversity of bacterial microflora of polluted soil and amendments conditions. Genetic fingerprinting showed that hydrocarbons stress led to depletion of the genetic resources of soil microflora and to a radical change in its qualitative composition. The amended stressed soils not only has a greater number of species present, but the individuals in the community are distributed more equitably among these species. Non-uniform marginal regain of community was clear with applied conditioner. Positive associations, however were observed with conditioner and phyto-assisted clean-up attempts.

Key words: Crude oil pollution, soil conditioners, microbial diversity, phytoremediation, environment.

INTRODUCTION

The oil industry has been a key sector of the Nigerian economy for over 50 years, but many Nigerians have paid a high price (UNEP, 2011) for this sector. It may therefore become pertinent to provide the foundation upon which necessary actions will follow to remedy the complex environmental and sustainable development issues facing people in the Niger Delta. A dynamic age with concomitant increases in crude oil exploration and production across the globe amidst other alternative sources of energy, requires an understanding of microbial diversity to design appropriate and sustainable polluted soil remediation techniques. Techniques devoid of basic considerations for microbial community structure are flawed (McArthur, 2006). Nwaichi et al. (2011) described the rhizospheric interactions with phytoremediation, the use of plants to remove, degrade or separate hazardous substances. Biodiversity is the extent of variation of life forms within a given ecosystem and are interrelated with genetic diversity. It is often used as a measure of the health of biological systems and has been employed to explain natural environment, nature conservation and extinction concern observed in the last decades of the

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Table 1. Initial soil character.

Identity	Polluted	Unpolluted (control)
PAHs (mg/kg)	0.424±0.009	0.077±0.002
THC (mg/kg)	378.3±5.001	64.8±0.821
BTEX (mg/kg)	<0.001	<0.001
As (mg/kg)	1.25±0.002	0.49±0.002
Cd (m/kg)	17.20±0.211	<0.001
Cr (mg/kg)	30.00±0.184	18.70±0.007
Fe (mg/kg)	20,642,50±18.652	16,657.50±24.127
Cu (mg/kg)	9.30±0.072	7.75±0.004
Pb (mg/kg)	86.20±4.440	45.30±2.008
TOC (%)	3.08±0.001	0.96±0.005
Cl^-	400±8.561	80±3.332
NO_3^- (mg/kg)	7.75±0.124	26.25±3.005
SO_4^{2-} (mg/kg)	215.00±5.112	305.00±8.190
N (%)	0.64±0.009	2.13±0.005
P (mg/kg)	0.67±0.002	26.30±2.883
K (cmol/kg)	1.612±0.087	0.068±0.001
Ca (cmol/kg)	<0.01	<0.01
Mg (cmol/kg)	4.721±0.461	0.682±0.011
Na (cmol/kg)	0.346±0.006	0.118±0.002
pH	3.85±0.052	5.45±0.088
Temp. (°C)	28.2±2.991	27.2±3.122

20th century. The word 'biodiversity' may have been coined by Rosen (1985) while planning the National Forum on Biological Diversity organized by the National Research Council (NRC) which was to be held in 1986, and first appeared in a publication in 1988 when entomologist E. O. Wilson used it as the title of the proceedings of that forum (Biodiversity, 2013).

Robe et al. (2003) described prokaryotes as the most ubiquitous organisms on earth, represented in all habitats, including soil, sediment, marine and terrestrial subsurface, animals and plant tissues. The further underscored their key role in the biogeochemical cycles of the biosphere and represent an enormous reservoir of novel valuable molecules for health or industry. Ecological diversity measurements (Sahney et al., 2010) are necessary to understand survival and adaptability of species.

Crude oil polluted soils suffer compaction (Nwaichi et al., 2010), which impedes growth, decreasing the ability of plants to take up nutrients and water (Parrish et al., 2004; Rezek et al., 2009). Soil conditioners, which are products added to soil to improve the soil's physical qualities, especially their ability to provide nutrition for plants, have relieved such soils as reported by Johnson et al. (2009) and Rezek et al. (2009), and in particular, may stimulate microbiological activity, increase nutrient levels, add more loft and texture to soil and improve plant survival rates to sustain natural cycle. Similar studies have few documentation on the fate on phytoremediation, especially of hydroarbon polluted sites.

Given recorded successes from molecular biological methodologies in microbial ecology studies, we attempted to utilize t-RFLP to characterize community dynamics and changes in community structure in response to changes in prevailing physicochemical parameters due to crude oil pollution and accompanying conditioners at phytoremediation sites.

MATERIALS AND METHODS

Description of study area

The study area consists of a two week old crude oil spilled (which caught fire afterwards) soil in Oshie community in the Niger Delta region of Nigeria. This community is a host to a major oil company in Nigeria. Eye witnesses suspect sabotage as the cause of spill. Soils (20 cm depth) from this site were randomly collected and bulked following a field survey, characterized and set up in a 90 days phytoremediation pot experiments (in replicates of three) using *Vigna subterranean* (Bambara), *Hevea brassilensis* (Rubber), *Cymbogonium citratus* (Lemon grass) and *Fimbristylis litoralis* (Fimbristylis). An agricultural soil in the same region with history of no pollution constituted control regimes. Chemical and physical characterization of control and polluted soils (Table 1) were done before the start of the experiment to determine appropriate soil conditioners to be used. Watering was based on need. At 90 days, plants were harvested and rhizopheric soils were collected for laboratory analyses. These samples were transported in ice chest coolers to Institute of Agrophysics Lublin Poland for analysis. Watering was on need basis. Different soil regimes ranging from planted polluted and unpolluted, and organic manure - amended

variants were subjected to preparations for various analyses discussed below.

Soil conditioning

Organic manure (poultry dung) and inorganic manure (NPK 20:10:10) were added for augumentation and comparison, and the amount was determined using the method of Akobundu (1987), C = (R × A)/Q (where C = amount of amendment, R = 2 = a constant, A = weight of soil, and Q = product weight of substance. Initial Physicochemistry for polluted and unpolluted soils. Air - dried screen (2 mm) soils were subjected to gas chromatographic (HP Gas Chromatograph 5890 Series ii, using dichloromethane as extraction solvent) analysis for Polycyclic aromatic hydrocarbons (PAH), Atomic absorption spectrophotometric analysis for heavy metals, Kjeldahl method for Total Nitrogen and portable meters as reported by Nwaichi et al. (2011).

Extraction of soil DNA

To avoid cross-contamination, Pre- and Post- activities including sample sorting and handling, DNA extraction, bench preparation for PCR, PCR implementation, its product visualization and storage, were physically seperated throughout the study period. Several attempts were made to optimize nucleic acid extraction method, which is important for description of microbial diversity. DNA was isolated from soil samples using FastDNA® SPIN kit for Feces and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA) following manufacturer's protocol. DNA concentration thereafter was determined using Nano Drop 2000 Spectrophotometer (Thermo Scientific). This kit included physical disruption method, glass bead homogenization and freezing-thawing, thus rendering confined bacteria available for lysis treatments (Frostegard et al., 1999), for a better DNA yields.

PCR for AOA (polymerase chain reaction - ammonia oxidizing archea)

Dilution of DNA to concentration of 2 ng/µl in nuclease free water was made and samples centrifuged in 'short' mode at 12000rcf. Into 0.1 ml PCR tubes, 28 µl reaction mix (15 µl SIGMA ReadyMixTM REDTaq® PCR Reaction mix with $MgCl_2$, 0.5 µl Primers AOA 19F and 0.5 µl AOA A643R and 12 µl kit water) was added to 2 µl diluted DNA, then centrifuged. A fluorophore, 6 - carboxyfluorescein (6 FAM) was used to label primer for AOA amplification. For effective hybridization of the PCR amplified 16S rDNA gene, PCR conditions on AB Applied Biosystems Veriti 96 Well Fast Thermocycler, were set to: Stage 1(Denaturation) -95^{o}C, 5 min; Stage 2 - 92°C, 45 s (Primer Annealing), 59°C, 30 s, 72°C, 1 min (Primer Extension) x 35cycles; Stage 3(Cycling concludes with final extension) - 72°C, 7 min, 4.0°C and ∞) was set. Sample was loaded, and run completely, then visualized on Agarose gel electrophoresis (AGE) as described by Traugott (2006). Samples reamplifications were done when some signs of contamination were observed.

PCR products purification

ExoSAP-IT® for ABI Affymetrix® USB® products (Source: Exonuclease 1- Recombinant) supplied in special buffer, was added to amplicones in the ratio of 2:5 in 0.1 ml PCR tubes and vortexed. In thermal cycler (37°C, 15 min, 80°C, 15 min), incubation was done. Centrifugation at 750 rcf for 4 min was performed before and after product transfer into catridges. Catridges were discarded and purified DNA (Young et al., 1993) was ready. DNA concentration was again measured to work out volumes.

Digestion of amplicons using restriction endonuclease

To 0.1 ml tube, 5µl of amplicone, 0.6 µl of Csp enzymes and 0.6 µl of TANGO buffer were added (following master mix method). Nuclease free water was added to 10 µl reaction volume while pipette - mixing and short centrifuged. Incubation in thermal cycler (37°C, 2 h: digestion; 65°C, 20 min: inactivation) followed.

Terminal restriction fragment length poly- morphism (t-RFLP) AOA

This was done using a DNA sequencer (AB Applied Biosystems HITACHI 3130). Master mix of 9 µl HiDi formamide and 0.5 µl Liz standard was centrifuged. To MicrAmpTM Optical 96-well Reaction Plate, 9.5 µl mix and add 1 µl of products after restriction were mixed, centrifuged at 800 rpm for 3 min to clear bubbles. Products were cooled on ice after incubation in Thermal block (95 C, 3 min for denaturation). Sequencer manufacturer's protocol was followed and all experiments were done in replicates of three. The size, in basepairs, of terminal restriction fragments (T-RFs) was determined thereof. T-RFs with a size < 40 bp and 1% area were excluded from analyses.

Statistical analyses

ANOVA, data (n=3) mean comparisons, Principal component and factor analyses were elaborately done using STATISTICA v 10.

RESULTS AND DISCUSSION

Initial soil asessment revealed that PAHs, Cd, As and Pb polluted soil and phytoremediation technique recovered the soil to a certain degree (data not shown) for plants and microorganisms to survive and thrive. Data obtained for control soil further shows that most soils in the Niger Delta are contaminated residually even when there is no history of pollution in the area (Table 1). High Cd levels may have arisen from complex nature of spill, and later, fire due to perceived sabotage. Genetic diversity, the level of biodiversity, refers to the total number of genetic characteristics in the genetic makeup of a species. Multivariate analysis of resolved t-RFLP fingerprints (Figure 1) showed nine active communities in Bambara - cultivated control soils and these were drastically reduced to 3 with crude oil pollution under same condition. LaMontagne et al. (2002) described its relevance in visualising relationships among fingerprints as done in this study. Although organic amendments only marginally increased community by one, species benefited in terms of growth. Presence of an internal standard (GeneScan-1000 ROX) in each sample, was useful in quantifying variation, not only in terms of the size, in basepairs of terminal restriction fragments, but also in terms of the relative proportions of each fragment in a community profile. For Rubber - cultivated regimes, species were severely impacted and a community shift was observed.

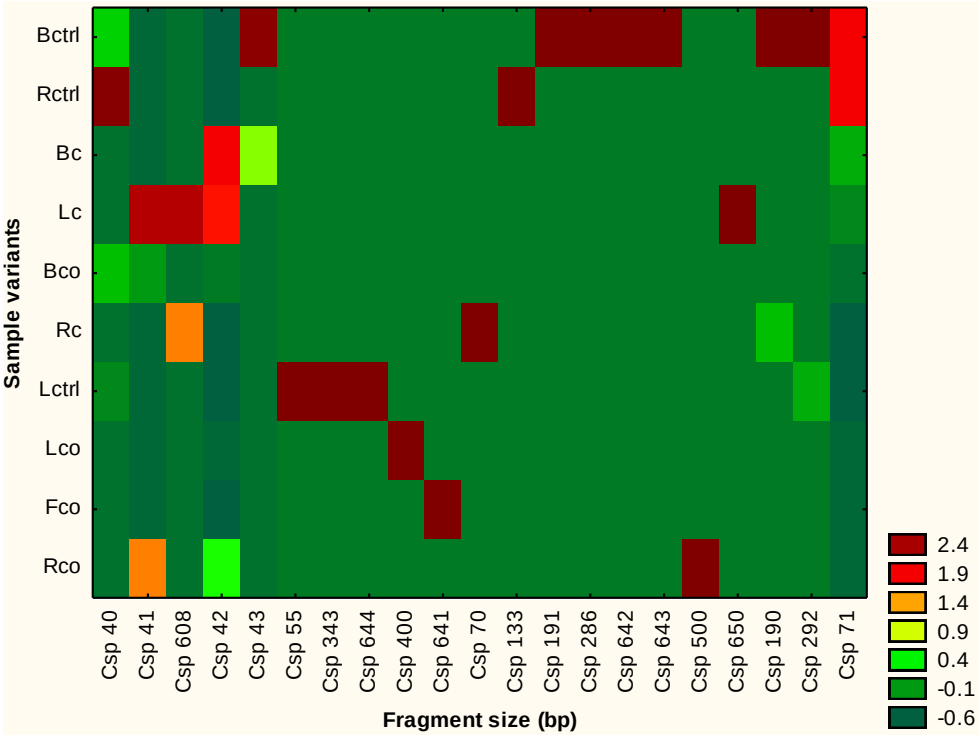

Figure 1. Multivariate analysis of t-RFLP profile. F, B, R and L denote soils planted with Fimbristylis, Bambara, Rubber and Lemon grass plants; attached ctrl, c, and co represent uncontaminated, contaminated and unamended, and contaminated and organic manure amended soils, respectively.

Lankau (2007) of the National Science Foundation found that diversity within a species is necessary to maintain diversity among species, and vice versa. Application of organic amendments indicated regain of a soil microbial community within 90 days. This could lead a loss of biological diversity (NBII, 2013). A significant reduction in community was also observed with contamination for Lemon grass nutrient - unamended cultivated soil but regain of community with organic conditioner, was marginal (10%) at 90 days. Consequently, the structure of the soil bacterial assemblage changes with hydrocarbon stress and a change of dominant forms occurs and the genetic diversity of prokaryotes decreases (Rivera and Lake, 2004). Depending on choice of plant for phytoremediation of polluted soil, considerable regain of communities could be established as seen with *C. citratus* and *F. Littoralis* - cultivated soil communities. For unamended and control regimes, fragments for Fimbristylis -cultivated soils were either < 40 bp or < 1% area and were excluded for analysis.

A more detailed analysis of the ecological parameters of the assemblage of prokaryotes (Figure 2) showed the highest genetic diversity, determined by Shannon's H index in control soil where Lemon grass was grown and the lowest in the contaminated but amended soil cultivated with Fimbristylis. To make these index numbers more biologically sensible (Tuomisto, 2010), they were converted to the effective number of types (ENT), which is the real biodiversity to allow for comparison of the biodiversity with other communities. This means that these extreme communities with Shannon index of 1.66 and 0.68 have equivalent diversity as communities with 5 and 2 equally-common species, respectively. Consistent high Shannon H values for Bambara cultivated rhizospheric soils may be be related to its family, leguminosae. From Figure 2, our results show that the diversity and evenness in organic manure amended contaminated regimes are much higher than in the unamended counterpart under similar conditions. Equitability index, EH, which is interpreted as the number of equally abundant species necessary to produce the same diversity as observed in a sample, showed dissimilar number of individuals in study community. Obasi et al. (2013) related high equitability values or Pileon's evenness index to relatively low human activities.

Nutrient recharge (Table 2) of the community had a beneficial effect and agrees with the findings of Patyka and Kruglov (2008), who identified increased species richness with systematic increase of manure. The amended stressed soils not only has a greater number of species present, but the individuals in the community are distributed more equitably among these species. Pearson's

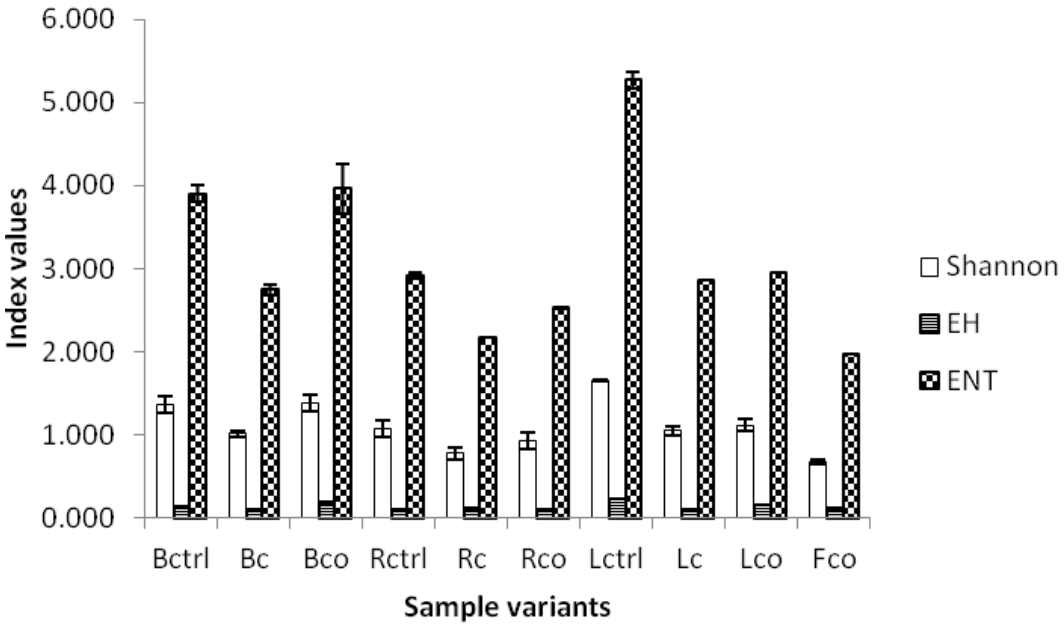

Figure 2. Ecological parameters of prokaryote assemblage of tested soils. EH, ENT, B, L, R and F denotes Equitability index, effective number of types of species, Bambara, Lemon grass, Rubber, and Fimbristylis - cultivated; attached ctrl, c, and co denotes control (unpolluted), crude oil polluted and organically amended - crude oil polluted agricultural soils respectively.

Table 2. Mean level of primary nutrients after phytoremediation.

Nutrient	Soil variants											
	Unpolluted				Polluted and unamended				Polluted but amended			
	F	B	R	L	F	B	R	L	F	B	R	L
NO_3-N (mg/kg)	10.02	21.40	10.98	10.66	26.40	44.20	26.15	10.45	32.42	40.60	32.89	18.00
P (mg/kg)	7.13	17.10	31.39	9.22	18.33	30.67	16.00	13.42	40.15	40.00	38.00	43.33
K (cmol/kg)	0.60	0.89	0. 86	1.49	0.39	0.54	1.94	1.06	0.99	1.57	2.28	2.14

B, L, R and F denotes Bambara, Lemon grass, Rubber and Fimbristylis - cultivated.

Table 3. Pearson's correlation coefficient of Cr removal versus total Expected number of species types, ENT.

Cr removal	ENT			
	Fimb	Bam	Rub	Lem
Fimb	-0.55			
Bam		-0.56		
Rub			-0.58	
Lem				-0.97

Fimb, Bam, Rub and Lem represent *F. Littoralis, V. Subterranea, H. Brasilensis* and *C. Citratus* respectively.

correlation coefficient of Cr removal versus total Expected number of species types, ENT (Table 3) shows moderate negative correlation *for F. Littoralis, V. Subterranea* and *H. Brassilensis* - cultivated soils, and near perfect negative correlation for soils cultivated with *C. citratus*. This implies that increase in performance of plants at remediation of polluted soil is tantamount to increased expected number of types of species. In other

words, increased contaminant load reduced the expected number of types of species in a soil community.

Conclusion

Different levels of disturbance gave different effects on abounding microbial diversity. In order to preserve biodiversity in a given distorted environment, it is important to understand the levels and types of available nutrients and inherent limitations. Abundance and evenness of the species present in study site, given different life - lines (organic amendments and phytoremediation) as indicated by Shannon index were minimally regained. We believe that there is a high tendency of genetic characteristics to vary with time. Natural nodule - level association could be explored in disturbed soil recovery. Generally, low equitability index, EH indicates that all species in the community are represented by a dissimilar number of individuals.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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