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Full Length Research Paper

Effect of phosphorus fertilization on arbuscular mycorrhizal fungi in the Bambara groundnut rhizosphere

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Tropical soils are highly leached, abundantly clayey and strongly acid, resulting in low mineral availability and especially phosphorus (P). The plants of this region must establish symbiotic relationships enabling them to strengthen their hydromineral nutrition. The aim of this work was to study the effect of P fertilizer application on the diversity of arbuscular mycorhizal fungi (AMF) under the rhizosphere of three Bambara groundnut (*Vigna subterranea*) landraces. To attain it, plants were grown in farm under different simple superphosphate (SSP) levels (0, 50, 100, 150 and 200 kg.ha^{-1} P_2O_5) in 2015 at Mendong and Soa districts. Experimental units were arranged in a randomized complete block design with three replications. Soil and root samples were taken from the rhizosphere of three randomly selected seeds holes per experimental unit at flowering (31 days after sowing) and fruition stages. At harvest, the roots were stored in 50% ethyl alcohol. They were stained with methyl blue and observed under an optical microscope. The soil samples allowed the trapping to be carried out in a greenhouse with sorghum as test plants. Results show that Bambara groundnut landraces were the host of several AMF species and were highly colonized with all showing hyphae, vesicles and even spores. High level of SSP (200 kg.ha^{-1} P_2O_5) significantly ($p<0.001$) reduced the intensity of mycorrhization as well as the diversity indices compared to the control. A density of AMF spores reaching 1930 was obtained in 100 g of trapping substrate. On the basis of morphological characteristics, 16 AMF species belonging to nine genera (*Glomus, Acaulospora, Gigaspora, Racocetra, Rhizophagus, Funneliformis, Septoglomus, Diversispora* and *Claroideoglomous*) were isolated and identified. *Rhizophagus intraradices* and *Septoglomus constrictum* were the most abundant. AMF species identified in this groundnut rhizosphere can be multiplied and used as biological fertilizer to increase its yield.

Key words: Arbuscular mycorhizal fungi (AMF) diversity, Cameroon, morphological characteristics, rhizosphere, root colonization, spore density, symbiosis, phosphate fertilization, Voandzou.

INTRODUCTION

In the world in general and in Africa in particular, about 795 million people are undernourished (one in four in sub-Saharan Africa) and suffer from insufficient energy intake associated with protein, vitamin and mineral deficiencies (WFP, 2016). Moreover, population projections predict an increase in world population. This demographic growth far exceeds that of agricultural production. Indeed, this agricultural production must increase by 70% for a world population of 2.3 billion inhabitants in 2050 (FAO, 2017). The challenge of agricultural research is to contribute to increasing crop yields while safeguarding the environment. Bambara groundnut (*Vigna subterranea* (L.) Verdc.) could play an important role because its seeds are highly caloric, rich in minerals, vitamins, proteins and amino acids (De Kock, 2013; Yao et al., 2015; Tsoata et al., 2017a).

In fact, seed legumes (Bambara groundnut) have an important socio-economic role in tropical Africa, where they are a tradition in the culinary habits of populations (Brink et al., 2006). Bambara groundnut is native to northeastern Nigeria and northern Cameroon (Begemann, 1988). Its world production was estimated at 216575 t in 2013. In Africa, its production has not varied considerably since 1993 and is about 300 000 t/year. In Cameroon, production increased from 5800 t in 1993 to 38075 t in 2014 (FAO, 2017), making it the third largest producer in the world. Bambara groundnut seeds are used as feed for humans, poultry and livestock (Brink et al., 2006).

They contain on average of 63% carbohydrates, 19% protein and 6.5% fat; these values are considered sufficient to make this legume a complete food (Bamishaiye et al., 2011) to consider for food security (De Kock, 2013; Yao et al., 2015). Bambara groundnut has medicinal properties well known to local populations (Brink et al., 2006). One of the main attributes of Bambara groundnut is its tolerance to poor soils (Temegne et al., 2015) and drought (Berchie et al., 2012; Tsoata et al., 2016; 2017b) as well as its ability to produce under conditions where groundnuts fail completely (Jideani and Diederiks, 2014). Despite this panoply of properties, its production remains low. This low production is reflected in rising market prices and scarcity of seeds (IRAD, 2013). This low production is due to diseases and pests but above all poverty of soil (FAO, 2003), in particular phosphorus (P) deficiency.

However, very little information exists on the biological factors that support the Bambara groundnut growth on poor soils. P is an essential mineral for living organisms which is after nitrogen an indispensable element to the good functioning of the plants (Morel et al., 2006). P is mainly extracted from phosphate (P_i) rock (natural P_i). Worldwide reserves of commercial natural P_i are currently estimated at 12 billion tons with an annual consumption of 132 million tons. These reserves are barely enough for a hundred years (Frossard et al., 2004). As a result, P was placed on the red list of raw materials by the European Commission in 2014 (CE, 2014).

Plant production is limited by low P availability due to inability to take P in orthophosphate ions form, either directly through roots or through arbuscular mycorrhizal fungi (AMF). Among the functional groups composing the telluric microflora, AMF play a major role in improving water and mineral nutrition. Thus, many plant species rely heavily on AMF for their survival, especially in the tropics, where the majority of soils are highly leached, clayey and highly acidic, resulting in low mineral availability, especially in P.

Many studies have also shown that AMF improve water and mineral nutrition of plants and in particular the Pi plants supply (Onguene et al., 2011; Taffouo et al., 2014). Some studies showed that phenological stages influence mycorrhizal activity (Mbogne et al., 2015; Johnson et al., 2016) but little is known about the chemical P fertilizer effect on it. Some work done in Cameroon on the mycorrhization of bambara groundnut did not take into account the AMF biodiversity under cultivation (Ngakou et al., 2012; Tsoata et al., 2015). Several researchers (Ngonkeu et al., 2003; Nwaga et al., 2003) recommend the use of indigenous (native) AMF as biological fertilizers because of their adaptation to local conditions. The objective of this work was to evaluate the AMF diversity in the Bambara groundnut rhizosphere under P_i fertilization.

MATERIALS AND METHODS

Plant material

Three Bambara groundnut landraces obtained from the producers were used: V1, the ivory cream (or white) seed coat; V2, the red seed coat and V3, the ivory cream seed coat with grey eyes (hilum) (V3). These landraces were chosen because they are among the most appreciated by the producers.

Study area

The study was conducted in Soa and Mendong localities. The Soa (N 03°58'647", E 011°34'361", altitude: 680 m) and Mendong (N 03°50'12 to 12.5", E 011°27'05.6 to 09", altitude: 717 m) sites are located in the Center region of Cameroon. They are characterized by precipitation from 1,617 to 1,800 mm/year. The average daily air temperature varies from 23 to 24°C (IRAD, 2008). They belong to

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Table 1. Physio-chemical properties of the soil of the experimental sites.

Site	Sand	Clay	Silt	OC	N	C/N	pH	P	CEC	Na^+	K^+	Mg^+	Ca^{2+}
	%					-		µg/g	cmol/kg				
Mendong	46.99	40.99	11.41	2.50	0.21	11.81	6.50	7.59	8.18	0.030	0.45	1.92	8.44
Soa	58.23	31.49	10.28	2.54	0.21	12.36	5.09	4.75	6.59	0.029	0.22	1.30	2.17

OC, Organic Carbon; N, total N; P, phosphorus (Bray 2); CEC, cation exchange capacity.

the agro-ecological zone of bimodal wet forest, characterized by acidic ferralitic soils. The area is characterized by a bimodal rainfall pattern, with four seasons: long rainy season from September to November, long dry season from December to February, short rainy season from March to June and short dry season from July to August. The previous crop of the Mendong study area was rice (*Oryza sativa* L.) with a predominance of species such as *Chromolaena odorata* L. and *Imperata cylindrica* L. The site of Soa was an old shrub fallow.

Soil sampling from the field

In each site, a composite sample of the top soil (0 to 15 cm depth) was collected from the experimental site with an auger before bed preparation following the transect method described by Okalebo et al. (2002). About 200 g of the samples were collected and afterward mixed to obtain a composite sample of the field.

Soil physical and chemical analyses

Physical and chemical properties were analyzed in the International Institute of Tropical Agriculture (IITA) soil laboratory of Nkolbisson (Cameroon). Soil sample was air-dried and ground to pass through a 2 mm sieve. For carbon (C) and nitrogen (N) analysis, soil was further fine ground to pass through a 0.5 mm sieve. Soil pH in water was determined in a 1:2.5 (w/v) soil: water suspension. Organic C was determined by chromic acid digestion and spectrophotometric analysis (Heanes, 1984). Total N was determined from a wet acid digest and analyzed by colorimetric analysis (Anderson and Ingram, 1993). P was extracted using Bray extractant and the resulting extract analyzed using the molybdate blue procedure described by Murphy and Riley (1962). Exchangeable cations - Ca, Mg, K and Na were extracted using the ammonium acetate (NH_4OAC, pH: 7) and determined by flame atomic absorption spectrophotometry. Cation exchange capacity (CEC) was determined using ammonium acetate. Results of the soil physico-chemical properties analysis are presented in Table 1. Mendong soil is weakly acidic (pH: 6.52) and Soa acidic (pH: 4.64). The soils of the two sites are poor in N and P in which Mendong soil is richer in P than in Soa with both sites have sandy-clay soil. The low C/N ratios at both sites reflect rapid decomposition of organic matter. It leads to a malfunctioning of the clay-humic complex.

Experimental design

The experimental set-up was a complete randomized block with three factors: phosphorus levels (0, 50, 100, 150 and 200 $kg.ha^{-1}$ P_2O_5) (Toungos et al., 2010; Nweke and Emeh, 2013), landraces (V1, V2 and V4) and sites (Mendong and Soa).

Experimental layout

In each site, after clearing and then plowing between 20 and 30 cm deep, the blocks and the experimental plots were delimited using a double decameter, a string and stakes. Each experimental unit consisted of one of three landraces of Bambara groundnut combined with one level of phosphorus. The distance between lines was 25 cm and in the line the spacing between the plants was 25 cm. A space of 25 cm was left at the edges of each unit. A total of 49 seed holes/4m m^2, that is, 1,470 seed holes for the 336 m^2 were seeded. Sowing was carried out on September 13, 2015 in Mendong and on September 26, 2015 in Soa. The sorted seeds were sown (3 to 5 cm deep) directly in the field at three to four seeds/seed hole. Thinning was kept as one to two vigorous seedlings per hole. The different levels of phosphorus fertilizer (P_2O_5) were applied two weeks after sowing. Urea (46% N) was supplied as a base fertilizer (25 gm^{-2}) to promote vegetative growth of plants. Weeding was done in order to eliminate weeds and reduce pest attacks and diseases. The first weeding was carried out one week after sowing and subsequently weeding and/or hoeing were carried out according to the frequency of grass growth.

Sampling

At flowering and fruition, the roots (three plants per treatment/landrace) were taken from the rhizosphere of the Bambara groundnut pods of each experimental unit at each site. About 5 g of fine roots attached to the main root were thus collected per seed hole and then stored in 50% of ethyl alcohol. After the plant harvest in each experimental unit, soil samples were collected and trapped in the greenhouse at the Institute of Agricultural Research for Development (IRAD).

Root colonization

In the laboratory, roots were thinned and stained (Phillips and Hayman, 1970) before microscopic observation. Approximately 3 to 5 g of each root sample (1 to 2 cm fragment length) of soil were placed in labeled test tubes containing 10 mL KOH (10%, W/V) to empty the contents of the cytoplasm in order to facilitate the observation of mycorrhizal structures. After 24 h, 10 mL of hydrogen peroxide was added to it during 15 min in order to perfect the clarification (Kormanik and McGraw, 1982). The tubes were emptied of the KOH and the roots were thoroughly rinsed three times with tap water. Then, 1% of hydrochloric acid (HCl) was poured into the roots in each tube and left for 15 min to acidify the cell contents. Next, the solution was removed. They were soaked in 10 mL of dye (lactic acid-glycerol-water-methyl blue). After 24 h, the dye (Koske and Gemma, 1989) was removed and three rinses were performed. Discoloration was achieved by introducing the bleach (lactic acid-glycerol-water) into the test tubes. The root fragments (10) were mounted on glass slide. The experiment was repeated three times for each sample. The observation was made with the photonic microscope (OLYMPUS, 100x). For each fragment, the abundance and diversity of the mycorrhizal structures (vesicles, extra and intracellular hyphae, auxiliary cells and spores) were noted. The frequency (F) and the intensity (I) of the mycorrhization were evaluated by the method of Trouvelot et al. (1986).

Table 2. Effect of stage of development on intensity and frequency of mycorhization.

Stage of development	Intensity (%)	Frequency (%)
Flowering	22.12±1.55[a]	41.83±2.55[a]
Fruition	24.00±1.63[a]	43.76±2.35[a]
p	0.181 ns	0.441 ns

Means±Standard error followed by the same letter in a column are not significantly different at the 5% threshold.

AMF trapping in a greenhouse

Approximately 500 g of soil from each sample was sandwiched between two layers of sterile sand in 1 kg plastic bags perforated at the bottom. The latter were seeded with four sorghum seeds (from IRAD) per sachet. These sorghum seeds were previously disinfected with 10% sodium hypochlorite for 15 min and rinsed three times with tap water. The bags were placed in unperforated bins to keep moisture. The plants were allowed to grow under these conditions for three months. Then the sachets were transferred to the shelter in order to provoke a water deficit which can induce sporulation in which after five weeks, the rhizosphere was removed for spore extraction.

AMF spore extraction and identification

For each treatment, a sample of 100 g of substrate taken from each trapping bag was used for the extraction of the spores by wet sieving following the method described by Gerdemann and Nicholson (1963) through a series of sieves ranging from 400 to 38 µm. The spore suspension contained in the sieves was centrifuged on a 50% sucrose gradient (Daniels and Skipper, 1982; Oehl et al., 2003). After rinsing, it was poured onto a filter paper with a grid surface to facilitate the counting of the spores. The spores were counted using a binocular magnifying glass (40× Magnification) according to their size, color, shape, ornamentation and characteristics of their walls and hyphal attachments. The average number of spores was expressed per 100 g of dry substrate. The AMF spores were mounted on glass slides in polyvinyl–lactic acid–glycerine (PVLG) with the Melzer reagent (Josserant, 1983) and identified on the basis of the morphological descriptions published by International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM). AMF types were classified to the genus rank and, if possible, to the species rank and named with the current taxonomy (Schüßler and Walker, 2010; Redecker et al., 2013).

Indices of diversity

The AMF diversity of each site (Castillo et al., 2016) was analyzed using the following indices: species richness (S: number of species in the study area), diversity index (H': Shannon-Wiener (1948) index; Pielou's (1966) evenness index (J'), Simpson's dominance index (*1*) and Simpson's (1949) diversity index (Ds). The indices were calculated by the following formulas:

$$H' = -\sum_{i=1}^{S} Pi \, Log \, Pi; \quad J' = H'/log(S);$$

$$I = \sum[ni(ni-1)]/[N(N-1)]; \quad Ds = 1-I$$

Where, Pi is the proportional abundance or percentage importance of the species (Pi = ni / N); ni is the number of individuals of a species in the sample, N the total number of individuals of all species in the sample and S is the total number of species.

Data analysis

The data were subjected to analysis of variance (ANOVA) using SPSS version 20 software. The Student-Newman-Keuls test was used to compare the means at the 5% threshold. Data that did not comply with the ANOVA assumptions (normality and homogeneity of variance tested through the Shapiro-Wilk's test and Brown and Kolmogrov-smirnov's test, respectively) were subjected to Kruskal-Wallis test. Their means were compared by the Mann-Whitney U test at the 5% threshold.

RESULTS

Effect of stage of development on mycorhization

The stage of development (flowering and fruition) did not influence the frequency and intensity of mycorrhization (Table 2).

Effect of P fertilization on mycorrhization and diversity index

Levels of P_2O_5 did not influence the frequency of mycorrhization (Table 3). The P_2O_5 levels greater than 150 $kg.ha^{-1}$ significantly ($p<0,001$) reduced mycorrhization intensity by 60%. The Shannon-Wiener (H'), Simpson (Ds) and Pielou (J') diversity indices were significantly ($p<0.001$) greater in control than the fertilized plots (Table 3). The dominance of Simpson (1) and the total number of spores were significantly ($p<0.001$) lower in the control treatment (Table 3).

Effect of Bambara groundnut landraces on mycorrhization and diversity index

The Bambara groundnut landraces bear arbuscular and vesicular mycorrhizae. The mycorrhizal structures observed in the roots were in the form of arbuscules, vesicles, spores, intra- or intercellular hyphae and auxiliary cells. Numerous external hyphae were also

Table 3. Effect of P levels on mycorrhization and AMF diversity index under Bambara groundnut rhizosphere at Mendong and Soa areas.

P_2O_5 (kg.ha^{-1})	Site	Intensity (%)	Frequency (%)	H'	J'	*I*	D_S	S
0	M	21.8±3.4^{ab}	48.1±4.5^{ab}	1.82±0.04^{bcd}	0.7±0.01^{bc}	0.24±0.01^{bc}	0.76±0.01^{bc}	14±0.3^{a}
	Soa	30.1±4.3^{a}	33.6±3.4^{bc}	2.17±0.01^{a}	0.82±0.01^{a}	0.14±0.01^{d}	0.86±0.01^{a}	15±0.2^{a}
Mean		25.9±2.9^{A}	40.8±3^{A}	1.99±0.05^{E}	0.76±0.02^{E}	0.19±0.01^{A}	0.81±0.01^{D}	14±0.2^{A}
50	M	19.5±3^{ab}	52.8±4.1^{a}	1.84±0.03^{bcd}	0.69±0.01^{bc}	0.23±0.01^{bc}	0.77±0.01^{bc}	15±0.3^{a}
	Soa	29.2±4^{a}	31.1±6.1^{c}	2.07±0.03^{a}	0.79±0.01^{ab}	0.17±0.01^{cd}	0.83±0.01^{ab}	14±0.2^{a}
Mean		24.4±2.5^{A}	41.9±4.1^{A}	1.96±0.04^{D}	0.74±0.02^{D}	0.2±0.01^{B}	0.8±0.01^{C}	15±0.2^{A}
100	M	21.3±3.6^{ab}	57.2±5.7^{a}	1.68±0.04^{d}	0.64±0.01^{c}	0.28±0.02^{ab}	0.72±0.01^{cd}	15±0.5^{a}
	Soa	25.3±2.9^{a}	29.4±4.9^{c}	1.7±0.11^{d}	0.65±0.04^{c}	0.32±0.04^{a}	0.68±0.04^{d}	14±0.4^{a}
Mean		23.3±2.3^{A}	43.3±4.4^{A}	1.69±0.06^{A}	0.64±0.02^{A}	0.3±0.02^{D}	0.7±0.02^{A}	15±0.3^{A}
150	M	25±3.1^{a}	60.8±3.7^{a}	1.74±0.13^{cd}	0.67±0.05^{bc}	0.27±0.05^{ab}	0.73±0.04^{cd}	14±0.2^{a}
	Soa	26.5±4.3^{a}	33.1±5.8^{bc}	1.96±0.06^{abc}	0.74±0.02^{abc}	0.21±0.02^{bcd}	0.79±0.02^{abc}	15±0.3^{a}
Mean		25.7±2.5^{A}	46.9±4.1^{A}	1.85±0.08^{B}	0.7±0.03^{B}	0.24±0.02^{C}	0.76±0.03^{B}	14±0.2^{A}
200	M	21.5±4.1^{ab}	46.8±5.5^{ab}	2.01±0.1^{ab}	0.76±0.01^{abc}	0.2±0.03^{bcd}	0.8±0.03^{abc}	14±0.2^{a}
	Soa	10.6±0.9^{b}	35±4.7^{bc}	1.81±0.08^{bcd}	0.69±0.03^{bc}	0.27±0.03^{ab}	0.73±0.03^{cd}	14±0.3^{a}
Mean		16.1±2.2^{B}	40.9±3.7^{A}	1.91±0.07^{C}	0.73±0.03^{C}	0.24±0.02^{C}	0.76±0.01^{B}	14±0.2^{A}
p (P_2O_5)		< 0.001***	0.513 ns	< 0.001***	< 0.001***	< 0.001***	< 0.001***	0.609 ns
p (Site)		< 0.001***	< 0.001***	< 0.001***	< 0.001***	< 0.001***	< 0.001***	0.552 ns
p (P_2O_5*Site)		< 0.001***	0.142 ns	< 0.001***	< 0.001***	< 0.001***	< 0.001***	0.108 ns

M, Mendong; H, Shannon-Wiener diversity index; J', Pielou's evenness index; I, dominance of Simpson; Ds, Simpson diversity index, S: species richness. Means±Standard errors followed by the same letter in a column are not significantly different at the 5% threshold.

Table 4. Effects of landraces on mycorrhization and AMF diversity index.

Land-race	Intensity (%)	Frequency (%)	H'	J'	I	DS	S
V1	26.77±2.01c	45.16±3.4a	1.87±0.05a	0.71±0.02a	0.24±0.02c	0.76±0.02a	15±0.16a
V2	22.5±1.77b	41.58±2.77a	1.90±0.05b	0.72±0.02b	0.22±0.02a	0.78±0.02c	14±0.14a
V3	19.91±1.98a	41.64 ±2.81a	1.86±0.05a	0.71±0.02a	0.23±0.02b	0.77±0.02b	14±0.19a
p	< 0.001***	0.408 ns	< 0.01**	< 0.05*	< 0.001***	< 0.001***	0.220

H, Shannon-Wiener diversity index; J', Pielou's evenness index; I, dominance of Simpson; Ds, Simpson diversity index; S, species richness. Means±Standard errors followed by the same letter in a column are not significantly different at the 5% threshold.

observed. The intensity of mycorrhization was higher at V1 and lower at V3; but the landrace did not affect mycorrhizal frequency (Table 4). V2 had the Shannon-Wiener (H'), Simpson (Ds) and Pielou (J') diversity indices higher than those of V1 and V3 (Table 4). The total number of spores of V1 significantly exceeded those of V2 and V3 (p<0.001) by 21 and 43%, respectively.

Effect of site on mycorrhization and diversity index

The frequency of mycorrhization was 1.6 times higher in Mendong than in Soa (Table 5). However, the intensity of mycorhization was higher at Soa than Mendong by 11.6%. The Shannon-Wiener (H'), Pielou (J'), Simpson (Ds) indices and the total number of spores were significantly (p<0.001) higher at Soa than Mendong (Table 5).

Identification of arbuscular mycorrhizal fungi

Bambara groundnut roots showed Mycorrhizal structures in the form of arbuscules, vesicles, hyphae, spores and auxiliary cells (Figure 1). Figure 2 shows some AMF spores isolated from the rhizosphere soil of Bambara

Table 5. Effect of study site on mycorrhization and AMF diversity index.

Sites	Intensity (%)	Frequency (%)	H'	J'	*I*	D_S	S
Soa	24.32±1.65[a]	32.44±2.23[b]	1.94±0.04[b]	0.74±0.01[b]	0.22± 0.02[b]	0.78±0.02[b]	14±0.12[a]
Mendong	21.8±1.53[b]	53.15±2.16[a]	1.82±0.04[a]	0.69±0.01[a]	0.24±0.01[a]	0.76±0.01[a]	14±0.15[a]
p	< 0.001***	< 0.001***	<0.001***	<0.001***	< 0.001***	<0.001***	0.557

H, Shannon-Wiener diversity index; J', Pielou's evenness index; I, dominance of Simpson; Ds, Simpson diversity index; S, species richness. Means±Standard errors followed by the same letter in a column are not significantly different at the 5% threshold.

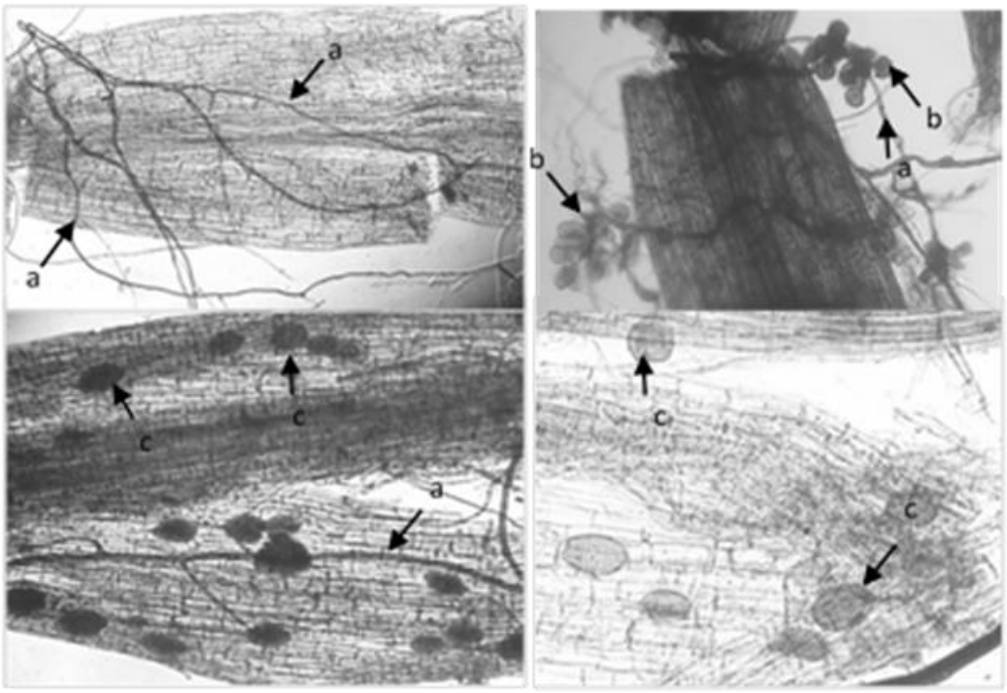

Figure 1. Mycorrhizal structures in Bambara groundnut roots. **a**, hyphae; b, auxiliary cells and c: vesicles.

groundnut. The 16 AMF species (Table 6) identified in the two sites could belong to six different families (Acaulosporaceae, Diversisporaceae, Entrophosporaceae, Gigasporaceae, Glomeraceae and Racocetraceae) and nine genera of *Glomeromycota* branch. The number of AMF spores of various species are presented in Table 7. The AMF diversity was identical in both sites.

DISCUSSION

High level of phosphate fertilizer (200 $kg.ha^{-1}$ P_2O_5) significantly reduced the intensity of mycorrhization. The application of this level of fertilizer might have increased the acidity of the soil. Indeed, the extreme acidity of soils could be the cause of the low density of spores observed in certain species of arbuscular mycorrhizal fungi (AMF) (Bivoko et al., 2013; Mbogne et al., 2015). Bhadalung et al. (2005) pointed out that chemical P fertilization reduces the total number of AMF spores in the long-term. They noted with maize that, only *Acaulospora* sp. maintains its number of spores unchanged under fertilization on the nine species listed, it is therefore insensitive to chemical fertilization.

On the other hand, they noted that the absolute number of spores of *Glomus* sp. and *G. geosporum* decreased in response to fertilization but not relative abundance. *Glomus* sp. and *G. geosporum* are therefore slightly sensitive to fertilization (Bhadalung et al., 2005). However, soils with low availability of phosphorus favor greater mycorrhizal colonization and possibly greater formation of spores (Smith and Read, 2008). The results of this study show that Glomeraceae and Entrophosporaceae are slightly sensitive, Acaulosporaceae are insensitive, Gigasporaceae and

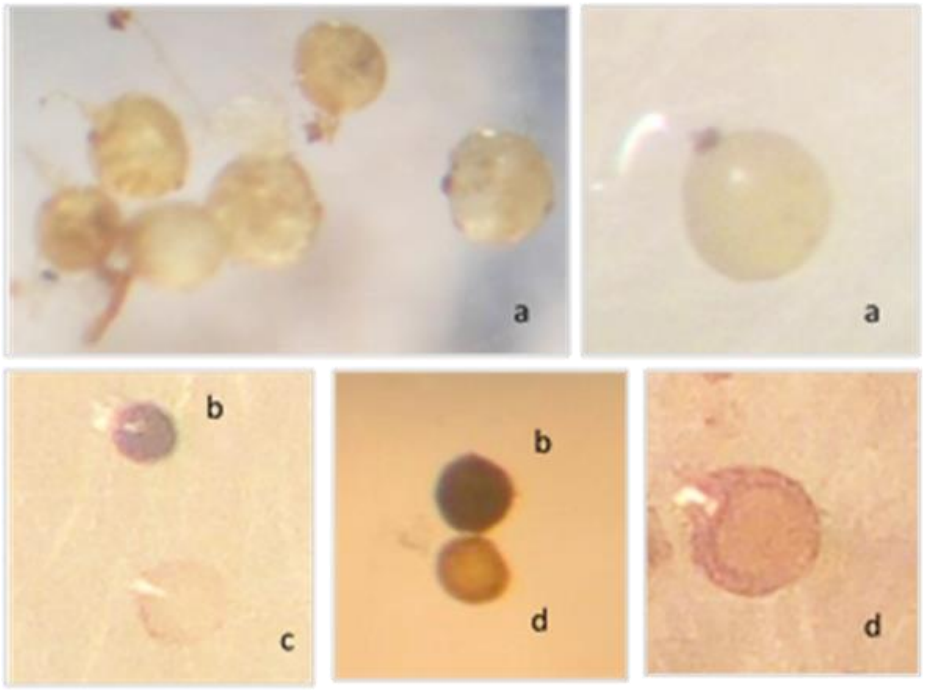

Figure 2. Arbuscular mycorrhizal fungi spores isolated from rhizosphere soil of Bambara groundnut. a, *Gigaspora margarita*; b, *Septoglomus constrictum*; c, *Acaulospora delicate*; d, *Claroideoglomus etunicatum*,

Table 6. AMF species identified in the study sites on the basis of morphological traits.

Family	Genera	Species
Acaulosporaceae	*Acaulospora* Gerd. and Trappe emend. Berch	*A. delicata* *A. laevis* *A. foveata* *A. tuberculta*
Diversisporaceae	*Diversispora* Walker C. and Schüßler A.	*D. eburnea*
Entrophosporaceae	*Claroideoglomus* Walker C. and Schüßler A.	*C. etunicatum*
Gigasporaceae	*Gigaspora* Gerd. and Trappe	*G. margarita*
	Gigaspora Gerd. and Trappe	*G. decipiens*
Racocetraceae Oehl, sieverd and Souza	*Racocetra* Oehl, Souza F.A. and Sieverd.	*R. coralloidea*
Glomeraceae	*Glomus* Tul. and Tul. C.	*G. ambisporum*
	Funneliformis Walker C. and Schüßler A.	*F. geosporum*
	Funneliformis Walker C. and Schüßler A.	*F. mossae*
	Rhizophagus Walker C. and Schüßler A.	*R. clarus*
	Rhizophagus Walker C. and Schüßler A.	*R. intraradices*
	Septoglomus Sieverd., Sliva G.A. and Oehl	*S. constrictum*
	Septoglomus Sieverd., Sliva G.A. and Oehl	*S. viscosum*

Diversisporaceae are highly susceptible to phosphate fertilization. Abbott and Robson (1991), Bagyaraj (2014) emphasize that Acaulospora species are commonly found in tropical soils and are well suited to soils with a

Table 7. Average of total number of AMF spores in 100 g of soil.

Genera	Study site									
	Mendong					Soa				
P_2O_5	0	50	100	150	200	0	50	100	150	200
Acaulospora	69	82	63	45	178	145	189	132	130	165
Claroideoglomus	19	24	15	11	67	43	56	31	33	38
Diversispora	33	45	39	28	54	68	99	81	71	80
Funneliformis	30	38	33	24	49	60	76	65	72	68
Gigaspora	0	1	1	0	1	2	2	3	3	2
Glomus	55	57	78	77	57	54	58	47	58	46
Racocetra	1	2	2	1	2	1	1	0	1	0
Rhizophagus	290	326	422	267	403	198	318	760	432	791
Septoglomus	193	257	247	122	206	134	234	184	227	174

pH below 5. This is similar to results obtained by Mbogne et al. (2015) who worked on the effect of fertilizer types on the endomycorrhizal biodiversity of pumpkins in Cameroon and Benin.

However, phosphate fertilization did not affect the frequency of mycorrhization in Bambara groundnut. Indeed, the simple superphosphate (P_2O_5) is a slow fertilizer whose solubilisation is progressive. This property could explain this result. Unlike other chemical fertilizers (NPK), it behaves like organic fertilizers (natural phosphate (rock), residual household waste, chicken droppings, etc. that do not reduce the frequency of AMF mycorrhization (Leyval et al., 2009, Mbogne et al., 2015).

This study highlights existence of a significant AMF diversity (16 species) in Mendong and Soa soils under Bambara groundnut culture. Nevertheless, the growing conditions of the Bambara groundnut could favor adaptation of species like *Rhizophagus intraradices* and *Septoglomus constrictum*. In fact, the Glomeraceae family is considered to be the most abundant of all AMF in the tropical arid zone (Maksoud et al., 1994) and is generally associated with acid soils (Abbott and Robson, 1991; Mbogne et al., 2015). It adapts to varied range of soils at different levels of nutrient availability (Straker et al., 2010).

According to Singh et al. (2008), the high competitiveness of Glomeraceae and their adaptive capacity, which allows them to establish better than other AMF families, could explain their predominance in the tropics. This adaptation is also due to their low-perturbable developmental cycle, which is unaffected by soil rehearsals compared to other families such as Acaulosporaceae, Gigasporaceae and Diversisporaceae (Oehl et al., 2003). Glomeraceae easily spread by spores, which are forms of AMF resistance under difficult conditions, while Acaulosporaceae, Gigasporaceae and Diversisporaceae propagate more with other propagule types such as extraracinar mycelial fragments and hyphae (Brito et al., 2012).

Several other studies (Johnson et al., 2013; Castillo et al., 2016) have noted the predominance of Glomeraceae in various tropical soils. Nevertheless, Acaulosporaceae were more abundant (four species) than Gigasporaceae (two species). This diversity of Acaulosporaceae is due to their tolerance to acid soils and their insensitivity to chemical fertilization. Species of this type are highly effective in P-uptake and transfer to the host plant, compared to Glomeraceae species (Jakobsen et al., 1992). The Gigaspora genus often predominates in sandy soils such as dunes (Lee and Koske, 1994).

However, majority of agricultural soils in southern Cameroon have a low percentage of sand. This could justify the low density of Gigaspora spores in the study sites. In addition, species of the genus Gigaspora produce large spores (260 to 440 µm for Gigaspora margarita) that require a longer developmental period than small spores (Hepper, 1984). They adapt more to changes in environmental conditions (Stutz and Morton, 1996) and are most often associated with wild plants than open-field crops (Gai et al., 2006). Species of the genus Gigaspora are able to cope with nutrient-rich environments (Liu et al., 2012).

The stage of development did not influence the frequency and intensity of mycorrhization. This result is contrary to those of Johnson et al. (2016) and Mbogne et al. (2015) who found that the frequency and intensity of mycorrhization are more important to fruition than flowering. The Bambara groundnut root infection process by AMF was certainly rapid and early so that the frequency and intensity of mycorrhization was already optimal at flowering.

Mycorrhization frequencies are around 50% and confirm the mycorrhizal status of the Bambara groundnut. The intensities of mycorrhization were low (less than 30%). This result could reflect the weak capacity of infection of the indigenous AMF of Cameroon. The landraces of Bambara groundnut used in this study seem to have root system with little ramification. However, the finer roots are the most likely to be infected. Furthermore, Duponnois et al. (2001) pointed out that mycorrhizal

infection of plants varies greatly from one plant to another but also within the same species. Nevertheless, Ngonkeu et al. (2003) noted that low root colonization does not imply a low symbiotic efficiency.

Trapping was necessary to observe the diversity of spores under Bambara groundnut culture. Indeed, several authors observed no spore after direct extraction from the native soil. However, after trapping, more than a dozen different spores of AMF were isolated in the same soil (Nwaga et al., 2003). The spore density was higher at Soa than at Mendong. Spore density varies greatly from one soil to another and the natural spatial distribution of AMF species is more closely related to environmental conditions, the complex structure of the soil rhizosphere constituents and/or the floristic composition (Begoude et al., 2016; Castillo et al., 2016).

Sixteen species in nine genera were identified. This specific richness is similar to that obtained in Cameroon and Benin by Mbogne et al. (2015) with pumpkins; as well as in Benin by Johnson et al. (2013) with cowpea. Indeed, the AMF communities' composition may vary from one region to another and from one type of habitat to another. In addition, there are obvious differences between ecosystems under different disturbance regimes (Öpik et al., 2006). For example, low AMF specific richness (only 5) was found in rhizospheric soils associated with pioneer plant species that grow at the mouth of Budi Lake in Chile (Medina et al., 2015). On the other hand, an enormous specific richness was found in biological tomato in a Mediterranean site with 58 species of AMF belonging to 14 genera (Njeru et al., 2015).

The number of genera obtained (nine) is contrary to those obtained by Mbogne et al. (2015) who noted four genera in the pumpkins rhizosphere by the classification of Schüßler et al. (2001), but similar to those of Johnson et al. (2013) who found eight genera under cowpea cultivation with the new classification of Schüßler and Walker (2010) and Redecker et al. (2013). The high number of genera is explained by the great progress made in the AMF identification by molecular tools. This work has led, in particular, to the division of *Glomus* genus in several genera, such as *Glomus*, *Funneliformis*, *Rhizophagus*, *Septoglomus* (Schüßler and Walker, 2010, Oehl et al., 2011, Redecker et al., 2013). The genera *Cetraspora*, *Dentiscutata*, *Racocetra* were also extracted from the genus *Scutellospora* (Morton and Msiska, 2010; Redecker et al., 2013).

At both sites, the average values of the Shannon-Wiener index ranged from 1.82 to 1.94. Effectively, this index can range from 0.45 for the AMF community present in an arable field (Daniell et al., 2001) to 3.0 in a forest (Becerra et al., 2011). According to Brower and Zar (1984), a high diversity community is characterized by low dominance (few species dominate AMF community). The morphotype V2 has a higher Shannon-Wiener index (1.90) than those of V1 (1.87) and V3 (1.86). The AMF diversity under Bambara groundnutt culture could be influenced by the genotype of the plant. Glomeraceae, Gigasporaceae and Acaulosporaceae are seemingly well-suited for the production of inocula in the humid agroecological zone.

The mean values of the Pielou's evenness index ranged from 0.69 to 0.74 in both sites, which means that each species is represented by almost the same number of individuals. The values of the Shannon-Wiener, Simpson and Pielou's evenness index are lower in plots with phosphate fertilizer. Indeed, the work of Mbogne et al. (2015) showed that chemical fertilizer (NPK) reduces diversity index values under pumpkins crop.

The total number of spores was significantly higher in Soa than in Mendong. Before the experiment, the Soa site (pH: 5.09) was an old shrub fallow, and that of Mendong (pH: 6.5) a young fallow. Some studies (Isobe et al., 2007; Mbogne et al., 2015) found a relationship between the AMF spores density and certain chemical properties of the soil like pH, available phosphorus and organic matter. Johnson (1991) and Mohammad et al. (2003) found that sporulation increases with soil pH and organic carbon.

The soils at both sites were acidic. Nevertheless, the optimum pH for AMF spore germination varies depending on the fungal species. In addition, Johnson et al. (2013) believe that the relationship between the density of AMF spores and the chemical properties of soils might not be stable, but could vary depending on the composition of the *Glomeromycota* community. Borriello et al. (2012) also pointed out that intensive plowing in conventional cropping systems negatively affects the AMF community and decreases the number of species thus reduces the sustainability of the system.

In this study, AMF diversity indices are negatively correlated with assimilable P. Johnson et al. (2013) also found that the species richness and AMF diversity associated with cowpea were negatively correlated with the P available in soils. Some species could be more sensitive to the available P and might become less abundant in soils with a high P level. The specific richness is also a reflection of the floristic richness of the previous cultivation of the various sites

Conclusion

The results obtained in this study show that AMF were present in the Bambara groundnut rhizosphere grown in Cameroon. All the landraces used formed the arbuscular mycorrhizae with characteristic structures such as arbuscules, hyphae, vesicles, auxiliary cells and spores. The spores were morphologically different in size, color, shape, presence or absence of the hypha and its mode of attachment to the spore. From these morphological criteria, 16 AMF species belonging to nine genera (*Rhizophagus*, *Septoglomus*, *Racocetra*, *Gigaspora*, *Acaulospora*, *Claroideoglomus*, *Diversispora*, *Funneliformis*

and *Glomus*) were identified. The species *Rhizophagus intraradices* was preponderant. The specific richness was identical in the two sites (Mendong and Soa), and between landraces. High level of chemical phosphate fertilizer (200 $kg.ha^{-1}$ P_2O_5) significantly reduced the intensity of mycorrhization. P fertilization reduced diversity index of AMF species in the Bambara groundnut rhizosphere. But the different P levels did not influence the frequency of mycorrhization and the specific richness. Thus, the choice of landraces, sites and especially P fertilizer level to be applied according to the initial richness of the soil is the factors to take into account to favor the establishment of the mycorrhizal symbiosis for a profitable culture of Bambara groundnut.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Distribution of *Aspergillus* and *Fusarium* ear rot causative fungi in soils under push-pull and maize monocropping system in Western Kenya

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It is imperative to establish the distribution and density of soil fungal communities as a requisite for formulating strategies for management of ear rot infections and mycotoxin contamination. In a two seasons study, short (SR) and long rainy (LR) seasons, we investigated the distribution of *Aspergillus* and *Fusarium* fungi causing ear rots and producing mycotoxins from 120 soil samples collected from maize fields under push-pull (PP) and maize monocrop (MM) systems in Western Kenya. Cultural methods were used for identification of *Aspergillus* and *Fusarium* species, while molecular techniques were used for confirmation of *Fusarium* section *Liseola*. Detection of total aflatoxins in cultures of section *Flavi* isolates was carried out by enzyme-linked immunosorbent assay (ELISA). A total of 338 fungi were isolated; 80% were identified as *Aspergillus* and 4.4% *Fusarium*. The distribution of fungi was significant with season but not cropping systems. The frequency of occurrence was higher during the LR (68.4%) than the SR (31.6%). In cropping systems, the frequency of occurrence of *Aspergillus flavus* was higher in MM (60.2%) than PP (39.8%). However, *Aspergillus parasiticus* was more frequent in PP (71.4%) than MM (28.6%); and during the SR (78.6%) than the LR (21.4%). Majority (81.3%) of *A. flavus* and *A. parasiticus* were toxigenic. There was low recovery of *Fusarium* species in soil samples. These findings show that soils from both cropping systems are potential for *Aspergillus* infection and aflatoxins contamination; however, low *Fusarium* distribution in soil suggest external inoculum source for *Fusarium* ear rot infections common in most maize fields in Western Kenya.

Key words: *Aspergillus*, *Fusarium* section *Liseola*, push-pull, soil.

INTRODUCTION

Fungi are part of diverse living components of soil, with several of them living as saprophytes and symbionts contributing to various soil services including structure formation, organic decomposition, recycling of major elements (for example carbon, nitrogen and phosphorus) and toxic removal (Aislabie and Deslippe, 2013). Pathogenic fungi also exist as major causal agents of soil borne diseases affecting roots, stalks, leaves and ears of

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various crops including maize (Shurtleff, 1980). Nevertheless, the presence of certain non-pathogenic (mainly saprophytes) or pathogenic fungi on grains, soils and other reservoirs are potential for ear rot infection and mycotoxin production, especially species in the *Aspergillus* and *Fusarium* genera (Horn et al., 1995; Pereira et al., 2011).

The *Aspergillus* genus is divided into sections (or subgenus groups) of which *Flavi* is most important in agriculture as cause of ear rot diseases and producer of aflatoxins (Gnonlonfin et al., 2011). Several species are classified under *Flavi*, but *Aspergillus flavus* Link*, Aspergillus parasiticus* Speare and *Aspergillus nomius* Kurtzman (Rodrigues et al., 2007) are prominent isolates in maize and soil samples. Amongst these species, *A. flavus* and *A. parasiticus* are prolific producers of aflatoxins with the former being the most abundant in both air and soil (Hedayati et al., 2007), hence affecting more of aerial crops like maize. On the other hand, *A. parasiticus* is mostly reserved in soils with high isolation frequency of peanuts fields (Garber and Cotty, 2014).

The filamentous fungus with equal importance in maize production is *Fusarium*. Most of its members are producers of three important agricultural mycotoxins which include: fumonisins, deoxynivalenol and zearalenone (D'Mello et al., 1999). They are also causative agents of root and ear rots in maize resulting in yield losses (Sutton, 1982). Three *Fusarium* species with high frequency of isolation in maize include: *Fusarium graminearum* Schwabe, *Fusarium verticillioides* (Sacc.) Nirenberg*, Fusarium proliferatum* (T. Matsushima) Nirenberg and *Fusarium subglutinans* (Wollenweb and Reinkings) P. E Nelson, T.A. Toussoun and Marasas (Leslie and Summerrel, 2006).

Soil is the primary habitat for *Fusarium* and *Aspergillus* species. The population of *Aspergillus* and *Fusarium* propagules in the soil (field) increases the risk for maize infections and mycotoxin contamination (Horn, 2003). In order to safeguard against losses, fungal distribution in food and soil ecology is imperative for effective formulation of prevention and control measures. In soil fungal ecology, cultural practices greatly encourage or discourage fungal distribution. For instance, rotation of susceptible crops like wheat with maize together (Schaafsma et al., 2005) increase fumonisin incidence. Addition of organic matter either by cultural practice through minimum tillage, or application of organic amendments increases *Aspergilli* propagules (Zablotowicz et al., 2007) while decreasing those of *Fusarium* in soil (Alakonya et al., 2008).

Among several strategies used for soil management in western Kenya, push-pull technology (PPT), a companion cropping system where maize or sorghum is intercropped with moth repellent (push) forage legumes in the genus *Desmodium,* edged with moth attractive (pull) grasses such as Napier grass (*Pennisetum purpureum* Schumach) or *Brachiaria*, is mostly adopted by smallholder farmers (Khan et al., 2011). Although, PPT is known for insect pest management, it contributes to soil health improvement which is potentially impactful on soil fungal community. The technology improves organic matter content of the soil, nitrogen fixation, overall improvement in soil macro- and micro arthropods and conservation of soil moisture (Khan et al., 2011). However, distribution of *Aspergillus* and *Fusarium* ear rot fungi in soil under push-pull remains unknown. In this context, the aim of this study was to investigate the level of soil-borne *Aspergillus* and *Fusarium* species in push-pull and maize monocrop plots in western Kenya.

MATERIALS AND METHODS

Field survey

The study sites included Kisumu, Siaya and Vihiga sub-counties (districts) of Western Kenya (Figure 1). Soils in these sites are generally vertisols, ferralsol and nitosols showing a natural decline in soil fertility predominantly manifested by occurrence of purple witch weed, *Striga hermonthica* (Del.) Benth. (Orobanchaceae), soil erosion and deficient nitrogen and phosphorus. However, heterogeneity in soil fertility exists amongst smallholder farms in the region where there is less investment in external inputs to restore soil fertility. Push-pull technology has been disseminated for pest control and soil fertility improvement for over 10 years in the region (Khan et al., 2011).

Sample collection

The sampling method of Horn and Dorner (1998) was adopted for soil sampling with slight modifications. Briefly, transect which runs 5 km from one push-pull cluster to the next was made. A total of 60 fields were sampled at maize silking period during the short and long rainy seasons of 2014 and 2015, respectively. In a cluster, four push-pull and maize monocrop fields were sampled by removal of 4 subsamples of soil with a sterile trowel from the top 4 to 6 cm of soil at intervals of 2 to 4 m. The soil subsamples collected from each field were mixed and placed in a paper bag and air dried at 25°C for 1 week. The soil was then carefully mixed and sieved through a no.10 USA standard sieve (2.00 mm opening) (Dual Manufacturing Company, Franklin Park, IL 60131, USA) and stored at 4°C.

Isolation of fungi

The dilution plate technique by Cotty (1994) and Leslie and Summerell (2006) were used for *Aspergillus* and *Fusarium* recovery, respectively. One gram of thoroughly mixed soil samples was suspended in 9 ml of distilled water. These resultant solutions were serially diluted to 10^{-3}. One milliliter of 10^{-2} and 10^{-3} were plated in quadruplicate in Petri dish (90 x 15 mm) containing a quarter strength potato dextrose agar (PDA) (HiMedia Laboratories Pvt. Ltd) amended with 30 mg chloramphenicol. The plates were then incubated at 31°C for 6 days in the dark for *Aspergillus* recovery, and at 25°C for 14 days for *Fusarium*. Colonies of *Aspergillus* and *Fusarium* that grew on each plate were counted and their population determined as colony forming unit (CFU) per gram and calculated as follows:

Total fungal colonies = Number of colonies x dilution factor/weight of soil (1 g)

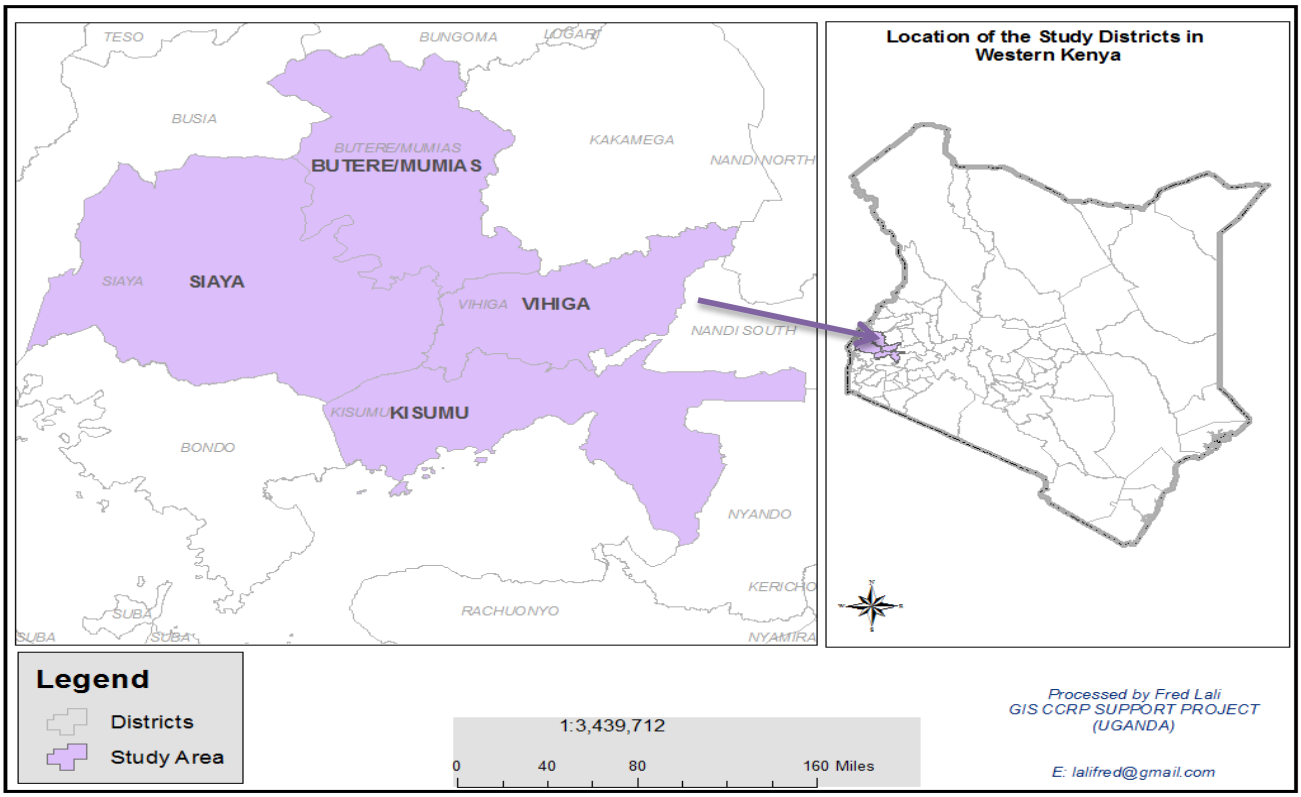


Figure 1. Map showing study districts in Western Kenya.

Colonies of *Aspergillus* and *Fusarium* were then sub-cultured on full strength PDA amended with 30 mg chloramphenicol.

Morphological identification of *Aspergillus* and *Fusarium*

The colonies on PDA identified as *Aspergillus* were transferred aseptically onto Czapek Dox Agar (CZ) (Oxoid Ltd, Basingstoke, Hampshire, England) plates and incubated at 31°C for five days. Their colony characteristics (colour and reverse) were observed. Those characterized to belong to *Aspergillus* section *Flavi* were confirmed on Aspergillus Flavus Parasiticus Agar (AFPA) base (HiMedia Laboratories Pvt. Ltd) plates incubated at 25°C for five days for positive orange reverse. Microscopic features such as: head serration, vesicle and conidia were observed in a compound light microscope (Carl Zeiss MicroImaging GmbH 37081, Gottingen, Germany) using keys by Klich (2002).

Fusarium colonies recovered were grown on PDA plates and observed for pigmentation on both top and reverse, and on Spezieller Nahrstoffarmer Agar (SNA) for macroconidial features. Further identification using species-specific primers was used for identification of *F. verticillioides, F. proliferatum* and *F. subglutinans*.

Molecular identification of *Fusarium* section *Liseola*

DNA extraction

Fusarium isolates, 13 in total, culturally identified to belong to *Fusarium* section *Liseola* were grown as monosporing cultures on PDA plates for seven days at room temperature. For each isolate, mycelium was harvested for total DNA extraction according to Gherbawy et al. (2001). One gram of freshly harvested mycelium was ground in liquid nitrogen with a mortar and pestle into a very fine powder. Fifty milligrams of the ground mycelium was transferred into 1.5 ml Eppendorf tube and mixed with 700 µL 2 x CTAB buffer. The contents of Eppendorf tube was incubated at 65°C for 30 min before addition of 700 µL of chloroform : isoamyl alcohol (24:1 v/v), and a brief mixing. The mixtures were then centrifuged at 10,000 *g* for 30 min and supernatant was transferred into another tube. Isopropanol, 700 µL in volume was added and mixed with the supernatant and left to chill overnight at -20°C. This content was centrifuged again at 10,000 *g* for 5 min, after which the supernatant was discarded and pellets washed twice in 1 mL of 70% ethanol and left to dry under a vacuum. The pellets were afterwards resuspended in 700 µL distilled water. The quality of DNA was evaluated in 1% agrose gel electrophoresis.

Detection of Fusarium DNA using species-specific primers

The following primer pairs, VER 1/2, PRO 1/2 and SUB 1/2 were used for identification of *F. verticillioides*, *F. proliferatum* and *F. subglutinans*, respectively in PCR assay according to Rahjoo et al. (2008): *F. verticillioides*, VER 1/2 (F: 5′-CTT CCT GCG ATC TTT CTC C-3′, R: 5′-AAT TGG CCA TTG GTA TTA TAT ATC TA-3′); *F. proliferatum*, PRO 1/2 (F: 5′-CTT TCC GCC AAG TTT CTT C-3′, R: 5′-TGT CAG TAA CTC GAC GTG TTG-3′); *F. subglutinans,* SUB ½ (F: 5′-CTG TCG CTA ACC TCT TTA TCC A-3′, R: 5′-CAG TAT GGA CGT TGG TAT TAT TAT ATC TAA-3′).

The PCR assay was done in a total volume of 25 µL of master mix comprising 5X buffer, 25 mM of each dNTP, 25 mM $MgCl_2$, 0.2 µL of Ampli Taq polymerase (Applied Biosystems, USA), 2.0 µl of

Table 1. Incidence of ear rot fungi recovered from maize fields in western Kenya.

Variable	Fungal genera				
	Aspergillus	***Fusarium***	***Trichoderma***	***Penicillium***	**Total fungi**
	n (%)				
District					
Kisumu (N=40)	106 (82.8)	2 (1.6)	4 (3.1)	16 (12.5)	128 (37.9)
Siaya (N=40)	88 (77.2)	7 (6.1)	13 (11.4)	6 (5.3)	114 (33.7)
Vihiga (N=40)	80 (83.3)	6 (6.3)	2 (2.1)	8 (8.3)	96 (28.4)
Season					
SR (N=60)	85 (84.2)	2 (2.0)	6 (5.9)	8 (7.9)	101 (29.9)
LR (N=60)	189 (79.8)	13 (5.5)	13 (5.5)	22 (9.3)	237 (70.1)
Cropping system					
PP (N=60)	130 (78.3)	8 (4.8)	7 (4.2)	21 (12.7)	166 (49.1)
MM(N=60)	144 (83.7)	7 (4.1)	12 (7.0)	9 (5.2)	172 (50.9)
Total (N=120**)**	274 (81.1)	15(4.4)	19 (5.6)	30 (8.9)	

n, Number of isolates; N, number of samples; SR, short rain; LR, long rain; PP, push-pull; MM, maize monocropping. The percentage (%) incidence was calculated based on the total counts across the table while incidence of fungi between variables was compared within a column in the table. The incidence of total fungi was significant with season (t (118) =5.513, p=0.001) and *A. flavus* (t (118) =2.1683, p<0.001).

each primer and 5 µL of fungal template DNA. Reactions were performed in Proflex PCR system thermocycler (Applied Biosystems, USA) under the following conditions: denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 50 s, annealing at 56°C for 50 s, extension at 72°C for 1 min, final extension at 72°C for 7 min with cooling at 4°C for final recovery of the samples. The amplified products were then visualized in 1.2% agarose gels stained with ethidium bromide.

Test for aflatoxigenicity

Twenty seven species identified belonging to *Aspergillus* section *Flavi* were grown on PDA at 31°C for a period of 7 days and total aflatoxin were extracted from their cultures according to method described by Rao et al. (1997). A whole sample comprising agar, mycelia and spores was ground in a blender for 5 min. Two grams of the blended mixture was used to extract total aflatoxins in 10 mL of 60% methanol. The mixture was then filtered (Whatman #1) and the filtrate analysed by using ELISA Kits for total aflatoxin (Helica Biosystem Inc.).

Data analysis

All analyses were done using R version 3.3.1. The incidences of fungi were presented in counts and percentages as score of total counts. The data for total fungi, *Aspergillus* and *A. flavus* were normalized by log *x*+1 transformation before mean comparison. However, means of *A. parasiticus, A. fumigatus, Aspergillus niger, Aspergillus terreus, Aspergillus tamarii* and *F. verticillioides* were compared with Mann-Whitney U test or Kruskal-Wallis test.

RESULTS

Distribution of fungi in soil

A total of 338 fungi in four genera were isolated from soil samples (Table 1). From these isolates, 80% were *Aspergillus*, 8.9% *Penicillium*, 5.6% *Trichoderma* and 4.4% *Fusarium*. The incidence of fungi with district was not statistically significant. However, the incidence of *Fusarium* was low in Kisumu (1.6%) than Siaya (6.1%) and Vihiga (6.3%). This was converse to *Penicillium* which had high (12.5%) incidence in Kisumu than Siaya (5.3%) and Vihiga (8.3).

There was significant difference (t (118) = 4.6018, p<0.001) in distribution of total fungi in short and long rainy season. SR had lower (29.9%) incidence than LR (70.1%). Similarly, the incidence of *Aspergillus* was significant (t (118) = 2.1683, p<0.001) in SR and LR season. Incidence of total fungi and in both PP and MM was not significant. However, *Penicillium* had high incidence in PP (12.7%) than MM (5.2%).

Identification of *Aspergillus* and *Fusarium* species

Three species belonging to *Aspergillus* section *Flavi* were identified by colony reverse on AFPA agar (Plate 1). The three species further identified on CZ based on their conidial colour and head serration were *A. Flavus;* yellow green surface and numerously biseriated [Plate 2 a(i) and a(ii)]; *A. parasiticus,* conifer green surface and mainly uniseriated [Plate 2 b(i) and b(ii)]; and *A. tamarii,* dark green surface and abundantly uniserate [Plate 2 c(i) and c(ii)]. Other *Aspergillus* species equally identified on PDA by other features were *A. terrei;* sand brown surface with columnar conidial ornamentation [Plate 2 d(i) and d(ii)]; *A. fumigatus,* blue grey surface and subglobose vesicle [Plate 2 e(i) and e(ii)]*;* and *A. nigri,* black surface and brownish, relatively long and smooth conidiophore [Plate 2 f(i) and f(ii)].

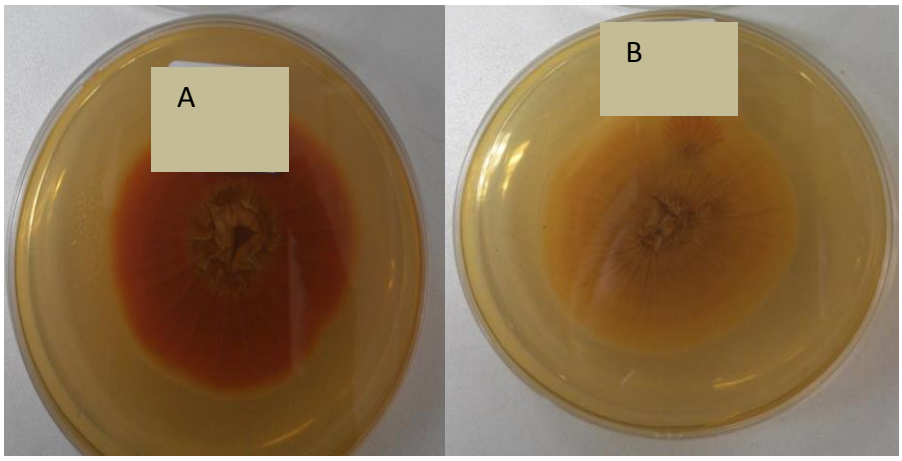


Plate 1. Colony reverses of two isolates in *Aspergillus* section *Flavi* showing bright orange (A) and yellow (B) colour after incubation on AFPA for 5 days at 25°C. Orange colour is positive for *Flavi* section.

There was low recovery of *Fusarium* species causing ear rots; however, 13 isolates recovered morphologically belong to *F. verticillioides* [Plate 2 g(i) and g(ii)]. Out of these isolates (13), 9 were positive (Figure 2) for *F. verticillioides* after molecular characterization with TEF-gene.

Incidence of *Aspergillus* and *Fusarium* species

The respective distribution of six *Aspergillus* and *Fusarium* species in push-pull and monocrop systems were as follows: *A. flavus,* 39.8 and 60.2%; *A. fumigatus,* 55.2 and 44.8%; *A. niger,* 35.6 and 64.4%; *A. parasiticus,* 71.4 and 28.6%; *A. terreus,* 53.3 and 46.7%; *A. tamari,* 20 and 80%; *F. verticillioides,* 50 and 50%; and *F. graminearum,* 66.7 and 33.3%, respectively (Table 2). A high incidence of total and individual fungi was observed during long than short rainy seasons respectively, except *A. parasiticus* which was abundant in short (78.6%) than long rainy season (21.4%). Majority of aflatoxigenic fungi were positive for aflatoxins (81.5%) with only 8.3 and 23.7% of *A. parasiticus* and *A. flavus,* respectively being atoxigenic (Table 3).

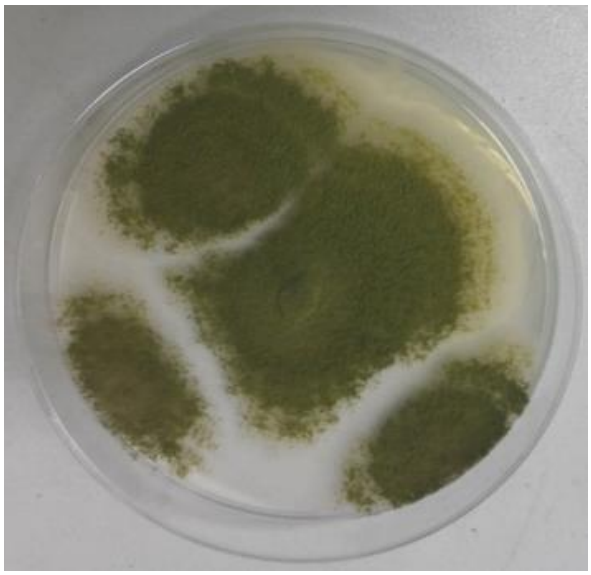
The population of *Aspergillus* and *Fusarium* species in soil

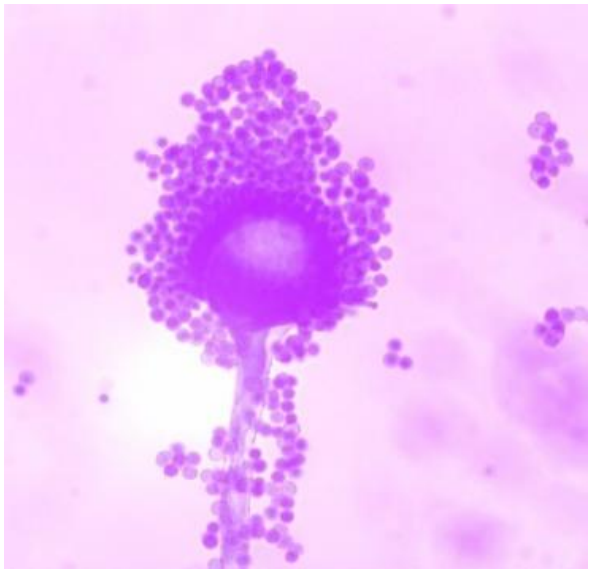
In general, there was no significant difference in population of total and individual fungal species between the two cropping systems (Table 4). However, low population of fungi was observed in push-pull (2,266.1 $CFUg^{-1}$) than in monocrop plots (2,499.9 $CFUg^{-1}$). *A. parasiticus* was the only species which had high population in push-pull (333.3 $CFUg^{-1}$) than in the monocrop system (133.3 $CFUg^{-1}$), with relatively small insignificant difference ($p<0.067$). During long rainy season, a significantly high population of *A. flavus*, *A. fumigatus*, *A. terreus* ($p<0.001$) and *A. parasiticus* ($p<0.05$) were also observed.

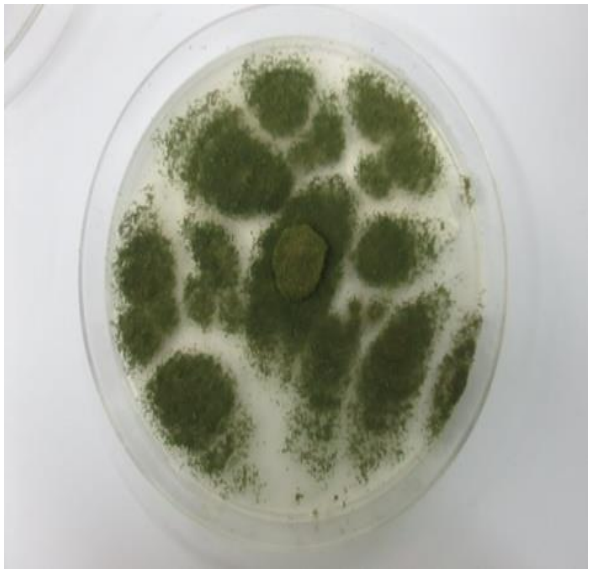
DISCUSSION

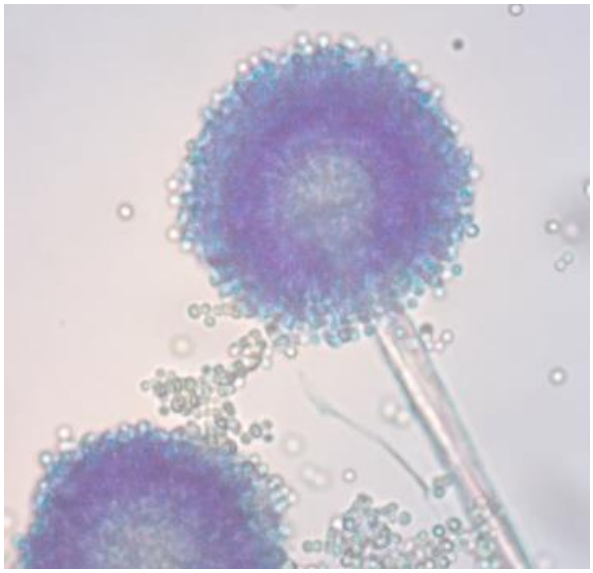
There was higher (averagely 80%) incidence of *Aspergillus* than other fungi in all the districts, cropping systems and seasons observed in this study. This corroborates findings of other studies in different agro-ecological areas in Kenya that reported relatively higher incidence of *Aspergillus* relative to other fungi (Okoth et al., 2012; Karanja, 2013). However, insignificant difference in incidence of *Aspergillus* between push-pull and maize monocrop systems contradicted the finding which showed significant increases in *Aspergillus* population with minimum tillage and organic matter amendments (Zablotowicz et al., 2007). Thus, more *Aspergillus* expected on a conserved system like push-push which improves organic matter content in the soil and reduces the amount of tillage was not observed. This observation could be explained on the basis that historically, and depending on the cropping season and amounts of rainfall, most farms in western Kenya more often have maize intercropped with food legumes such as common bean (*Phaseolus vulgaris* L.) and peanuts (*Arachis hypogaea* L.) (Mudavadi et al., 2001). Such edible legumes provide beneficial ecological services of soil improvement through addition of organic matter and nitrogen fixation that could increase *Aspergillus* incidence in the soil (Mudavadi et al., 2001).

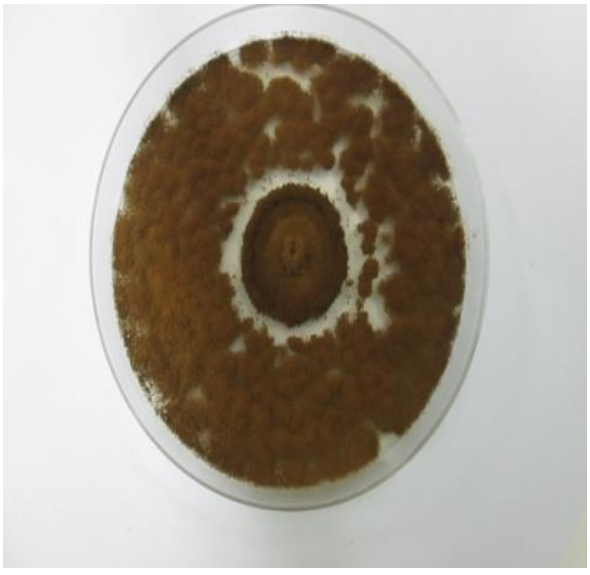
Soil as the main reservoir for both *A. flavus* and *A. parasiticus* has relatively higher frequency of the former

a(i) 

a(ii) 

b(i) 

b(ii) 

c(i) 

c(ii) 

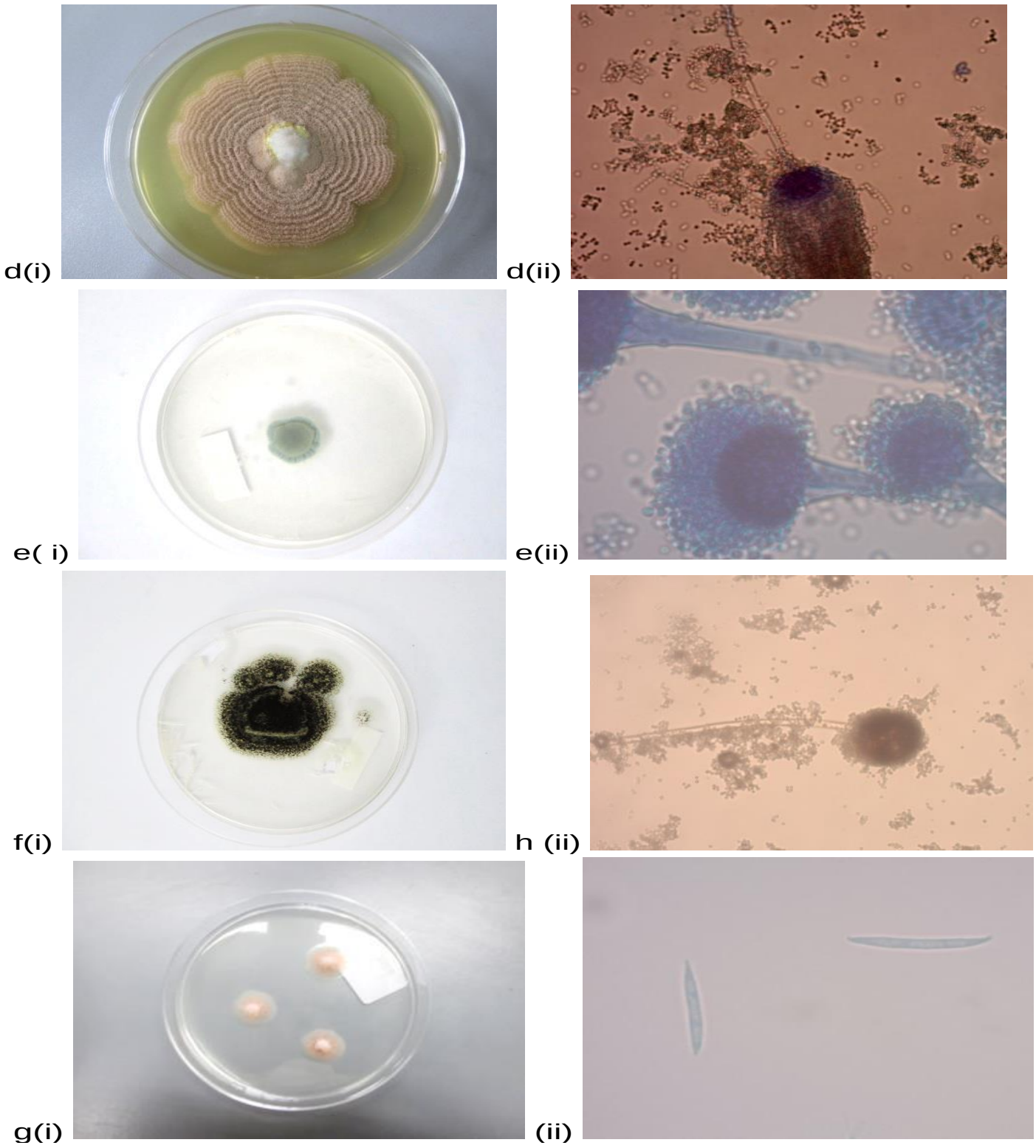


Plate 2. The cultural and morphological traits of the 6 *Aspergillus* species *and Fusarium verticillioides* growing in PDA and CZ after 7 days of incubation. a(i) *A. flavus* greenish yellow surface on CZ; a(ii) a biseriate conidial head with a globose vesicle of *A. flavus* (Mg=1000×); b(i) *A. parasiticus* ivy green surface on CZ, b(ii) *A. parasiticus* with uniserate, globose and conidia in chains (Mg=1000x); c(i) *A. tamarii* dark brown surface on PDA, c(ii) globose vesicle, as observed under the microscope (Mg=1000×); d(i) *A. terreus* sand brown surface on PDA; d(ii) columnar conidial ornamentation in *A. terreus* (Mg=500×); e(i) *A. fumigatus* blue grey surface on CZ; e(ii) *A. fumigatus* subglobose vesicle (1000x); f(i) *A. niger* black surface; f(ii) brownish, relatively long and smooth conidiophore of *A. niger* (Mg=400×); g(i) *Fusarium verticillioides* surface on PDA; g(ii) *Fusarium verticillioides* macroconidia (Mg=1000x).

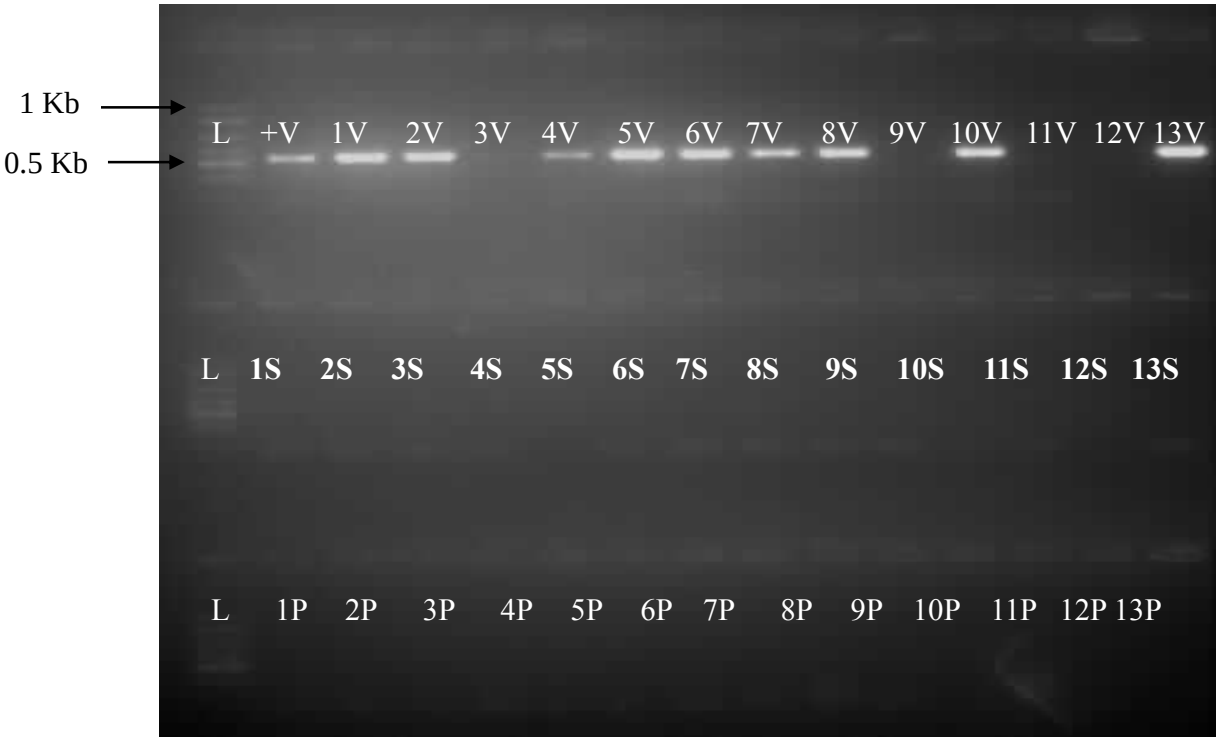


Figure 2. Gel electrophoresis of PCR amplified translation elongation factor-1 alpha gene (611 bp) on 13 isolates of *Fusarium* section *Liseola*. Isolates denoted as V, *F. verticillioides*; S, *F. subglutinans*; and P, *F. proliferatum*. Lane L, 1 kb base pair ladder; +, positive control for *F. verticillioides*. Electrophoresis was performed on 1.2% agarose gel.

Table 2. Population of *Aspergillus* and *Fusarium* spp. in different cropping systems and seasons.

Fungi species	Cropping system		Season	
	Push-pull maize monocrop		Short rain	Long rain
A. flavus	39 (39.8)	59 (60.2)	31 (31.6)	67 (68.4)
A. fumigatus	37 (55.2)	30 (44.8)	12 (17.9)	55 (82.1)
A. niger	16 (35.6)	29 (64.4)	9 (24.3)	28 (75.7)
A. parasiticus	20 (71.4)	8 (28.6)	22 (78.6)	6 (21.4)
A. terreus	16 (53.3)	14 (46.7)	2 (6.7)	28 (93.3)
A. tamarii	1 (20.0)	4 (80.0)	1 (20.0)	4 (80.0)
F. verticillioides	9 (50.0)	9 (50.0)	2 (11.1)	16 (88.9)

n, number of isolates (%); raw percentages calculated based on counts within district, cropping system and season.

Table 3. Percentage of selected section *Flavi* isolates tested for aflatoxigenicity.

Species	Number of isolate	Toxigenic (%)	Atoxigenic (%)
A. flavus	15	73.3	23.7
A. parasiticus	12	91.7	8.3
Total	27	81.5	18.5

Aspergillus species than the latter (Klich, 2007). However, the frequency of *A. parasiticus* is comparatively higher and more endemic in soils where peanut or sugarcane is grown relative to that under maize (Garber

Table 4. Population (CFU g^{-1}) of *Aspergillus* and *Fusarium* species in different cropping systems and seasons.

Fungi	Cropping system			Season		
	PP (mean $CFUg^{-1}$)	MM (mean $CFUg^{-1}$)	P-value	LR (mean $CFUg^{-1}$)	SR (mean $CFUg^{-1}$)	P-value
A. flavus	650.0	983.3	0.405	1,116.6	516.7	0.0012
A. fumigatus	616.7	500.0	0.330	916.7	200.0	0.001
A .niger	266.7	483.3	0.090	433.3	316.7	0.550
A. parasiticus	333.3	133.3	0.067	100.0	366.7	0.054
A. terreus	266.7	233.3	0.464	466.7	33.3	0.0001
A. tamarii	16.7	66.7	0.311	66.7	16.7	0.311
F. verticillioides	116.0	100.0	0.761	200.0	16.7	0.0045
Total	2,266.1	2,499.9	0.856	3300.0	1466.9	0.001

PP, Push-pull; MM, maize monocrop; $CFUg^{-1}$, colony forming unit per gram of soil; LR, long rainy season; SR, short rainy season; significance level (p=0.05).

and Cotty, 2014). Although, not measured in the current study, soil temperature has been reported to influence incidence of these fungi, with lower temperatures favoring *A. parasiticus* relative to *A. flavus* (Horn, 2005). Optimally, *A. parasiticus* grow at temperature of 22°C, while *A. flavus*, at 30 to 37°C (Horn, 2005). This cool soil temperature is encouraged by cultural practices such as cover cropping, reduced tillage (Sławiński et al., 2012), and wet season (Horn et al., 1995). In the push-pull system, *Desmodium* provides soil cover for a longer period due to its perennial nature as compared to annual edible intercrop legumes common (bean and peanuts) in western Kenya. The push-pull system also manifest limited tillage practices during land preparation and weeding for conservation, and from cover cropping of *Desmodium,* respectively. This explain probable low soil temperature in PP thus higher population of 71.4% was observed on *A. parasiticus* in soil samples from push-pull as compared to 28.6% in maize monocrop systems. The long dry spells which increases soil temperature in long rainy season than the short rainy season (Mugalavai et al., 2008) also account for low (21.4%) population of *A. parasiticus* during the long rainy season relative to 78.6% during the long rainy season in this study.

Several studies on aflatoxin production have reported fewer incidences of non-aflatoxin (atoxigenic) producers amongst *A. parasiticus* isolates (Tran-Dinh et al., 2009; Barros et al., 2006), except in few cases (Okoth et al., 2012; Salano et al., 2016). The current study supports these findings as 8.3% of *A. parasiticus* isolates as compared to 23.7% of *A. flavus* were positive for aflatoxin production. With more aflatoxigenic fungi, the merit of conserved systems in increasing soil agricultural sustainability might also expose crops to aflatoxin contamination by increasing their *A. flavus* propagules in soils (Zablotowicz et al., 2007). However, contamination of maize is not entirely dependent on the population of *A. flavus* in the soil since maize intercropping which encourages more *A. flavus* has shown low aflatoxin contamination as compared to sole cropping system (Mutiga et al., 2015). Therefore, as revealed in these studies, intercrops are able to reduce *Aspergillus* infections and contamination through other factors such as increased soil nitrogen and limiting insect damage (Brun, 2003).

The frequency of *A. parasiticus* or ratios of *A. flavus/A. parasiticus* (4:1) in this study suggest the potential levels of contamination in maize. Studies show that *A. parasiticus* is comparatively a poor colonizer of aerial plants like maize (Horn, 2003) and have low spore density in air (Horn et al., 1995) than *A. flavus* (Hedayati et al., 2007). Indeed, study by Angle et al. (1982) observed almost complete infection of maize ears with *A. flavus* despite high incidence of both *A. parasiticus* and *A. flavus* in soil. Therefore, increased frequency of occurrence of *A. parasiticus* in push-pull relative to maize monocrop warrants further investigation.

The observations of this study presented *A. terreus*, *A. niger* and *A. fumigatus* as equally abundant in soil, with respective 30, 45 and 69 isolate counts as compared to 98 of *A. flavus*. This observation corroborates reports of most studies on distribution of microflora in the soil (Horn et al., 1995; Horn, 2005). However, they contradicted study by Salano et al. (2016) which reported higher (55) count of *A. niger* than *A. flavus* (26) in eastern province of Kenya. The high presence of these species portends less impact on grain quality as they are not chief producers of agriculturally important mycotoxins (D'Mello et al., 1999; Gnonlonfin et al., 2011). However, recent studies have reported production of fumonisin and ochratoxins A by *A. niger* (Mogensen et al., 2010; Palencia et al., 2010) and territrem by *A. terreus* (El-Sayed Abdalla et al., 1998), while *A. fumigatus* is known causal agent of invasive aspergilosis (Hedayati et al., 2007)

The observations in this study illustrated low (18) isolate count of *Fusarium* section *Liseola* and no *F. graminearum* isolates in soils. This was similar to the study by Okoth and Siameto (2010) on soils in maize fields. The most plausible explanation for this occurrence could be their inherent scarcity (Okoth and Siameto,

2010) or effects of organic matter in the soil (Alakonya et al., 2008) from intercropping systems common in western Kenya. But importantly, low soil *Fusarium* incidence indicated more *Fusarium* infection from aerial spores and external sources.

The cultural identification in *Fusarium* section *Liseola* is demanding and limiting (Summerell et al., 2003), thus molecular methods are used for confirmation. In molecular identification of *F. verticillioides* using translation elongation factor 1-alpha (TEF) gene, 140 isolates culturally identified as *F. verticillioides*, 133 and 4 isolates were confirmed as *F. verticillioides* and *F. proliferatum,* respectively (Rahjoo et al., 2008). Therefore, further identification of species in *Fusarium* section *Liseola* using TEF genes is more accurate and reliable. Evidentially, in this study, 13 isolates were initially identified by cultural characteristics as *F. verticillioide;* 9 isolates were positive for *F. verticillioides* using TEF gene. However, *F. proliferatum* and *F. subgluinans* were not present amongst the isolates.

In conclusion, seasons had significant influence on distribution of *Aspergillus* and *Fusarium* fungi in soil, while cropping system did not. The high *Aspergillus* fungi in the soil in this study show that soil fungal community within the field is a potential risk for aspergillus ear rot infection and aflatoxin contamination, while the low frequency of *F. verticillioides* and *F. graminearum* in the soil samples suggest external inoculum as important for both gibberella and fusarium ear rot infection in the field.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Morphophysiological and molecular characterization of wild yeast isolates from industrial ethanol process

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Wild yeasts are commonly found during the fermentation process, displaying different survival and competition strategies that commonly enable their successful spread in the medium. Identifying these yeasts by biological and molecular monitoring, and knowing their traits is vital for the fermentation yield, and this can be done by simple methods such as differential mediaplating, growth rate evaluation and DNA sequencing. The aim of this work was to perform morphophysiological and molecular characterization of 14 yeast isolates from a bioethanol plant in the State of Sao Paulo, Brazil. This was done by employing different culture media to assess the growth and the morphophysiological characteristics of the isolates. The molecular characterization was also done in order to identify the samples in intra-specific levels, compared to the reference strains. The *Saccharomyces cerevisiae* CAT-1 and PE-2, Brazil's two main commercial strains, were used as reference. The results suggest that a single ethanol-producing unit may display a highly diversified microbiome, with the occurrence of distinctive wild yeast strains disclosing diverse morphophysiological traits, as observed in the differential media plating and growth rate assay results. The molecular characterization shows that these yeast isolates differ from the reference strains, as observed in interdelta-based PCR fingerprint banding patterns. These findings are a statement of the yeast diversity found in the fermentation process, and are of interest for the ethanol industry, being that many of the commercial strains were firstly isolated from the local biome.

Key words: Bioethanol, differential media, growth rate, molecular characterization, morphology, *Non-Saccharomyces*, *Saccharomyces cerevisiae.*

INTRODUCTION

Brazil is the second largest producer of bioethanol in the world (Lucena et al., 2010). Most Brazilian industrial processes utilize the Melle-Boinot method, where by yeast cells are recovered from the process and subjected to an acid wash treatment before starting a new fermentation cycle (Amorim et al., 2011). Most commercial *Saccharomyces cerevisiae* strains utilized in the Brazilian processes are not the result of induced

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Table 1. Identification of yeast isolates according to date of sampling.

Sampling date	Isolate	Sampling period of crop season
03 May 2013	A1 to A5	Beginning
28 May 2013	B1	Middle
29 June 2013	C1, C2, C4	Middle
10 October 2013	D1 to D5	End

genetic modifications. In fact, they come from selection processes of autochthonous yeast isolates that showed desirable characteristics to that particular fermentation process (Amorim et al., 2011). The commercial yeast strains mostly employed in several Brazilian ethanol plants are: CAT-1 (from Catanduva Mill); SA-1 (from Santa Adelia Ethanol Plant) and PE-2 (from Pedra Agroindustrial) (Basso et al., 2008).

Several factors may negatively affect the fermentative yield, but bacteria and wild yeasts spoilage are the most frequent causes (Basso et al., 2008; Amorim et al., 2011). Even under controlled process conditions, in which the domesticated yeasts are more prevalent, recent comparative genomic studies have demonstrated cases of spoilage in ethanol plants where native yeast strains were found to indeed overlap those inoculated ones. These wild yeasts can be found in different matrices, from wine making-related environments to tree barks, fruits and even seawater (Duina et al., 2014; Barbosa et al., 2016).

Nonetheless, several non-*Saccharomyces* strains have been isolated from bioethanol plants by using differential culture media, including specimens of the genera *Candida*, *Brettanomyces*, *Trichosporon*, *Pichia*, *Dekkera* and *Hansenula* (Ceccato-Antonini and Silva, 2000). This gives a brief indication about the diversity of the local microbiota commonly found in those fermentation units. Wild yeasts can be introduced into the fermentation process through the raw material and water, or even become adapted residents due to lack of good practices and sanitation. Yeast strains with undesirable characteristics, such as flocculation or excessive foam production, are considered potential spoilage agents, constituting a major problem to the bioethanol industry (Basso et al., 2008).

Wild yeasts may become dominant in the process, occasionally displaying desirable technological traits, and can be often isolated and used for subsequent fermentations. The identification of those emerging yeast strains through biological monitoring is vital for the fermentation yield, and such control can be performed from the simplest culture media plating methods to other more complex and costly ones (Priest and Campbell, 2003).

The origin of those wild yeasts and their natural habitat still remains unknown, even though recent studies have shown a relationship to the surroundings of the sugarcane fields, perhaps dispersed by birds or insects, exudates of trees, and water (Stefanini et al., 2012; Beato et al., 2016). High incidence of wild yeasts is usually associated with significant reduction of fermentation yield, increased processing time, and viscosity (Ceccato-Antonini, 2010).

Both wild and industrial yeast strains shows similar metabolism, making it difficult to control contamination (Amorim et al., 2011), and a solution to this problem would be to better understand the behavior of those wild microorganisms. Monitoring the permanence of selected yeasts and controlling the growth of wild strains are primary parameters for saving inputs used to control the negative effects of spoilage in the fermentation process (Amorim et al., 2011).

Therefore, the aim of this study was to evaluate the morphophysiological and molecular traits of yeast isolates in comparison to commercial strains, shedding light on the diversity of yeasts found in ethanol-producing unit environments.

MATERIALS AND METHODS

Sampling and yeast strains

Samples were directly collected from fermentation tanks during the 2013 to 2014 harvest season (Table 1), in a bioethanol plant in Piracicaba, State of São Paulo, Brazil. The samples were serially diluted in sterile 0.9% (wt/v) NaCl solution and aseptically inoculated on Petri dishes containing YEPD medium (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$ glucose; 18 $g.L^{-1}$ agar) plus chloramphenicol (100 $\mu g.L^{-1}$) and tetracycline (100 $\mu g.L^{-1}$). Plates were incubated at 30°C for 48 h. Fourteen distinctive morphotypes were selected for the subsequent assays.

Isolates were maintained in 16% (v/v) glycerol solution at -80°C. The commercial *S. cerevisiae* strains namely, CAT-1 and PE-2 (LNF Latino Americana, Bento Gonçalves, Brazil) were used as reference; these strains were chosen as they are the two most prominent amongst the commercial strains utilized in Brazil. The isolates and reference yeast strains will be named "samples".

Molecular characterization

Genomic DNA samples were obtained as follows: cells from YEPD medium were resuspended in 1 mL saline solution, and centrifuged at 2046 × *g* for 3 min. The pellet was resuspended in lysis buffer (Tris 500 mM, pH 8.0; *β*-mercaptoethanol 100 mM); followed by the addition of sterile glass beads (Sigma-Aldrich, Brasil LTDA) subsequently, it was homogenized by vortexing, incubated at 100°C for 15 min, and homogenized again. The supernatant containing the

DNA was obtained by centrifugation and stored at -20ºC for further analysis.

Identification of yeast isolates was carried out by deploying PCR amplification of the D1/D2 domain of 26S rDNA utilizing the Sanger sequencing method, as previously described by Kurtzman and Robnett (1998). The resultant PCR products (~600 bp) were purified with charge switch PCR clean-up kit (Invitrogen, USA), according to manufacturer's instruction, and sequenced in the Laboratory of Animal Biotechnology (College of Agriculture 'Luiz de Queiroz', Piracicaba, Brazil).

The chromatograms were analyzed with the software Chromas-Pro (version 1.49; Technelysium Pty Ltd, Australia), and the BLASTn search tool (National Library of Medicine, National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/blast)) for comparison of sequences included in the GenBank database [NCBI (http://www.ncbi.nlm.nih.gov/genbank)] for species determination.

Genetic fingerprinting of *S. cerevisiae* isolates was performed by simple PCR amplification based on the variable interdelta region, using the primers delta-12 (5'-TCAACAATGGAATCCCAAC-3') and delta-2 (5'-GTGGATTTTTATTCCAAC-3'). PCR conditions and analysis of results were realized according to Xufre et al. (2011).

Morphophysiological characterization

One yeast colony from YEPD growth medium was resuspended in saline solution and analyzed under microscopic optical microscope (Axioscopte 40, Zeiss, Germany). Three differential culture media were employed to discriminate morphological and physiological traits of yeast samples: 1) WLN (Wallerstein Laboratory, BD Difco™); 2) BiGGY (Bismuth-Sulfite-Glucose-Glycine-Yeast Extract, BD Difco™); 3) Nagai medium (20 $g.L^{-1}$ glucose; 1.5 $g.L^{-1}$ peptone; 1.5 $g.L^{-1}$ yeast extract; 1.5 $g.L^{-1}$ potassium sulfate; 1.5 $g.L^{-1}$ ammonium sulfate; 1.0 $g.L^{-1}$ magnesium sulfate; 12 $g.L^{-1}$ Agar).

The WLN and BiGGY media were prepared according to manufacturer's instructions, and Nagai medium according to Nagai (1963), using a mixture of dyes (15 $mg.L^{-1}$ trypan blue; 8 $mg.L^{-1}$ eosin). The isolates were punctually inoculated in the plates with a platinum needle, and incubated at 30°C for up to five days. All microbiological analyses were performed in triplicate.

Distinct morphological characteristics such as shape, color and texture were analyzed to classify the isolates cultivated in WL medium. Analyzes on BiGGY and Nagai media considered the color displayed by the colonies after growth, which were further numerically classified and statistically analyzed. Hidrogen sulphide (H_2S) production was detected by the color of the colonies, done through qualitative evaluation, assigning values on a continuous scale based on colony coloration on BiGGY medium, according to Neto and Mendes-Ferreira (2005), namely: 1) white; 2) Beige; 3) light brown; 4) dark brown; 5) black.

The presence of petite cells (brilliant purple colony stained) and normal yeast cells (grayish violet colony stained) indicated in the Nagai medium was also evaluated through the assignment of values on a continuous scale, namely: 1) no growth; 2) both normal and petite cells; 3) normal cells; 4) petite cells.

Growth rate of isolates in different media was determined by a microplate reader (Tecan Infinite M200, RChisto), with capacity for a 96 microwell plate. Each sample was analyzed in four different growth media namely YEPD (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$ glucose), YPSac2 (10 $g.L^{-1}$ yeast extract; 10 $g.L^{-1}$ peptone; 20 $g.L^{-1}$ sucrose), MCC5 (sterilized sugarcane juice at 50 $g.L^{-1}$ TSS) and MMel5 (clarified molasses at 50 $g.L^{-1}$ TSS). The analyses were conducted in triplicates.

Each microwell was inoculated with 50 µL of a yeast cell suspension, obtained from the dilution of a single colony previously grown in YEPD medium in saline solution. The optical density (OD) of the inoculum was monitored spectrophotometrically (Femto 700S) at 600 nm, in order to achieve 0.1 OD. Along with the inoculums, 50 µL of the growth media was added (2 x concentrations). A negative control consisting of 50 µL of growth media and 50 µL of saline solution (9g L^{-1}) was added to every plate. Cell growth was carried out at 30ºC for 24 h, with optical density readings (OD) at 600 nm measured every 2 h.

Statistical analysis

All of the experiments were performed in triplicates. Table data represents the mean values ± standard deviation (n = 3). The qualitative data referring to the morphophysiological analyses were transformed into quantitative ones through the assignment of values. The interaction between the growth rate results was evaluated by clustering according to similarity. The statistical analyses of the standardized values were performed using the SAS statistical program (Statistical Analysis System, version 9.3).

RESULTS

Molecular characterization

The 14 yeast isolates were identified as *S. cerevisiae* by analysis of 26S rDNA sequencing (intra-specific similarity > 99%) using GenBank database search. The interdelta-based PCR fingerprints from the samples are shown in Figure 1, disclosing the band patterns obtained for each sample strain.

Isolates A1, A2, A4 and A5, obtained at beginning of sugarcane harvest, presented a similar banding arrangement (main-pattern 1), which was distinct from the ones observed for A3, B1 and reference strains. Isolates B1, C1, C2 and C4 (mid harvest season) showed similar band patterns to those observed for D2-D5 and PE-2 (main-pattern 2). Isolates A3 and D1 in particular showed distinct interdelta amplification bands.

Morphophysiological characterization

First of all, the cell morphology of YEPD grown yeast samples was assessed. It was observed that the cell morphology was predominantly rounded or oval shaped, with the presence of budding cells. Only the isolate D1 differed by cell multilateral budding tendency, suggesting it has a flocculent pattern under the assayed conditions, as well as in fermentation systems.

The samples cultivated in differential media were characterized as to colony morphotypes. The data of growth in WLN, BIGGY and Nagai media are shown in Table 2. The growth of the sample strains in WLN medium provided colonies of varied sizes, and with different coloration, allowing differentiating variations of morphological aspect like texture, elevation and appearance of the surface of the colony. Isolates from early harvest season, namely A1 and A2 presented smooth colony morphology in WLN medium, however, the isolate A5 was not able to grow in this specific

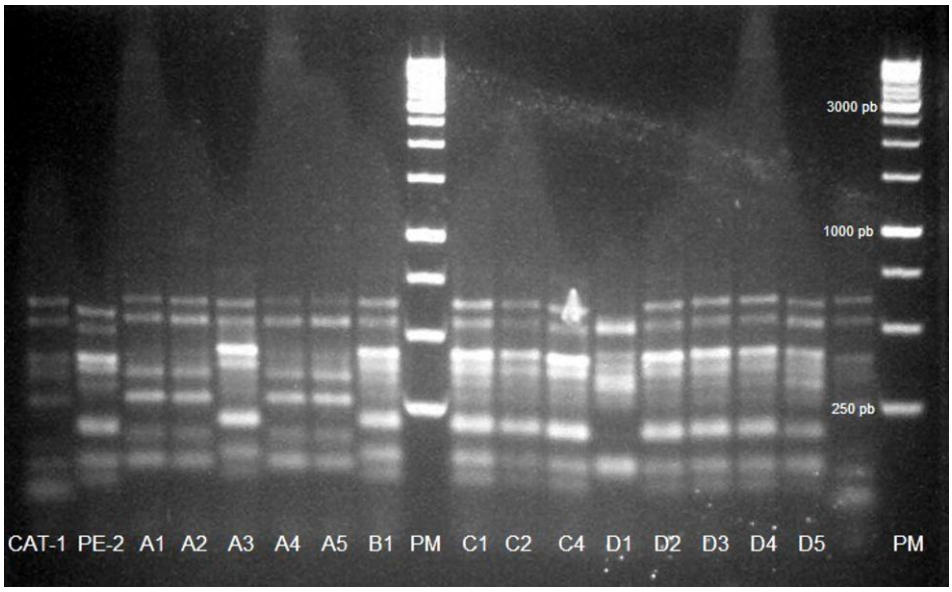

Figure 1. Interdelta banding patterns of yeast isolates and reference strains.

medium (Table 2).

Mid-harvest isolates, namely C1, C2 and C4 also presented smooth colony morphology, like the end of harvest isolates D2 and D4. Isolates A1, A2 and reference strains showed similar colony morphologies. Isolates A3, A4, B1, D1, D3 and D5 displayed rough edges with opaque texture in the assayed conditions. Isolates A3 and A4 also displayed rough edges, but are different in morphology from each other. The mid-season isolates namely C1, C2 and C4 are analogous morphotypes, but with divergent traits from the related isolate B1. End-season isolates, D1 and D5 are related in morphotype, while D2, D3 and D4 displayed a distinct profile (Table 2). Both colony and cellular morphology of indigenous and industrial strains can vary in response to environmental stimuli.

The utilization of BiGGY culture medium to identify production of H_2S in yeast strains was validated by studies that linked the dark colonies obtained directly to the strain's capability to produce H_2S (Zambonelli et al., 1964; Jiranek et al., 1995). The assayed isolates were mainly characterized as low hydrogen sulfide producers, with the exception of isolates A4 and A5, who were not able to grow in these conditions, and isolate C2, who was characterized as moderate hydrogen sulfide producer (Table 2).

The Nagai medium allows the detection of petite cells, with reduced respiratory capacity, leading to very small colonies (Nagai, 1963). The presence of those mutant cells in the medium can be perceived by smaller sizes and brilliant purple color, while the normal cells grow with grayish violet shades. Even distribution of normal and petite cells between the isolates and commercial strains was observed (Table 2).

The analysis of the appearance of isolates cultured in this medium through the assignment of continuous scale values showed that five of the isolates (A1, A2, A5, C4 and D3) presented respiratory deficient cells in the assayed conditions, five of the isolates (A3, A4, B1, C1, and D4) showed the presence of normal cells, and four isolates (C2, D1, D2 and D5) were characterized by the presence of both normal and deficient cells. Both reference strain, namely CAT-1 and PE-1 presented normal cells under assayed conditions (Table 2).

Growth rate evaluation

The samples growth rates were evaluated in four distinct media (Table 3). The mean maximum specific growth rate (μ_{max}) of the samples grown in YEPD medium was 0.409±0.054, superior to that observed for the PE-2 reference strain. Overall, early season samples had a better performance in this medium. In the YPSac medium, the mean μ_{max} of the samples was 0.484±0.047, while PE-2 was 0.440; highlighting the samples A3 (0.530), C2 (0.539) and D3 (0.538).

As for the MCC medium, the mean μ_{max} of the samples was 0.208±0.041 and PE-2 was 0.186; in general all the samples performed poorly in this medium. In the MMel medium, the mean μ_{max} of the samples was 0.443±0.212 and PE-2 was 0.338; in particular the mid-season samples presented mean μ_{max} of 0.650, highlighting the samples C1 (0.992), C2 (0.717) and D4 (0.688). It is noteworthy that the performance of sample D1 in all media utilized is being lower than all the samples and both reference strains. Although the sugarcane juice has in its composition sucrose and glucose, the least expressive results were obtained in MCC medium.

Table 2. Morphophysiological characteristics of yeast colonies in different media*.

Colony name	WLN medium						BiGGY medium		Nagai medium	
	Diameter (mm)	Texture	Color	Surface	Edge	Elevation	Diameter (mm)	Value [1]	Color[2]	Value [3]
A1	5	Shiny	Light green	Smooth	Smooth	Convex	6	2	Brilliant purple	3
A2	5	Shiny	Light green	Smooth	Smooth	Convex	5	2	Brilliant purple	3
A3	7	Opaque	Beige	Rough	Rough	Convex	8	2	Grayish violet	2
A4	6	Shiny	Beige	Rough	Rough	Convex	n/g	n/g	Grayish violet	2
A5	n/g	n/g	n/g	n/g	n/g	n/g	n/g	n/g	Brilliant purple	3
B1	7	Opaque	Beige	Rough	Rough	Convex	9	2	Grayish violet	2
C1	6	Opaque	Beige	Smooth	Rough	Convex	10	1	Grayish violet	2
C2	5	Opaque	Beige	Smooth	Rough	Convex	6	3	Brilliant purple	1
C4	5	Opaque	Beige	Smooth	Rough	Convex	4	2	Brilliant purple	3
D1	10	Opaque	Beige	Rough	Rough	Convex	4	2	Brilliant purple	1
D2	10	Opaque	Beige	Smooth	Rough	Convex	5	2	Brilliant purple	1
D3	6	Opaque	Beige	Rough	Rough	Convex	5	2	Brilliant purple	3
D4	7	Opaque	Beige	Smooth	Rough	Convex	5	2	Grayish violet	2
D5	10	Opaque	Beige	Rough	Rough	Convex	6	2	Grayish violet	1
CAT-1	8	Shiny	Beige	Smooth	Smooth	Convex	5	2	Grayish violet	2
PE-2	6	Shiny	Beige	Smooth	Smooth	Convex	5	2	Grayish violet	2

*Cultivation conditions: 30ºC for 48 h; (n/g) no growth; [1]Numerical value attributed to colony color after growth: (0) white, (1) beige, (2) light brown, (3) dark brown, (4) black; [2]Presence of petite cells (brilliant purple colony stained); normal yeast cells (grayish violet colony stained).[3] (0) no growth, (1) both normal and petite cells, (2) normal cells, (3) petite cells.

Table 3. Maximum growth rate and optical density of yeast isolates and reference strains grown in different media*.

	YEPD			YPSac			MCC			MMel		
Isolate	μ_{max} (h^{-1})	OD_{max}	time (h)	μ_{max} (h^{-1})	OD_{max}	time (h)	μ_{max} (h^{-1})	OD_{max}	Time (h)	μ_{max} (h^{-1})	OD_{max}	Time (h)
A1	0.440	0.442	4	0.472	0.748	10	0.272	0.320	12	0.347	0.680	14
A2	0.440	0.416	4	0.477	0.743	10	0.267	0.282	12	0.278	0.687	14
A3	0.461	0.449	4	0.530	0.860	8	0.212	0.350	12	0.371	0.720	14
A4	0.434	0.389	4	0.473	0.744	10	0.164	0.277	14	0.323	0.694	14
A5	0.401	0.398	4	0.455	0.718	10	0.198	0.299	12	0.312	0.696	14
B1	0.370	0.365	4	0.466	0.784	8	0.201	0.416	20	0.506	0.665	12
C1	0.382	0.349	6	0.469	0.787	10	0.246	0.374	20	0.992	0.612	14
C2	0.444	0.367	6	0.539	0.817	8	0.259	0.349	20	0.717	0.650	14
C4	0.415	0.358	4	0.523	0.795	8	0.194	0.433	20	0.390	0.672	14

Table 3. Contd.

D1	0.249	0.265	4	0.361	0.659	8	0.133	0.241	8	0.259	0.605	10
D2	0.385	0.358	4	0.467	0.731	8	0.167	0.300	14	0.318	0.584	14
D3	0.450	0.415	4	0.538	0.84	8	0.199	0.472	22	0.394	0.679	14
D4	0.439	0.401	4	0.528	0.839	8	0.215	0.470	20	0.688	0.750	14
D5	0.417	0.352	4	0.482	0.730	8	0.185	0.237	8	0.309	0.615	12
Mean	0.409	0.380	4.3	0.484	0.771	8.7	0.208	0.344	15.3	0.443	0.665	13.4
SD	0.054	0.047	0.73	0.047	0.056	0.99	0.041	0.079	4.9	0.212	0.047	1.2
CAT-1	0.391	0.471	6	0.372	0.810	10	0.306	0.257	12	0.304	0.619	16
PE-2	0.352	0.358	4	0.442	0.780	10	0.186	0.106	22	0.338	0.673	12

*Cultivation conditions: 30ºC for 24 h in microplates. μ_{max}, maximum specific growth rate; OD_{max}, maximum optical cell density; time (h) at which OD_{max} was reached.

Interaction between analyzed characteristics

The values of μ_{max}, OD_{max} and time (Table 3) were submitted to cluster analysis by similarity using origin software (OriginLab, 2017), which allowed to define 2 groups besides the isolated D1 and reference lines that were separated (Figure 2). The reference lines and the samples D1, C1 and C2 are separated from the rest of the samples forming a large group. This means that most of the samples were similar to each other but different from the reference strains.

DISCUSSION

The variety of yeast strains found during the industrial fermentation process is highly correlated to the conditions of which they are exposed (Silva-Filho et al., 2005), including the raw material quality and the ethanol concentration reached during the process, among other factors (Amorim et al., 2011).

Several studies aimed to characterize native *S. cerevisiae*, showing that most of those strains found in bioethanol plants are found in the surrounding environment, and are brought into the fermentation process along with the substrates (Beato et al., 2016; Sampaio and Gonçalves, 2008). Contrariwise, some isolates were found to derive from genetic modifications, a clonal differentiation that occurs during the fermentation process. This mainly occurs due to the unique large-scale fed-batch process, which utilizes acid cell recycling and submits the cells under a great deal of stress (Della-Bianca et al., 2013). Yeast isolates subjected to harsh conditions tend to develop genetic-induced physiological traits that make them more resistant, including the activation of genes responsible for cell wall integrity and oxidative stress response (Elsztein et al., 2011). Chromosomal rearrangements referred to as adaptive evolution were reported for the industrial PE-2 strain, under industrial-related environments and extended laboratory storage conditions (Argueso et al., 2009). This can be observed in the results obtained by the analysis of 26S rDNA sequencing (Figure 1), where isolates presented distinct banding patterns when compared to the reference strains. This can be caused by the emergence of a native strain in the process, or due to adaptive evolution, as mentioned by Argueso et al. (2009), Burke (2012) and Xufre et al. (2011). The results made it possible to identify groups of wild strains that are probably of a common origin, most likely like the local microbiota, as similarly observed by Kishkovskaia et al. (2017).

Adaptive evolution in yeast populations is driven by genetic accumulation (Burke, 2012). The dynamics involving mutations in populations depend on accumulation, natural selection, competition within the population itself and overall fitness (Lee and Marx, 2013; Bergström et al., 2014). Understanding native yeast population and evolution dynamics is important in order to comprehend how the stressing factors of the fermentation process impact the commercial yeast strains go through and how they interact with competitive invasive strains (Payen et al., 2014). Additionally, repetitive DNA sequences may display inter-specific patterns, punctual variations in position and number of such regions constitutes a genetic fingerprinting that permits to identify and differentiate yeast strains or clonal variants from a given local microbiota (Xufre et al., 2011).

One isolates namely D1, displayed flocculent patterns during cell morphology assays, which

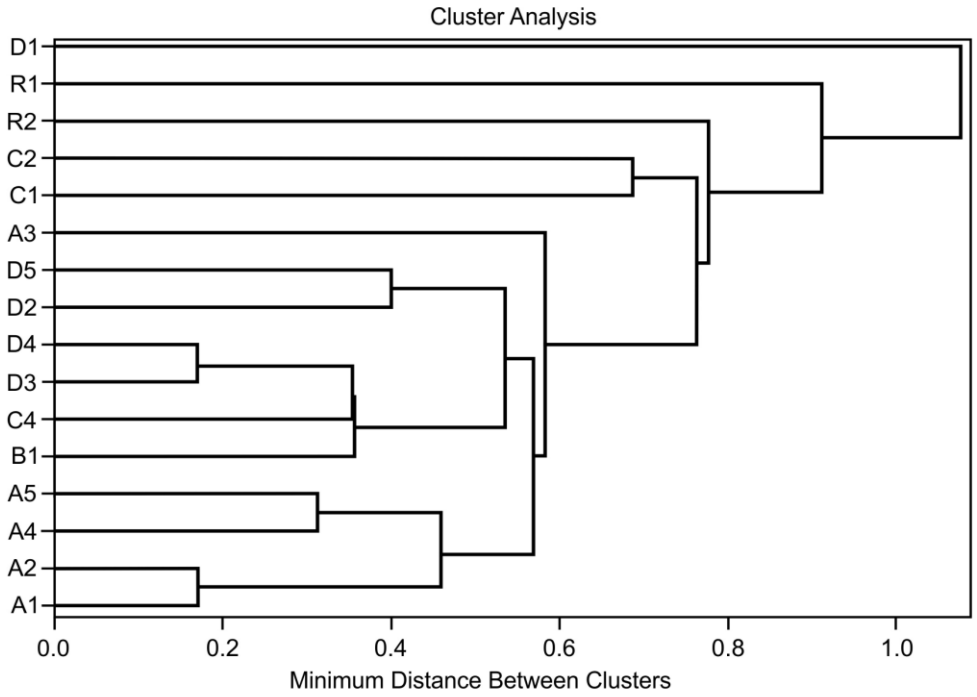

Figure 2. Cluster analysis from the values of μ_{max}, OD_{max} and time.

can be a sign of cell-cell adhesion, known as flocculation, which in turn can be an indicator of stress. It is used by the cell as a form of protection. This mechanism is activated by cell-surface adhesions; the flocculin (Flo) proteins are activated in response to stressing conditions (El-Kirat-Chatel et al., 2015). This response is mainly regulated by environmental conditions such as changes in pH and ethanol concentration or nitrogen and glucose depletion (Braus et al., 2003; Rossouw et al., 2015; Reis et al., 2016; Stewart, 2009). On the other hand, some *S. cerevisiae* strains can show sexual aggregation patterns, described as co-flocculation and cell chain formation, derived from the failure of the bud to separate itself from its mother cell, resulting in an aggregation of approximately 30 to 50 cells, unable to regroup after being mechanically dispersed (Soares, 2010). The results observed for isolate D1 point out in the direction of it being a native yeast strain that emerged in the industrial process; taking into consideration that this particular sample showed a difference banding pattern (Figure 1) and distinct growth rate performance (Table 3).

The response of *S. cerevisiae* strains to Brazil's unique fermentation process is yet poorly understood, but it is hypothesized that an enhanced stress response, and adaptive capacity play an important role in this. Transcriptional studies that investigated industrial strains such as CAT-1 and PE-2 showed genome-wide responses to environmental stress and acid wash treatment, as well as ethanol-induced stress. These adaptive characteristics are an insight of what to look for in native strains, so as to select them for industrial use (Brown et al., 2013).

The results obtained in this study revealed the diversity of morphophysiological traits of fermenting yeast isolates found in an ethanol-producing unit (Table 2), coexisting with the commercial strain pitched at the beginning of the process. These emerging wild yeasts can either overgrow, or act in consortium carrying out the fermentation with no damage to the production (Beato et al., 2016). In many cases, however, wild yeasts may display undesirable characteristics that can damage the ongoing production. The utilization of plating techniques to determine morphophysiological traits is a valuable and cost-effective tool to discriminate strains that might exhibit desirable characteristics for fermentation processes (Palmann, 2001).

Concerning the growth in different media, a study conducted by Casalone et al. (2005) described that, among a thousand colonies grown on YEPD medium, 2.5% exhibited the rough colony phenotype. *S. cerevisiae* strains that exhibit this trait are often associated with disturbances in the fermentation process (Andrietta et al., 2011).The morphological pattern of commercial strains grown in YEPD medium supplemented with rose Bengal (Moreira et al., 2015) shows similar results for the morphological patterns of CAT-1 and PE-2 strains, with shiny colonies, smooth surface and edges and convex elevation, as observed in Table 2.

However, the isolates, mainly early harvest and late harvest season, showed rough colony morphology,

displaying the presence of diverse *S. cerevisiae* in this particular fermentation process. Previous investigations show that the acid treatment utilized in the Brazilian industry can inhibit the growth of the “rough strains” to a certain degree, and can also be applied as a tool to avoid spoilage and minimize damages caused by rough strains, due to their peculiar metabolic profile (Reis et al, 2013). However, the so-called “rough strains” are usually variants of smooth colony *S. cerevisiae*.

The WLN medium is commonly employed to isolate and identify yeasts, molds and bacteria. This method was originally developed for monitoring yeast populations during brewing processes (Green and Gray, 1950), but shows reliable results in monitoring native flora fermentation (Palmann, 2001), as most yeast typically occurring in those systems can be distinguished by colony color and morphology (Jespersen and Jakobsen, 1996). The isolates displayed diverse morphotypes when grown in this medium, with the exception of isolate A5. Mid-season isolates displayed predominantly smooth colony surface, differing from the end-season isolates, which displayed more rough morphotypes (Table 2). This can be caused by adaptive evolution, caused by the cell recycling process, among other stressing factors (Bergström et al., 2014).

It was previously proposed that BiGGY agar should be used for the identification of species of the genus *Candida* spp., whilst simultaneously offering an indirect measurement of hydrogen sulfide production by fermenting yeasts. This compound is a common by-product of alcoholic fermentation, and once its production is a strain-dependent trait (Giudici et al., 1993), the utilization of this differential medium allows the characterization of wild yeast isolates in comparison to reference strains (Neto and Mendes-Ferreira, 2005). The sulfite reductase activity in commercial *S. cerevisiae* and non-*Saccharomyces* strains was documented by Mendes-Ferreira et al. (2002), showing the diversity of characteristics found in yeast strains. The results obtained for the isolates and reference strains showed predominantly low sulfite reductase activity (Table 2), which is a trait of interest for both the ethanol industry and beverages industry, since the interest in utilizing wild yeasts is growing constantly.

When analyzing the growth rate in different medium, the qualitative analysis of the isolates and reference strains growth on microplates indicates that they presented different performances, depending on the medium utilized. YEPD medium is considered good for cell growth and longevity, because of the yeast extract and peptone present in the composition (Table 3). Studies show that the nutrient composition in growth media directly impacts the growth rate and longevity of yeast cells: yeast grown in culture media with different ratios of nutrients presented diverse life spans (Wu et al., 2013); high nitrogen concentration is not mandatory to achieve maximum growth rate, however, it is shown to influence the fermentation performance due to higher biomass concentration (da Cruz et al., 2002; Barbosa et al., 2014). Khoja et al. (2015) found that sugarcane molasses are a better fermentation medium for the bacteria *Zymomona mobilis*, which performed better in this particular medium.

Conclusions

The yeast isolates showed distinct morphological characteristics among each other and were compared to the reference strains. Same observation was made with the molecular characterization, which showed intra-specific differences for all *S. cerevisiae* isolates. These isolates also differed from the reference strains, thus this necessitates constant monitoring from the producers, in order to verify the yeast cells that initiates fermentation which will be the ones to complete it. Despite the small sample sizes, a diversity of molecular morphophysiological traits has been revealed. This work show morphophysiological and molecular characterization of yeast to be useful to industry as it contributes to the selection of new suitable strains for alcoholic fermentation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Short Communication

Screening of rural scavenging birds for the presence of detectible protective Newcastle disease antibodies in some selected rural communities of Plateau State

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A sero-prevalence study was conducted to determine the level of detectible protective antibody titre using the haemagglutination (HA) and haemagglutination inhibition (HI) tests. Serum samples were collected from four hundred and eighty seven unvaccinated rural scavenging chickens from five selected rural communities, including Du, Chaha, Chakarum, Chele and Ngo'hong in Jos South Local Government Area of Plateau state. The result revealed a very low mean titre ≤2 log_2, which explained the unusually high morbidity and mortality rate experienced during seasonal outbreaks. There is an urgent need to initiate control measures in rural areas through the establishment of a strategic vaccination programme against new castle disease outbreaks. This will reduce the incidence of the disease to a large extent.

Key word: Newcastle disease, detectible protective antibody, rural scavenging birds.

INTRODUCTION

Newcastle disease(ND) caused by a virus of genus Avulovirus, subfamily Paramyxovirinae of the family Paramyxoviridae, is an acute, highly contagious rapidly spreading viral disease of domestic poultry and other wild species of birds of all ages (Seal et al., 2003; Aldous, 2003, Haque et al., 2010; Iran et al., 2013). Since ND was first reported in Nigeria in 1953 at Ibadan, the disease has become the most important viral disease of chickens and widely spread throughout the country with annual epidemics being recorded in highly susceptible poultry flocks (Aliyu et al., 2015). The high genetic diversity of the virus could have contributed to the increasing rate of the disease (Aliyu et al., 2015). In Nigeria, ND is generally well-recognised by farmers in both local and exotic breed (Lawal et al., 2015).

Village poultry products have ensured household

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Table 1. Sero-prevalence of Newcastle disease antibody in free range domesticated chicken in Du, Ngohong, Chaha, Chakarum and Chele in Jos south local government area, Plateau State.

Village	Number of birds screened	Vaccination record/history	Mean HI titres
Chaha	130	Nil	≤2 $\log_2$
Ngohong	72	Nil	≤2 $\log_2$
Du	57	Nil	≤2 $\log_2$
Chakarum	155	Nil	≤2 $\log_2$
Chele	73	Nil	≤2 $\log_2$
Total	487		

food security as it supplies high quality animal protein (meat and egg) which where used as food, petty cash derived from sales of poultry products, poverty alleviation and create jobs for rural dwellers (ILRI, 2014; Mulugeta et al., 2013).

Village poultry species could play a significant role in the epidemiology and transmission of the infection to the more susceptible commercial exotic chickens or immune deficient village poultry species especially when reared together or in close proximity.

The disease may therefore be considered a threat to successful village poultry production system (Lawal et al., 2016). Nigeria has an estimated poultry population of 140 million with backyard poultry production accounting for more than 60% of the total flock with an asset value of > 5.75 billion US Dollars (Nnadi et al., 2010).However, annual loss due seasonal outbreak of ND discourages the rural dwellers from investing in scavenging backyard poultry farming.

Though, it is source of cheap animal protein and a profitable means of income generation for rural dwellers, seasonal outbreak of ND challenge in rural scavenging poultry farming, limits derivable profitability and productivity of rural scavenging poultry farming. Morbidity and mortality rate of up to 100% is highly probable in ND immune deficient flock, especially, if challenged by wild virulent strain. Thus, a strategic vaccination control programme against Newcastle disease is imperative in rural scavenging poultry population especially in countries where ND is endemic and application of ND bioexclusion and biocotainment measures is impossible.

MATERIALS AND METHODS

Blood sample

Blood sample was aseptically collected from the wing vein; usually 0.5 to 1 ml was collected per bird with needle and syringe. The collected blood sample was kept in syringes and allowed to clot. The clotted blood was left overnight at room temperature for complete serum separation. The separated serum was harvested and frozen at -20°C for subsequent antibody immune profiling using HI test.

Haemagglutination and haemagglutination inhibition

Haemagglutination and haemagglutination inhibition tests were carried out on the collected sera samples. A 1% suspension of chicken red blood cells (RBCs) was prepared for use in haemagglutination (HA) and haemagglutination inhibition (HI) tests according to Office International des Epizootics (OIE, 2010). The HA titres of standard NDV antigen was determined as described by Allan and Gough (1974) and diluted to contain 4HA units. The reconstituted antigen containing the 4HA units was used in the determination of test sera titres in a HI test. The HI titre for each screened sera was determined and expressed in $\log_2$, and the geometric mean titre calculated for each village that was sampled. Any HI titre of ≥2 $\log_2$ was considered positive.

RESULTS

The results in Table 1 show that protective ND antibody was completely absent in all the screened sera samples collected from the five villages that was sampled (all the screened sera samples were negative). This result is in agreement with the flock history, as none of the sampled birds had history of ND vaccination.

DISCUSSION

Economic analysis estimates that the current annual financial loss as a result of New Castle Disease outbreak in rural chicken in Nigeria amounts to $38,695,652.2 million dollars annually (International Livestock Research Institute (ILRI) 2013). ND control by vaccination is widely practiced world over, but in developing countries, vaccination of rural scavenging flock is rarely practiced and most commercially available vaccine are geared towards the control of ND for large commercial flock.

The result in Table 1 shows that the screened free range chickens all lacked protective antibody against ND virus. Though, the $NDVI_2$ vaccine is available and at an affordable price, rarely do rural farmer vaccinate their chickens. These birds are usually allowed to roam free and fend for themselves, by scavenging around, thus, allowing them to come in close contact with wild feral unvaccinated birds, infected birds and contaminated formites.

A common practice amongst the rural scavenging poultry farmers is the introduction of birds from other flock without history of previous contact with infected birds. These birds are usually given as gift from ceremonies or are sometimes purchased by the farmer to increase his or her stock. The farmers expect maximum output from these birds in terms of income generation, increased egg production and meat yield, but persistent annual outbreak of the disease and consequent losses minimizes this expected yield.

Current vaccines come mainly in large doses and are targeted towards large commercial flocks with little relevance in village flocks which are often small, scattered, multi-aged, and free-roaming with minimal control. However, in most rural settings, the lack of a strategic control measures against this devastating poultry disease outbreak has constantly impeded the development, of rural scavenging poultry farming, though its economic benefit is enormous.

However, ND I-2 vaccine has undergone laboratory tests in several countries and has proved to be protective against local virulent strains of the ND virus (Alders and Spradbrow, 2001b). In Vietnam, after extensive laboratory and village trials, it has been officially recognized as the ND vaccine for village chickens (Tu et al., 1998). In Tanzania, it has given protection for at least two months after vaccination (Wambura et al., 2000).

A mean titre of ≤2 $\log_2$ in the screened flock implies that in the event of an outbreak and depending on the virulence of the virus, flock morbidity and mortality could be up to 100%.

Conclusion

Vaccination has been reported as the only safeguard against endemic ND (Usman, 2002), thus a strategic mandatory vaccination of rural poultry flock against ND will mitigate against seasonal outbreak of ND in rural scavenging flock. The result from this shows that the essence of vaccination of rural scavenging poultry flock cannot be over emphasized since it remains the only option especially in countries where ND is endemic.

CONFLICT OF INTERESTS

There is no conflict of interests in preparation of the manuscript.

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