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Screening for Mycotoxins and Major Bioactive Molecules in Mould Infested Brown Variety (BV) and Speckled Flecked Spotted Variety (SFSV) *Vigna subterranea* (Bambara Nut) in Nigeria

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors designed the study. Author PTN performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors PTN and AAB managed the analyses of the study. Author PTN managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: This research was carried out with the sole aim of screening for mycotoxins and identifying the major bioactive molecules in two varieties of mould infested *Vigna subterranea.*
Place and Duration of Study: Department of microbiology, University of Calabar, Cross River State Nigeria was used for this research within the space of four months.
Methodology: Two varieties of *Vigna subterranea* were collected into a clean sealed container from major markets in South Eastern Nigeria. Samples were properly labeled and conveyed to the laboratory. After four months, samples were blended and dissolved in methanol and ethanol at a ratio of 80:20 volumes / volume (v/v). Bioactive molecules and mycotoxins were screened for using Gas chromatography/ Mass spectrometry.
Result: There was high level of similarities in major bioactive molecules of fatty acids, sugars, amino acids, phenols, alcohol and antioxidants in the two varieties. The pH and moisture content

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were within unfavourable range for mycotoxin production. Moisture content of the varieties was 2.46% and 8.03% while the pH was 7.01 and 6.9 respectively. Some biomolecules were peculiar to only one variety. Brown Variety (BV) and Specked Flecked Spotted Variety (SFSV) had Oleic acid with peak percentage area of 37.91% and 34.40% as the highest bimolecules respectively. Palmitic acid had percentage area above 15% in the two varieties.

Conclusion: This study juxtaposes the fact that mycotoxin production in *V. subterranea* is a synergistic effect of mould contamination and the necessary favourable environment.

Keywords: Vigna subterranean; mycotoxin; antioxidants; mould; fatty acids; sugars; amino acids.

1. INTRODUCTION

Mycotoxins are poisonous secondary metabolites produced by toxigenic moulds. These metabolites are usually of low molecular weight. Filamentous fungi have been identified as major culprits of mycotoxins production [1]. The purpose for the secretion or production of mycotoxin is not yet fully understood. However, it is believed that the poisonous nature of mycotoxins could serve as a defensive mechanism to organisms that secrete them. Numerous types of mycotoxins have been traced to toxigenic moulds as their sources. These mycotoxins range from highly toxigenic aflatoxins, ochratoxins, patulin, zearalenone, fumonisins, trichothecenes to other mycotoxins of lesser toxigenic effects to human and animal health. An outstanding attribute of mycotoxins and their sources is that, more than one mycotoxin could be produced by one mould and as well, two or more moulds may produce same type of mycotoxin [1]. Mycotoxins such as aflatoxins, ochratoxins, zeralenone, fumonisins, patulin, and trichothecenes have been found associated with food contamination in Indian communities [2]. Various works have disclosed that pre and post harvest factors contributes to the total mycotoxin produced in any agricultural product [3]. Environmental factors relating to storage conditions that are however not beyond control are major reason for mycotoxin production [1]. The Water activity, pH, temperature, moisture content, time of storage and presence of insect pest activities are major contributor to the proliferation of toxigenic mould on any substrate where their spores lands [4,5].

The mycogenera and mycotoxins associated with nearly all globally recognized food stuffs and animal feeds have been researched. Some crops although not globally recognized, because of some peculiar traits and qualities have also attracted the attention of researchers. Bambara nut (*Vignea subteranea)* is an indigenous crop of African origin with so great outstanding qualities, but very little research has been carried out over the centuries, since it's recognition as a food [6]. *Vigna subterranea* has been identified by different names all over Africa. History has it that the crop originated from the North Eastern states of Nigeria to the other parts of the country, excluding the riverine and swampy zones of the country.Nigerians are the largest producers of bambara nut in Africa [6,7].

Bambara nut has an appealing flavor which has highly influenced its demand from the local markets where producers sell them. It has high nutritive value as other popularly consumed African legumes. Bambara nut is capable of growing in arid land where Ground nut *(Arachis hypogea),* maize *(Zea mays* L.) and sorghum *(Sorghum bicolar)* have failed [7]. The seeds coat of this crop do not maintain homogenity in morphological atrributes. According to [6], the seed colours, sizes, pigmentation,pod shape,and other characteristics tend to vary from one geographical location to another. However, there are about seven known varieties of bambara nut in Nigeria. These varieties are: Black variety (kernel is usually between small to medium size),Red variety (kernel is usually large size), Cream/black eye variety (kernel is usually large size), Cream/brown variety (large moderate size), Cream/no eye variety (very small pod and kernel), Speckled/flecked/spotted variety (purple colour predominates but the kernel is small), Brown variety (variation is between light and dark brown and the kernel are medium size to large) [8]. In line with the world's population demand for nutrient rich food stuff, *V. subtteranea* is nutritionally endowed with major food classes. About 63% carbohydrate, 6.5% oil and 19% protein is present in bambara nut seed. No harm is associated with consumption of nut at different stages of maturity (so far it's well processed) [9,7,6].

2. METHODOLOGY

2.1 Sampling and Study Site

Vigna subterranea was collected into a clean dry container and conveyed to the laboratory from some major traders of in South Eastern Nigeria. These samples were kept in a cool and dried environment afterwards conveyed to microbiology laboratory in University of Calabar [10].

2.2 Mould Growth

Samples were kept in the laboratory for four months and observed for development of mould growth with no form of additional substance to promote or inhibit their growth.

2.3 Culture and Identification

2.3.1 Media preparation and plating procedure

Sabouraud Dextrose Agar (SDA) was prepared according to manufacturers guide and supplemented with amoxicillin at a concentration of 50µg/1000ml before pouring into plates. Surface plating method was used for growing moulds at ambient temperature for 5-7 days [11,4].

2.3.2 Purification, identification and characterization of isolates

For proper observation of cultural morphology, the discrete fungal colonies were randomly picked up and sub-cultured into SDA in order to obtain an absolute pure culture. Hence, slide cultures were prepared from 5-7 days old plates of pure culture for microscopic morphology. The prepared culture slides were viewed under x40 objective. Identification was chiefly based on macroscopic and microscopic morphology and some physiological observation.

2.4 Determination of Moisture Content and pH

The pH meter was used to determine the pH of the varieties after preparing the blended seeds (flour) and making solution with deionized water according to standard procedures.

Moisture content was determined using the formula shown below:

$$\text{Moisture content} = \frac{\Theta_2 - \theta_3}{\Theta_2 - \theta_1}$$

Such that:

θ_1= Weight of empty petri dish
Θ_2=Weight of sample + petri dish before drying
θ_3= Weight of sample + petri dish after drying [12].

2.5 Analysis for Bioactive Molecules and Mycotoxins

The two varieties of *V. suterranea* (Brown variety (BV) and Speckled Spotted Variety (SFSV)) blended with a properly cleaned up and disinfected blender. After blending each sample, the blender was cleaned up and disinfected again to avoid any false positive or negative results. After proper blending of samples, 1 gram of Bambara nut flour was dissolved in methanol and ethanol at a ratio of 80:20 volumes / volume (v/v). Five milliliters of each sample dissolved was aseptically collected into a plane sample bottle and properly labeled. Samples were properly masked to avoid any alteration of the indigenous constituents of the sample. Afterwards, samples were analyzed for the presence of mycotoxin through a stationary column with a carrier helium gas. All bioactive constituents of *V. subterranea* were quantified using mass spectrometer. From the result obtained, the retention time (RT) of peaks from the total ion current (TIC) were used to identify the compound with molecular weight equal to the molecular weight stated in the spectrometric graph of current against mass charge ratio.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Mould growth, moisture content and pH

From the study, it was identified that fungi species that contaminated the Brown Variety (BV) and Speckled Flecked Spotted Variety (SFSV) *V. subterranea* were majorly species of *Aspergillus niger, Penicillium spp, Rhizopus nigrican and Mucor spp.* The moisture content as well as the pH was determined to know the amount of moisture and the ratio of hydrogen ion to hydroxyl ion concentration that is present in these varieties as represented in Table 1.

3.1.2 Bioactive molecule analysis in BV and SFSV of *V. subterranea*

The numbered peaks of the chromatogram in Figs. 1 and 2 represents the major bioactive

compounds present in the varieties. The Retention time, molecular weight, Initial time, Final time, percentage area, of major bioactive molecules are represented in Tables 2 and 3.

3.1.3 Co-occorrence of major bio-molecules in BV and SFSV *V. subterranea*

During the study, it was observed that about thirteen bio-molecules co-occurred in the two varieties of *V. subterranea.* The percentage of these molecules have been represented in bars as shown in the bar chart in Fig. 3.

Table 1. Moisture content and pH level of *V. subterranean*

Varieties of *V. subterranea*	Percentage (%) moisture content	pH of samples
SFSV	2.46	7.01
BV	8.03	6.91

KEY: SFSV- Speckled flecked spotted variety, BV- Brown variety

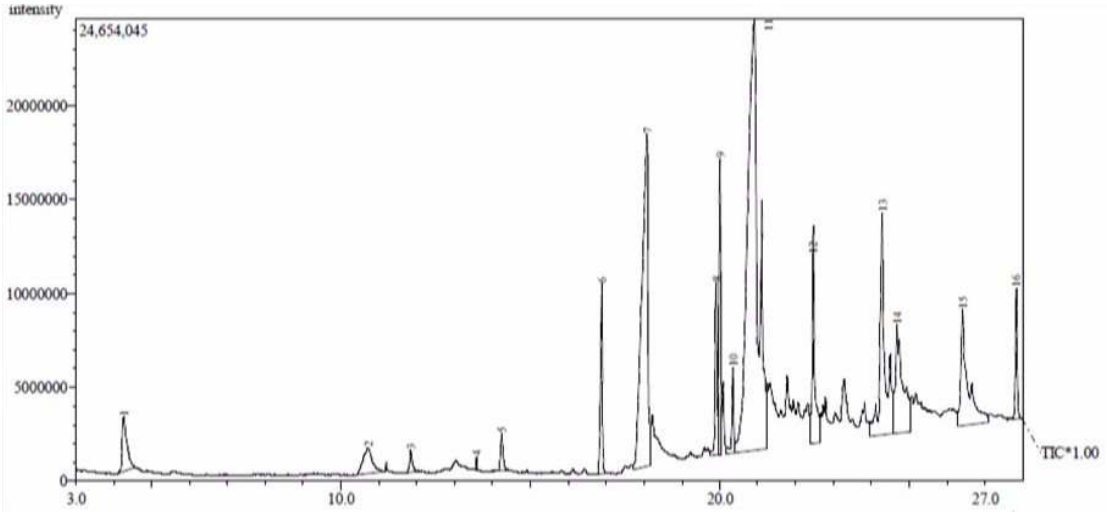

Fig. 1. Chromatogram of major bioactive molecules present in brown variety (BV)

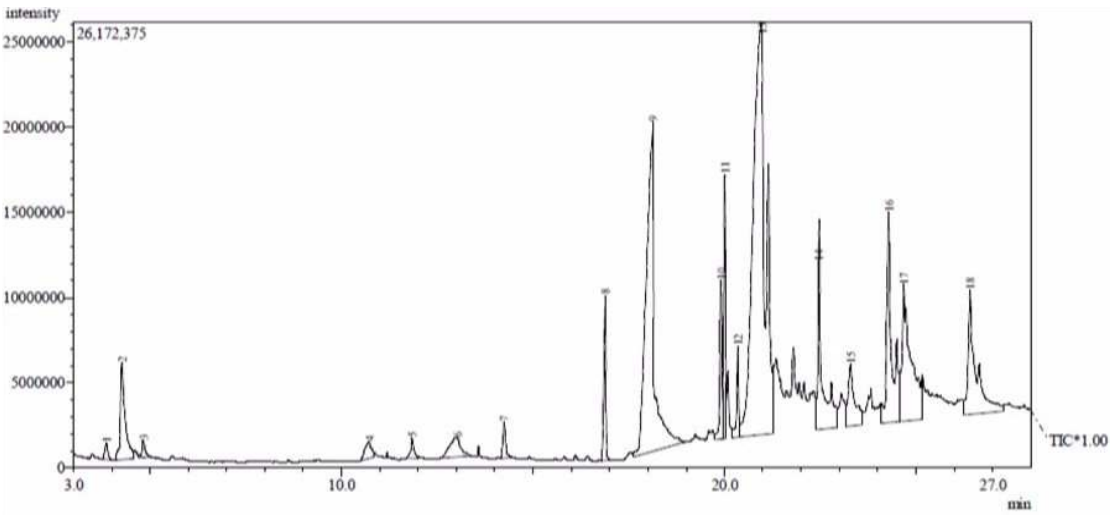

Fig. 2. Chromatogram of major bioactive molecules present in speckled flecked spotted (SFSV)

Table 2. Measuring parameters (peaks, retention time, initial time, final time and percentage area) of major bioactive molecules present in brown variety (BV)

Peak #	Name of compound	Molecular weight	Retention time (RT)	Initial time (IT)	Final time (FT)	Area %
1	Phenol	94	4.259	4.108	4.558	2.16
2	1,3-propanediol	151	10.719	10.425	11.067	1.96
3	Undecanoic acid	186	11.843	11.725	12.000	0.51
4	Methyltetradecanoate	242	13.578	13.517	13.642	0.13
5	Tetradecanoic acid	228	14.242	14.158	14.425	0.74
6	Pentadecanoic acid	270	16.883	16.775	17.033	3.17
7	n-Hexadecanoic acid	256	18.082	17.717	18.175	16.59
8	9,12-octadecadienoic acid	294	19.905	19.758	19.950	2.75
9	11-octadecenoic acid	296	20.006	19.950	20.050	4.43
10	Octadecenoic acid	298	20.346	20.175	20.400	1.30
11	Oleic acid	282	20.915	20.400	21.225	37.02
12	2-Amino-4-furan-2-yl-7-methyl-5-oxo-4H	270	22.475	22.400	22.650	4.52
13	9-Octadecinal	266	24.294	23.950	24.575	9.19
14	Hexadecanoic acid	330	24.685	24.575	25.025	6.72
15	2-methyl-Z	280	26.419	26.275	27.100	6.67
16	Squalene	410	27.836	27.600	27.975	2.14

Table 3. Measuring parameters (peaks, retention time, initial time, final time, area and percentage area) of major bioactive molecules present in speckled flecked spotted variety (SFSV)

Peak #	Name of compound	Molecular weight	Retention time (RT)	Initial time (IT)	Final time (FT)	Area %
1	Benzaldehyde	106	3.856	3.767	4.033	0.33
2	Phenol	94	4.261	4.075	4.575	3.22
3	Benzyl Alcohol	108	4.820	4.717	5.008	0.40
4	1,2-cyclopentanediol	116	10.718	10.492	10.858	0.77
5	Undecanoic acid	186	11.850	11.700	12.008	0.46
6	Beta-d-manofuranoside	194	12.968	12.608	13.400	1.52
7	Tetradecanoic acid	228	14.247	14.150	14.467	0.67
8	Pentadecanoic acid	270	16.885	16.783	17.042	2.37
9	n-hexadecaanoic acid	256	18.128	17.617	18.950	18.62
10	9,12-octadecadienoic acid	294	19.909	19.750	19.950	2.13
11	11-octadecenoic acid	296	20.009	19.950	20.050	3.36
12	Octadecanoic acid	298	20.348	20.200	20.408	1.26
13	Oleic acid	282	20.964	20.408	21.275	34.40
14	Benzene, 1- bromo-2-ethyl	184	22.479	22.400	22.950	5.66
15	Eicosanoic acid	312	23.299	24.100	23.600	2.73
16	9-octadecenal	266	24.695	23.175	24.575	7.09
17	Hexadecanoic acid	330	24.691	24.575	25.150	8.37
18	(E)-13-Docosenoic acid	338	26.422	26.250	27.275	6.64

3.1.4 Mass spectra of bio-molecules of high percentage area in BV and SFSV *V. subterranean*

From the mass spectrometric analysis, Oleic acid, n-Hexadecanoic acid, 11-octadecenoic acid and 9-octadecenal were the four bio-molecules which had the highest percentage. Their structures are represented in Figs. 4-7.

3.2 Discussion

The analysis of the samples showed that the major bioactive compounds were more of organic

acids. Fatty acids, sugars, amino acids, phenols and alcohol varied in the two varieties. Amongst these fatty acids, were numerous unsaturated fatty acids (including the essential; linoleic acid

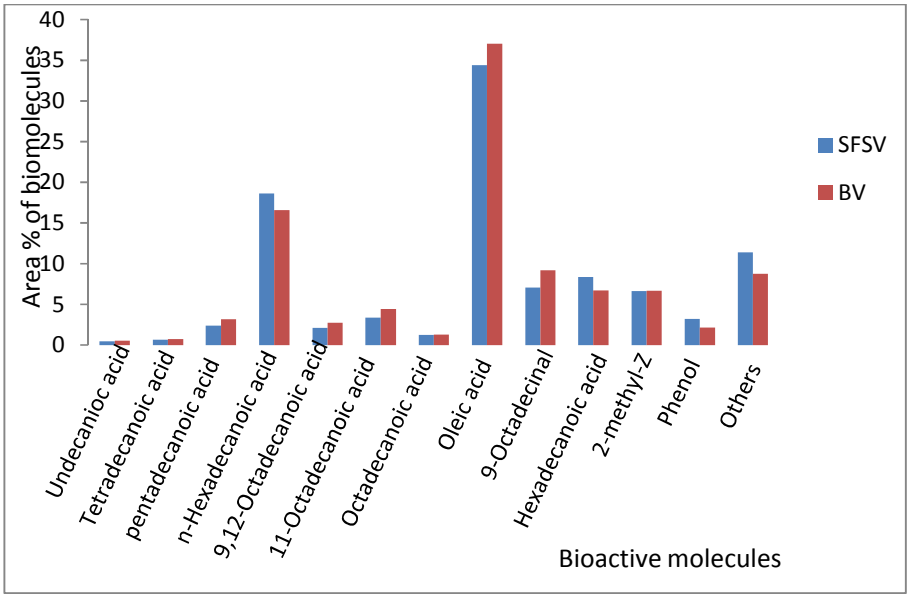

Fig. 3. Comparative analysis of major bioactive molecules in brown variety and speckled flecked sported variety of *Vigna subterranea*

Key: BV-brown variety; SFSV-Speckled Flecked Spotted Variety

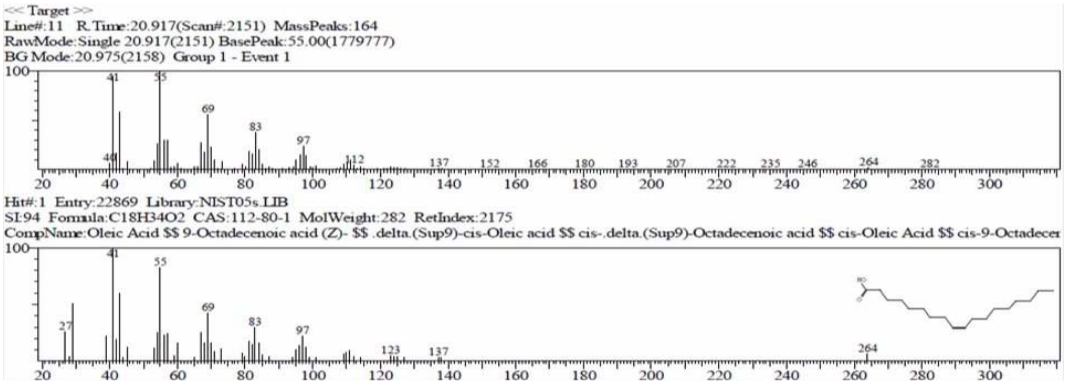

Fig. 4. Mass spectra and molecular structure of oleic acid

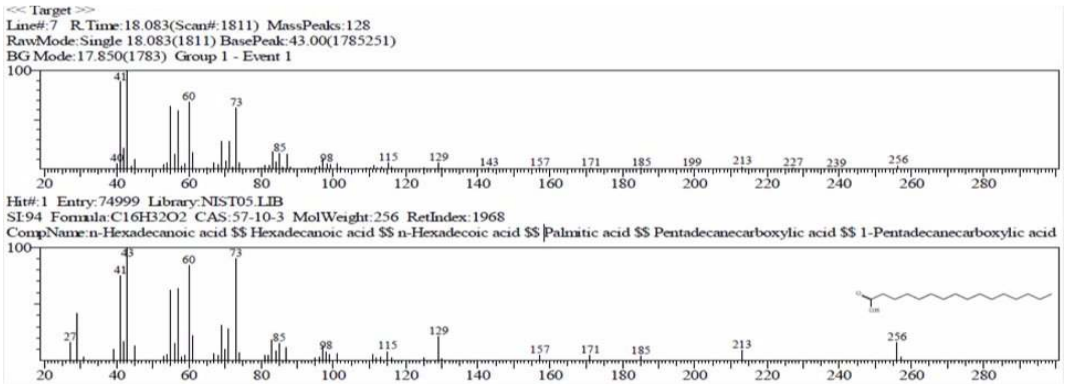

Fig. 5. Mass spectra and molecular structure of n-hexadecanoic acid

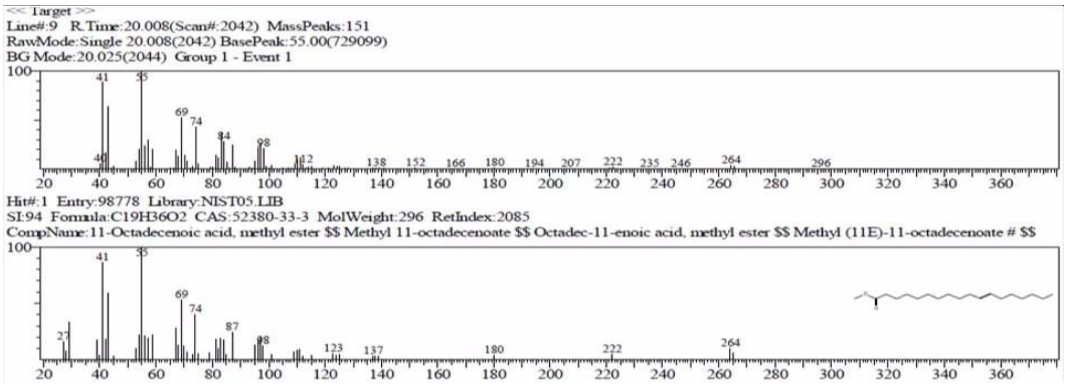

Fig. 6. Mass spectra and molecular structure of 11-octadecenoic acid

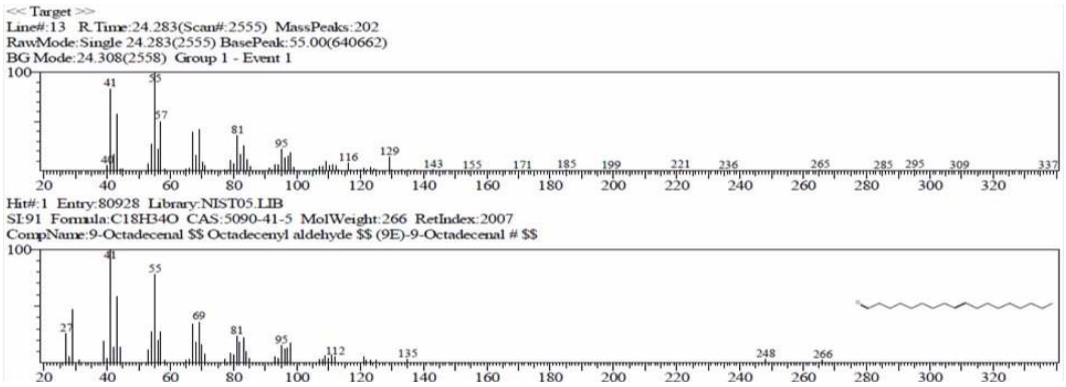

Fig. 7. Mass spectra and molecular structure of 9-octadecenal

and alpha-linoleic acid). A healthy nut will present the essential food classes. In addition, the Brown variety is one of the few plants that contain the antioxidant Squalene. Squalene, though a metabolite and precursor of cholesterol, it reduces the production of unhealthy cholesterol and destroys cancer cells in the blood according to [13]. The occurrence of similar biomolecules (fatty acids, sugars, amino acids, phenols, and alcohols) is likely responsible for the similarities in the physical and chemical properties (such as close range of moisture contents and pH) in the varieties. On the other hand, the dissimilarity in the percentage abundance or absence of some compounds in a variety may have contributed to some of the variations in organoleptic properties.

Aspergillus niger is a known ochratoxin A and fumonisins producer according to the studies by [14]. However, this result indicates that, BV and SFSV varieties of *Vigna subterranea* posses resistance to mycotoxin production by *Aspergillus niger, Penicillium spp Mucor spp* and *Rhizopus nigrican*. It is therefore worthy to say that the absence of mycotoxin may be attributed to some unfavorable factors associated with the storage and internal properties of the samples studied; since mycotoxin production is synergistic effect of favourable condition to fungi contaminants. This result agrees with the findings of [15] who stated that the best range for mycotoxin production is within the range of 4.0-4.5 and that the pH range from 5.5 and above reduce or inhibit mycotoxin production. The high growth of fungal species with no mycotoxin production within the pH of the varieties agrees with the findings of [16]. The moisture content for favorable mould growth and mycotoxin production (<14.5%) fell below the range stated by [17].

Furthermore, the antifungal activities of some of the fatty acids (hydroxyl and methyl fatty acids) identified in the varieties of *V. subterranea* cannot be silenced. Palmitic acid (16.59% in BV, and 18.62% in SFSV), Lauric acid and Myristic acid have antifungal properties against *Aspergillus* and *Penicillium* which is the major toxigenic group identified [18].

4. CONCLUSION

Vigna subterranea is rich in fatty acids, sugar and amino acid. Mycotoxin production in *V. subterranea* is a synergistic effect of mould contamination and the necessary favourable environment. Fungal contamination of *V. subterranea* with no favourable pH, moisture content and temperature will likely not favour mycotoxin production. Little variations in the bioactive components of the varieties may ameliorate the resistance of varieties to spoilage organism or mycotoxin production by moulds.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Mohamed EZ. Impact of mycotoxin on humans and animals. Journal of Saudi-Chemical Society. 2011;15:129-144.
2. Gahukar RT. Food adulteration and contamination in India: Occurrence, implication and safety measures. International Journal of Basic and Applied Science. 2014;3:47-54.
3. Stoev SD. Food safety and increasing hazard of mycotoxin occurrence in foods and feeds. Journal of Critical Review for Food Science and Nutrition. 2013;53:887-901.
4. Nwankwo JI, Ogunbodede TT, Ukpai EG. Mycogenera of stored cereal grains in ogbete main market, Enugu State, South East Nigeria. International Journal of Current Microbiology and Applied Science. 2015;4:875-883.
5. Kristian FN. Mycotoxin production by indoor moulds. Journal of Fungal Genetics and Biology. 2003;(39):103-117.
6. Aviara NA, Lawal AA, Atiku AA, Hague MA. Bamabara groundnut processing, storage and utilization in North Eastern Nigeria. Continiental Journal of Engineering Sciences. 2013;8:28-36.
7. Hillocks RJ, Bennett C, Mponda OM. Bambara nut: A review of utilization, market potential and crop improvement. African Crop Science Journal. 2012;(20):1-16.
8. Okonkwo SI. Opara MF. The analysis of bambara nut (*Vandezia subterranea* (L) thouars) for sustainability in Africa. Research Journal of Applied Science. 2010;(5):394-396.
9. Anonymous. Bambara groundnut: Effect of packaging material and oil type on sensory properties and microbial stability. Available:http://Www.Doublegist.Com/Bambara-Groundnut-Effect-Packaging-Material-Sensory-Properties/
   (27/6/2016)
10. Abriba C, Lennox JA, Asikong BE, Asitok A, Ikpoh SI, Henshaw EE, Eja ME. Isolation of aflatoxin producing species of *Aspergillus* from foodstuffs sold in Calabar markets, Cross River state Nigeria. Journal of Microbiology and Biotechnology Research. 2013;(3):8-13.
11. Nnaji PT, Akan AB. Assessment of post-harvest insect and mould infestation of *Vigna subterranea.* 2016;13(1). *(in press).*
12. Aguoru CU, Onda MA, Victor T, Ogbonna IO. Characterisation of moulds associated with processed garri stored for 40 days at ambient temperature in Makurdi, Nigeria. African Journal of Biotechnology. 2014;13:673-677.
13. William L, Bikul D, Kelly GS. Role of squalene in health. Natural Sources for Health Science for Life.
14. Soares C, Calado T, Venacio A. Mycotoxin production by Aspergillus niger aggregate strains isolated from harvested maize in three Portuguese region; 2013.
15. Brzonkalik K, Hummer D, Cyldatk C, Neuman A. Influence of pH & Carbon to nitrogen ratio on mycotoxin production by alternaria alternate in submerged cultivation; 2012.

16. Al-Gabri H, Ye C, Zhang Y, Khan S, Lin H, Zheng T. Effect of carbon, nitrogen and Ph on the growth of *Aspergillus paraciticus* & aflatoxin production; 2012.
17. Trenk HL, Hartman PA. Effects of moisture content & temperature on aflatoxin production in corn. Applied Microbiology. 1970;5:781-784.
18. Altieri C, Cardillo D, Bevilacqua A, Sinigalia M. Inhibition of *Aspergillus* species and *Penicillium* species by fatty acids and thin monoglycerides. Journal of Food Protection. 2007;(5):1206-1212.

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