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VOLATILE ORGANIC COMPOUNDS PRODUCTION DURING SPOILAGE OF AFRICAN HORNED CUCUMBER FRUITS

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ABSTRACT: Microorganisms associated with the production of volatile organic compound in spoilt African horned cucumber have been isolated by standard plate count (SPC) and identified. The mean aerobic colony count observed for bacteria ranged from 3.3 to 8.3 x 10^7 CFU/g while those of fungi ranged from 4.0 x 10^3 to 1.1 x 10^4 CFU/g. The organisms isolated and identified included three species of bacteria (*Bacillus lentus, Bacillus firmus* and *Paenibacillus alveii*) while three of fungi (*Rhizopus stolonifer, Mucor circinelloides*, and *Monascus ruber*). GC-MS analysis revealed the presence of 11 volatile organic compounds in the healthy ripe African horned cucumber and 21 volatile organic compounds in spoilt African horned cucumber. Six volatile organic compounds were common to spoilt and healthy African horned cucumber while 1,2,3, trimethyl benzene, Decane 1,4-Dimethyl-2-ethyl benzene, tetralin, 6- methyl tetralin, phenyl benzene (Lemonene), 2,3-dimethylnaphthale (Guajen), (1-Ethyl-2-methyl-1-propenyl) benzene, Diphenyl methane (Ditan), Hexadecane, Heptadecane, 6-octadecanoic acid (Z) and 1-Nondecanol were unique to spoilt African horned cucumber fruits. This study suggests that these unique volatile organic compounds could provide baseline knowledge for curbing postharvest losses and the volatile organic compound could form the basis for constructing a metabolomics database for Nigeria.

Keywords: GC-MS, spoilage organisms, metabolomics, post-harvest losses, volatile organic compounds

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INTRODUCTION

Cucumis metuliferus belongs to the family *Cucurbitaceae*, and is a monoecious, climbing, annual herb that can be grown practically anywhere, provided the season is warm (Benzioni et al., 1993). The plant is endemic to the semiarid regions of Southern and Central Africa (Morton, 1987), the fruits are ovoid berries of 8-10 cm long and 4 - 5 cm in diameter, reddish orange at maturity, hanging, covered with strong spiny outgrowths; and the seeds are embedded in the mesocarp which is emerald green and consists of juicy, bland – tasting tissues. It is commonly known as African horned cucumber, melano, Jelly melon, and more recently, kiwano. The fruits occur in two forms the bitter and non-bitter forms, mostly in the wild state. The bitter form contains cucurbitacins (triterpenoids), which are highly toxic compound (Teuscher and Lindequist, 1994). The non-bitter form has been found to be less toxic and has also been widely cultivated (Enslin et al., 1954; Andeweg and De Bruyn, 1959).

The fruits of the non-bitter form have been claimed to cure HIV/AIDS positive patients in and around Jos of Plateau State, Nigeria (Wannang, 2007). It has been reported that the fruits and seeds of *Cucumis metuliferus* are eaten raw as supplements by local populations of Africa (Bruecher, 1977; Keith and Renew, 1975). Reports have also shown that the seeds can be ground into fine flour, made into an emulsion with water, and then eaten to expel parasites from the body (Chiej, 1984).
In some areas of Plateau state of Nigeria, the fruit pulp of the plant is used by traditional healers for the management of various ailments including peptic ulcer disease, diabetes mellitus, hypertension and HIV/AIDS (Wannang, 2007). It is believed that the fruit pulp is useful in all diseases-hence the local name kanda (literally meaning 'stop it before it comes'. ie as a vaccine). It is an adjunct in the treatment of 'watery sperm' in males. The plant is widely eaten by many African populations as food supplements as it is also claimed to possess anti-helmintic activity (Bruecher, 1977; Keith and Renew, 1975; Chiej, 1984). The fruit pulp was found to possess alkaloids, flavanoids, glycosides (Jimam, 2008), which are known to produce biological activity.
Post-harvest diseases of fruits and vegetables caused by fungal and bacterial pathogens result in significant economic losses. One of the limiting factors in reducing losses is the non-availability of an efficient early detection system for the presence of the disease (Prithiviraj et al., 2004). Several sensitive systems like ELISA and PCR based methods have been developed for detecting plant diseases (Schaad and Frederick, 2002; Somai et al., 2002; Jeong et al., 2003). However, such methods are not at the disposal of most developing countries Nigeria in Particular. The volatiles of several fruits and vegetables have been extensively studied to detect and discriminate diseases (de Lacy Costello et al., 1999; Kushalappa et al., 2002). The research work is aimed at: Isolating and identifying bacteria and fungi from spoilt and healthy African horned cucumber fruits extract and identify volatile organic compounds from the diethyl ether extract of spoilt and healthy African horned cucumber fruits.

MATERIALS AND METHODS

Sample Collection

Ten each of spoilt and healthy intact ripe African horned cucumber fruits were collected from major areas were the fruits are found in Sokoto metropolis. Samples were collected into clean polythene bags and transported immediately to the research laboratory of Usmanu Danfodiyo University, Sokoto for further analysis.

Isolation of microorganisms

Bacteria were isolated by transferring an aliquot of 0.1 ml of a serially diluted (10^6) sample of spoilt mango fruits in test tubes onto molten nutrient agar plates and incubated at 37ºC for 24 hours. The colonies that emerged were sub-cultured continuously until a pure culture is obtained.

On each of the dried Potato Dextrose Agar plates to which streptomycin had been incorporated were inoculated in the processed samples using sterile forceps. The inoculated plates were incubated upright at room temperatures for 5 days. All observed colonies were sub-cultured to obtain pure cultures (ICFM, 2007).

Identification of Bacteria and Fungi

The bacterial isolates were identified following series of biochemical test as described by Holt et al. (1994). For the fungi, the growth rate, colour, texture, colonial morphology and diffusible pigmentation of each sample were examined macroscopically. For fungi, tease mount using lactophenol cotton blue was adopted and microscopic features such as spore and hyphae morphology were observed and compared with the standard colour atlas as described by Ochei and Kolhatkar (2000).

Extraction of volatile Metabolites

Volatile compounds were extracted using general purpose solvent Parliment (1997) as described by Ibrahim et al. (2011). Extraction of volatile compounds was done by direct solvent extraction method. Ten grams of spoilt African horned cucumber fruits and healthy ripe African horned cucumber fruits were weighed into bottles and saturated with 20 ml of diethyl ether. They were allowed to stand at room temperature for 24 hours, filtered using Whatman No. 1 filter Paper and the filtrate was collected in sterile bottles, closed tightly before the GC-MS analysis.

Gas chromatography-Mass spectrometry (GC-MS) analysis

GC-MS analysis was performed using GC-MS-QP2010 plus (Shimadzu, Japan) equipped with flame ionization detector (FID). The injection was conducted in split less mode at 250 ^{0}C for 3min by using an inlet of 0.75mm i.d to minimize peak broadening. Chromatographic separations were performed by using DB-WAX analytical column 30 m 0.25 mm, 0.25mm (J&W scientific, Folsom C.A) with helium as carrier gas at a constant flow rate of 0.8 ml/Min. The oven temperature was programmed at 60 ^{o}C for 5min, followed by an increase (held for 5 min), and finally at 10^{0}C/min to 280 ^{o}C (held for 10min). The temperature of the FID was set to 250 ^{o}C. MS operating conditions (electron impact ionization mode) were an ion source temperature of 200 ^{o}C, ionization voltage of 70 eV and mass scan range of m/z 23- 450 at 2.76 scans/s.

Identification and quantification of volatile Metabolites

The chromatographic peak identification was carried out by comparing their mass spectra with those of the bibliography data of unknown compounds from the NIST library mass spectra database on the basis of the criterion similarity (SI)>800 (the highest value is 1,000). According to the method of (Wanakhachornkrai and Lertsiri, 2003) approximate quantification of volatile compounds was estimated by the integration of peaks on the total ion chromatogram using Xcalibur software (Vienna, VA). The results are presented as the peak area normalized (%).

Statistical analysis

The data sets were expressed as mean $\pm$ standard deviation (n=3). Analysis of variance (ANOVA) was done using One-Way Analysis of Variance to test for the difference in means. Paired Sample T-Test was used to test for the significance between samples at α (0.05) level of significance using the SPSS for Windows, version 15.0. (Chicago IL, USA).

RESULTS

A study on viable bacterial count of unspoilt and spoilt African horned cucumber was conducted and the result presented in Table 1. From the result, spoilt African horned cucumber had the highest viable count of 8.3 x 10^7 CFU/g while unspoilt had 3.3 x 10^7 CFU/g.

The viable count of fungi isolated from unspoilt and spoilt African horned cucumber was evaluated and the result presented in Table 2. From the result spoilt African horned cucumber 1.1 x 10^4 and 4.0 x 10^3 CFU/g.

Biochemical characterization of bacterial isolates from spoilt African horned cucumber revealed that the organisms were *Bacillus lentus, Bacillus firmus,* and *Paenibacillus alvei.* While the morphological and microscopic characterization of fungi isolated from spoilt and unspoilt African horned cucumber fruits revealed the presence of *Rhizopus stolonifer* as the only fungus isolated from healthy African horned cucumber fruits while *Mucor circinelloides* and *Monascus ruber* was isolated from spoilt sample.

GC-MS analysis of the diethyl-ether extract of healthy and spoilt African horned cucumber was evaluated and the resulted presented in Table 3. From the result, a total of 11 and 21 volatile organic compounds were identified in the healthy and spoilt fruits with Nonane (47.84%) being the predominant in healthy fruits followed by 1,6- Methano [10] Annulene (8.60%) and least 4, phenylbut-3-ene-1-yne (1.12%) while spoilt fruits had 1,6- Methano [10] annulene (25.58%) as the predominant followed by Nonane (12.44%) and least among them are Diphenylmethane (Ditan) (0.87%), 6- octadecanoic acid (Z) (0.87%) and 1-Nonadecanol (0.87%).

Table 1: T-test result of viable counts (x10^7) of bacteria isolated from African horned cucumber.

Treatments	Viable bacterial counts (x 10^7)
Spoilt	$8.300^a \pm 12.73$
Unspoilt	$3.300^b \pm 5.66$
T-value (P-value)	5.077(0.037)

Table 2: T-test result of viable counts (x10^3) of fungal isolated from African horned cucumber

Treatments	Viable bacterial counts (x 10^3)
Spoilt	$11.00^a \pm 2.83$
Unspoilt	$4.00^b \pm 2.83$
T-value (P-value)	2.475(0.132)

Table 3: Result of GC-MS analysis of spoilt African horned Cucumber

RT-1 (min)	Compound	Peak Area (%)	
		Spoilt	Healthy
4.00	Octane	-	5.16
4.70	Ethylcyclohexane	-	1.37
6.71	Nonane	12.44	47.84
7.23	2,2–Dimethlvaleraldehyde (2,2-Dimethyl Pental)	-	2.24
9.41	1,2,3-Trimethybenzene	2.44	-
9.41	Isopropylbenzene	-	2.91
10.12	Decane	5.15	-
10.67	Tetracyclo [3.3.1.0 (2,8).0(4,6)]-non-2-ene	1.13	1.19
12.50	1,4-Dimethyl-2-ethylbenzene	1.49	-
13.65	1-Isopropyl-2-methylbenzene	1.03	-
14.75	Tetralin	2.18	-
15.32	4-phenybut-3-ene-1-yne	2.72	1.12
17.93	6-Methyltetralin	1.18	-
18.68	1,6 – Methano [10] annulene	25.58	8.60
20.90	Phenylbenzene (Lemonene)	1.38	-
21.36	2,3-Dimethylnaphthalene (Guajen)	1.39	-
21.95	(1-Ethyl-2-methyl-1-propenyl) benzene	5.38	-
23.10	Diphenylmethane (Ditan)	0.87	-
24.95	Hexadecane (Cetane)	5.05	-
25.35	1-Ethyl-2-(1-Phenylethyl) benzene	3.29	-
25.95	Heptadecane	3.59	-
27.90	Hexadecanoic acid (Palmitic acid)	3.15	5.14
29.02	6-Octadecenoic acid (Z)	0.87	1.69
29.21	Octadecanoic acid (Strearic acid)	1.76	7.44
30.99	1-Nonadecanol	0.87	-

[1] Retention time (RT) on DB-WBX column in GC-MS.

DISCUSSION

This study revealed that African horned cucumber had a high aerobic colony count and this might be attributed to factors such as the environment which include exposure of the African horned cucumber to air, soil, post production operations and personal hygiene of foods handlers (Ray and Bhunia, 2007, Jay et al., 2005; Beuchat, 1996). Exposure of the foods to air or dust at the point of sale is likely to increase the counts of the bacteria as virtually most of the bacteria are carried in aerosols by dust and air (Food and Drug Administration, 2009). Through microbial quality limit is not applicable to fruits of cucumber (Gilbert, et al., 2000), this count is higher enough to pose microbial hazard making the safety of this fruits questionable.

The bacterial microflora associated with spoilt African horned cucumber in this study were *Bacillus lentus, Bacillus firmus* and *Paenibacillus alvei*. The source of these organisms could probably be from soil particularly because the fruits are in contact with soil during harvesting / packaging containers handlers, air and dust (Jay et al., 2005). Many researchers (Marchett et al., 1992; Garg et al.,1990; Magnusson et al., 1990 ; Carlin et al., 1989 ; Nguyen-the and Prunier, 1989) have determined that 80-90% of mesophilic bacteria in the aerobic plate counts of vegetables are Gram negative rods, *Pseudomonas* spp was not isolated and the only genera in this work is gram positive organism *Bacillus.* This probably is explained by the fact that *Bacillus spp* are able to overcome some of the intrinsic and extrinsic parameters that could have checked their population due to their ability to form spores. *Rhizopus stolonifer* was the only fungus isolated from African horned cucumber while *Mucor circinelloides* and *Monascus ruber* was isolated from spoilt samples. The source of these organisms could probably be from soil particularly because the fruits are in contact with soil, air and dust. This result will be of paramount importance in curbing post harvest losses.
Several compounds were unique to diseased African horned cucumber fruits, which could be qualitatively used to discriminate disease fruits. 1,2,3, trimethyl benzene, Decane 1,4-Dimethyl-2-ethyl benzene, tetralin, 6- methyl tetralin, phenyl benzene (Lemonene), 2,3-dimethylnaphthale (Guajen), (1-Ethyl-2-methyl-1-propenyl) benzene, Diphenyl methane (Ditan), Hexadecane, Heptadecane, 6-octadecanoic acid (Z) and 1-Nondecanol were unique to spoilt African horned cucumber fruits. Octane (5.16%), ethylcyclohexane (1.37%), 2,2–Dimethlvaleraldehyde (2.24%), Isopropylbenzene (2.91%) and 9-Octadecenoic acid (Z) 1.69% were unique to healthy mango fruits. These unique metabolites could be used as biomarkers to detect the presence of the pathogens identified in this study.

Six volatile organic compound were unique to healthy and spoilt African horned cucumber fruits which include Nonane (12.44; 47.84%), Tetracyclo [3.3.1.0 (2,8).0(4,6)]-non-2-ene(1.19%; 1.13%), 4-Phenylbut-3-ene-1-yne (1.12%; 2.27%), 1,6-Methano[10] Annulene (8.60%; 25.58%), Hexadecanoic acid (Palmitic acid) (5.14; 3.15%), Octadecanoic acid (Strearic acid) 7.44; 1.76%. An increase in the relative abundance of 4-Phenylbut-3-ene-1-yne, Nonane and 1,6-Methano[10] Annulene. However, a reduction was observed in the relative abundance of Octadecanoic acid (Strearic acid). The presence and/or absence of the above volatile organic compounds and the differences in their relative abundance could be considered for qualitative discrimination of healthy and spoilt African horned cucumber fruits especially when unique compounds are absent and mixed infections, especially in the same lesion, are present. Similar result had been reported by Moalemiyan et al. (2006) who observed, that certain volatile metabolites were common to stem-end rot and anthracnose diseases of mango fruits.

The fatty acids detected in the spoilt and healthy fruits were octadecanoic acid, Octadecanoic acid and Hexadecanoic acid (Palmitic acid). The hydroxyl form of 9- Octadecenoic acid (Z) that is hydroxy fatty acids (HFA) have been described as multifunctional molecules that have a variety of applications (Bódalo et al., 2005), and they and their derivatives are used in cosmetics, paints and coatings, lubricants and in the food industry (Bódalo et al., 2005).

They are useful chemical intermediates in the synthesis of fine chemicals and pharmaceuticals (Bódalo et al., 2005). Moreover, some of them may protect plants against microbial infection, although the mechanism of these antimicrobial effects is poorly understood (Suzuki et al., 2005). The importance of these esters has been described to contribute to food aroma with the fact that esters with low carbon atoms are highly volatile as precursors (Izco and Torre, 2000; Nogueira et al., 2005). In addition to imparting a fruity floral character, esters can diminish or mask the sharpness of unpleasant free amino acid-derived notes (Yanfang and Wenyi, 2009).

Conclusion

This study suggests that these unique volatile organic compounds could provide baseline knowledge for curbing postharvest losses and the volatile organic compound could form the basis for constructing a metabolomics database for Nigeria.

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