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Volatile Metabolites Profiling for Discriminating Tomato Fruits Inoculated with some Bacterial Pathogens

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ABSTRACT: The volatile metabolites of tomato fruits inoculated with three bacterium isolated from spoilt tomatoes were investigated using gas chromatography/mass spectrometry. Differences in the number and amount of volatile metabolites were observed. The study yielded a total of 66 different volatile metabolites. Healthy ripe tomato fruits yielded twenty-eight metabolites predominated among them are Oleic acid (10.89%), methyl cis-9-octadecenoate (7.73%), octadecanoic acid (9.83%) and the least was 2, 3-Heptanedione (0.32%). Tomato fruits inoculated with *Listeria monocytogenes* and *Brevibacillus laterosporus* yielded 14 volatile metabolites each while that inoculated with *Bacillus megaterium* yielded 16 volatile metabolites. Among them only octadecanoic acid and oleic acid occurred relatively consistently. An increase in the amount of these consistent metabolites was recorded as compared to the amount in the healthy ripe tomato fruits with fruits inoculated with *Bacillus megaterium* recording the highest value for octadecanoic acid (30.22%) and oleic acid (23.80%). Two compounds were common to *Brevibacillus laterosporus* and *Bacillus megaterium*; three compounds were common to *Listeria monocytogenes* and *Bacillus laterosporus;* while four were common to *Listeria monocytogenes* and *Bacillus megaterium*. Eleven metabolites were unique to *Bacillus megaterium* while ten were unique to *Brevibacillus laterosporus* and eight metabolites were unique to *Listeria monocytogenes*. The differences in these volatile metabolites can be potentially exploited for detecting and discriminating pathogens of tomato during post-harvest handling, storage and the possible outbreak of food borne disease after further validation under commercial conditions.

Key Words: Disease diagnosis, disease detection, GC-MS, post-harvest pathogen, Volatile compound, *Listeria monocytogenes* .

INTRODUCTION

Microbial Spoilage is manifested by a variety of sensory cues such as off-colours, off-odours, off- flavours, softening of vegetables and fruits, and slime. However, even before it becomes obvious, microbes have begun the process of breaking down food molecules for their own metabolic needs. Sugars and easily digested carbohydrates are used first, plant pectins are degraded. Then proteins are attacked, producing volatile compounds with characteristic smells such as ammonia, amines, and sulfides. This may be accompanied by the production of a wide range of metabolites which includes: alcohols, sulphur compounds, ketones, hydrocarbons, fluorescent pigments, organic acids, esters, carbonyls, diamines (Doyle, 2007).

Microbial product quality or shelf-life indicators are organisms and/ or their metabolic products whose presence in given foods at certain levels may be used to assess existing quality or, better, to predict shelf-life. These may be: the spoilage organisms themselves, or their metabolic products (Doyle, 2007).

Traditionally, fresh fruits and vegetables have not been considered high-risk foods in terms of causing food-borne illness, especially when compared with foods of animal origin (meat, dairy products and seafood). The general assumptions have been that the pH of fruits and vegetables was too low to support the growth of human disease-causing pathogens, and that the natural barriers of the fruits and vegetables would prevent microbes from entering and subsequently growing inside the food (Beuchat, 1996; Madden, 1992). Nonetheless, the relationship between fresh produce and so-called "travellers' diarrhoea" has frequently been observed; this ailment is sometimes contracted when people travel away from their home country and encounter different foods with different microflora (Kenny, 2002). Several outbreaks of human listeriosis in North America and Europe have been documented in recent years. Most cases have been caused by the consumption of foods from animal origin, particularly soft cheeses, but at least one outbreak has been attributed to the consumption of cabbage contaminated with *Listeria monocytogenes* (Beuchat and Brackett, 1991). In another outbreak involving hospital

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patients, epidemiologic evidence suggested that raw celery, tomatoes, and lettuce may have been vehicles of *L. monocytogenes* infection (Beuchat and Brackett, 1991).
Post harvest losses include the rotting of produce and damage during storage, packaging and transportation which leads to consumer rejection (Kader and Rolle, 2004). Most losses and wastes occur in the latter part of the food chain through excessive processing, packaging and marketing (FAO, 2008). Post harvest loss can be defined as a measurable quantitative and qualitative loss of a given product at any moment along the post harvest chain (De lucia and Assemato, 1994). Post harvest loss is much more painful and costlier than pre harvest loss both in terms of money and man-hours (Dasgupta and Mandal, 1989). Post harvest losses which decrease returns of fruits and vegetables occur mainly because of lack of infrastructure, poor handling and marketing knowhow (Prigojin, et al., 2004). The magnitude of losses depends on the nature of the commodities, the condition of the produce at the time of collection, distance travelled and the nature of the road network. The principal causes of losses are physiological deterioration, mechanical damage and pathological damage (Kader and Rolle, 2004). According to Adeoye et al. (2009); pathological damage constituted the greater percentage (44%) of losses in a particular variety of tomatoes in Ibadan. The occurrence of bacteria in spoilt tomato fruits and have been reported (Goldoni et al., 1992). This study is aimed at identifying disease-discriminatory volatile metabolites released from tomato fruits inoculated with *Listeria monocytogen; Bacillus megaterium* and *Bacillus Laterosporus.* The study will also profile the volatile metabolites using GC-MS analysis to discriminate/detect the presence of *Listeria monocytogenes; Bacillus megaterium* and *Bacillus Laterosporus* in spoilt tomato fruits.

MATERIALS AND METHODS

Sample Collection

Spoilt and healthy intact ripe tomatoes fruits were purchased from Kasuwan Daji, in Sokoto metropolis. Samples were collected into polythene bags and immediately transported to the laboratory for the analysis.

Bacterial inoculum preparation

Bacteria used in this work were isolated from spoilt tomato fruits obtained within sokoto metropolis. The bacterial isolate were identified following series of biochemical test as described by Holt et al. (1994) and maintained on nutrient agar slants. The colonies were subculture onto molten nutrient agar plates and incubated at 37 ^{o}C for 24 hours. The inoculum was prepared as described by Opara and Odibo (2009) with little modification. It was prepared by suspending the young active colonies from the culture into sterile distilled water, By serial dilution and plating, the number of viable colonies in the inoculum was found to be 3.2 x 10^6 CFU/ml for *Bacillus megaterium;* 2.5 x 10^6 CFU/ml for *Brevibacillus laterosporus;* and 2.2 x 10^6 CFU/ml for *Listeria monocytogenes,* was used as inoculum for the subsequent pathogenecity test.

Inoculation of tomato fruit (Pathogenecity test)

This was done as described by Opara and Odibo (2009) with little modification. Mature ripe and intact tomato fruits were sterilised with cotton wool soaked in 70% ethanol. When dry, 0.5 ml of inoculum suspension was injected into the fruit and the needle puncture hole was sealed with Vaseline to prevent drying and contaminations. A metal spatula was flamed, dipped into Vaseline and quickly smeared over the punctured holes. The spatula was flame sterilized between treatments. The inoculated fruits were kept in a closed humid chamber lined with damp filter paper and incubated at 27°C for 7-10 days.

Extraction of volatile compound

Volatile compounds were extracted using general purpose solvent as described by (Parliment, 1997). Extraction of volatile compounds was done by direct solvent extraction method as described by Ibrahim et al., 2011. Two gram of spoilt tomatoes was weighed into a bottle and saturated with 20ml of diethyl ether. It was allowed to stand at room temperature for 24 hours, filtered using Whatman filter Paper and the filtrate was collected in a sterile bottle, 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. Chromatography separations were performed by using DB-WAX analytical column 30m 0.25mm, 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 10 min). 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 volatile compounds

The chromatography 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 (%).

RESULTS

Bacteria microflora of spoilt tomatoes fruits which include *Listeria monocytogenes, Bacillus megaterium* and *Brevibacillus laterosporus* were inoculated into ripe healthy and intact tomato fruits. Volatile metabolite profile of healthy tomato fruits was determined by GCMS analysis of its diethyl ether extract and the result presented in Table 1. From the result, twenty-eight metabolites were determined. Predominated among them are Oleic acid (10.89%), methyl cis-9-octadecenoate (7.73%), octadecanoic acid (9.83%) and the least was 2, 3-Heptanedione (0.32%).

Table 1: Result of GC/MS analysis of ripe healthy tomatoes fruit

RT[-1] (min)	Compounds metabolites	Peak area normalized (%)
3.83	2-Ethylhexane (3-methylheptane)	5.78
4.461	Ethylcyclohexane	1.63
5.11	2-Methyl-4,6-octadiyn-3-one	2.98
6.34	5,6-Dimethylundecane	6.16
7.17	3-Hexen-2-one/(3E)3-Hexen-2-one	1.15
7.46	2,2-Dimethylbutane	1.26
7.87	1,2-Diphenyl-1-butanone	1.65
8.94	Isopropylbenzene (2-phenylpropane)	5.08
9.64	3,5-Dimethyloctane	7.28
10.16	2-Phenyl-3-buten-1-ol	2.55
10.44	2,4,4-Trimethylhexane	0.71
10.87	Benzoylcarboxaldehyde (Phenylglyoxal)	0.91
12.33	Endo-tricyclo [5.2.1.0(2.6)] decane	1.80
12.95	2,4-Dimethyl-3-hexanone	1.64
14.16	Benzene acetic acid,2-phenylethyl ester	1.59
14.72	Cyclopentacycloheptene (Azulene)	1.42
16.05	2,3-Heptanedione(Acetyl valeryl)	0.32
18.02	1,6-Methano[10] annulene	3.97
18.44	1-Naphthaleneacetic acid, methyl ester	3.19
21.38	N(Dimethylsulfonio)methanesulfonimidoate	0.42
26.69	Methyl tridecanoate	2.05
27.09	Cis 9-Octadecanoic acid	7.73
27.33	Methyl-15-methylhexadecanoate(methyl isohepta-decanoate)	3.28
27.59	Octadecanoic acid	9.83
28.52	Methyl cis-9-octadecenoate	9.83
28.75	Oleic acid	10.89
29.07	Methyl 2-ethyl-2-methyllicosanoate	2.40
30.65	Tetradecahydrobenzo[a] cyclodecene	2.50

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

Volatile metabolite of spoilt tomato fruits inoculated with *Listeria monocytogenes* was determined by GCMS analysis of its diethyl ether extract and the result presented in Table 2. The result revealed that fourteen volatile metabolites were produced. Predominated among them are octadecanoic acid (19.82%), 1-Hexadecanol (17.01%) and cyclopentacycloheptene (1.94%) having the least value.

Table 2: Result of GC/MS analysis of ripe healthy tomato fruit inoculated with *Listeria monocytogenes* (Lm2)

RT[-1] (min)	Compounds metabolites	Peak Area Normalized (%)
3.83	4-Methyloctane (Isononane)	9.08
6.34	Undecane	10.21
8.74	1,2,3-Trimethylbenzene	6.27
9.65	3,7-Dimethylundecane	11.84
12.33	N-Acrylonitrylaziridine	2.44
12.96	2,3-Heptanedione (Acetyl valeryl)	2.62
14.17	1-Methyl-3-beta-phenylthyl-2,4,5-trioxoimidazolidine	2.05
14.72	Cyclopentacycloheptene (Azulene)	1.94
18.02	2-Isopropenyl-5,5-dimethyl-1,3-dioxane	6.23
18.44	1-Napthaleneacetic,methyl ester	4.99
27.60	Octadecanoic acid (Hystrene S-97)	19.82
28.76	1-Hexadecanol (Adol52)	17.01
29.65	3-methylcyclopentan-1,2-diol	3.16
30.65	1-Hexyl-1-nitrocyclohexane	2.34

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

Volatile metabolite of spoilt tomato fruits inoculated with *Bacillus megaterium* (Bm_2) was determined by GCMS analysis of its diethyl ether and the result presented in Table 3. From the result, it revealed that sixteen metabolites were produced, predominated by octadecanoic acid (30.22%), oleic acid (23.80%) and pyrrole-2- carboxaldehyde (0.99%) was the least.

Table 3: Result of GC/MS analysis of ripe healthy tomato fruit inoculated with *Bacillus megaterium* (BM2)

RT[-1] (Min)	Compounds	Peak Area Normalized (%)
3.84	3,3-Dimethylhexane	5.34
6.37	2-Ethylnonane	7.10
8.95	Acetophenone (Hypnone)	2.84
9.67	3,7-Dimethylnonane	8.05
10.19	Cis-propenylbenzene	1.74
12.36	Pyrrole-2-carboxaldehyde	0.99
12.98	2,2-Dimethyl-3-hexanone	1.44
14.19	1-Methyl-3-beta-phenylethyl-2,4,5-trioxoimidazolidine	1.33
14.74	4-Isothiazolecarboxamide	0.83
18.04	1,6-Methano[10] annulene	4.65
18.46	1H-indene,1-ethylidene	3.32
27.61	Octadecanoic acid	30.22
28.76	Oleic acid	23.80
29.66	3-Methylcyclopentan-1,2-diol	3.02
30.66	1-Hexyl-1-nitrocyclohexane	3.81
32.00	3-Penten-2-one,4-methyl (isopropyldene-acetone/Mesityloxide)	1.53

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

Volatile metabolite of spoilt tomato fruits inoculated with *Brevibacillus laterosporus* (Bl_1) was determined by GCMS analysis of its diethyl ether extract and the result presented in Table 4. The result revealed that fourteen volatile

metabolite were produced, predominated among them are octadecanoic acid (21.05%), oleic acid (18.33%) and 5 - (3-oxohexyl)-2-pyrrolidinone (1.46%) having the least.

Table 4: Result of GC/MS analysis of ripe healthy tomato fruit inoculated with *Brevibacillus laterosporus*

RT[1] (min)	Compounds	Peak Area Normalized (%)
3.84	2-Ethylhexane (3-Methylheptane)	10.02
6.35	Decane	8.57
8.94	2-(3-Hydroxy-2-nitrocyclohexyl)-1-phenylethanone	4.27
9.64	Undecane	9.02
12.3	2-Norbornane carbonyl chloride	1.55
18.01	1-Methylbutyl2-[(trifluoroacetyl)amino]	4.27
18.43	1-Napthalene acetic acid, methylpropanoate ester	3.50
27.60	Octadecanoic acid (Hystrene S-97)	21.05
27.81	Ethyldodecanoate (Ethyl laurate)	3.54
28.76	Oleic acid	18.33
28.93	Ethyl oleate	6.55
29.10	Ethyl hexadecanoate	5.33
29.65	5-(3-oxohexyl)-2-pyrrolidinone	1.46
30.65	Bicyclohexyl,2-methyl-,trans.	2.52

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

DISCUSSION

The GCMS analysis carried out is the first study to provide data on the composition of the diethyl ether extract volatile metabolites of tomatoes inoculated with bacterial pathogens and provides the basis for discriminating the post harvest diseases caused by *Listeria monocytogenes, Bacillus megaterium,* and *Brevibacillus laterosporus*. Several compounds were unique to a disease/inoculation, which could be qualitatively used to discriminate diseases studied here, in unknown disease samples. Decane, 2-(3-Hydroxy-2-nitrocyclohexyl)-1-Phenylethanone, 2-Norbornane, carbonylchloride, 1-Methylbutyl,2-[(trifluoroacetyl)amino], Ethyldodecacanoate (ethyl laurate), Ethyl oleate, Ethyl hexadecanoate, 5-(3-oxohexyl)-2-pyrrolidinone and Bicyclohexyl,2-methyl-,trans were unique to *Brevibacillus laterosporus* inoculated tomatoes, which could be discriminated from *Bacillus megaterium* ones, which also produced unique metabolites, . 3,3-Dimethylhexane, 2-Ethylnonane, Acetophenone (Hypnone), 3,7-Dimethylnonane, Cis-Propenylbenzene, Pyrrole-2-aldehyde, 2,2-Dimethyl-3-hexanone, 4-Isothiazole Carboxamide trioxoimidazolidine, 1,6-methano[10] annulene, 1H-Indene,1-ethylidene, and 3-Penten-2-one,4-methyl(Isopropylidene-acetone/mesityloxide) and *Listeria monocytogenes* ones, which also produced unique metabolites, 4-methyloctane (Isononane), 1,2,3-Trimethylbenzene (Hemimellilene), 3,7-Dimethylundecane, 1-Hexadecanol (Adol 52), 2-Isopropenyl-5,5-dimethyl-1,3-dioxane and N-Acrylonitrylaziridine (3-Aziridinoacrylonitrile). These unique metabolites can be used as biomarkers to detect the presence of these bacteria in spoilt tomato fruits. Disease-specific metabolites have been detected in other diseased fruits and vegetables. In a study on apples, methyl acetate was found to be unique to fruits inoculated with *Botrytis cinerea*, 4-methyl-1-hexene to fruits inoculated with *Mucor piriformis*, 2-methyltetrazole and butyl butanoate to fruits inoculated with *Penicillium expansum*, and 3,4-dimethyl-1-hexene and fluorethene to fruits inoculated with *Monilinia* sp. (Vikram et al., 2004a). 1-Pentanol and ethyl boronate were also reported to be unique for bacterial soft rot of carrot (Vikram et al., 2006). One (1)-pentanol and ethyl boronate, were detected in *L. theobromae* inoculated mangoes alone, while thujol was observed only in *C. gloeosporioides* inoculated mangoes (Moalemiyan et al., 2006). Acetyl hydrazide, propylcarbamate, propenyl bromide, acetone, 1-ethenyl-4-ethyl benzene, thiirane and 1-(methylthio)- E-1-propene were unique to onion bulbs inoculated with *Botrytis allii*, while 3-bromo furan was specific to bulbs inoculated with *E. carotovora* subsp. *carotovora* (Prithiviraj et al., 2004). Also, 4-mercapto-3- (methylthio)-c-(thiolactone)-crotonic acid and 1-oxa- 4, 6- diazacyclooctane-5-thione were unique to *Fusarium oxysporum*-inoculated onions (Prithiviraj et al., 2004). Seven unique compounds, viz. 1-pentanol, 3-methylbutanol, 2-methylpropanol, 2, 3-butanedione, ethyl boronate, isopentyl methyl ether and ethane ethoxy were detected in carrots (cv. Vita Treat) inoculated with *E. carotovora* subsp. *carotovora* (Vikram et al., 2006). The use of unique compounds for disease/pathogen discrimination may be valid if the lesions are spatially separated, but their uses when the diseases occur together in the same lesion remain to be validated.

The metabolites undecane and 1-Napthalene acetic acid, methyl ester were common to *Listeria monocytogenes* and *Brevibacillus laterosporus* inoculated tomatoes, but were absent in the healthy tomatoes. *Listeria monocytogenes* inoculated tomatoes had the highest amount of undecane (10.21%) than *Brevibacillus laterosporus* inoculated tomatoes which had 9.02%. The absence of these compounds in healthy tomatoes agrees with the findings of Yilmaz, (2001). The presence and/or absence of the above metabolites and the differences in their relative abundance could be considered for qualitative discrimination of *Listeria monocytogenes* and *Brevibacillus laterosporus*, especially when unique compounds are absent and mixed infections, especially in the same lesion, are present.

Several compounds were common to *Bacillus megaterium* and *Listeria monocytogenes* inoculated tomatoes. The metabolites 3-methylcyclopentan-1,2-diol, 1-Hexyl-1-nitrocyclohexane and 1-methyl-3-beta-phenylethyl-2,4,5 trioxoimidazolidine were common to tomatoes inoculated with the two genera of bacteria. Tomatoes inoculated with *Listeria monocytogenes* had the highest amount of 1-methyl-3-beta-phenylethyl-2,4,5 trioxoimidazolidine (2.05%) and 3-methylcyclopentan-1,2-diol (3.16%) while *Bacillus megaterium* inoculated tomatoes had the highest relative

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abundance of 1-Hexyl-1-nitrocyclohexane (3.81%). Even though these compounds were common to *Bacillus megaterium* and *Listeria monocytogenes* inoculated tomatoes, the differences in their relative abundance can help to detect and discriminate diseases/ *Bacillus megaterium* and *Listeria monocytogenes*, especially in the absence of unique or other disease-discriminatory compounds (Moalemiyan et al., 2006).

Octadecenoic acid (Hystrene S-97) was produced in tomatoes inoculated with the pathogens and in healthy fruits. *Bacillus megaterium* and *Brevibacillus laterosporus* inoculated tomatoes produced higher amount of octadecenoic acid (Hystrene S-97) (30.33% and 21.05%) than *Listeria monocytogenes* (19.82%) inoculated tomatoes while healthy tomatoes had the least (7.73%). The increase in the relative abundance of octadecenoic acid (Hystrene S-97) observed in *Bacillus megaterium* and *Brevibacillus laterosporus* inoculated tomatoes could probably be from the tomatoes seed oils and microorganisms (Hayes, 1996). Even though this compound was common to all the isolates, the differences in their relative abundance can help to detect and discriminate diseases/toxigenic bacteria, especially in the absence of unique or other disease-discriminatory compounds. Some of the hydroxyl form of the above compounds may protect plants against microbial infection, although the mechanism of these antimicrobial effects is poorly understood (Suzuki et al., 2005).

The volatile metabolite, oleic acid was common to tomato fruit inoculated with *Bacillus megaterium* and *Brevibacillus laterosporus* and healthy tomato fruit. Even though this compound is common to both the inoculated and healthy tomato fruit, there was a difference in their relative abundance. *Bacillus megaterium* inoculated fruit had the highest relative abundance (23.80%) compared to *Brevibacillus laterosporus* inoculated tomato fruit (18.33%) while healthy tomato fruit had the lowest relative abundance (10.89%).

Over 400 different aroma volatile compounds were identified in tomato fruit (Petro-Turza, 1987). Variation was observed in the number and occurrence of some volatile metabolites. Some compounds were detected only in healthy tomatoes. The inconsistency of exogenous metabolites among replicates has been reported in earlier studies on other crops (Prithiviraj et al., 2004; Vikram et al., 2004a, Vikram et al., 2004b; Lui et al., 2005). Such variation is also not unusual in endogenous metabolic profiling studies (Roessner et al., 2001; Dixon et al., 2002). Misidentification of metabolites using the NIST library, especially using mass ions in the limited range of 46–300 *m/z,* maturity stage of tomatoes, extraction solvent and method could attribute to the variation in number and occurrence of metabolites. Reactions among different volatiles and also between volatiles of fruits or vegetables have been reported as other potential reasons for variability in volatile profiles among replicates (Hamilton-Kemp et al., 1996).

Conclusion

The pathogen/disease-specific volatile metabolites, unique and common to *Listeria monocytogenes, Bacillus megaterium* and *Brevibacillus laterosporus*, reported here, could be used as biomarkers to discriminate diseases/pathogens even when more than one disease is present, and software could be developed for user friendly applications under commercial conditions but this has to be tested before commercial application. However, additional studies involving other cultivars, physical, environmental factors influencing volatile dynamics, larger sample size, etc. are required before recommendation for commercial use.

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