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Volatile metabolites profiling to discriminate diseases of tomato fruits inoculated with three toxigenic fungal pathogens

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The volatile metabolites of tomato fruits inoculated with three toxigenic fungi isolated from spoilt tomatoes were profiled using gas chromatography/mass spectrometry. Differences in the number and amount of volatile metabolites were observed. The study yielded a total of 52 different volatile metabolites. Healthy ripe tomato fruits yielded twenty-eight metabolites predominated among them were oleic acid amide (10.89%), 9-octadecenoic acid (9.83%), methyl cis-9-octadecenoate (7.73%), and the least was 2, 3-Heptanedione (0.32%). Tomato fruits inoculated with *A. niger* yielded 11; *A. flavus* yielded 15 different volatile metabolites while that inoculated with *F. oxysporum* yielded 8 volatile metabolites. Among them only 5 volatile metabolite occurred relatively consistent in fruits inoculated with *A. niger* and *A. flavus* while adogen 73 and 9-Octadecenoic acid (Z) occurred relatively consistently in fruits inoculated with the three fungi. Hexadecanoic acid and 6-Methyl-2,4-di - tert - butyl – phenol was common in fruits inoculated with *F. oxysporum* and *A. niger* with that of *A. niger* having the highest value (9.67%) for Hexadecanoic acid while fruits inoculated with *F. oxysporum* had highest (2.66%) for 6-Methyl-2,4-di - tert - butyl – phenol. Ten metabolites were unique to *A. flavus* while *A. niger* and *F. oxysporum* had 4 metabolites unique to each of them. This study suggests that these unique metabolites can be used as biomarkers to detect tomato diseases/pathogen or toxigenic fungi at an early stage of disease progression and to manage tomato diseases in storage and outbreak of food borne disease, after further validation under commercial conditions.

Keywords: Disease detection, disease diagnosis, GC-MS, Metabolomics, post-harvest pathogens

Estimates of production losses in developing countries are hard to evaluate. Postharvest losses of fruit and vegetables in some African countries have been estimated to reach 50% (FAO, 2008). Both qualitative and quantitative losses occur in horticultural commodities between harvest and consumption (Kader and Rolle, 2004), hence minimizing post harvest losses of already produced food is more sustainable than increasing production (Kader and Rolle, 2004). 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 suitable for storage facilities as they involve destructive sampling and the machines are sometimes not available in 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). Potato tubers, cv. Maris Piper and Russet Burbank, produce many volatile compounds. Volatile production by many other fruits and vegetables has been extensively studied with a view to detecting disease occurrence in order to reduce losses in storage. Volatile metabolites produced by diseased potato, onion, citrus, raspberry, peach and other crops have been studied using gas chromatography and gas chromatography–mass spectrometry (Kushalappa et al., 2002; De Lacy Costello et al., 1999; Kallio and Salorinne, 1990; Ouellette et al., 1990; Wilson and Wisniewski, 1989; Pauli and Knoblauch, 1987; Davis and Smoot, 1972). Many compounds were found to be disease-specific, e.g. potatoes infected with *Phytophthora infestans* produced butanal, 3-methyl butanal, undecane and verbenone, while those infected with *Fusarium coeruleum* produced 2-pentyl furan and capaene (De Lacy Costello et al., 2001).

The occurrence of fungi in spoilt tomato fruits has been reported (Ghosh, 2009). Among the fungi, it was found that *Aspergillus niger* and *Fusarium spp* were the most occurring in spoilt tomatoes with a few samples containing *Penicillium spp*. These fungi are the source of highly potent mycotoxins which can cause severe food poisoning resulting in fatal outcome (Ghosh, 2009). The objectives of this study were to identify disease-discriminatory volatile metabolites released from tomato fruits inoculated with *Fusarium oxysporum; Aspergillus niger* and *Aspergillus flavus* and profile the volatile metabolites using GC-MS analysis to discriminate/detect the presence of these toxigenic fungi in spoilt tomato fruits.

MATERIALS AND METHODS

Sample Collection

Fresh, matured, ripe and healthy (intact) tomato fruits were purchased in a local market within the main campus of Usmanu Danfodiyo University Sokoto.

Fungal inoculum preparation

Fungi used in this work were isolated from spoilt tomato fruits obtained within Sokoto metropolis and maintained on potato dextrose agar slants. The spores were subculture onto molten potato dextrose agar slant and incubated at room temperature for 5 days. Inoculum preparation was done as described by Negi and Banerjee (2006). For inoculum preparation, 25 ml of sterile distilled water was added to the 5-day-old slant grown on potato dextrose agar slant and scraped aseptically with inoculating loop. Zero point five (0.5) ml of this suspension, having spore concentration of approximately 1.3×10^7 cells/ml, was used as inoculum for the subsequent pathogenecity test.

Pathogenecity test of isolates on healthy tomato fruits

This was done according to the method of Kutama, *et al.* (2007). Intact and matured tomato fruits were surface sterilized with 1 % Sodium Hypochlorite and rinsed with sterile distilled water. One side of each of the replicates was carefully punctured with a sterile scalpel beyond the epidermal layer. The identified isolates were introduced into the punctured portions with a sterile needle and sealed with sterile molten Vaseline petroleum jelly to avoid being contaminated by opportunistic micro organisms. All samples were incubated at room temperature (22-28^{o}C) with enough

moisture for 5-7 days with daily observations for spoilage symptoms.

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. Two gram of spoilt mango fruits and healthy ripe mango fruits 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 No. 1 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 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 (%).

RESULTS

Fungi microflora of spoilt tomatoes fruits were isolated and identified. The microflora includes *Fusarium oxysporum; Aspergillus niger* and *Aspergillus flavus*. They were inoculated into ripe healthy and intact tomato fruits. Volatile metabolite profile of healthy ripe tomato fruits was determined by GC-MS analysis of its diethyl ether extract and the result presented in Table 1. From the result, twenty-eight metabolites were determined. Predominated among them were Oleic acid (10.89%), 9-octadecenoic acid (9.83%) methyl cis-9-octadecenoate (7.73%), and the least was 2, 3-Heptanedione (0.32%).

GC-MS analysis of the diethyl ether extract of tomato fruits inoculated with *Fusarium oxysporum* was conducted and the result presented in Table 2. From the result, eight metabolites were determined predominated by 1, 2- dimehyl benzene (14.05%) followed by methyl cis octadec-11-enoate (7.95%), isopropyl benzene (7.47%) and adogen 73 was the least (0.73%).

GC-MS analysis of the diethyl ether extract of tomato fruits inoculated with *A. flavus* was conducted and the result presented in Table 3. From the result, fourteen metabolites were determined predominated by 9-octadecenoic acid (Z) (13.41%) followed by octane (11.15%), nonane (11.03%) and butylated hydroxytoluene was the least (0.83%).

GC-MS analysis of the diethyl ether extract of tomato fruits inoculated with *A. niger* was conducted and the result presented in Table 4. From the result, fourteen metabolites were determined predominated by 9-octadecenoic acid (Z) (21.58%) followed by decane (12.93%), octane (10.80%) and 1-methylene-1H- indene was the least (1.68%).

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

RT-1 (min)	Volatile 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	9-Octadecenoic acid	9.83
28.52	Methyl cis-9-octadecenoate	9.83
28.75	Oleic acid amide (Adogen 73)	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.

Table 2: Result of GC/MS analysis of ripe tomato fruits inoculated with *Fusarium oxysporum*

RT-[1](Min)	Volatile metabolites	Peak Area Normalized (%)
3.21	1,2 - Dimethylbenzene	14.05
3.35	Isopropylbenzene (Cumol)	7.47
3.83	6-Methyl-2,4-di - tert - butyl – phenol	2.66
4.69	Methyl 14-methylpentadecanoate	3.97
4.89	Hexadecanoic acid	1.68
5.14	Methyl cis-octadec-11- enoate	7.95
5.33	9 – Octadecenoic acid (Z)	1.24
5.53	Oleic acid amide (Adogen 73)	0.71

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

Table 3: Result of GC/MS analysis of ripe tomato fruits inoculated with *Aspergillus flavus*

RT-1 (Min)	Volatile metabolites	Peak Area Normalized (%)
3.83	Octane	11.15
6.36	Nonane	11.03
8.98	1,2,3- Trimethylbenzene	5.97
9.67	Decane	10.83
10.20	Tetracyclo [3.3.1.0(2,8).0(4,6)] -one-2-ene	2.63
	Tricyclo [5.2.1.0(sup2,6)] decane	2.69
12.36	Tetrahydromaphthalene (Tetraline)	2.03
14.21	4 - phenyl but - 3 - ene - 1 –yne	2.39
18.08	1,6-methano[10] annulene	9.68
18.50	1,8- Dimethyl naphthalene	1.24
21.43	Butylated Hydroxytoluene	0.83
23.24	Pentadecanecarboxylic acid	6.21
27.61	9-octadecenoic acid (Z)	13.41
28.74	Oleic acid amide (Adogen 73)	4.81
29.74	(6Z, 9Z)-6,9-pentadecadien - 1-ol	1.11

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

Table 4: Result of GC/MS analysis of ripe tomato fruits inoculated with *Aspergillus niger*

RT-1(Min)	Volatile metabolites	Peak Area Normalized (%)
3.83	Octane	10.80
6.98	2-(3-Hydroxy 1-2-nitrocyclohexyl) -1-phenylethanone	4.39
9.67	Decane	12.93
12.99	Oxalic acid, isobutyl pentyl ester	2.53
14.78	1-Methylene -1H-Indene	1.68
18.08	1,6 – Methano[10] annulene	9.60
18.50	(1E)-1-Ethylidene – 1H- indene	6.94
23.25	6-Methyl-2,4-di-tert –butyl-phenol	2.38
27.61	Hexadecanoic acid	9.67
28.7	9-Octadecenoic acid (Z)	21.58
29.99	Oleic acid amide (Adogen 73)	3.54

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

DISCUSSION

This is the first study to provide data on the composition of the diethyl ether extract volatile metabolites of tomatoes inoculated with pathogens and provides the basis for discriminating the postharvest diseases caused by *F. oxysporum, A. flavus* and *A. niger*. Several compounds were unique to a disease/inoculation, which could be qualitatively used to discriminate diseases studied here, in unknown disease samples. 1,2–Dimethylbenzene, Isopropyl benzene (Cumol), Methyl 14-methylpentadecanoate and Methyl cis-octadec-11-enoate were unique to *F. oxysporum* inoculated tomatoes, which could be discriminated from *A. flavus* ones, which also produced unique metabolites, Nonane, 1,2,3-Trimethylbenzene; Tetracyclo

[3.3.1.0(2,8).0(4,6)] -one-2-ene; Tricyclo [5.2.1.0(sup2,6)] decane; Tetrahydro-naphthalene (Tetraline); 4-phenyl but-3-ene-1-yne; 1,8-Dimethyl naphthalene; Butylated Hydroxytoluene; Pentadecanecarboxylic acid and (6Z, 9Z)-6,9-pentadecadien-1-ol and *A. niger* ones, which also produced unique metabolites, 2-(3-Hydroxy 1-2-nitrocyclohexyl) -1-phenylethanone; Oxalic acid, isobutyl pentyl ester; 1-Methylene -1H-Indene and (1E)-1-Ethylidene – 1H-indene. Butylated Hydroxytoluene and (6Z, 9Z)-6,9-pentadecadien-1-ol detected in commercialised tomatoes fruits in our earlier studies (Ibrahim et al., 2010), are characteristic to the presence of *A. flavus*. These unique metabolites can be used as biomarkers to detect the presence of these toxigenic fungi 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 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 Hexadecanoic acid and 6-Methyl-2, 4-di-tert –butyl-phenol were common to *F. oxysporum* and *A. niger* inoculated tomatoes, but were absent in the healthy tomatoes. *A. niger* inoculated tomatoes had the highest amount of Hexadecanoic acid (9.67%) than *F. oxysporum* inoculated tomatoes which had 1.68%. 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 *F. oxysporum* and *A. niger*, especially when unique compounds are absent and mixed infections, especially in the same lesion, are present.

Oleic acid amide (Adogen 73) and 9-octadecenoic acid were produced in tomatoes inoculated with the pathogens and in healthy fruits. *A. flavus* and *A. niger* inoculated tomatoes produced higher amount of 9-octadecenoic acid (21.58% and 13.41%) than healthy tomatoes (9.83%) while *F. oxysporum* inoculated tomatoes had the least 1.24%. The increase in the relative abundance of 9-octadecenoic acid observed in *A. flavus* and *A. niger* inoculated tomatoes could probably be from the tomatoes seed oils and microorganisms (Hayes, 1996). Healthy tomatoes had the highest relative abundance of Adogen 73 (10.89%) while *F. oxysporum* inoculated tomatoes had the least 0.71%. Even though these compounds were common to all the treatments, the

differences in their relative abundance can help to detect and discriminate diseases/toxigenic fungi, 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).

Several compounds were common to A. *flavus* and *A. niger* inoculated tomatoes. The metabolites Octane, Decane and 1, 6–Methano [10] annulene were common to tomatoes inoculated with the two toxigenic fungi. Tomatoes inoculated with A. *flavus* had the highest amount of Octane (11.15%) and 1, 6 –Methano [10] annulene (9.68%) while *A. niger* inoculated tomatoes had the highest relative abundance of decane (12.93%). Even though these compounds were common to A. *flavus* and *A. niger* inoculated tomatoes, the differences in their relative abundance can help to detect and discriminate diseases/ A. *flavus* and *A. niger*, especially in the absence of unique or other disease-discriminatory compounds (Moalemiyan et al., 2006).

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

Many diseases are important in storage; the outbreaks are often from a single disease. The toxigenic fungi/disease-specific volatile metabolites, unique and common to only *F. oxysporum, A. flavus* and *A. niger*, reported here, could be used as biomarkers to discriminate diseases/ toxigenic fungi even when more than one disease is present, but this has to be tested before commercial application.

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