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Volatile Mass Ion and Metabolite Profiling to Detect and Discriminate Fungal Diseases in Different Mango Cultivars

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ABSTRACT

Gas chromatography and mass spectrometry was used to analyze the volatiles in the headspace of mango cultivars 'Tommy Atkins', 'Keitt' and 'Kent' inoculated with *Alternaria alternata*, *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*. A total of 251, 255 and 224 volatile peaks were detected in the three cultivars 'Tommy Atkins', 'Keitt' and 'Kent', respectively. The volatile compounds were identified based on mass spectral match using NIST library. Thirty compounds were detected relatively consistently among replicates in 'Tommy Atkins' while the relatively consistent compounds in 'Keitt' and 'Kent' were 34 and 20, respectively. Several of these compounds were specific to a disease/inoculation or they varied in their abundances when being present in all treatments. In 'Kent', 2-butenoic acid, methyl ester was specific to *Colletotrichum* while propanoic acid, ethyl ester and 2-propenoic acid, 2-methyl-, ethyl ester was specific to *Lasiodiplodia*. 1-butanol and propanoic acid, ethyl ester was specific to both *Colletotrichum* and *Lasiodiplodia* in 'Tommy Atkins' cultivar while octanoic acid, methyl ester was specific to *Colletotrichum* in 'Keitt'. Overall rate of disease classification based on metabolite profiles was poorer than discriminability of mass ion fingerprints. The average error rates for resubstitution and cross validation for separate metabolite profiles of each cultivar or pooled data of cultivars was higher than 40%. The utmost shortcoming of metabolite profile based discriminant models was their high rate of false negatives. The discriminant models developed on mass ion fingerprints correctly classified almost hundred percent of the entries both in resubstitution and cross validation procedures. The disease specific metabolite profiles and mass ion fingerprints produced could be used to differentiate cultivars and has the potential to be used in early detection of postharvest diseases of mango fruits after validation under commercial conditions.

Keywords: *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Lasiodiplodia theobromae*, mango, mass ion fingerprinting, metabolite profiling

INTRODUCTION

Mango is an economically important crop produced in over 90 countries worldwide. It is estimated that about 77% of global mango production comes from Asia followed by 13% from Americas and 9% from Africa (FAOSTAT 2007). The world production of mango was estimated at 28.51 million metric tons in 2005 and India is the largest producer of mangoes which accounts for 38.6% of total world production. The other leading producers of mango are China, Thailand, Mexico, Indonesia, Pakistan, Brazil, Philippines, Nigeria and Egypt (FAOSTAT 2007).

Mango diseases are one of the most important causes of postharvest losses. The main postharvest diseases of mango include anthracnose (*Colletotrichum gloesporioides*), stem-end rot (*Lasiodiplodia theobromae*) and alternaria rot (*Alternaria alternata*) (Barkai-Golan 2001). In anthracnose the lesions are observed at first on the surface of the fruit which later move into the flesh. The spore masses are produced and its color changes from salmon pink to dark brown in humid conditions (Fitzel and Peak 1984). The disease is most severe following periods of wet weather. The losses caused by stem-end rot are more profound during transit and storage and also in situations where anthracnose is not well managed (Johnson *et al*. 1993). A dark rot develops from the stem end as fruit ripens after harvest. Stem end rot may be more serious than anthracnose in fruits from drier areas. The symptoms of alternaria rot can be distinguished from anthracnose by darker, more limited firm lesions. In advanced stages of decay olive brown spores can be found within lesions. The fruit shows development of small black spots around lenticels, which can grow and coalesce to form large spot that covers a large part of the fruit. At first, decay does not extend into the fruit, but as symptoms develop, the disease progresses into the pulp which darkens.

Early detection of diseases in storage can reduce significant post-harvest losses in storage conditions. More sophisticated diagnostic methods and techniques like polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immuno-sorbant assay (ELISA) are now available for disease detection (Meyer *et al*. 2000; Mathioudakis *et al*. 2009; Abarshi *et al*. 2010). However, their feasibility in detecting diseases under storage conditions is widely reduced due to the need for destructive collection of a large number of samples. A method for detecting diseases which involves non destructive sampling is necessary for stored fruits and vegetables. An alternative to this could be the use of gas chromatography/mass spectrometry by trapping and quantification of volatile compounds in the storage headspace. Several researchers have identified and used volatile metabolites released from infected fruits and vegetables in developing techniques for detection and discrimination of stored products (Prithiviraj *et al*. 2004; Lui *et al*. 2005; Vikram *et al*. 2005).

Studies conducted on mango fruits have shown that there are more than 300 metabolites belonging to several chemical groups namely monoterpenes, sesquiterpenes, esters, ketones, alcohols, organic acids, aliphatic hydrocar-

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bons and aromatics (Shibamato and Tang 1990; Winterhalter 1991). In mango aroma is affected by various factors like cultivar, production environment, fruit maturity at harvest and storage conditions. There are reports stating that overall flavor of fresh and processed fruits are influenced by aroma volatile compounds (Lalel *et al*. 2003; Singh *et al*. 2004). Volatile metabolites released from two mango cultivars infected with two different diseases were identified in an earlier study (Moalemiyan *et al*. 2006; Moalemiyan *et al*. 2007). In the present trial the volatiles released from three different mango cultivars inoculated with three important fungal pathogens were studied.

MATERIALS AND METHODS

Fungal cultures and inoculum production

The cultures of *Alternaria alternata*, *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae* were obtained from Brazil. The fungal pathogens were stored at 4°C until further use. The fungal cultures were grown on potato dextrose agar (Difco Laboratories, Detroit, MI, USA) and incubated at 22°C for a week and then exposed to near UV light for sporulation. The fungal spore suspensions were prepared from 7 to 10 day-old cultures, by flooding the plates with distilled water. The suspension was vortexed and filtered through two layers of a cheese cloth. The concentration of spores in the suspension was adjusted to 10^5 spores ml^{-1} for *Alternaria alternata* and *Colletotrichum gloeosporioides* and 10^4 spores ml^{-1} for *Lasiodiplodia theobromae* using a haemocytometer.

Inoculation and incubation

Mango fruits of cultivars Tommy Atkins, Keitt and Kent produced in Mexico were obtained from a mango distributing company (Aliments, IMAX Foods Inc., Montreal, Quebec). Apparently disease-free, uniform in size, and mature green mangoes were selected and surface sterilized in 1% sodium hypochlorite solution for 15 min, and rinsed with distilled water. Six holes were made at the equatorial region with a cork borer (3 mm diameter, 3 mm depth). Each hole was inoculated with 30 µL of *Alternaria*, 30 µL of *Colletotrichum* or 20 µL of *Lasiodiplodia* spore suspension using a micropipette. The inoculated mangoes were placed in 2 L glass jars, one fruit per bottle, with 13 mL of water at the bottom to create a humidity saturated atmosphere and incubated at 20°C in the dark. Stainless steel supports were used to suspend mangoes above water surface and an aluminum foil was used to cover the mouth. After 24 h, the water was removed. The blocks containing all the treatments were conducted eight times, at weekly intervals.

Volatile accumulation and analysis

The inoculated mangoes were transferred into new sampling jars at the end of each incubation period (3 and 6 days for *Alternaria*, *Colletotrichum*, *Lasiodiplodia* and controls). A magnetic stirrer was placed at the bottom to agitate headspace gas to establish equilibrium while gas sampling. The bottles were flushed with pure dry air, covered tightly with 0.02 mm Teflon film (ENFLO Canada LTD, Montreal), and incubated at 20°C in dark for 150 min. The headspace gas was sampled and analyzed using a portable GC/MS system (Model: Hapsite -10122: Inficon, Syracuse, NY, USA) (Vikram *et al*. 2004a). The GC/MS was programmed to sample headspace air for 15 s at the rate of 100 mL min^{-1} and 25 mL of the headspace air was preconcentrated in a carboxen trap (15 mg) which was heated to 225°C to desorb volatiles. Compounds were separated using a 30 m long SPB-5 capillary column with 0.32 mm internal diameter (Inficon, NY, model: 930-489-G8). Nitrogen was used as the carrier gas at a flow rate of 3 ml min^{-1}. The column was heated to 50°C followed by a ramping of 3°C min^{-1} for 50 min, when the temperature reached 200°C, it was held for 2 min. Mass ions were scanned at the rate of 1.08 s per mass decade over a mass range of 46-300 m/z.

Disease severity assessment

The diameter of diseased mango tissue was measured 3 and 6 days after inoculation (DAI) (for *Alternaria*, *Lasiodiplodia* and *Colletotrichum*). At 6 DAI, the mango was cut at the site of inoculation and the depth of diseased tissue was measured, from which the volume of the diseased tissue was calculated ($\pi r^2 h$). Disease specific discoloration or rot was considered as diseased tissue.

Experimental design, compound identification, and data analysis

The experiment was designed as a randomized complete block design and consisted of five main treatments, designated as inoculation/disease: non-wounded-non-inoculated (N-control), wounded-non-inoculated (W-control), inoculated with *Alternaria alternata* (*Alternaria*), inoculated with *Lasiodiplodia theobromae* (*Lasiodiplodia*), and inoculated with *Colletotrichum gloeosporioides* (*Colletotrichum*). Two observations were made at two incubation times (3 and 6 DAI) for *Colletotrichum*, *Lasiodiplodia*, *Alternaria*, N-control and W-control to represent disease severity levels. The experimental unit consisted of one mango fruit per bottle. The entire experimental block was conducted 8 times, at weekly intervals, thus 8 blocks and two observations for each block.

The initial output of GC/MS consisted of scans and mass ion abundances which were further processed by the Saturn software to derive the peak abundance and the retention time. The metabolites and their abundances were exported in to an EXCEL spreadsheet and sorted using the pivot procedure to develop a list of compounds detected in this study. The NIST mass spectral search program (version 2.0) was used to tentatively identify the compounds. The peaks with an abundance of greater than 1000 (quadrupole analyzer output) were retained for further analysis.

The volatiles which occurred (frequency) in four or more observations (out of 16 observations = eight blocks x two incubation times for *Lasiodiplodia*, *Alternaria*, *Colletotrichum*, N-control, and W-control treatments) were considered relatively consistent. The consistent metabolites unique or common to a few but not to all inoculation/diseases were separated and designed as disease discriminatory. The initial output of GC/MS was also used to derive the metabolic fingerprints. The abundance of each mass ion for all the scans of the chromatogram was summed, normalized by dividing the abundance of each mass ion with the total abundance for 150 mass ions (46-195 m/z) and designated as the metabolic fingerprint. The abundance of relatively consistent metabolites, excluding those that were unique to an inoculation/disease, were normalized by dividing each by the total for all the consistent metabolites and designated as the metabolic fingerprint.

Statistical analysis

The relatively consistent metabolites and the mass ion fingerprints were subjected to discriminant analysis using the DISCRIM procedure of SAS (Johnson 1998). The discriminant analysis was to find models with a reasonably accurate capability of classifying entries (disease × mango cultivar combination) into their respective groups (Johnson 1998). An accurate classification must theoretically have an error rate of zero or near to zero; however for practical purposes a fair classification with some extent of errors may also be satisfactory. Misclassification probability combined with risk of probable future losses is of critical importance in decision making for both market managers and disease management of post harvest diseases.

The modeling approach is based on development of separate discriminant models for each disease × mango cultivar combination. The following steps were employed to determine the misclassification probability for a model through validation procedure. The following steps are employed. In resubstitution, each observation enters all the models and based on the discriminant value it would be assigned to a group. Basically misclassification probability of a model at this level of validation is lower as the model is validated in its data space. To validate further however jackknifing (cross validation) was used, where the procedure starts with removing the first observation vector; rebuilding a discriminant

Table 1 Volatile metabolites and their average abundances ($\times 10^5$) detected in the headspace gas of mango fruits (cv. 'Kent') inoculated with water or three plant pathogens.[a]

RN[b]	Compounds	*Alternaria*	*Colletotrichum*	*Lasiodiplodia*	N-control	W- control	Inoculation
8	2-Propenoic acid, 2-methyl-, ethyl ester			107			L
36	Propanoic acid, ethyl ester			40			L
7	2-Butenoic acid, methyl ester, (*Z*)-		7				C
13	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-		2	204			CL
17	Butanoic acid, ethyl ester		84	209			CL
29	Ethyl acetate		4	81			CL
32	Hexanoic acid, ethyl ester		24		24		CN
31	Furan, 2-methyl-		84	9	128		CLN
44	α-Pinene	7			41	3	ANW
45	α-Phellandrene			25	4	31	LNW
40	Styrene		8	4397	3	4	CLNW
39	Silanol, trimethyl	14	10		17	20	ACNW
33	Limonene	65	29	49		15	ACLW
9	3-Carene	4419	12113	4928	2441	2659	ACLNW
12	Benzene, tert-butyl-	7	6	7	9	5	ACLNW
20	Chloroform	27	7	2	17	22	ACLNW
26	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	62	176	69	277	34	ACLNW
28	Ethanol	15303	21671	25585	7692	346	ACLNW
30	Ethylbenzene	11	5	1	6	4	ACLNW
42	Toluene	37	41	25	63	38	ACLNW

[a] **A**: *Alternaria alternata*; **C**: *Colletotrichum gloeosporioides*; **L**: *Lasiodiplodia theobromae*; **N**: non-wounded-non-inoculated control; **W**: wounded-non-inoculated control.
[b] **RN** = Metabolite reference number of the metabolites tentatively identified in this study (see **Supplementary Table**).

model based on the remaining data, and using the new rule to classify the first removed observation. Finally a summary table of resubstitution and cross-validated data is created by repeating the model development and validation cycle with rest of the observation vectors.

RESULTS

Cultivar susceptibility to the pathogens

The highest volume of mango flesh destroyed was by *Lasiodiplodia* on mango cultivar 'Tommy Atkins' followed by 'Keitt' and 'Kent' (**Fig. 1**), while *Colletotrichum* caused the highest loss to 'Keitt' with a significant reduction of losses to both 'Kent' and 'Tommy Atkins' ($p=0.05$). *Alternaria* caused the lowest losses to all three cultivars as compared to the other two fungal pathogens (**Fig. 1**).

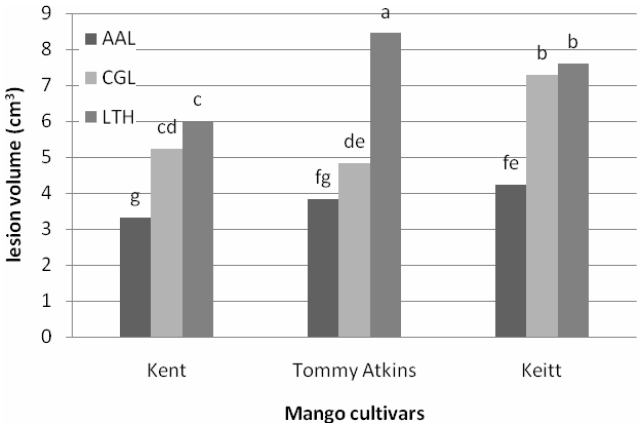

Fig. 1 Bar graph of disease severity of the causal agents of three fungal diseases on each of three mango cultivars 'Keitt', 'Kent' and 'Tommy Atkins'. Bar labels show the results of Duncan's multiple range test at α = 0.05, Bars with the same letter are not significantly different at 5%. **AAL** = *Alternaria alternata*, **CGL** = *Colletotrichum gloeosporioides* **LTH** = *Lasiodiplodia theobromae*.

Metabolite profiles and volatile markers of cultivar × disease

A total number of 730 metabolites were detected in all treatments with 251, 255 and 224 volatile peaks in 'Tommy Atkins', 'Kent' and 'Keitt' cultivars, respectively. The relative abundance of compounds ranged between 1×10^5 and 2.3×10^{10}. Twenty compounds were found to appear relatively consistently among replicates in 'Kent' mangoes inoculated with various treatments (**Table 1**). Propanoic acid ethyl ester and 2-propenoic acid, 2-methyl-, ethyl ester were unique to 'Kent' mangoes inoculated with *Lasiodiplodia*. Similarly, 2-butenoic acid, methyl ester were unique to 'Kent' mangoes inoculated with *Colletotrichum*. Ethyl acetate, butanoic acid, ethyl ester and bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene were specific to *Lasiodiplodia* and *Colletotrichum*. Thirty four compounds were found to appear relatively consistently among replicates in 'Keitt' mangoes inoculated with various treatments (**Table 2**). Octanoic acid, methyl ester was unique to 'Keitt' mangoes inoculated with *Colletotrichum*. Similarly, boronic acid, ethyl- was specific to *Lasiodiplodia* and *Colletotrichum* while 1,4-pentadiene was specific to non-wounded-non-inoculated and wounded-non-inoculated 'Keitt'mangoes. Thirty compounds were found to appear relatively consistently among replicates in 'Tommy Atkins' mangoes inoculated with various treatments (**Table 3**). 1-butanol and propanoic acid, ethyl ester were specific to 'Tommy Atkins' mangoes inoculated with *Lasiodiplodia* and *Colletotrichum*.

Disease discriminability of metabolite profiles

A metabolic fingerprint of each specific entry (cultivar × disease combination) consisting of 43 relatively consistent volatiles was determined based on the abundance of mass ions (46–195 m/z) and subjected to discriminant analysis (DA) through PROC DISCRIM of SAS 8.2. The entries were grouped according to disease in separate cultivars and also in the data produced by pooling all cultivars. The accuracy of classification of entries into their respective groups was evaluated for discriminant analysis model by computing error rates of classification based on resubstitution and jack-knife procedures.

The mean of the rate of classification of entries into their relevant group (cultivar and disease) was globally higher than 50% with the best results for cultivar 'Keitt' with 63.8 and 57.5% resubstituted and cross validated metabolite based classification rates. The average error rates for resubstitution and cross validation when data of each cultivar was used separately to develop separate models for each cultivar were 41.27 and 44.27%, respectively while the error rates increased to 43. 70 and 52.40% when metabolite

Table 2 Volatile metabolites and their average abundances ($\times 10^5$) detected in the headspace gas of mango fruits (cv. 'Keitt') inoculated with water or three plant pathogens.[a]

RN[b]	Compounds	*Alternaria*	*Colletotrichum*	*Lasiodiplodia*	N-control	W-control	Inoculation
35	Octanoic acid, methyl ester		18				C
15	Boronic acid, ethyl-		5	57			CL
4	1,4-Pentadiene				1	1	NW
2	1,2,4-Benzenetricarboxylic acid, 1,2-dimethyl ester		1		1	1	CNW
3	1,4-Cyclohexadiene, 1-methyl-		13		4	5	CNW
11	Acetic acid, methyl ester		7	5	1	8	CLNW
21	Cycloheptane, bromo-		8	1	5	4	CLNW
22	Cyclohexane		4	2	4	7	CLNW
23	Cyclohexane, 1,3-dimethyl-, *cis*-		36	34	29	39	CLNW
24	Cyclohexane, ethyl-		1	1	1	2	CLNW
25	Cyclohexane, methyl-		59	53	63	72	CLNW
31	Furan, 2-methyl-		3	3	23	8	CLNW
41	Terpinolene		418	621	367	325	CLNW
43	Trichloromonofluoromethane		5	2	14	16	CLNW
18	Butanoic acid, methyl ester	7	8	4		1	ACLW
6	2-Butenoic acid, ethyl ester	151	16	50	1	9	ACLNW
7	2-Butenoic acid, methyl ester, (*Z*)-	13	85	20	1	11	ACLNW
8	2-Propenoic acid, 2-methyl-, ethyl ester	267	351	450	28	305	ACLNW
9	3-Carene	25573	28587	28711	19081	18595	ACLNW
45	α-Phellandrene	95	7	67	54	42	ACLNW
44	α-Pinene	115	177	265	162	163	ACLNW
12	Benzene, tert-butyl-	32	5	1	1	1	ACLNW
13	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	483	150	103	178	175	ACLNW
17	Butanoic acid, ethyl ester	853	179	422	14	135	ACLNW
20	Chloroform	58	12	15	18	12	ACLNW
28	Ethanol	7579	1283	14821	31	412	ACLNW
29	Ethyl acetate	253	32	103	1	846	ACLNW
30	Ethylbenzene	3	4	10	7	7	ACLNW
32	Hexanoic acid, ethyl ester	1091	341	467	27	303	ACLNW
33	Limonene	514	509	713	482	385	ACLNW
36	Propanoic acid, ethyl ester	184	19	98	1	27	ACLNW
39	Silanol, trimethyl-	95	24	16	30	33	ACLNW
40	Styrene	218	127	944	3	1	ACLNW
42	Toluene	55	150	161	149	148	ACLNW

[a] **A**: *Alternaria alternata*; **C**: *Colletotrichum gloeosporioides*; **L**: *Lasiodiplodia theobromae*; **N**: non-wounded-non-inoculated control; **W**: wounded-non-inoculated control.
[b] **RN** = Metabolite reference number of the metabolites tentatively identified in this study (see **Supplementary Table**).

fingerprints of all cultivars were pooled to develop general discriminant models for each disease (**Table 4**). According to our results on disease classification of three cultivars, 'Keitt' showed the best volatile based-classification among the three mango cultivars (with 57.5 total rate of cross validated classification). The rate of cross validated classification on both separate cultivars and pooled data of all cultivars belonged to *Alternaria alternata* treatment with a mean rate of classification of 91.9% with only 8.1% misclassification). The rate of correct cross-validated classification for *A. alternata* ranged from 93.8% (in 'Tommy Atkins') to 100% (in both 'Keitt' and 'Kent') with slightly lower classification rate of 73.8% (for pooled data of all cultivars). The problem with metabolic profile based DA models was their high false negatives, i.e. the high rates of misclassification of wounded water inoculated control (W-control) into other groups.

Mass ion fingerprinting of cultivar × disease

The discriminant models developed based on mass ion fingerprints of each entry correctly classified 100% of entries (with a mean of error rate = 0%) into their respective disease-cultivar groups when DA models were developed separately. The cross-validation results also showed an excellent capability for the separate DA models to correctly classify 100% (error rate = 0) of the observations into their groups (**Table 4**). When the data for both 'Keitt' and 'Tommy Atkins' cultivars were pooled and a global DA model was developed, the results however showed a reduced classification rate (80.6% correct classification based on resubstitution and cross validation with a global error rate of 19.4%), yet the results pictured a far superior classification as compared to near 50% mean error rates for metabolite fingerprint based classification.

The DA model developed on pooled data for two mango cultivars for the *Alternaria alternata* treatment was the best and classified 100% of entries into this treatment correctly (without any error) in both resubstitution and cross validation runs. Although the DA model for *C. gloeosporioides* treatment was better than the others for disease discrimination, it was slightly worse than the pooled DA model for *A. alternata* and the resubstitution and cross validation classification rates were 96.9% (with only 3.1 misclassification of *A. alternata* into other groups) (**Table 4**).

DISCUSSION

This is the first study to provide data on the headspace volatile metabolite composition of three different mango cultivars inoculated with three different pathogens. The study provided the basis for discriminating postharvest diseases caused by *Alternaria alternata*, *Colletotrichum gloesporioides* and *Lasiodiplodia theobromae* in 'Kent', 'Keitt' and 'Tommy Atkins' mangoes. Early detection of these postharvest diseases based on the symptoms they cause on the fruits is not easy. The amount of injury caused to the fruits in each of these pathosystems showed a similar pattern however not adequate to discriminate pathogen/cultivar entries. *C. gloesporioides* and *L. theobromae* cannot be differentiated based on fruit disease severity on neither of the 'Keitt' and 'Kent' mangoes. On the other hand the injury to fruit flesh on 'Tommy Atkins' mango caused by the three fungi could be differentiated based on disease severity and *A. alternata* always caused the lowest loss to all mango cultivars (**Fig. 1**). The present study revealed that there are

Table 3 Volatile metabolites and their average abundances ($\times 10^5$) detected in the headspace gas of mango fruits (cv. 'Tommy Atkins') inoculated with water or three plant pathogens.

RN[b]	Compounds	*Alternaria*	*Colletotrichum*	*Lasiodiplodia*	N-control	W-control	Inoculation
1	1-Butanol		56	59			CL
36	Propanoic acid, ethyl ester		70	95			CL
40	Styrene	48	2765	2701			ACL
7	2-Butenoic acid, methyl ester, (*Z*)-		131		15	41	CNW
35	Octanoic acid, methyl ester		3		21	17	CNW
6	2-Butenoic acid, ethyl ester		163	232	2	19	CLNW
8	2-Propenoic acid, 2-methyl-, ethyl ester		2271	1256	49	532	CLNW
13	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-		1776	3261	1068	3329	CLNW
16	Butanoic acid, 2-methyl-, ethyl ester		13	138	2	1	CLNW
17	Butanoic acid, ethyl ester		1193	1314	70	115	CLNW
23	Cyclohexane, 1,3-dimethyl-, *cis*-		32	38	53	15	CLNW
25	Cyclohexane, methyl-		63	50	59	46	CLNW
27	(*E*)-3-Caren-2-ol		1014	684	363	580	CLNW
29	Ethyl Acetate		278	245	6	354	CLNW
32	Hexanoic acid, ethyl ester		3787	1878	317	1281	CLNW
34	Octanoic acid, ethyl ester		424	79	13	63	CLNW
20	Chloroform	20		31	1	6	ALNW
30	Ethylbenzene	19		3	2	2	ALNW
5	2,4,6-Octatriene, 2,6-dimethyl-	875	285	905	84	872	ACLNW
9	3-Carene	85827	92578	144753	53375	63080	ACLNW
10	3-Pentanone	1	3	9	7	6	ACLNW
45	α-Phellandrene	1541	307	379	276	453	ACLNW
44	α-Pinene	23610	25452	48093	14925	20173	ACLNW
14	Borane-methyl sulfide complex	187	328	16	274	323	ACLNW
19	Camphene	882	536	1334	417	1383	ACLNW
26	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	989	2332	3796	1276	5186	ACLNW
28	Ethanol	14292	9152	41008	290	242	ACLNW
31	Furan, 2-methyl-	19	52	9	35	35	ACLNW
33	Limonene	8062	4520	7941	2307	3803	ACLNW
42	Toluene	184	351	210	176	161	ACLNW

[a] **A**: *Alternaria alternata*; **C**: *Colletotrichum gloeosporioides*; **L**: *Lasiodiplodia theobromae*; **N**: non-wounded-non-inoculated control; **W**: wounded-non-inoculated control.
[b] **RN** = Metabolite reference number of the metabolites tentatively identified in this study (see **Supplementary Table**).

Table 4 Percentage of entries correctly classified into their group by re-substitution, and cross-validation of discriminant analysis models as well as their respective error rates based on metabolic and mass ion fingerprints.

Molecular response	Mango cultivar(s)	Validation method	% of Observations classified into their group*						
			A	C	L	N	W	Total	Error
Classification based on Mass ion fingerprint	Both Tommy Atkins and Keitt	cross validation	100	96.9	56.3	81.3	68.8	80.6	19.4
		resubstitution	100	96.9	62.5	78.1	65.6	80.6	19.4
	Tommy Atkins	cross validation	100	100	100	100	100	100	0.0
		resubstitution	100	100	100	100	100	100	0.0
	Keitt	cross validation	100	100	100	100	100	100	0.0
		resubstitution	100	100	100	100	100	100	0.0
Classification based on Metabolic Profile	All cultivars pooled	cross validation	73.8	28.6	45.2	50.0	40.5	47.6	52.4
		resubstitution	81.0	47.6	50.0	52.4	52.4	56.7	43.3
	Tommy Atkins	cross validation	100	25.0	31.3	43.8	75.0	55.0	45.0
		resubstitution	93.8	31.3	75.0	75.0	12.5	57.5	42.5
	Keitt	cross validation	93.8	31.3	75.0	75.0	12.5	57.5	42.5
		resubstitution	93.8	31.3	93.8	62.5	37.5	63.8	36.3
	Kent	cross validation	100	25.0	31.3	43.8	75.0	55.0	45.0

A: *Alternaria alternata*; **C**: *Colletotrichum gloeosporioides*; **L**: *Lasiodiplodia theobromae*; **N**: non-wounded-non-inoculated control; **W**: wounded-non-inoculated control.

several compounds which are unique to a pathosystem (disease/inoculation). These compounds could be qualitatively used in discrimination of diseases.

2-Butaneoic acid, methyl ester was unique to *Colletotrichum* inoculated 'Kent' mangoes while propanoic acid, ethyl ester and 2-propenoic acid, 2-methyl-ethyl ester were unique to *Lasiodiplodia* inoculated 'Kent' mangoes. Similarly, Octanoic acid methyl ester was unique to *Colletotrichum* inoculated 'Keitt' mangoes. The above mentioned compounds can be used as a tool to diagnose the respective diseases. In an earlier study compounds like 1-pentanol, 3-methylbutanol, 2-methylpropanol, 2,3-butanedione, ethyl boronate, isopentyl methyl ether and ethane ethoxy were reported to be unique for bacterial soft rot of carrot caused by *Erwinia carotovora* subsp. *carotovora* (Vikram *et al*. 2006). Similarly, Laothawornkitkul *et al.* (2010) found the presence of three marker volatiles namely (*E*)-2-hexenal, 5-ethyl-2(5H)-furanone and benzene-ethanol in potato plants infected with *Phytophthora infestans* (Mont.) de Bary after analyzing the headspace samples using gas chromatography - flame ionization detector (GC-FID). In another study Rizzi *et al*. (2009) detected the presence of volatiles like isobutyl-acetate, 3-methyl-1-butanol and, 3-octanone in maize seeds infected with *Fusarium graminearum* and *F. moniliforme* by using headspace samples of infected maize seeds in a gas-chromatograph equipped with a capillary column TR-WAX.

Disease-specific volatile metabolites have been used earlier in discrimination of diseased fruits and vegetables. In 'McIntosh' apples, fluoroethene and 3,4-dimethyl-1-hexene were specific to blue mold rot caused by *Penicillium expansum* while butanoic acid butyl ester, 4-methyl-1-hexene and 2-methyltetrazole were specific to mucor rot caused by *Mucor piriformis* (Vikram *et al*. 2004a). Correspondingly, acetic acid methyl ester and fluoroethane were specific to gray mold rot and brown rot, respectively.

In potato tubers, acetic acid ethenyl ester was unique to *Erwinia carotovora* subsp. *atroseptica* while cylcohexene diazene and methoxy-(1,1-dimethyl-2-dihydroxy-ethyl)-amine were unique to *Erwinia carotovora* subsp. *carotovora* and 2,5-norbornadiene and styrene were unique to *Fusarium sambucinum* (Lui *et al*. 2005). Disease specific volatiles have also been detected in onions (Prithiviraj *et al*. 2004; Vikram *et al*. 2005), carrots (Vikram *et al*. 2006), different apple cultivars (Vikram *et al*. 2004b), cereal grains (Magan and Evans 2000) and mangoes (Moalemiyan *et al*. 2006, 2007).

In the present trial several metabolites were common to a few but not all treatments. 1-Butanol and propanoic acid, ethyl ester was common to *Colletotrichum* and *Lasiodiplodia* inoculated 'Tommy Atkin' mangoes while styrene was common to *Alternaria*, *Colletotrichum* and *Lasiodiplodia*. These compounds were detected only in pathogen inoculated treatments but not in both the controls. These volatiles were detected in an earlier study in pathogen inoculated mangoes but absent in healthy mangoes (Narain and Galavao 2004; Moalemiyan *et al*. 2006). Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene, butanoic acid, ethyl ester and ethyl acetate were detected in 'Kent' mangoes inoculated with *Colletotrichum* and *Lasiodiplodia* while boronic acid, ethyl- were detected in 'Keitt' mangoes inoculated with same pathogens (Moalemiyan *et al*. 2007). These volatiles were not detected in 'Kent' and 'Keitt' mangoes inoculated with *Alternaria* and can be used in discrimination of these diseases. 2-Butenoic acid, methyl ester and octanoic acid, methyl ester were also common to *Colletotrichum*, N-control and W-control in 'Tommy Atkins' mangoes while 1,2,4-benzenetricarboxylic acid, 1,2-dimethyl ester and 1,4-cyclohexadiene, 1-methyl- were common to the same treatments in 'Keitt' mangoes. All the above mentioned compounds can also be used in discriminating diseases of mango under conditions where unique compounds are not very consistent or absent (Moalemiyan *et al*. 2006, 2007).

It is reported that butanoic acid methyl ester is a characteristic aroma compound present in 'Keitt' mangoes (Pino *et al*. 2005). Butanoic acid methyl ester and octanoic acid methyl ester were detected only in pathogen inoculated 'Keitt' mangoes but absent in healthy ones (Moalemiyan *et al*. 2007). These compounds were in higher levels in pathogen inoculated treatments and so were detected while their absence in healthy mangoes could be ascribed to the fact that the compounds with lower abundances were not identified in the present trial (Pino *et al*. 2005; Moalemiyan *et al*. 2007). Although some compounds were present in all treatments, their abundance was higher in pathogen inoculated mangoes than in controls. The abundance of 2-propenoic acid, 2-methyl-, ethyl ester, butanoic acid, ethyl ester, (*E*)-3-caren-2-ol, hexanoic acid, ethyl ester, octanoic acid, ethyl ester, 3-carene, limonene and ethanol were several folds higher in pathogen inoculated 'Tommy Atkins' mangoes than in controls. Similarly, the abundance of terpinolene, 2-butenoic acid, ethyl ester, 3-carene, butanoic acid, ethyl ester, ethanol, hexanoic acid, ethyl ester, propanoic acid, ethyl ester and styrene were several folds higher in pathogen inoculated 'Keitt' mangoes than in controls. In the same manner, the abundance of styrene, limonene, 3-carene and ethanol were higher in pathogen inoculated treatments compared to control. All these compounds can be used in discrimination of diseases and also in conditions where there is absence of unique compounds. The presence of styrene, detected in all treatments in 'Keitt' mangoes, is in agreement with earlier studies and their abundance was several times higher in pathogen inoculated treatments than in controls (Pino *et al*. 2005; Moalemiyan *et al*. 2007). In all the three cultivars of mango ('Tommy Atkins', 'Keitt' and 'Kent'), 3-carene, α–pinene and ethanol were the most abundant metabolites in the present trial which is in confirmation with the studies done on mango by other workers (MacLeod and Snyder 1985; Malundo *et al*. 1997; Moalemiyan *et al*. 2006, 2007). Other metabolites detected in our study in 'Tommy Atkins', 'Kent' and 'Keitt' mangoes like toluene, ethyl acetate, limonene, terpinolene, 2-methylfuran and camphene have earlier been detected by other workers in these mangoes (Narain and Galavao 2004; Moalemiyan *et al*. 2006, 2007). Butanoic acid ethyl ester and ethyl 2-methyl butanoate are mango aroma esters and have been detected in all the three mango cultivars in the present study and reported by earlier workers (MacLeod and Snyder 1985; Narain and Galavao 2004; Moalemiyan *et al*. 2006, 2007). The presence of butanoic acid ethyl ester in other mango cultivars like 'Baladi', 'Kensington Pride' and several from Cuba has been reported in Egypt, Cuba and Australia (Engel and Tressl 1983; Bartley and Schwede 1987; Pino *et al*. 1989). These compounds were present in higher amounts in infected mangoes than the healthy ones in our study.

In the present study, the discriminant models (DA) based on metabolic fingerprints using abundance of 150 mass ions classified observations to their respective treatments better than those based on abundance of relatively consistent metabolites. Similar results were obtained when DA models were developed for 'Keitt' and 'Tommy Atkins' cultivars in an earlier study with two plant pathogens (Moalemiyan *et al*. 2006, 2007). The lower classification rate in models developed using abundance of relatively consistent metabolites could be due to the fact that unique metabolites with high discriminability were excluded from the analysis and also owing to the fact that not all peaks detected in the study were included in the analysis. Depending on the treatments DA-models correctly classified up to 100% of the observations. Marsili (1999) used multivariate analysis to discriminate different abiotic and biotic factors responsible for milk spoilage. For cross-validation of DA-models in our study we have used jack-knife method. The cross-validation method used in this study is unbiased although it does not eliminate the necessity of the commonly preferred test validation of the models which is done by using a data set not used in model development. The test-validation of DA-models was done using a data set which was not used in developing the model. DA-models and principal component analysis has been used earlier to discriminate five diseases of onion bulbs (Vikram *et al*. 2005). The models developed based on relative abundance of mass ions are very easy to develop and can be automated for user-friendly applications. However, the applicability of compound-based models depends on correct identification of compounds. The advantage of DA based discrimination in the absence of unique metabolite markers is the capacity of automation of the method which brings about rapid and early detection of the diseased storage produce.

The methods devised in the present trial to discriminate mango diseases in different cultivars can be used by storage managers to make a decision on the length of product storage or in choosing which lots can be stored for longer period of time. The methods developed needs to be validated under commercial conditions to assess the quality of mangoes either before harvest, at harvest or during storage. In addition volatiles can be collected in air tight containers by placing the samples for up to 3 hours and then GC/MS can be used to trap and analyze volatiles. The discriminant models developed based on mass ions can be used to create algorithms as the main core of computer based packages. These programs may be installed on computers attached to a portable GC/MS and make possible real time disease identification based on discriminatory mass ion. One great advantage of such method is its independence from convoluted and challenging process of compound identification.

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Supplementary Table Names (based on NIST library) of the metabolites detected in the headspace gas of mango fruits (cultivars 'Kent', 'Keitt' and 'Tommy Atkins') inoculated with water or three plant pathogens

RN	Compounds	*Alternaria*	*Colletotrichum*	*Lasiodiplodia*	N-control	W-control	Inoculation	Cultivar
1	1-Butanol		56	59			CL	Tommy Atkins
2	1,2,4-Benzenetricarboxylic acid, 1,2-dimethyl ester		1		1	1	CNW	Keitt
3	1,4-Cyclohexadiene, 1-methyl-		13		4	5	CNW	Keitt
4	1,4-Pentadiene				1	1	NW	Keitt
5	2,4,6-Octatriene, 2,6-dimethyl-	875	285	905	84	872	ACLNW	Tommy Atkins
6	2-Butenoic acid, ethyl ester	151	16	50	1	9	ACLNW	Keitt
6	2-Butenoic acid, ethyl ester		163	232	2	19	CLNW	Tommy Atkins
7	2-Butenoic acid, methyl ester, (*Z*)-		7				C	Kent
7	2-Butenoic acid, methyl ester, (*Z*)-	13	85	20	1	11	ACLNW	Keitt
7	2-Butenoic acid, methyl ester, (*Z*)-		131		15	41	CNW	Tommy Atkins
8	2-Propenoic acid, 2-methyl-, ethyl ester			107			L	Kent
8	2-Propenoic acid, 2-methyl-, ethyl ester	267	351	450	28	305	ACLNW	Keitt
8	2-Propenoic acid, 2-methyl-, ethyl ester		2271	1256	49	532	CLNW	Tommy Atkins
9	3-Carene	4419	12113	4928	2441	2659	ACLNW	Kent
9	3-Carene	25573	28587	28711	19081	18595	ACLNW	Keitt
9	3-Carene	85827	92578	144753	53375	63080	ACLNW	Tommy Atkins
10	3-Pentanone	1	3	9	7	6	ACLNW	Tommy Atkins
11	Acetic acid, methyl ester		7	5	1	8	CLNW	Keitt
12	Benzene, tert-butyl-	7	6	7	9	5	ACLNW	Kent
12	Benzene, tert-butyl-	32	5	1	1	1	ACLNW	Keitt
13	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-		2	204			CL	Kent
13	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	483	150	103	178	175	ACLNW	Keitt
13	Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-		1776	3261	1068	3329	CLNW	Tommy Atkins
14	Borane-methyl sulfide complex	187	328	16	274	323	ACLNW	Tommy Atkins
15	Boronic acid, ethyl-		5	57			CL	Keitt
16	Butanoic acid, 2-methyl-, ethyl ester		13	138	2	1	CLNW	Tommy Atkins
17	Butanoic acid, ethyl ester		84	209			CL	Kent
17	Butanoic acid, ethyl ester	853	179	422	14	135	ACLNW	Keitt
17	Butanoic acid, ethyl ester		1193	1314	70	115	CLNW	Tommy Atkins
18	Butanoic acid, methyl ester	7	8	4		1	ACLW	Keitt
19	Camphene	882	536	1334	417	1383	ACLNW	Tommy Atkins
20	Chloroform	27	7	2	17	22	ACLNW	Kent
20	Chloroform	58	12	15	18	12	ACLNW	Keitt
20	Chloroform	20		31	1	6	ALNW	Tommy Atkins
21	Cycloheptane, bromo-		8	1	5	4	CLNW	Keitt
22	Cyclohexane		4	2	4	7	CLNW	Keitt
23	Cyclohexane, 1,3-dimethyl-, cis-		36	34	29	39	CLNW	Keitt
23	Cyclohexane, 1,3-dimethyl-, cis-		32	38	53	15	CLNW	Tommy Atkins
24	Cyclohexane, ethyl-		1	1	1	2	CLNW	Keitt
25	Cyclohexane, methyl-		59	53	63	72	CLNW	Keitt
25	Cyclohexane, methyl-		63	50	59	46	CLNW	Tommy Atkins
26	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	62	176	69	277	34	ACLNW	Kent
26	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	989	2332	3796	1276	5186	ACLNW	Tommy Atkins
27	(*E*)-3-Caren-2-ol		1014	684	363	580	CLNW	Tommy Atkins
28	Ethanol	15303	21671	25585	7692	346	ACLNW	Kent
28	Ethanol	7579	1283	14821	31	412	ACLNW	Keitt
28	Ethanol	14292	9152	41008	290	242	ACLNW	Tommy Atkins
29	Ethyl Acetate		4	81			CL	Kent
29	Ethyl Acetate	253	32	103	1	846	ACLNW	Keitt
29	Ethyl Acetate		278	245	6	354	CLNW	Tommy Atkins
30	Ethylbenzene	11	5	1	6	4	ACLNW	Kent
30	Ethylbenzene	3	4	10	7	7	ACLNW	Keitt
30	Ethylbenzene	19		3	2	2	ALNW	Tommy Atkins
31	Furan, 2-methyl-		84	9	128		CLN	Kent
31	Furan, 2-methyl-		3	3	23	8	CLNW	Keitt
31	Furan, 2-methyl-	19	52	9	35	35	ACLNW	Tommy Atkins
32	Hexanoic acid, ethyl ester		24		24		CN	Kent
32	Hexanoic acid, ethyl ester	1091	341	467	27	303	ACLNW	Keitt
32	Hexanoic acid, ethyl ester		3787	1878	317	1281	CLNW	Tommy Atkins
33	Limonene	65	29	49		15	ACLW	Kent
33	Limonene	514	509	713	482	385	ACLNW	Keitt
33	Limonene	8062	4520	7941	2307	3803	ACLNW	Tommy Atkins
34	Octanoic acid, ethyl ester		424	79	13	63	CLNW	Tommy Atkins
35	Octanoic acid, methyl ester		18				C	Keitt
35	Octanoic acid, methyl ester		3		21	17	CNW	Tommy Atkins

Supplementary Table (Cont.)

RN	Compounds	*Alternaria*	*Colletotrichum*	*Lasiodiplodia*	N-control	W-control	Inoculation	Cultivar
36	Propanoic acid, ethyl ester			40			L	Kent
36	Propanoic acid, ethyl ester	184	19	98	1	27	ACLNW	Keitt
36	Propanoic acid, ethyl ester		70	95			CL	Tommy Atkins
39	Silanol, trimethyl	14	10		17	20	ACNW	Kent
39	Silanol, trimethyl-	95	24	16	30	33	ACLNW	Keitt
40	Styrene		8	4397	3	4	CLNW	Kent
40	Styrene	218	127	944	3	1	ACLNW	Keitt
40	Styrene	48	2765	2701			ACL	Tommy Atkins
41	Terpinolene		418	621	367	325	CLNW	Keitt
42	Toluene	37	41	25	63	38	ACLNW	Kent
42	Toluene	55	150	161	149	148	ACLNW	Keitt
42	Toluene	184	351	210	176	161	ACLNW	Tommy Atkins
43	Trichloromonofluoromethane		5	2	14	16	CLNW	Keitt
44	α -Pinene	7			41	3	ANW	Kent
44	α -Pinene	115	177	265	162	163	ACLNW	Keitt
44	α -Pinene	23610	25452	48093	14925	20173	ACLNW	Tommy Atkins
45	α-Phellandrene			25	4	31	LNW	Kent
45	α-Phellandrene	95	7	67	54	42	ACLNW	Keitt
45	α-Phellandrene	1541	307	379	276	453	ACLNW	Tommy Atkins

A: *Alternaria alternata*; **C**: *Colletotrichum gloeosporioides*; **L**: *Lasiodiplodia theobromae*; **N**: non-wounded-non-inoculated control; **W**: wounded-non-inoculated control.

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