

## USE OF VOLATILE METABOLITE PROFILES TO DISCRIMINATE FUNGAL DISEASES OF CORTLAND AND EMPIRE APPLES

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### ABSTRACT

The volatile metabolites from the headspace gas of apple fruits, cvs Cortland and Empire, inoculated with water or four different fungi, *Botrytis cinerea* Pers., *Penicillium expansum* Link, *Mucor piriformis* Fischer and *Monilinia* sp, were profiled using gas chromatography/mass spectrometry (GC/MS). A total of 1081 different peaks were detected. The number of compounds that occurred in abundance  $\geq 10^5$  and relatively consistently in 6 replicates over 3 incubation periods was 34 and 36, in Cortland and Empire, respectively. Of the consistent metabolites in Cortland, 19 were specific to one or more diseases/inoculations, including five that were unique to apples inoculated with different pathogens. In Empire, 15 compounds were specific to one or more diseases/inoculations, including 3 that were unique to single pathogens. In Cortland, dimethyl ether and propanal were specific to *Penicillium*, while acetic acid methyl ester and styrene were common only to *B. cinerea* and *Monilinia*. Similarly, in Empire the compounds 3,4-dimethyl-1-hexene, butanoic acid-2-methylpentyl ester, and 2-methyl propyl hexanoate were common only to *B. cinerea*, *M. piriformis* and *Monilinia*, respectively. A factor analysis, considering 29 relatively consistent metabolites for both cultivars, discriminated all the disease/inoculations. The disease/ inoculation discriminatory metabolites or groups of these metabolites based on factor models could be used for the early detection of apple diseases in storage. However, for commercial application of the system scale-up studies and validation under practical condition is required.

*Key words:* disease diagnosis, post-harvest pathogens, metabolomics, electronic nose.

### INTRODUCTION

Apple is one of the most frequently consumed fruits and is a major fruit crop cultivated worldwide. China is the leader in apple production followed by the United States of America, Turkey, Italy, Poland, France, Germany, Argentina and Japan. Apple production in Canada is 514,333 ton with major contributions coming from the provinces of Quebec, Ontario, British Columbia, Nova Scotia and New Brunswick (Statistics Canada, 2003). Apples are often stored for 6-10 months after harvest wherein they are subjected to attack by various pathogens. Detection of diseases in stored apples is very difficult, especially in controlled atmosphere storage, where apples are kept in closed chambers for long duration of time. *Botrytis cinerea* Pers., *Penicillium expansum* Link., and *Mucor piriformis* Fischer are some of the most common and important post-harvest pathogens of apple (Pierson *et al.*, 1971; Michailides and Spotts, 1990). *B. cinerea*, the causal agent of gray mold rot in apple, produces dry lesions which becomes soft as the rot advances. The blue mold rot caused by *P. expansum* is characterized by production of soft watery brown spots. In Mucor rot caused by *M. piriformis* the lesions are formed on the fruit surface and the skin turns dark brown with a pale brown margin. The brown rot caused by *Monilinia* sp. shows the development of a small circular brown spot as the first indication of fruit infection. The detection of these diseases in storage is often possible only at an advanced stage and any intervention at this stage will not significantly reduce losses. Controlled atmosphere storage method which consists of combining low temperature and oxygen with levels of carbon dioxide in order to suppress the metabolic activities of fruits in storage is one of the most important advances since beginning of use of mechanical cooling methods in fruit and vegetable storage (Blankenship, 1985; Hardenburg *et al.*, 1986; Blanpied, 1987). But under these conditions, the detection of diseases by visual observations is not possible since the storage room is closed for longer periods of time, in addition to apples are buried in the pallet boxes. Thus, there is a need to develop sensitive, rapid and cost effective methods for the detection and identification of pathogens. Such a

knowledge base can ultimately help reduction of unexpected storage losses.

Rotten smell of apples has been used as a means to detect diseases in storage. Volatiles produced by rotting fruits and vegetables have been identified and used by many researchers in order to develop a technology to detect and discriminate diseases in stored products. The production of volatile metabolites from diseased carrot, citrus, onion, peach, potato, raspberry and other crops have been reported by various workers using Gas Chromatograph, and Gas Chromatograph/Mass Spectrometer (GC/MS) (Davis and Smoot, 1972; Pauli and Knoblauch, 1987; Wilson and Wisniewski, 1989; Kallio and Salorinne, 1990; Ouellette *et al.*, 1990; de Lacy Costello *et al.*, 1999; Kushalappa *et al.*, 2002). The inoculation of potato with *Phytophthora infestans* and *Fusarium coeruleum* produced disease specific compounds such as butanal, 3-methyl butanal, undecane, verbenone, 2-pentyl furan and capaene (de Lacy Costello *et al.*, 2001).

Many compounds produced by apples have been identified. These were primarily acetaldehydes and esters of formic, acetic and hexanoic acids (Power and Chestnut, 1920). During the late 1940s investigations at the Eastern Regional Laboratory (USDA) enlarged the listing of compounds that contribute to apple aroma (White, 1950). Later Meigh (1956) identified six alcohols, five aldehydes, three ketones and six esterified acids in intact apples. Over 300 compounds contributing to apple flavor and aroma from many different cultivars have been reviewed (Dimick and Hoskin, 1983). But to date no work has been published on production of disease specific volatiles in apples.

Though volatiles have been detected in diseased fruits and vegetables the occurrence of these compounds appears to be quite inconsistent. In spite of such variations, modeling approach has been taken to discriminate diseases. In potato, using neural network modeling of GC retention time and peak area data it was possible to discriminate four diseases (Kushalappa *et al.*, 2002). However, for lack of identity of compounds the use of GC retention time alone to detect and discriminate diseases is not enough and confirmation based on compound identity is needed for higher assurance sought by users. Therefore a study was undertaken to analyze volatiles of apple cultivars Cortland and Empire inoculated with a few important fungal pathogens using GC/MS technology with an ultimate goal to develop a disease detection system for use in storage.

## MATERIALS AND METHODS

**Preparation of apples for inoculation.** The two cultivars of apples, namely Cortland and Empire, harvested from a single orchard were obtained from Stevenson

Orchards. Apparently disease free apples were selected and surface sterilized with 0.5% sodium hypochlorite solution for 3 minutes, rinsed with sterile water and dried under laminar flow hood. Five wounds, 4 mm in diameter and 3 mm deep, were made around the equatorial region with a cork borer.

**Pathogen cultures.** The fungal cultures, namely *Botrytis cinerea* (isolate #B-27), *Penicillium expansum* (isolate #1790), *Mucor piriformis* (isolate #563) and *Monilinia* sp. (isolate #1683), were obtained from the Agriculture and Agri-Food Canada, Summerland, British Columbia. They were grown on potato dextrose agar at 22°C for 7 days and stored at 4°C. Seven-ten day old sub-cultures were flooded with 0.05% Tween 80 in distilled water and filtered through two layers of cheese-cloth. The concentration of spores in the suspension was adjusted to  $10^5$  ml<sup>-1</sup> conidia using a haemocytometer.

**Inoculation and incubation.** The apples were inoculated with different pathogens/water (Tween 0.05%) by placing 20 µl of fungal spore suspension into each wound using a micropipette. They were placed singly on a stainless steel support in a 1 l wide mouthed glass Mason jars containing 10 ml of sterile distilled water for creating saturated atmosphere to facilitate initial establishment of infection and were incubated at 20°C. After 24 h the water was removed and the bottle air was continuously flushed with laboratory air, which bubbled through a flask containing water to provide moist air at the rate of 0.04 m<sup>3</sup> h<sup>-1</sup>.

**Accumulation of volatiles and GC/MS analysis.** The apples were removed from Mason jars at 2, 4 and 6 days after inoculation (DAI) and placed in one liter wide mouthed glass bottles with caps lined with Teflon coated septa, flushed with pure dry air and incubated at 20°C for 30 min for volatile accumulation. The headspace gas was sampled and the volatiles were analyzed using HAPSITE (INFICON, Syracuse, New York, USA). The HAPSITE was programmed to sample headspace air for 30 s at the rate of 100 ml min<sup>-1</sup>. After sampling the apples were returned to Mason jars and humid air was flushed continuously. The entire experiment was conducted six times on different days.

HAPSITE is a GC/MS system equipped with a hand held sniffing probe attached to a terminal stainless steel needle, 21 gauge and 10 cm long which could be inserted into a septa bottle. The headspace air was pre-concentrated in a tube trap containing 15 mg carboxen and the sample was then thermally desorbed at 225°C. The gas passed through a GC with a capillary column SPB-5 (Supelco, Bellfonte, CA, USA) 30 m, 0.32 mm internal diameter with 1.0 µm film coating (and configured in a heating coil, INFICON - 930-489-G8). The carrier gas used was volatile organic free nitrogen at a flow rate of

3 ml min<sup>-1</sup>. The temperature of the column was maintained at 50°C isothermal for 4 minutes followed by a ramping of 3°C min<sup>-1</sup> for 26 min, and 14.4°C min<sup>-1</sup> for 5 min, when the temperature reached 200°C. The GC was interfaced to a MS equipped with a quadrupole analyzer. The mass spectrum was scanned at the rate of 1.04 s per mass decade over a mass range of 46-300 *m/z*. NIST mass spectral search program (Version 2.0) was used for tentative identification of compounds. The amounts of volatiles were expressed as mass-ion abundance (relative response of quadrupole detector). A mixture of bromopentafluorobenzene and 1,3,5-tris-(trifluoromethyl) benzene, programmed to inject known amount automatically from a canister (INFICON, Syracuse, NY, USA), was used as an internal standard to calibrate the GC/MS.

**Disease severity assessment.** The diameter and depth of diseased apple tissue was measured on 2, 4 and 6 DAI (Day After Inoculation), from which the volume of diseased tissue was calculated.

**Experimental design and data analysis.** The experiment was designed as factorial, with 6 main factors of pathogens/inoculations: non-wounded-inoculated with sterile water (N-control), wounded-inoculated with sterile water (W-control), inoculated with pathogens *B. cinerea*, *P. expansum*, *M. piriformis* and *Monilinia* sp. and 3 sub-factors of incubation times 2, 4 and 6 DAI. Each experimental unit consisted of one apple and the entire experiment was conducted six times. The data output consisted of compounds and their abundance of mass ions. The metabolites, which occurred in two or more replicates, were used to calculate the frequency and average abundance of metabolites (out of six replicates x three incubation times = 18). The compounds identified were also grouped according to their chemical functional group, for which average abundance was calculated which was divided by the average total abundance and multiplied by 100 to derive normalized or percent abundance. The compounds occurring in one or more diseases/inoculations, but not in all 6 main treatments, were sorted and designated here as disease/inoculation discriminatory metabolites.

**Statistical analysis.** The average abundance of relatively consistent but excluding unique compounds for each of Cortland and Empire cultivars were averaged per cultivar and normalized by dividing abundance of each metabolite by the total abundance for all compounds for both the cultivars. The proportion of abundance values, averaged for 2 cvs for 29 relatively consistent compounds for different disease/inoculation treatments were subjected to factor analysis using principal component method by means of procedure FACTOR in SAS (version 8.02, SAS Institute Inc., Cary, NC, USA).

A set of variables that are linear combinations of original proportion of abundance were produced. The new variables were independent of each other and ranked according to the amount of variance. After initial factor extraction an orthogonal varimax rotation was used to estimate the factor loadings. Factor scores were calculated and projected for all treatments on the plane of factor 1 and 2. The first 5 factors associated with the 29 relatively consistent metabolites were used to describe the different diseases/inoculations. Scatter plot was developed based only on factors 1-3.

## RESULTS

**Disease progress.** Visible rotting symptoms were observed in Cortland and Empire apples inoculated with *P. expansum* and *B. cinerea* on 2 DAI, which increased over days of incubation. In cultivar Cortland, the lesions from *B. cinerea* and *P. expansum* were 1.3 and 0.6 cm in diameter, respectively, on 2 DAI, which increased to 3.6 and 2.2 cm at 4 DAI and the lesions coalesced at 6 DAI. The inoculation of *M. piriformis* and *Monilinia* sp. produced only a slight brownish discoloration around the inoculation site on 6 DAI in both the cultivars. The non-wounded control and wounded control remained disease free throughout the six-day period. The range of volume of disease observed for Cortland, from 2 to 6 DAI, were: 1.7-32.9 cm<sup>3</sup> for *B. cinerea* and 0.2-14.1 cm<sup>3</sup> for *P. expansum*. In cv Empire, the lesions from *B. cinerea* and *P. expansum* were 1.6 and 1.1 cm in diameter, respectively, on 2 DAI which increased to 3.2 and 2.1 cm at 4 DAI and the lesions coalesced at 6 DAI. The range of volumes of disease observed for cv Empire were: 3.3-29.5 cm<sup>3</sup> for *B. cinerea*, 0.9-9.7 cm<sup>3</sup> for *P. expansum*, 0.8-3.3 cm<sup>3</sup> for *M. piriformis* and 1.3-2.4 cm<sup>3</sup> for *Monilinia* sp..

**Volatile metabolic profile.** The headspace gas analyses of apples of cv Cortland inoculated with water or pathogens yielded a total of 538 different volatile metabolites with their abundance varying from 10<sup>5</sup>-7.2·10<sup>9</sup>. Most of the volatiles eluted within 20 min and 99% within 25 min. The number and abundance of compounds varied with treatment, incubation time and replicates. The incubation times (2, 4 and 6 DAI) did not show a particular general trend in volatile production, though there were some increasing or decreasing trend in abundance of certain compounds within a disease/inoculation, but were inconsistent among replicates (data not shown). Therefore, the volatile compounds produced at 2, 4 and 6 DAI were combined and were classified into different chemical groups. The number and average abundance of metabolites for different chemical groups are presented in Table 1. The highest number of volatile compounds, 136, was detected from wounded control treatment followed by *P. expansum*,

**Table 1.** Number and normalized abundance (%) of different chemical groups of compounds<sup>a</sup> detected in Cortland and Empire apples inoculated<sup>b</sup> with different pathogens/water.

Group	N-control	W-control	<i>Botrytis cinerea</i>	<i>Mucor piriformis</i>	<i>Monilinia sp.</i>	<i>Penicillium expansum</i>
<b>CORTLAND</b>						
Alcohol	6 (5.5)	10 (3.1)	7 (6.4)	13 (4.2)	9 (5.2)	11 (6.8)
Aliphatic	33 (23.8)	37 (16.9)	27 (9.2)	30 (23.2)	31 (3.6)	30 (8.0)
Alkene	3 (1.0)	5 (3.8)	4 (4.6)	2 (0.1)	9 (2.2)	7 (1.4)
Aromatic	14 (5.9)	10 (6.3)	11 (8.5)	16 (2.8)	10 (1.8)	18 (2.6)
Ester	24 (62.9)	30 (69.6)	29 (63.6)	31 (67.0)	32 (66.7)	29 (68.7)
Nitrogenous	18 (0.8)	30 (0.1)	21 (0.2)	13 (2.6)	23 (20.1)	20 (1.5)
Sulfur	6 (0.1)	8 (0.1)	6 (0.1)	4 (0.1)	10 (0.2)	11 (0.2)
Other	6 (T)	6 (0.1)	7 (7.4)	9 (T)	6 (0.2)	8 (10.8)
Total	110 (150)	136 (201)	112 (94)	118 (222)	130 (162)	134 (117)
<b>EMPIRE</b>						
Alcohol	10 (5.1)	12 (12.9)	11 (2.4)	13 (6.3)	13 (3.1)	7 (6.3)
Aliphatic	48 (30.9)	36 (34.6)	20 (25.5)	36 (19.6)	26 (18.2)	25 (32.4)
Alkene	13 (6.1)	8 (0.4)	10 (0.2)	12 (0.1)	5 (0.2)	4 (0.6)
Aromatic	15 (0.2)	14 (0.4)	12 (0.1)	13 (0.1)	8 (4.2)	18 (0.1)
Ester	45 (46.2)	37 (47.3)	42 (52.9)	43 (46.6)	40 (42.5)	43 (56.6)
Nitrogenous	26 (1.4)	21 (0.1)	14 (18.3)	20 (9.7)	16 (10.4)	21 (3.6)
Sulfur	15 (0.1)	8 (T)	10 (0.1)	12 (T)	6 (8.9)	7 (0.1)
Other	4 (T)	6 (4.3)	9 (0.5)	9 (17.6)	14 (12.5)	7 (0.3)
Total	176 (226)	142 (212)	128 (192)	158 (274)	128 (491)	132 (181)

<sup>a</sup> Normalized abundance = (Average abundance/total abundance for all compounds)100 - the values in parenthesis; T = traces of abundance <10<sup>5</sup>; Total = Total number of relatively consistent compounds detected and in parenthesis are the total mass ion abundance (quadrupole detector output) = x10<sup>8</sup> - based on average per replicate x incubation time.

<sup>b</sup> N-control = non-wounded-water-inoculated control; W-control = Wounded-water-inoculated control.

*Monilinia sp.*, *M. piriformis*, *B. cinerea* and non-wounded control that yielded 134, 130, 118, 112, and 110 volatile compounds, respectively. The number of esters was the highest for all the diseases/inoculations tested here. Maximum number of alkenes and esters were detected in *Monilinia sp.* inoculated, while aliphatic and nitrogenous compounds were more in wounded control. *P. expansum* inoculation resulted in higher number of aromatic and sulfur compounds while *M. piriformis* inoculated had the highest number of alcohols. The normalized abundance of chemical groups of compounds varied among the diseases/inoculations (Table 1). Higher proportion of alkenes (4.6%) and aromatics (8.5%) were detected in *B. cinerea* while higher proportion of alcohols (6.8%) and sulfurs (0.2%) in *P. expansum* inoculation. *M. piriformis* inoculations recorded nitrogenous (2.6%) compounds in higher proportions than others. Similarly, wounded control and non-wounded control recorded high proportions of esters (69.6%) and aliphatics (23.8%), respectively.

A total of 643 volatile metabolites were obtained in cv Empire apples inoculated with water or pathogens, with their abundance varying from 10<sup>5</sup>–1.7·10<sup>9</sup>. The number and average abundance of metabolites for different chemical groups are shown in Table 1. The highest number of volatile compounds detected was from non-wounded control treatment, 176, followed by *M. piriformis*, wounded control, *P. expansum*, *B. cinerea* and

*Monilinia sp.* which yielded 158, 142, 132, 128 and 128 volatile compounds, respectively (Table 1). In general, the highest contribution of compounds came from esters. Maximum number of aliphatics, alkenes, sulfurs and nitrogenous compounds were detected in non-wounded control, while largest number of alcohols was found in *M. piriformis* and *Monilinia sp.* inoculated ones. Largest number of aromatic compounds was found in apples inoculated with *P. expansum*. Variation among the diseases/inoculations was observed with respect to normalized abundance of chemical groups of compounds (Table 1). Higher proportion of aromatics (4.2%) and sulfurs (8.9%) were detected in *Monilinia sp.* while the proportion of alcohols (12.9%) and aliphatics (34.6%) were higher in wounded control. Non-wounded control recorded higher alkenes (6.1%) while *B. cinerea* and *P. expansum* recorded nitrogenous compounds (18.3%) and esters (56.6%) in proportions higher than other treatments.

#### Disease/inoculation specific volatile metabolites.

The headspace gas analysis of both Cortland and Empire, inoculated with pathogens/water yielded a total of more than 1,000 compounds, but many were inconsistent among incubation times and replicates. The volatile metabolites, which occurred in at least 3 out of 18 (6 replicates over 3 incubation periods) are presented in Tables 2 and 3.

**Table 2.** The abundance<sup>a</sup> and frequency of volatile metabolites detected in Cortland apples non-inoculated and inoculated with four different fungal pathogens.

RT mm:ss	Chem-Group	Compounds	N-control	W-control	<i>Botrytis cinerea</i>	<i>Mucor piriformis</i>	<i>Monilia</i> sp.	<i>Penicillium expansum</i>	Disease/Inoc <sup>b</sup>
01:05	Ether	dimethyl ether						9(3)	P
01:10	Aldehyde	propanal						2(3)	P
01:19	Ester	acetic acid methyl ester		6 (8)					B
02:13	Aliphatic	2-methyltetrazole		1408 (3)					W
07:24	Aliphatic	oxirane, (1-methylbutyl)-	76 (3)						N
08:13	Aromatic	styrene					23 (5)		M
07:22	Alkene	1-hexene			116 (5)			38(5)	BP
13:22	Ester	butanoic acid butyl ester					299 (3)	1963(3)	MP
01:04	Aliphatic	fluoroethene		3 (3)	9 (3)				WB
18:22	Ester	heptanoic acid ethyl ester			146 (3)			3(3)	BP
04:16	Ester	2-methylbutanoic acid methyl ester			96 (14)		13 (5)	99(10)	BMP
18:11	Ester	hexanoic acid propyl ester		344 (3)			6 (4)	5 (3)	WMP
01:52	Ester	propanoic acid methyl ester			13 (10)		8 (5)	82 (7)	BMP
03:18	Aliphatic	4-methyl-1-hexene	5 (4)	9 (4)			14 (5)	4 (5)	NWMP
01:05	Alcohol	ethanol			18 (5)	7 (5)	1 (6)	5 (6)	BUMP
09:50	Ester	hexanoic acid methyl ester			201 (15)	187 (3)	104 (5)	254 (8)	BUMP
18:44	Ester	propanoic acid hexyl ester		358 (5)	135 (3)	558 (3)		465 (3)	WBUP
01:22	Sulfur	thiirane	1 (4)		1 (4)		1 (4)	4 (4)	NBMP
14:02	Ester	acetic acid hexyl ester		2 (4)	426 (6)	754 (4)	461 (6)	2 (3)	WBUMP
02:12	Alcohol	1-butanol	365 (12)	18 (11)	22 (12)	542 (15)	289 (12)	19 (11)	NWBUMP
03:21	Alcohol	2-methyl-1-butanol	589 (13)	4 (15)	13 (14)	9 (14)	380 (14)	8 (12)	NWBUMP
07:32	Alcohol	1-hexanol	827 (9)	796 (8)	830 (8)	804 (9)	1075 (8)	813 (17)	NWBUMP
07:26	Aliphatic	2H-pyran-2-one, tetrahydro-3,6-dimethyl	72 (3)	172 (7)	93 (4)	109 (10)	136 (10)	37 (5)	NWBUMP
02:09	Alcohol	3-methyl-4-penten-2-ol	15 (5)	52 (6)	45 (4)	36 (4)	17 (4)	43 (4)	NWBUMP
06:40	Ester	2-methylbutanoic acid ethyl ester	1448 (18)	1189 (18)	1517 (18)	1243 (18)	974 (18)	1402 (18)	NWBUMP
04:59	Ester	butanoic acid ethyl ester	683 (18)	569 (18)	1184 (18)	1195 (18)	1753 (18)	998 (18)	NWBUMP
02:59	Ester	butanoic acid methyl ester	394 (13)	437 (16)	655 (18)	638 (18)	450 (17)	645 (18)	NWBUMP
08:38	Ester	butanoic acid propyl ester	2017 (13)	1257 (13)	18 (6)	2824 (14)	1928 (6)	1402 (9)	NWBUMP
01:45	Aliphatic	chloroform	2 (3)	1 (13)	18 (3)	6 (3)	5 (4)	3 (5)	NWBUMP
01:42	Ester	Ethyl acetate	53 (14)	56 (13)	224 (15)	62 (14)	59 (15)	58 (16)	NWBUMP
13:22	Ester	hexanoic acid ethyl ester	1102 (18)	765 (13)	1512 (18)	1580 (18)	637 (18)	903 (18)	NWBUMP
08:42	Ester	pentanoic acid ethyl ester	7 (6)	23 (5)	504 (5)	19 (3)	8 (10)	11 (7)	NWBUMP
03:47	Ester	methyl-2-ethyl propanoate	2096 (9)	1 (5)	521 (8)	2 (10)	1805 (16)	4 (14)	NWBUMP
02:51	Ester	propanoic acid ethyl ester	548 (18)	727 (18)	701 (18)	677 (18)	215 (18)	799 (18)	NWBUMP

<sup>a</sup> Average abundance = average of 18 replicate x incubation periods; abundance x 105<sup>b</sup> Disease/inoculation = N-control = Non-wounded control; W-control = Wounded control; B = *Botrytis cinerea*; U = *Mucor piriformis*; M = *Monilia* sp.; P = *Penicillium expansum*.

The numbers outside the bracket is for abundance and the ones inside the bracket is for frequency of compounds. Some discriminatory compounds found were detected once or two times in other groups and their frequencies are not included in the above list.

**Table 3.** The abundance<sup>a</sup> and frequency of volatile metabolites detected in Empire apples inoculated with four different fungal pathogens.

RT min:ss	Chem-Group	Compounds	N-control	W-control	<i>Botrytis cinerea</i>	<i>Mucor piriformis</i>	<i>Monilinia sp.</i>	<i>Penicillium expansum</i>	Disease/Inoc <sup>b</sup>
18:28	Alkene	3,4-dimethyl-1-hexene			1 (3)				B
20:24	Ester	butanoic acid- 2-methyl-pentyl ester				422 (3)			U
22:35	Ester	2-methyl propyl hexanoate					1 (3)		M
02:10	Aliphatic	oxetane, 3,3-dimethyl-	245 (3)						N
01:12	Aliphatic	trimethylene oxide		1 (3)					W
01:52	Ester	propanoic acid methyl ester			11 (10)				BP
01:26	Alcohol	1-propanol		7 (3)	6 (3)				NWB
02:09	Alcohol	3-methyl-4-penten-2-ol	1 (3)	39 (3)		50 (3)	1 (4)		WUM
01:05	Alcohol	ethanol			6 (4)	1 (4)	1 (4)	6 (6)	BMP
02:58	Ester	methyl-2-methyl propanoate					58 (5)	3 (3)	UMP
05:17	Ester	propanoic acid propyl ester	10 (4)	20 (3)		48 (4)			WNU
01:19	Ester	acetic acid methyl ester			22 (10)	2 (4)	1 (4)	7 (12)	BUMP
09:50	Ester	hexanoic acid methyl ester			167 (15)	2 (3)	9 (3)	107 (13)	BUMP
02:13	Aliphatic	2-methyltetrazole	697 (3)	704 (3)	665 (3)	437 (4)	660 (4)		NWBUM
02:59	Ester	butanoic acid methyl ester		84 (3)	147 (14)	101 (7)	5 (7)	131 (14)	WBUMP
02:12	Alcohol	1-butanol	10 (13)	34 (14)	41 (15)	35 (13)	36 (12)	795 (15)	NWBUMP
03:21	Alcohol	2-methyl-1-butanol	1 (9)	4 (14)	424 (18)	780 (17)	5 (18)	353 (17)	NWBUMP
07:50	Ester	2-methyl-1-butyl acetate	1646 (18)	785 (18)	849 (18)	798 (18)	710 (18)	1479 (18)	NWBUMP
05:24	Ester	acetic acid butyl ester	1553 (18)	2058 (18)	2183 (18)	307 (17)	2088 (18)	913 (18)	NWBUMP
14:02	Ester	acetic acid hexyl ester	1399 (18)	2510 (18)	1667 (18)	944 (18)	1004 (18)	1088 (18)	NWBUMP
09:20	Ester	acetic acid pentyl ester	1107 (12)	1381 (18)	935 (13)	944 (18)	1194 (15)	1028 (14)	NWBUMP
06:40	Ester	2-methylbutanoic acid ethyl ester	1151 (18)	460 (18)	914 (18)	667 (18)	866 (18)	1099 (18)	NWBUMP
04:16	Ester	2-methylbutanoic acid methyl ester	114 (13)	1 (14)	45 (17)	150 (16)	2 (15)	5 (18)	NWBUMP
10:50	Ester	2-methylbutanoic acid propyl ester	1252 (17)	739 (16)	328 (14)	927 (16)	1838 (14)	169 (13)	NWBUMP
13:22	Ester	butanoic acid butyl ester	1984 (11)	898 (11)	4085 (10)	737 (13)	1358 (10)	1387 (7)	NWBUMP
04:59	Ester	butanoic acid ethyl ester	1225 (18)	1101 (18)	801 (18)	1550 (18)	1041 (18)	1330 (18)	NWBUMP
08:38	Ester	butanoic acid propyl ester	2598 (18)	2281 (18)	892 (11)	931 (18)	3061 (14)	1350 (14)	NWBUMP
15:20	Ester	butyl 2-methylbutanoate	2216 (18)	898 (18)	895 (18)	1195 (18)	792 (18)	3020 (18)	NWBUMP
01:42	Ester	ethyl acetate	108 (16)	403 (18)	375 (17)	366 (18)	346 (18)	604 (18)	NWBUMP
22:42	Ester	hexanoic acid butyl ester	53 (8)	1340 (15)	103 (9)	74 (15)	102 (14)	99 (13)	NWBUMP
13:22	Ester	hexanoic acid ethyl ester	1840 (18)	1932 (18)	1482 (18)	1121 (18)	1027 (18)	132 (18)	NWBUMP
18:11	Ester	hexanoic acid propyl ester	1983 (14)	1573 (18)	996 (14)	1595 (18)	1480 (18)	1897 (17)	NWBUMP
09:11	Ester	propanoic acid butyl ester	1138 (16)	2198 (18)	3254 (11)	689 (17)	747 (16)	2638 (14)	NWBUMP
02:51	Ester	propanoic acid ethyl ester	6 (17)	1841 (18)	683 (18)	630 (18)	847 (18)	954 (18)	NWBUMP
18:44	Ester	propanoic acid hexyl ester	1701 (8)	1882 (13)	2828 (10)	1744 (12)	14 (18)	4576 (10)	NWBUMP
01:22	Sulfur	thiirane	2 (5)	1 (13)	2 (13)	2 (14)	2 (13)	2 (14)	NWBUMP

<sup>a</sup> Average abundance = average of 18 replicate x incubation periods; abundance x 105

<sup>b</sup> Disease/inoculation = N-control = Non-wounded control; W-control = Wounded control; B = *Botrytis cinerea*; U = *Mucor piriformis*; M = *Monilinia sp.*; P = *Penicillium expansum*.

The numbers outside the bracket is for abundance and the ones inside the bracket is for frequency of compounds. Some discriminatory compounds found were detected once or two times in other groups and their frequencies are not included in the above list.

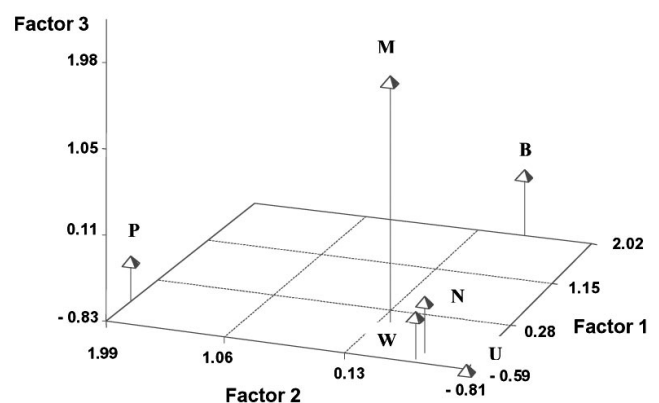
**Cortland.** A total of 34 volatile compounds were relatively consistently detected in all the disease/inoculations and the compound with the highest abundance ( $7.1 \cdot 10^9$ ) was butanoic acid butyl ester, found in wounded control. The frequency of occurrence of different compounds varied among diseases/inoculations. The disease specific compounds that occurred most frequently were 1-hexene, ethanol, propanoic acid hexyl ester, 2-methylbutanoic acid methyl ester, propanoic acid methyl ester, hexanoic acid propyl ester, 4-methyl-1-hexene, hexanoic acid methyl ester, acetic acid hexyl ester and thiirane (Table 2). Out of 34 consistent compounds, only 19 were specific to one or more diseases/inoculations, including 6 that were unique to single disease. *P. expansum* produced dimethyl ether and propanal while *B. cinerea* and *Monilinia* sp. produced acetic acid methyl ester and styrene, respectively. 2-methyltetrazole and 1-methylbutyl oxirane were specific for wounded control and non-wounded control, respectively. 1-hexene and heptanoic acid ethyl ester were detected in *B. cinerea* and *P. expansum* whereas butanoic acid butyl ester was identified in *B. cinerea* and wounded control.

**Empire.** A total of 36 relatively consistent volatile compounds were detected in apple cv Empire in all the diseases/inoculations. The compound accounted for the highest abundance ( $1.7 \cdot 10^9$ ) was 3,3-dimethyl oxetane found in *M. piriformis*. The disease specific compounds that occurred most frequently were ethanol, propanoic acid methyl ester, 3-methyl-4-penten-2-ol, methyl-2-methyl propanoate, propanoic acid propyl ester, acetic acid methyl ester, hexanoic acid methyl ester, 2-methyltetrazole and butanoic acid methyl ester (Table 3). Out of 36 relatively consistent compounds, only 15 were specific to one or more diseases/inoculations, including 5 that were unique to single disease. 3,4-dimethyl-1-hexene, butanoic acid-2-methyl-pentyl ester, 2-methyl propyl hexanoate, 3,3 dimethyl oxetane and trimethylene oxide were specific for *B. cinerea*, *M. piriformis*, *Monilinia* sp., non-wounded control and wounded control, respectively. Propanoic acid methyl ester was detected in *B. cinerea* and *P. expansum*. Ethanol was detected in *B. cinerea*, *Monilinia* sp. and *P. expansum* whereas methyl-2-methyl propanoate was found in *M. piriformis*, *Monilinia* sp. and *P. expansum*.

In the present study we observed that the compounds like ethanol, acetic acid methyl ester, propanoic acid methyl ester and hexanoic acid methyl ester were specific to apples inoculated with pathogens. These compounds were not detected in water inoculated control treatments.

**Disease/inoculation discrimination based on metabolite abundance.** Factor analysis, using principal component method, based on normalized abundance for 29 relatively consistent metabolites (out of 34 compounds 5 compounds were common to both cvs) (Table

4) discriminated all the six different diseases/inoculations (Fig. 1). Five factors explained 100% of the total variance, with a maximum of 35% by factor 1. The factor scores varied for different diseases/inoculations (scores for factors 1-3, out of 5, shown in Fig. 1). Highest factor scores were observed for: factor 1 for *B. cinerea*, factor 2 for *P. expansum*, factor 3 for *Monilinia* sp., factor 4 for N-control and factor 5 for W-control. Low scores of all the factors were observed for *M. piriformis*. The inspection of eigen vector loadings, for 29 compounds (names listed in Table 4), showed that the production of compounds like acetic acid methyl ester, heptanoic acid ethyl ester, fluoroethene, 3,4-dimethyl-1-hexene, ethanol, 1-hexene and 2-methylbutanoic acid methyl ester were governed by factor 1 (*B. cinerea*), while factor 2 (*P. expansum*) explained another aspect of the pathosystem which was the production of dimethyl ether, propanal, butanoic acid butyl ester, and propanoic acid methyl ester. Similarly, the production of 2-methyl propyl hexanoate, styrene, methyl-2-methyl propanoate and 4-methyl-1-hexene were dependent on factor 3 (*Monilinia* sp.) while 3,3-dimethyl oxetane and 1-methylbutyl oxirane were dependent on factor 4 (N-control). The compounds hexanoic acid propyl ester and trimethylene oxide were dependant on factor 5 (mainly W-control). Interestingly, the distance between non-wounded control and wounded control was shorter than the pathogen inoculated ones, which were classified away from the water-inoculated controls, except for *M. piriformis*. Among pathogen inoculated, the apples inoculated with *B. cinerea* can be differentiated from other pathogens by increased production of compounds such as acetic acid methyl ester, heptanoic acid ethyl ester, 3,4-dimethyl-1-hexene and 1-hexene (Table 4).



**Fig. 1.** Projections of factor scores, in the space of factors 1-3, from factor analysis (using principal component method) of proportion of abundance of mass ions for 29 disease/inoculation discriminatory compounds to discriminate different diseases/inoculations of Cortland and Empire apples: N = Non-wounded-water-inoculated control; W= wounded-water-inoculated control; B = inoculated with *Botrytis cinerea*; M = *Monilinia* sp.; U = *Mucor piriformis*; P = *Penicillium expansum*.

**Table 4.** Eigenvector loadings<sup>a</sup> and eigenvalues of principal components derived from proportion of abundance of 29 relatively consistent metabolites for apples inoculated with pathogens/water.

Metabolites	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Acetic acid methyl ester	0.99216*	0.01376	-0.07335	-0.03413	-0.09424
Heptanoic acid ethyl ester	0.99061*	-0.11369	-0.06231	0.01818	-0.03930
3,4-Dimethyl-1-hexene	0.98917*	-0.12709	-0.05955	0.01932	-0.03822
1-Hexene	0.98878*	0.09342	-0.10289	0.00042	-0.05480
Fluoroethene	0.95933*	-0.18031	-0.09522	-0.03427	0.19219
Ethanol	0.94769*	0.08110	-0.12154	-0.16552	-0.23054
2-Methylbutanoic acid methyl ester	0.84618*	0.51937	-0.01540	-0.5420	-0.10517
Hexanoic acid methyl ester	0.77867	0.34988	0.03299	-0.32973	-0.40181
Butanoic acid methyl ester	0.73021	0.16056	-0.45860	-0.47978	-0.02263
1-Propanol	0.64985	-0.32991	-0.21688	0.14130	0.63391
Dimethyl ether	-0.08927	0.97286*	-0.18369	-0.08415	-0.06889
Propanal	-0.08927	0.97286*	-0.18369	-0.08415	-0.06889
Butanoic acid butyl ester	-0.13646	0.97240*	0.10182	-0.11439	-0.11118
Propanoic acid methyl ester	0.28701	0.94559*	-0.03929	-0.09678	-0.11215
Thiirane	-0.00423	0.75781	0.11947	0.59071	-0.25000
Propanoic acid propyl ester	-0.50089	-0.57594	-0.55926	-0.14532	-0.28899
2-Methyl propyl hexanoate	-0.15413	-0.05446	0.97188*	-0.09731	-0.13874
Styrene	-0.15413	-0.05446	0.97188*	-0.09731	-0.13874
Methyl-2-methyl propanoate	-0.16093	-0.03287	0.96945*	-0.10640	-0.14784
4-Methyl-1-hexene	-0.40690	-0.09844	0.82698*	0.35713	0.11537
3,3-Dimethyl-oxetane	-0.26106	-0.19388	-0.18426	0.91873*	-0.12745
1-Methylbutyl-oxirane	-0.26106	-0.19388	-0.18426	0.91873*	-0.12745
3-Methyl-4-penten-2-ol	-0.39406	-0.49381	-0.43498	-0.63865	0.06156
Propanoic acid hexyl ester	-0.18120	0.11213	-0.64034	-0.73122	-0.09939
Hexanoic acid propyl ester	-0.20438	-0.19304	-0.11110	-0.22187	0.92704*
Trimethylene oxide	-0.19663	-0.20288	-0.13798	-0.21581	0.92443*
2-Methyltetrazole	-0.18327	-0.58804	0.11115	0.49927	0.59916
Butanoic acid- 2-methyl-pentyl ester	-0.28808	-0.39455	-0.40640	-0.54078	-0.55112
Acetic acid hexyl ester	0.18470	-0.44174	0.39268	-0.51862	-0.58957
Eigen Values	10.02	6.48	5.31	4.27	2.92
Variance	0.35	0.22	0.18	0.15	0.10
Cumulative variance explained (%) <sup>b</sup>	0.35	0.57	0.75	0.90	1.00

<sup>a</sup> Are the high levels of eigenvector loadings of the compounds on the corresponding factor; the sum of the product of eigenvector loadings and metabolite abundance will give the factor scores (see Fig. 1).

<sup>b</sup> Cumulative per cent of total variance explained by factors.

## DISCUSSION

The study reported here considered multiple criteria based on volatile metabolic profile to detect and discriminate apple diseases/inoculations: a) abundance of compounds in different chemical groups; b) disease/inoculation discriminatory metabolites; c) model based on factor analysis considering combinations of normalized abundance of 29 relatively consistent compounds. We have reported here many disease/inoculation discriminatory volatile metabolites, from Cortland and Empire apples, using GC/MS to analyze the headspace gas. Though more than 1000 metabolites were detected, a total of 34 (29 irrespective of cultivars) metabolites were unique to one or more diseases/inoculations. Of this 11 were unique to a single disease/inoculation. In cultivar

Cortland, all the inoculations except *M. piriformis* produced at least one unique volatile, whereas *P. expansum* produced two unique volatile compounds. All the inoculations except *P. expansum* produced one unique volatile in the cultivar Empire. The uniqueness of a compound to a given disease/inoculation was only based on compounds that were more frequent. However, not all the compounds were really unique and some occurred in others in <11% of the times, implying 2 or more samples must be analyzed to discriminate diseases/inoculations. The compounds that were unique to an inoculation and not detected in any other were: styrene and acetic acid methyl ester produced by *Monilinia* sp. and *B. cinerea*, respectively in Cortland. In both the cultivars, there were several metabolites, which were common only to 2-5 inoculation-agents. These

volatiles also can be considered for use in discriminating the groups. In our study, however, we were not able to detect a compound, which was unique to a pathogen irrespective of cultivars. This suggests that more complex models are required involving various metabolites to discriminate diseases/inoculations, especially when more than one cultivar is involved.

The compounds like ethanol, acetic acid methyl ester, propanoic acid methyl ester and hexanoic acid methyl ester were produced by pathogen-inoculated treatments in the present study. These compounds produced exclusively by pathogens and not by non-wounded control and wounded control could be used in disease detection studies for identification of diseased and non-diseased apples.

The normalized abundance of chemical groups of compounds also can be used to discriminate different diseases/inoculations. Though no disease/inoculation discriminatory chemical groups were found the relative abundance varied among inoculations. Maximum number of alkenes and esters were detected in *Monilinia* sp. inoculated, while largest number of aliphatic and nitrogenous compounds was found in wounded control. The *P. expansum* inoculation recorded largest number of aromatic and sulfur compounds while *M. piriformis* had the highest number of alcohols. In general, we observed highest abundance of esters. Many other workers have reported high abundance of esters (Flath *et al.*, 1967; Panasiuk *et al.*, 1980; de Pooter *et al.*, 1983; Mattheis *et al.*, 1991a; Thedy *et al.*, 2003). About 66% of total abundance of compounds produced in Cortland was esters while in Empire it was 48%. Twelve out of 14 volatile compounds identified in Renetta Canada apples were esters of which 6 were different butanoic acid esters (Thedy *et al.*, 2003). In the present study, we were able to detect 6 and 8 different butanoic acid esters in the apple cultivars Cortland and Empire, respectively.

We have detected here many volatile metabolites from non-diseased apples. Even though there is no report of disease discriminatory metabolites in the literature many volatiles have been reported in non-diseased apples. The Cortland and Empire apple cultivars in our study yielded 136 and 176 volatiles from N- and W-control treatments, respectively. In Delicious apple, 56 volatile compounds contributing to apple aroma were detected. About 300 compounds contributing to apple flavor and aroma from different cultivars has been reviewed earlier (Dimick and Hoskin, 1983). In our study, even though the number of volatile compounds produced in inoculated apple cultivars was relatively fewer, some volatiles were distinctly different from the water-inoculated controls. The main interest of many workers in the study on volatiles in apple has been the detection of aroma volatiles. In McIntosh apples, the aroma has been attributed to the presence of hexanal, 2-hexanal, ethyl propionate, ethyl 2-methyl propionate, methyl bu-

tyrate, methyl 2-methyl butyrate, ethyl butyrate, ethyl 2-methyl butyrate and ethyl pentanoate (Panasiuk *et al.*, 1980). In the present experiment involving the two cultivars, we were able to identify several esters, alcohols and aldehydes. In most apple cultivars, the three volatile esters namely butyl acetate, 2-methyl butyl acetate and hexyl acetate are considered as major contributors to the characteristic apple like aroma and flavor (Dimick and Hoskin, 1983). Although volatile profiles vary with cultivar (Kakiguchi *et al.*, 1986), there appears to be some similarity in the major esters. This was also the case observed in our study involving the cultivars Cortland and Empire. In Rome apples, ethyl acetate, hexyl acetate, hexyl butyrate and butyl 2-methyl butyrate were major ester components of the volatile profile (Fellman *et al.*, 1993). In Bisbee Delicious apples, the major ester constituents included hexyl acetate, butyl acetate, 2-methyl butyl acetate, ethyl acetate, ethyl 2-methyl butyrate and pentyl acetate (Mattheis *et al.*, 1991a,b) while hexyl acetate, butyl acetate, and 2-methyl butyl acetate were also prominent in Golden delicious apples (Brackmann *et al.*, 1993).

The relationship among diseases/inoculations and cultivars in volatile production in apples is very complex, thus requiring complex models to discriminate them. Factor analysis models discriminated all five diseases/inoculations with high probability. This model can be used for both cultivars but the samples of each cultivar have to be analyzed separately. The sum of the product of eigenvector loadings and the normalized abundance of the respective compound for an unknown disease would give factor scores which could be plotted in the graph (Fig. 1) to discriminate/diagnose the unknown disease. Such models based on normalized abundance of combinations of compounds could be used to discriminate diseases/inoculations under commercial conditions after further validation. However, for user friendly and efficient commercial applications computer models have to be developed.

The pathogens *B. cinerea* and *P. expansum* produced higher amounts of disease than *M. piriformis* and *Monilinia* sp. in both the cultivars. *M. piriformis* and *Monilinia* sp. were able to produce slightly higher levels of disease in cultivar Empire than in Cortland. This indicates that the cultivar Cortland might be relatively more resistant than Empire to *M. piriformis* and *Monilinia* sp. Apple cultivars have been shown to vary in their susceptibility to different pathogens. Granny Smith and Braeburn apples were more susceptible to *M. piriformis* than Royal Gala (Spotts *et al.*, 1999). Thus, the relation of volatile metabolites to resistance in apple to different pathogens may make disease discrimination across cultivars difficult.

Although in both the cultivars we have reported many disease discriminatory volatile metabolites, they were not consistent among replicates. These kinds of

variations are not rare in studies involving metabolites and the changes of this nature in metabolite production could be attributed to variations in host, pathogen and environmental factors (Fiehn *et al.*, 2000; Roessner *et al.*, 2001; Dixon *et al.*, 2002). Some of the variations also could be due to miss identification of compounds without abundance data on mass ions <45, which were excluded mainly because of background noise due to moisture in our samples. Thus, in our study highly volatile metabolites were not detected. Highly volatile compounds were also considered to be not discriminatory of milk spoilage due to microbial contamination (Marsili 1999).

Multiple criteria, based on unique compounds, compounds specific to more than one disease/inoculation, abundance of chemical groups of compounds, factor analysis models based on normalized abundance of combination of different metabolites, etc., must be considered to develop an efficient system to discriminate diseases of apple under commercial conditions. Software for microcomputers, with different options/criteria, can be developed for user-friendly applications under commercial conditions. The storage managers could use the method developed in the present study for detection and discrimination of apple diseases at an early stage of disease progress. They can also use this knowledge base to manage apple diseases in storage. This knowledge base on disease potential could be used in making several sensible management decisions like how long the product can be stored and also for choosing of specific lots for longer storage. One of the major problems could be inconsistency of occurrence of disease discriminatory compounds. The probability of correct diagnosis can be increased by increasing the number of samples analyzed for each lot or field in question. Under commercial storage conditions often only one disease becomes a major issue, however, occurrence of more than one disease is possible. The disease specific volatile metabolite markers or combinations of discriminatory metabolites could be used to discriminate diseases even when more than one disease is present, however, their validity must be tested under commercial conditions before recommendation. To automate the detection procedures and also for practical purposes it is advisable to study and develop models for many diseases commonly occurring in a given locality. The application of such a tool to detect diseases in large storage facilities is only possible after undertaking scale up studies.

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