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Volatile metabolite profiling for the discrimination of onion bulbs infected by *Erwinia carotovora* ssp. *carotovora*, *Fusarium oxysporum* and *Botrytis allii*

B. Prithiviraj[1,*], A. Vikram[1], A.C. Kushalappa[1,**] and V. Yaylayan[2]
[1]*Plant Science Department,* [2]*Food Science and Agricultural Chemistry, Macdonald Campus of McGill University, 21111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada H9X 3V9;*
[*]*Present address: Department of Horticulture and Landscape Architecture, Colorado State University, Fort Collins, CO 80523, USA;* [**]*Author for correspondence (Phone: +514-398-7851 ext. 7867; Fax: +514-398-7897; E-mail: ajjamada.kushalappa@mcgill.ca)*

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Abstract

The volatile metabolites of the headspace gas of onion bulbs inoculated with three different pathogens, *Erwinia carotovora* ssp. *carotovora*, *Fusarium oxysporum* and *Botrytis allii*, were profiled using gas chromatography/mass spectrometry. Differences in the number and amount of volatile metabolites were observed. Two hundred and fifty three volatile metabolites were detected in bulbs inoculated with three pathogens or sterile distilled water. On day three, 202 volatile metabolites were observed, compared to 166 on day six. Of the 253 compounds, however, only 59 occurred relatively consistently over replications, of which 25 compounds were specific to one or more pathogens, including 10 that were unique to a pathogen. Metabolites such as 1-Oxa-4,6-diazacyclooctane-5-thione and 4-mercapto-3-(methylthio)-ς-(thio-lactone)-crotonic acid were exclusive to onions inoculated with *F. oxysporum*. Acetone, acetic acid-hydrazide, propylcarbamate, 1-bromo-1-propene, thiirane, 1-(methylthio)-*E*-1-propene and 1-ethenyl-4-ethyl-benzene were specific to *B. allii*. 3-bromo-furan was specific to *E. carotovora* ssp. *carotovora*. Sterile water-inoculated bulbs produced 3,3′-dioxy-1,2-propanediol-tetranitrate. Highest amount of sulfurs was found in pathogen-inoculated, while highest amounts of terpenes, aromatics and aliphatics were found in sterile distilled water-inoculated bulbs. The possible use of these differences in the volatile metabolites for detecting and discriminating diseases of onion in storage is discussed.

Abbreviations: GC/MS – gas chromatograph/mass spectrometer.

Introduction

Onion (*Allium cepa*) is a major crop world-wide. Three major types of onion bulbs are recognized: red, white and yellow/brown. In Eastern Canada, Spanish yellow type is the most popular and is stored year round. However, post-harvest diseases 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. 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.

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, produced many volatile compounds

and the number increased following inoculation with a pathogen (de Lacy Costello et al., 1999; 2001; Kushalappa et al., 2002). The sulfur containing compounds constituted the major proportion of onion headspace gas (Kallio and Salorinne, 1990; Kallio et al., 1994). However, there is no systematic study on the volatile metabolite profiling of onion infected by fungal or bacterial pathogens. In this paper, we report the differential volatile production in onion bulbs inoculated either with the bacterial pathogen *Erwinia carotovora* ssp. *carotovora* causing soft rot or with two fungal pathogens, *Fusarium oxysporum* and *Botrytis allii*, which cause basal and neck rots, respectively. Further, we have attempted to discriminate the pathogens/diseases using volatile metabolites as markers.

Materials and methods

Plant material

Bulbs of onion cv. Fortress (yellow or brown type) were stored at $4 \pm 2\,^{\circ}$C with a relative humidity of 80–95% in a cold room at the Horticultural Research Facility, Macdonald Campus of McGill University for 2–4 months. Before use, dry skins were removed, basal roots were trimmed and the bulbs were surface sterilized by dipping in 1% sodium hypochlorite solution for 10 min.

Pathogens

Cultures of *F. oxysporum*, *B. allii* and *E. carotovora* ssp. *carotovora* were obtained from the Plant Diagnostic Lab, St. Foy, Quebec. The fungi were maintained on potato dextrose agar (Difco Laboratories, Detroit, USA). *E. carotovora* ssp. *carotovora* was maintained on tryptic soy agar (Difco Laboratories, Detroit, USA). For the production of macroconidia, *F. oxysporum* was grown on a glucose onion agar (glucose 10 g, onion pieces 5 g, agar 15 g and distilled water 1 l) for 5–7 days and exposed to near UV light (UV-A) for 2 days to induce sporulation (Hite, 1973; Leach, 1962).

Inoculation and incubation

Spore suspensions (10^5 spores ml^{-1}) of *F. oxysporum* and *B. allii* were made with sterile distilled water containing 0.02% Tween-80 (Fisher Biotech, New Jersey, USA). Cell suspension (10^{-7} cfu ml^{-1}) of *E. carotovora* ssp. *carotovora* was made in distilled water. Eight holes (5 mm diameter and 3 mm deep) were made equidistantly at the equatorial region of onion bulbs using a sterile cork borer. Twenty microliters of each pathogen suspension was placed into these holes. Sterile distilled water served as the control. The inoculated onion bulbs were placed on a stainless steel support in 89 mm wide mouth glass bottle with Teflon coated septa lined cap (I-Chem, New Castle, USA) containing about 10 ml sterile distilled water to maintain a saturated atmosphere to facilitate infection (Kushalappa et al., 2002) and held at $20 \pm 2\,^{\circ}$C in the dark for 24 h. During incubation water was drained from the bottle and the headspace flushed with dry pure air every 24 h.

Volatile accumulation and GC/MS analysis

The headspace gas was accumulated for 24 ± 2 h before sampling. The samples were analyzed 3 and 6 days after inoculation (dai). Volatile metabolites were sampled and analyzed using HAPSITE, a portable gas chromatograph/mass spectrometer (GC/MS) system (INFICON, Syracuse, NY, USA). The HAPSITE was equipped with a hand held sampling-probe attached to a terminal stainless steel needle, 18 gauge and 15 cm long (Popper and sons Inc, NY, USA), which was inserted into a septa bottle. One hundred milliliters of headspace gas was sampled from a septa bottle, after stirring with a magnetic stirrer. The collected headspace gas was passed through a pre-concentrator tube trap containing 15 mg carboxen (INFICON, Syracuse, NY, USA). The sample was thermally desorbed at $225\,^{\circ}$C. The GC analysis was performed using a 30 m long capillary column SPB-5 (Supelco, Bellfonte, CA) with 0.32 mm internal diameter and 1.0 μm film coating (and configured in a heating coil, INFICON – 930-489-G8). Volatile organic compound-free nitrogen was used as the carrier gas at a flow rate of 3 ml min^{-1}. The column temperature was held at $50\,^{\circ}$C for 4 min followed by a ramping of $3\,^{\circ}$C min^{-1} upto $200\,^{\circ}$C. The GC was interphased to a MS equipped with a quadrupole analyzer, and the mass spectrum was scanned at the rate of 0.8 s per mass decade over a mass range of 46–300 m/z. The compounds were tentatively identified using a NIST mass spectral search program (Version 2) (INFICON, Syracuse, NY, USA). The amount of each volatile metabolite was expressed as relative abundance of mass-ions (relative response

of mass ion detector = relative concentration of compounds; for absolute concentration of compounds calibration curve has to be established for each compound).

Disease severity assessment

The diameter of diseased tissue was measured at 3 and 6 dai. Disease specific discoloration or rot was considered diseased tissue.

Experimental design and data analysis

The experiments consisted of eight treatments, four inoculations (sterile distilled water or three pathogens) with two incubation times (24 and 48 h) for each. The entire experiment was conducted three times, providing 24 volatile samples. For each treatment, the average abundance of mass-ions of each compound was calculated by summing the abundance (for compounds with relative abundance $\geq 10^5$ and occurred in ≥ 2 replicates/incubation time of at least one of the four inoculations) for replicates and dividing by three. The compounds were classified based on their functional group. For each group the average and the relative abundance (proportion of total amount produced) were calculated for each treatment.

Results

Disease severity

Disease severity varied among pathogen inoculations and incubation time. No visible rot was observed 3 dai, except for small discolored area for *E. carotovora* ssp. *carotovora*. The diameter of tissue diseased 6 dai were 1.46 cm for *E. carotovora* ssp. *carotovora*, 1.47 cm for *B. allii* and 1.33 cm for *F. oxysporum*.

Volatile metabolite dynamics

Two hundred and fifty three compounds were detected in onion bulbs inoculated with sterile distilled water or three pathogens, across all treatments and replicates (24 samples). Two hundred and two volatile compounds were detected 3 dai; it reduces to 166 at 6 dai (details not shown). However, only 59 compounds occurred relatively consistently (occurring in ≥ 2 out of 6 replicates/incubation times) (Table 1). Among treatments *B. allii* inoculated bulbs produced the most compounds, about twice or more than the others.

The metabolites detected belonged to different broad groups based on their chemical and biological

Table 1. Average abundance of mass-ions ($\times 10^5$) of volatile metabolites* consistently produced by bulbs of onion cv. Fortress inoculated with sterile distilled water, *E. carotovora* ssp. *carotovora*, *F. oxysporum* and *B. allii*

RT (h : m : s)	Group	Compounds	SDW	BAL	ECC	FOX	TREAT
01 : 25.7	Aliphatic	Acetic acid-hydrazide		2			B
01 : 24.9	Aliphatic	Propylcarbamate		3			B
11 : 05.3	Aliphatic	1-bromo-1-propene		1			B
01 : 18.0	Aliphatic	Acetone		55			B
18 : 35.2	Aromatic	1-ethenyl-4-ethyl-benzene		29			B
01 : 34.1	Sulfur	Thiirane		8			B
03 : 20.1	Sulfur	1-(methylthio)-*E*-1-propene		96			B
14 : 38.0	Aliphatic	3-bromo-furan			1		E
20 : 24.2	Sulfur	1-Oxa-4,6-diazacyclooctane-5-thione				11	F
20 : 28.8	Sulfur	4-mercapto-3-(methylthio)-ç-(thio-lactone)-crotonic acid				2	F
01 : 53.4	Sulfur	1-propanethiol		51	31		BE
00 : 51.9	Aliphatic	Methyl hydrazine		12		7	BF
08 : 57.1	Aliphatic	Phenyl-butanedioic acid		1		1	BF
11 : 09.0	Aromatic	à-(1-phenylaminoethyl)-benzenemethanol		2		5	BF
20 : 23.4	Sulfur	Prop-1-enyl dithiopropanonate		46		5	BF
13 : 43.5	Terpene	á-myrcene		7		6	BF
09 : 00.2	Alcohol	à,á-dimethyl-benzeneethanol			3	29	EF
09 : 21.7	Aliphatic	Heptanal			3	26	EF
00 : 55.8	Aliphatic	Dimethyl ether			17	8	EF
08 : 03.4	Aliphatic	2-methyl-4,6-octadiyn-3-one			1	1	EF
15 : 30.2	Terpene	1-methyl-5-(1-methylethenyl)-, *R*-cyclohexene			16	259	EF

Table 1. (Continued)

RT (h : m : s)	Group	Compounds	SDW	BAL	ECC	FOX	TREAT
01 : 11.9	Alcohol	Ethanol		11	1	8	BEF
01 : 18.0	Aliphatic	1,1-dimethylethyl-urea		50	34	14	BEF
14 : 14.2	Aromatic	4,4-dimethyl-1,3-diphenyl-1-(trimethylsilyloxy)-1-pentene		1	4	1	BEF
15 : 13.3	Terpene	3,7,7-trimethyl-, [1S-(1à,3à,6à)]-bicyclo[4.1.0]hept-4-en-3-ol		76	28	9	BEF
00 : 53.5	Alcohol	3,3′-dioxy-1,2-propanediol-tetranitrate	9				S
13 : 00.5	Aliphatic	4-methylene-1-(1-methylethyl)-cyclohexene	35	15			SB
08 : 54.8	Aromatic	Styrene	11	3			SB
14 : 22.6	Aromatic	à,4-dimethyl-benzenemethanol	3	18			SB
11 : 06.1	Sulfur	Methyl-1-propenyl-disulfide	2	134			SB
20 : 23.5	Sulfur	2-ethylidene[1,3]dithiane	53	71			SB
18 : 36.1	Aromatic	1-[(4-methoxyphenyl)methyl]-3,3-dimethyl-4-phenyl-2-azetidinone	3		9		SE
19 : 51.2	Aromatic	2,3-butanedionylidene bis(o-aminobenzyl alcohol)	6		15		SE
01 : 18.1	Aliphatic	N,N-dimethyl-1-Butanamine	11	55	32		SBE
14 : 39.5	Terpene	2-methyl-5-(1-methylethyl)-bicyclo[3.1.0]hexan-2-ol	11	16	50		SBE
03 : 50.8	Sulfur	Dimethyl disulfide	1	32		3	SBF
19 : 52.8	Sulfur	2-(thiocarboxy)hydrazide-*O*-methyl ester-acetic acid	33	25		114	SBF
04 : 26.1	Aliphatic	1,3,5-cycloheptatriene	6		3	1	SEF
09 : 32.4	Sulfur	2,3-dimethyl-thiophene	26		16	16	SEF
09 : 32.5	Sulfur	3,4-dimethylthiophene	12		7	29	SEF
19 : 28.9	Sulfur	Bis(1-methylethyl) disulfide	211		28	1	SEF
11 : 37.6	Terpene	á-phellandrene	9		18	8	SEF
00 : 55.8	Acid	Formic acid	20	8	26	20	SBEF
15 : 37.9	Alcohol	1-methyl-4-(1-methylethenyl)-acetate-cyclohexanol	149	30	77	128	SBEF
10 : 47.7	Alcohol	endo-bicyclo[3.3.1]nonan-3-ol	2	1	3	3	SBEF
01 : 27.2	Aliphatic	Oxybis chloro-methane	8	89	266	5	SBEF
01 : 57.2	Aliphatic	Chloroform	553	62	107	164	SBEF
18 : 34.5	Aliphatic	2,3,5-trimethyl-4-methylene-2-cyclopenten-1-one	1	1	2	1	SBEF
15 : 34.8	Aliphatic	1,3-cycloheptadien-1-ylmethyl-ketone	18	7	2	11	SBEF
19 : 56.7	Aromatic	2-(dimethylamino)-1-phenyl-3-heptanone	8	22	8	2	SBEF
15 : 22.6	Aromatic	1-(4-methylphenyl)-1-pentanone	1	1	1	2	SBEF
15 : 22.6	Aromatic	1-methyl-2-(1-methylethyl)-benzene	111	70	22	77	SBEF
10 : 45.3	Sulfur	Methyl propyl disulfide	26	581	39	140	SBEF
19 : 29.0	Sulfur	Dipropyl disulfide	326	6031	872	1809	SBEF
19 : 47.4	Sulfur	[1,3]Dithiane-2-thione	2	72	4	5	SBEF
19 : 52.8	Sulfur	2-ethyl-5-chloro-1,3,4-thiadiazole	97	542	7	100	SBEF
10 : 58.4	Terpene	1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene	3164	1260	690	5	SBEF
11 : 04.6	Terpene	2-methyl-5-(1-methylethyl)-bicyclo[3.1.0]hex-2-ene	6	10	13	23	SBEF
16 : 32.4	Terpene	3-carene	4	268	341	119	SBEF
Total Acids			20	8	26	20	
Total Alcohols			160	42	84	168	
Total Aliphatics			632	353	468	239	
Total Aromatics			143	146	59	87	
Total Sulfurs			789	7689	1004	2235	
Total Terpenes			3194	1637	1156	429	
Total of all metabolites (relative abundance = amount)			9876	19748	5594	6356	
Total of all metabolites (number)			34	43	36	40	

*The metabolites produced in <2 out of 6 replicates/incubation periods (3 and 6 dai) and abundance $<10^5$ are not included in this table; the compound identification is tentative based on NIST library match; because the structural identification is difficult the compounds are grouped into broad categories; SDW = sterile distilled water; ECC = *E. c. carotovora*; FOX = *F. oxysporum*; BAL = *B. allii*.

functions: acids (1), alcohols (5), aliphatics (18), aromatics (10), sulfurs (17) and terpenes (8). Though the number of compounds in different groups did not vary much among treatments, the amount of compounds varied drastically. Highest abundance of aliphatics, aromatics and terpenes were detected in sterile distilled water-inoculated bulbs, whereas the highest abundance of sulfurs was detected in pathogen-inoculated bulbs. *B. allii* inoculated bulbs produced the highest abundance of sulfurs, followed by *F. oxysporum* and *E. carotovora* ssp. *carotovora* inoculated bulbs. The levels of disease severity were not directly related to the amount of volatiles or sulfur volatiles produced, by different pathogens. Next to sulfur, terpene was the most abundant compound and the highest amount was detected in sterile distilled water-inoculated bulbs. The relation of sulfur to terpene varied among treatments: SDW = 0.2, ECC = 0.9, FOX = 5.2 and BAL = 4.7. Since these compounds are produced in high abundance their relationships could be used to discriminate fungal diseases tested here.

Pathogen/disease discriminatory volatile metabolites

Forty two volatile metabolites were specific to inoculation with one or more agents, including 25 that were common to one or more pathogens and 10 compounds unique to a pathogen. When compounds were found in more than one pathogen, several were in higher abundance in a pathogen than the others. *B. allii* produced seven unique metabolites, with acetone, 1-(methylthio)-*E*-1-propene and 1-ethenyl-4-ethyl-benzene in relatively high abundance. *F. oxysporum* produced two unique metabolites of which 1-Oxa-4,6-diazacyclooctane-5-thione was produced in relatively high abundance. *E. carotovora* ssp. *carotovora* produced one unique compound, 3-bromo-furan, but in relatively low abundance. The sterile water-inoculated bulbs produced one unique metabolite. *B. allii* inoculated bulbs produced more methyl 1-propenyl disulfide than the sterile water. *F. oxysporum* produced high amounts of 1-methyl-5-(1-methylethenyl)-*R*-cyclohexene as compared to very low by *E. carotovora* ssp. *carotovora*. These pathogen-specific metabolites could be used in discriminating diseases or pathogens of yellow onions. Though, some volatiles were produced by all inoculations, the abundance was very high for some. *B. allii* produced the highest abundance of dipropyl disulfide followed by *F. oxysporum*. Very high amounts of 1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene were produced by sterile water and *B. allii* inoculated bulbs.

Discussion

Fifty nine compounds were relatively consistently detected in high abundance in inoculated onion bulbs. The number and relative amount of compounds produced varied among treatments. Of the 59 compounds, 25 were disease discriminatory, including 10 that were unique to a pathogen. Only a few compounds were detected in high abundance: dipropyl disulfide, bis(1-methylethyl) disulfide, methyl propyl disulfide, 2,3-dimethyl-thiophene, 2-ethyl-5-chloro-1,3,4-thiadiazole and 2-(thiocarboxy)hydrazide-*O*-methyl ester-acetic acid. Not all the pathogens produced these compounds, and when more than one pathogen produced the abundance of some varied drastically among them. Park et al. (2001) found lower amounts of sulfur compounds and higher amounts of ketones in decayed onion bulbs compared to healthy bulbs. Several other workers have reported volatiles of onion, but most of the published works are related to food science and flavor chemistry in particular (Kallio and Salorinne, 1990; Abbey et al., 2001). Several sulfur compounds in the headspace of onions have been reported: propanethiol, dimethyl disulfide, methyl propyl disulfide, dipropyl disulfide, methyl *cis*-propenyl disulfide, methyl *trans*-propenyl disulfide, *cis*-propenyl propyl disulfide, *trans*-propenyl propyl disulfide, 2,4-dimethyl thiophene and 3,4-dimethyl thiophene (Boelens et al., 1971; Mazza, 1980; Kallio et al., 1994). Twenty seven prominent aroma compounds have been identified including dipropyl disulfide, methyl propyl disulfide, 1-propenyl propyl disulfide (*E* and *Z*), 1-propanethiol and 2-methyl pentanol. Volatile composition has been used to differentiate between cultivars Jumbo and Lafort, though the abundance of compounds such as dimethyl disulfide, methyl 1-propenyl disulfide (*E* and *Z*), dipropyl disulfide and 1-propenyl propyl disulfide varied significantly. Volatiles detected using electronic nose have been used to discriminate flavor and aroma characteristics amongst macerated garlic, leek, shallot, bulb onion and spring onion (Abbey et al., 2001).

It should be noted that the composition of the volatile metabolites varied over replications, with many compounds detected only once in three replications of day three or six. Some of the variation may be due to the identity of the compound which is only based on

NIST library search. Thus, the identity of compounds detected in this study should be considered preliminary. Despite the variation, several compounds were consistent over replications which can be used as markers for detecting and discriminating diseases of onion bulbs in storage. Variation is apparently inherent while studying the metabolites, and this should not deter workers from taking up such studies (Fiehn et al., 2000). The abundance of volatiles in our study varied among the three replicates from 20–40% for different pathosystems, confirming high variation in volatile metabolite studies (Kallio and Salorinne, 1990).

Detection and discrimination of post-harvest diseases on the basis of differences in the volatiles in headspace gas of potato tubers has been studied extensively. de Lacy Costello et al. (2001) reported that butanal, 3-methylbutanal, undecane and verbenone were specific to *Phytophthora infestans* and 2-pentyl furan and copaene to *Fusarium coeruleum*. Four different pathogens of potato tubers have been discriminated by neural network modeling of the GC retention time of headspace volatile compounds (Kushalappa et al., 2002). Similarly, volatiles have been used to discriminate diseases of carrots (Ouellette et al., 1990) and mycotoxigenic organisms in cereal grains (Keshari and Magan, 2000; Magan and Evans, 2000). However, to our knowledge, there are no reports in the literature regarding the changes in the volatile metabolite profile in onion infected by fungal or bacterial pathogens. This study has generated fundamental knowledge on the composition of headspace volatile metabolites of onion bulbs inoculated by pathogens, and provides the basis for discriminating post-harvest diseases caused by *E. carotovora* ssp. *carotovora*, *F. oxysporum* and *B. allii* in storage. In our study the *E. carotovora* ssp. *carotovora* inoculated onion bulbs produced eight sulfur compounds whereas the *E. carotovora* ssp. *carotovora* inoculated potato tubers produced only one sulfur compound (de Lacy Costello et al., 1999). Though portable GC/MS (HAPSITE) could be used directly in storage rooms to detect diseases of onion bulbs, the method developed here must be validated before commercial use.

Forty two volatile metabolites were specific to one or more inoculation agents. These compounds, in different combinations, could be used to discriminate diseases or groups of a few diseases. Though some compounds were common to two or more pathogens the abundance among them varied. Pathogen inoculation elicited production of 25 volatile metabolites which were not detected in sterile distilled water-inoculated bulbs. Though some compounds were common to all the treatments their abundance varied among them. Use of all these variations, in different combinations, to develop a rule based model(s) to discriminate onion diseases can be quite a challenge. Alternatively, these data could be used to develop statistical or neural network models to discriminate onion diseases (Kushalappa et al., 2002), however, in this study there are only three replicates and more number of replications is required to develop a stable model. Pathogen inoculation produced high abundance of sulfur compounds, whereas the sterile distilled water-inoculated produced very low abundance of sulfurs but high abundance of terpenes. The regulation of abundances of these two groups of compounds, relation of sulfur to terpene, over time in storage also can be exploited as a tool to detect the occurrence of fungal diseases. The knowledge base generated here could be used to develop rule based models to discriminate diseases and software could be developed for user-friendly applications under commercial conditions. However, additional studies involving other cultivars, physical environmental factors influencing volatile dynamics, larger sample size, etc. are required before recommendation for commercial use.

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