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Direct analysis of nectar and floral volatile organic compounds in hybrid onions by HS-SPME/GC–MS: Relationship with pollination and seed production

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ABSTRACT

In this study, a simple and solvent-free method using headspace solid-phase microextraction (SPME) and gas chromatography–mass spectrometry (GC–MS) was developed for determination of the volatile compounds from fresh flowers and nectar of male sterile and fertile lines of *Allium cepa* L. The SPME parameters were studied; the optimum conditions of 85 μm carboxen/polydimethylsiloxane (CAR/PDMS), extraction temperature of 30 °C and extraction time of 30 min were obtained. The analytical method was applied to characterize different onion lines according to the chemical composition of the volatile fraction of nectar and flowers. On the other hand, it was determined which odor components contribute to the pollination of onions lines and relate the content of specific analytes with foraging behavior and seed production. More than 90 compounds were identified. The samples studied showed differences in the volatile profiles of flowers and nectar among the different lines. The results demonstrated that headspace SPME–GCMS is a simple, rapid and solvent-free method suitable for analysis of volatile compounds emitted from onion plants. Furthermore, a great difference was found for the number of bee visits and seed yield among the onion lines analyzed. This study demonstrates that the combination of chemical information and statistical analysis is able to differentiate onion male sterile lines from fertile lines, as well as among male sterile lines. Repellent compounds, such as dioxolanes, piperidines and organosulfurs may contribute negatively to pollination, since the higher content of these analytes, the less bee visits and seed production yield.

1. Introduction

Onion (*Allium cepa* L.) is a worldwide important vegetable crop. It is an allogamous species that requires insect pollination in order to produce seeds. Among insects, honey bees (*Apis mellifera* L.) have been reported to be the most efficient and major onion pollinators, due to their specific instinctive behavioral features that affect both pollen and nectar collection and efficiency in pollen transfer. Consequently, any significant increase in onion seed production depends heavily on pollination efficiency; especially for hybrid onion seed production [1].

In Argentina, onion along with garlic, are the main fresh vegetables exported. Two types of onion varieties are usually grown around the world; open pollinated (OP) and first generation (F1) hybrids. In order to produce F1 hybrid seed in onion, it is necessary to cross a male-sterile line with a fertile one. Cytoplasmic-genic male sterility (CMS) systems are used to produce hybrid-onion seed. Two different CMS systems have been described in onion, termed S- and T-cytoplasm [2,3]. Field observations indicate that F1 hybrid seed yield are much lower than open pollinated varieties seed yields, with a decrease of up to 60% [4]. These differences in yield can be due to pollination problems.

Pollination systems are biological markets in which animals choose between "products" (flower species) on the basis of quality (e.g. nectar sugar quantity), and in which plants might compete for "customers" (pollinators). It is now clear that flower visitation (and therefore plant fitness) can be affected by multiple factors that are beyond the control of the individual plant or species [5]. One function of floral phenotype is to signal, or 'advertise', the presence of a nutritious or reproductive reward to animal pollinators [6]. Flower attributes such as size, color, flower organs, nectar volume and composition, and amount of pollen are considered to be important factors attracting honeybees and thus can affect visitation frequency [7].

Plants release a large variety of volatile organic compounds (VOCs) into the surrounding atmosphere. In addition to simple gases, such as oxygen, carbon dioxide and water vapor, plants emit an enormous wealth of different metabolites [8]. The primary functions of airborne VOCs are

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to defend plants against herbivores and pathogens, to attract pollinators, seed dispersers, and other beneficial animals and microorganisms, and to serve as signals in plant–plant communication. Chemically, VOCs belong to the large group of terpenoids (homo-, mono-, di-, sesquiterpenoids), fatty acid derivatives, benzenoids, phenylpropanoids, and amino acid derived, as well as certain alkanes, alkenes, alcohols, esters, aldehydes, and ketones. Today more than 1700 volatile compounds have been isolated from more than 90 plant families, constituting approximately 1% (w/w) of all plant secondary metabolites [41].

Recent studies indicate that the chemical components contributing to the fragrance of flowers play an important role in the honeybee attractiveness to flowers [7]. Olfactory signals are rapidly learned, indicating that foraging behavior results from the association of plant chemicals acting as chemosensory cues for the bees. In addition, vast evidence indicates that the olfactory signals may be the dominant factor controlling bee behavior. Consequently bee behavior is controlled by the integration of perceived cues, color and/or fragrance, and the amount of a reward, pollen and nectar [9]. On the other hand, very little is known about the origin of the metabolites that are commonly found in nectar. Given that flower scents are known to have both positive and negative consequences for interactions between plants and floral visitors, the function of VOCs in nectar is probably similarly complex [10,11].

Many different analytical methods have been developed to determine volatile constituents present in flowers, honey and different parts of plants [12–18]. Moreover, there are many research papers in which the potential of VOCs analysis in combination with chemometrics, was used to achieve a correct classification of food samples from different origins according to their geographical origin, variety and aging [19–21].

Solid-phase micro-extraction (SPME) is a rapid, solvent free and cost effective extraction technique. It combines extraction and preconcentration in one step, which is realized by a modified syringe-like device that utilizes a polymeric extraction phase. This technique provides significantly more rapid sample preparation than the majority of traditional methods [22]. Several fiber coatings are commercially available for the extraction of volatile compounds. The principle of headspace SPME involves partition and equilibrium of analytes among the coating of the fiber, the sample and its headspace [14]. There are various studies addressing the odors of different *Allium* parts [23–25], and the effects of *Allium* key compounds on pollination [26]. Storsberg et al., (2004) [42] reported that the distribution of the cysteine sulfoxides as well as the volatile secondary metabolites in onion hybrids is not uniform. The chemical analysis of cysteine sulfoxides and volatile sulfur-containing substances is shown to be a useful tool for breeding purposes as it allows an effective selection with regard to optimal distribution and amount of valuable constituents. At the moment, despite its advantages, to our knowledge there are no reports on the application of SPME–GC–MS for the determination of VOCs in flowers and onion nectar.

The aims of this study were (i) to propose an analytical method for the determination of volatile compounds in onion flowers and nectar by headspace solid-phase microextraction (HS-SPME)–gas chromatography/mass spectrometry, (ii) to apply the optimized methodology for the analysis of VOCs in flower and nectar in different onion male sterile and fertile lines (iii) to characterize different lines according to the chemical composition of the volatile fraction of nectar and flower onion (iv) to correlate the content of specific analytes with number of visits and seed production.

2. Materials and methods

2.1. Plant materials

An open pollinated cultivar (OP), Valcatorce INTA, [27] as well as three male sterile lines (MSL 1, MSL 2, MSL 3 from Enza Zaden) were cultivated in a randomized complete block design with three replicates for each cultivar under a cage (4 × 8 m) in order to isolate the materials from other pollinators, at the Institute of Horticulture (Agronomic Faculty, UNCuyo, Mendoza, Argentine). The plants flowered from November to December, 2011. Flowers were picked at the middle of November, at 50% of flowering as recommended by Mena Granero et al. (2004). Samples were picked always at the same time. At midday, ten umbels per plot were randomized chosen to pick flower samples. Onion nectar was obtained in blossom. Analyses were performed immediately after sampling. Three separate vials of 500 μg of fresh flowers and 3 vials of 200 μl of nectar were collected and sampled, thus providing a sample size of 3 for each onion line.

2.2. Instrumentation and software

All of the SPME fibers, which included Stable Flex™ polydimethylsiloxane/divinylbenezene (PDMS/DVB, 65 μm), polydimethylsiloxane (PDMS, 100 μm), Stable Flex™ carboxen/polydimethylsiloxane (Car/PDMS, 85 μm) and Stable Flex™ divinylbenzene/carboxen/polydimethylsiloxane (Car/PDMS/DVB, 50/30 μm) SPME-support, and holder manual for SPME were purchased from Supelco (Bellefonte, USA). Magnetic stirrer bar and 10 mL glass vial with a PTFE-faced silicone septa were supplied by Varian (Lake Forest, CA, USA). Stirring was made with magnetic stirrer Ret Control Visc IKAMAG Safety Control (IKA, Wilmington, USA). Before use, fibers were conditioned following the instructions from manufacturers, and cleaned at 250 °C for 5 min.

GC–MS analyses were performed on a Varian CP-3800 gas chromatograph with a Saturn 2200 Ion Trap Mass Spectrometric detector (Varian, Walnut Creek, CA, USA). The system was operated by Saturn GC–MS Workstation software Version 6.41. The column was a Factor Four™ capillary column VF-5MS (50m × 0.25 mm I.D., with 0.25 μm film thickness; Varian, Lake Forest, CA, USA).

2.3. Sample preparation and HS-SPME analytical procedure

The yield and repeatability of the extraction was affected by factors as the type of stationary phase of the fiber, sample composition, sample volume, extraction temperature, and extraction time. Previous most commonly employed methods of extraction are microcapillary tubes, filter paper wicks, washing in a known volume of distilled water, rinsing with successive rinses of a known volume of distilled water, and microliter syringe or capillary glass tubes. Such approaches cannot be treated as a suitable method to apply for tiny flowers and small volumes of nectar as in onion [28–31]. Thus, these methods were not considered further. In order to obtain the nectar in the most natural way, and preserve it in similar conditions as it is in the plant we found that the most effective way of extraction was to separate freshly opened flowers from umbels, removing anthers, filaments and peduncle, and immediately centrifuging (13,000 rpm, 30 min, 4 °C) into a 1.5 ml microtube.

Two hundred microliters of nectar per aliquot were diluted (1:5) with pure water. Nectar samples were spiked with an internal standard solution to obtain a final concentration of 1 μg mL^{-1}. On the other hand, 500 μg of flowers from freshly opened flowers were sampled per aliquot, avoiding contamination from other flower parts. Both samples were placed into a 10 mL glass screw-top vial with polytetrafluoroethylene/silicone septa and placed on the magnetic stirrer (1000 rpm). They were allowed to equilibrate for 30 min at 30 °C. Then, the SPME fiber was exposed on headspace mode (2 cm) during 30 min. In this study four different fibers of PDMS, PDMS/DVB, CAR/PDMS and DVB/CAR/PDMS were evaluated to determine which fibers most effectively extracted volatile compounds from fresh flowers and nectar of different onion cultivars. After extraction, the SPME fiber was withdrawn into the needle, removed from the vial and inserted into the injection port of GC–MS. All the analyses were performed in triplicate. Qualitative analysis of the constituents was based on comparison of the obtained mass spectra with those of reference compounds in the NIST Mass Spectral Search Program (NIST Version

2.0). Semiquantitative analysis was performed by means of the internal standard method; which is an accurate strategy for comparative purposes when a large number of analytes are involved [32].

2.4. Chromatographic conditions

The oven temperature program was: 40 °C (2 min), 4 °C min^{-1} to 100 °C (1 min), 3 °C min^{-1} to 220 °C (5 min), 30°min^{-1} to 280 °C (10 min). The 1079 injector was equipped with a glass insert for SPME and the temperature was programmed isothermally at 220 °C. Injection was split/splitless mode (split ratio 1/50, 2 min). The carrier gas flow-rate (Helium 6.0, Linde, Buenos Aires, Argentina) was constant at 1 mL min^{-1}. The electron impact energy was 70 eV. Transfer line and ion trap temperature were 200 °C and manifold temperature was 40 °C. Mass spectrometry acquisition was carried out using the continuous scanning mode (5 μscan s^{-1}) from *m/z* 30 to *m/z* 300.

2.5. Assessment the foraging behavior of bees onto onion flowers

In the present study *Apis mellifera* L. was used as pollinator. At 10% of flowering the hives were introduced into the cage. The hives were placed 1 m apart along the field margin. Since that time the number of bees visiting each plot was recorded by a visual counting method for 1 min from each side of the plot. The mean of these 3 observations constituted a reading for each line. The number of bee visits/umbel/min, was recorded every day except cloudy days, three times a day 9:00, 12:00 and 15:00 h, up to 100% bloom.

Simultaneously, air temperature was recorded with temperature sensors placed at the height of the inflorescences; solar radiation was recorded with a radiometer (Kavadivice).

When fruit set was accomplished, umbels were harvested and dried. Seeds were extracted manually and weighed in order to estimate seed yield. Relationships between seed yield and frequency of honey bee visits and volatile compounds were estimated.

2.6. Statistical analysis

Volatile organic compound from nectar and flowers of different onion lines were expressed as μg per g. All data was reported as the mean of three replications. Data obtained were saved in ASCII format, and transferred to a personal computer for subsequent manipulation by Statgraphics Plus Version 5.0 program (Manugistic Inc., Rockville, MD, USA).

3. Results and discussion

3.1. SPME extraction conditions

Several HS-SPME conditions were investigated to determine the most suitable conditions for the analysis of volatiles in onion flowers and nectar. The yield and repeatability of the extraction was affected by factors as the type of stationary phase of the fiber, sample composition, sample volume, extraction temperature, and extraction time. In order to determine optimal extraction conditions, the effects of changing the selected parameters were studied with other conditions constant. In this step of method development, the real samples of nectar and flowers were investigated. The variation coefficients (CV) for optimization of extraction conditions were calculated as relative standard deviations of absolute peak areas for the triplicate analyses of real samples.

3.1.1. Effect of the dilution of sample on the SPME extraction

Previous most commonly employed methods of extraction are microcapillary tubes, filter paper wicks, washing in a known volume of distilled water, rinsing with successive rinses of a known volume of distilled water, and microliter syringe or capillary glass tubes. Such approaches cannot be treated as a suitable method to apply for tiny flowers and small volumes of nectar as onion has [24–27]. Thus, these methods were not considered further. In order to obtain the nectar on the most natural way, and preserve it in similar conditions as it is in the plant we found that the most effective way of extraction was to separate freshly opened flowers from umbels, removing anthers, filaments and peduncle, and immediately centrifuging (13,000 rpm, 30 min, 4 °C) into a 1.5 ml microtube.

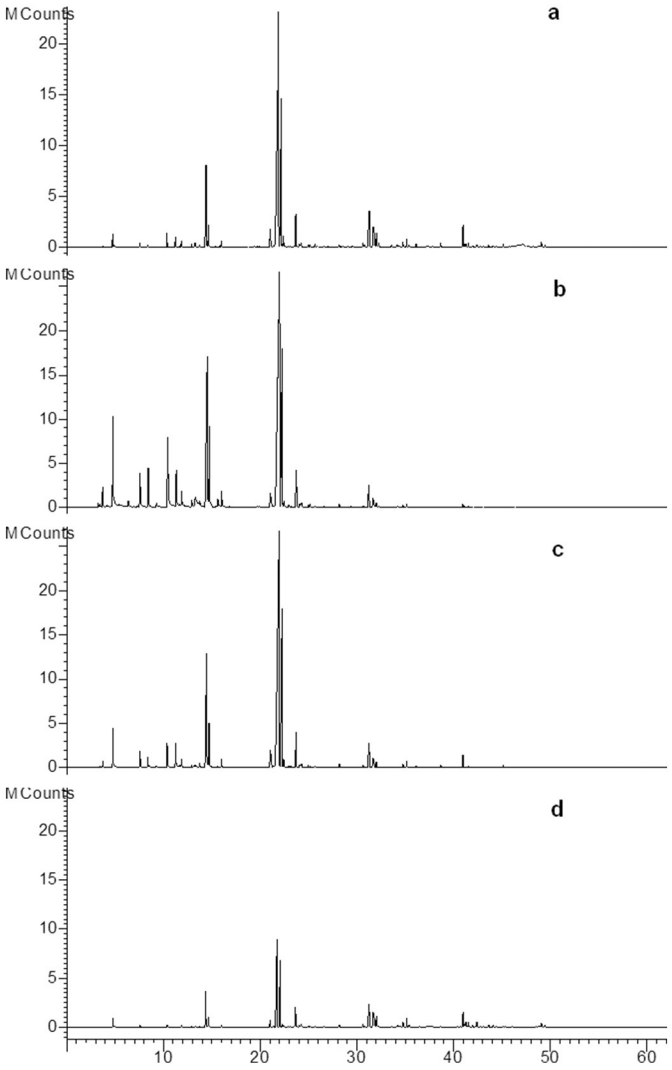

Fig. 1. TIC of headspace volatile compounds from fresh flowers of an onion line by four different SPME fibers ((a) 65 μm PDMS/DVB; (b) 85 μm CAR/PDMS; (c) 50/30 μm CAR/PDMS/DVB; (d) 100 μm PDMS).

In order to examine the effect of dilution of nectar samples with water on the extraction process, samples were prepared with 1 ml total volume, where nectar and water were mixed in the following weight ratios: 1:0, 1:1 and 1:5. As Plutowska et al., [28], reported for honey samples, the composition of the sample played an important role in the extraction of volatiles. Apparently more amount of volatile compounds is obtained from diluted nectar than from undiluted nectar. Highly-volatile compounds easier passed into a headspace phase from a water solution. Moreover, it was very difficult to obtain satisfactory repeatability of extraction using undiluted nectar samples (data not shown). The best results were obtained for a dilution of 1:5. So, this dilution was selected for further studies.

3.1.2. Selection of optimal type of fiber

The SPME sensitivity and selectivity depended mainly on the value of the partition coefficient for analytes between the fiber coating and

Table 1
Identification of the main volatiles from nectar and flowers of different onion lines.

			Nectar				Flower			
n°	Compound identified	Rt	OP	MSL 1	MSL 2	MSL 3	OP	MSL 1	MSL 2	MSL 3
1	Dimethylamine	3.17	0.11	0.07	–	–	–	–	–	0.04
2	Cyclobutylamine	3.28	–	–	–	–	0.12	–	–	–
3	2,3-Butanediol	3.42	–	–	–	–	–	0.31	0.28	0.31
4	2,4-Pentanediol	3.54	–	–	–	–	–	0.13	0.08	0.23
5	2-Methyl-2-propanamine	3.68	0.83	1.12	1.08	0.89	1.13	1.19	1.57	1.39
6	Methyl sulfide	3.85	0.19	0.10	0.64	–	–	–	–	–
7	1-Propanethiol	4.03	–	–	–	–	0.11	–	0.04	0.14
8	2-Methyl-2,3-pentanediol	4.17	0.05	0.04	–	–	0.14	0.09	0.22	0.27
9	2-Methyl-1,4-pentadiene	4.37	–	–	–	–	0.09	0.09	0.11	0.11
10	2-Methyl thiirane	4.56	0.11	0.14	0.15	0.29	0.10	0.08	–	0.16
11	Isopropyl hydrogen trithiocarbonate	4.71	–	–	–	–	1.79	3.14	7.67	4.92
12	Allyl mercaptan	4.82	–	–	–	–	0.53	0.64	0.95	0.52
13	2-Methyl-thiirane	4.86	–	–	–	–	1.25	1.74	2.03	1.65
14	(2E,4Z)-2,4-hexadiene	5.43	–	–	–	–	0.37	0.12	0.11	0.48
15	2,3-Dimethyl oxirane	6.37	–	–	–	–	0.16	0.21	0.39	0.52
16	1-(Methylthio)-, (E)-propane	6.68	–	–	–	–	–	0.03	–	0.07
17	1-(Methylthio)-, (Z)-propane	6.92	0.07	0.07	–	–	0.03	0.07	0.10	0.11
18	1-(Methylthio) 1-propene	7.30	–	–	–	–	0.04	0.12	0.17	0.13
19	1,1′-Dithiobis piperidine	7.50	–	–	–	–	–	1.60	3.78	2.59
20	5-Methyl pyrimidine	7.60	–	–	–	–	3.20	–	–	–
21	Dimetyl disulfide	7.80	0.31	0.17	1.68	1.81	–	–	–	–
22	2-Pentyn-1-ol	7.88	–	–	–	–	0.43	–	–	–
23	1,6-Heptadiyne	8.41	–	–	–	–	5.53	7.63	8.06	2.81
24	Octanal	9.26	0.11	0.12	0.49	–	0.42	0.25	0.21	0.29
25	4-Methyl-3-pentenal	10.42	18.42	17.67	32.97	20.91	15.57	7.71	9.25	6.33
26	(2E)-2-hexenal	11.26	–	–	–	–	3.17	0.73	2.12	2.13
27	1,2,3,6-Tetrahydro-1-methyl pyridine	11.61	–	–	–	0.11	–	–	–	–
28	1-Methyl-2-propyl-cyclohexane	11.74	–	–	–	0.07	–	–	–	–
29	1-(Ethynylsulfinyl) propane	11.87	–	–	–	–	0.82	0.60	0.94	0.94
30	1-(Propylthio)-1 propene	12.91	–	–	–	–	0.65	0.37	0.45	0.36
31	3,4-Dimethylthiophene	13.30	6.67	3.15	12.05	3.52	5.07	3.67	4.39	3.44
32	Methyl propyl disulfide,	13.75	–	–	–	–	0.42	0.33	0.53	0.51
33	2-Methyl-1-(2-propynyl) piperidine	14.26	15.07	5.30	8.52	1.61	13.08	14.99	27.25	20.70
34	1,3-Dithiane	14.67	–	–	6.89	1.39	4.89	7.09	12.55	10.40
35	Methyl 2-propenyl disulfide	15.40	–	–	–	–	0.11	0.10	0.19	0.24
36	Propanethioic acid, S-propyl ester	15.60	–	–	–	–	0.44	0.14	0.24	0.21
37	Dimethyltrisulfide	16.00	13.55	12.70	29.48	26.37	0.91	1.45	2.96	2.14
38	2,4-Nonadiyne	16.83	0.08	–	0.16	–	–	–	–	–
39	Octanal	17.11	–	–	–	–	0.06	0.08	0.06	0.12
40	2-Methyl-1,3-dioxolane	17.72	–	–	–	–	0.02	0.01	0.02	0.06
41	3,8-Dimethyl-1,2,4-triazolo[4,3-a] pyridine	18.22	–	–	–	–	–	0.02	–	0.00
42	2,2-Bis(methylthio)- propane	18.29	–	–	–	–	–	0.05	0.11	0.08
43	Carene	18.80	–	–	–	–	0.01	–	–	–
44	Neodecanoic acid	19.14	–	–	–	–	0.03	–	–	–
45	N,N-Dimethyl-1,3,4-thiadiazol-2-amine	19.25	–	–	–	–	0.03	–	–	–
46	2-(Methylthio) thiophene	19.41	–	–	–	–	0.02	–	–	–
47	Isopropyl propyl disulfide	19.76	–	–	–	–	0.03	–	–	–
48	2-Ethyl-1,3-dioxolane	19.95	2.84	3.84	4.99	0.31	0.08	0.10	0.10	0.17
49	3-[(1-Methylethyl)thio] propanoic acid	20.20	–	–	–	–	0.01	–	–	–
50	di-2-Propenyl disulfide	20.48	–	–	–	–	0.02	–	–	–
51	2,2-Dibenzothiazole	20.61	–	–	–	–	0.03	0.04	0.00	0.07
52	2-Butyl-1-octanol	20.94	0.09	0.05	0.17	–	–	–	–	–
53	2-Dimethylamino-1-phenyl-3-heptanone	21.02	0.13	–	0.40	0.13	2.45	1.69	2.85	2.50
54	Z-4-Dodecenol	21.51	0.69	0.01	0.89	0.19	0.56	0.78	–	0.65
55	Dipropyl disulfide	21.78	6.57	1.10	5.27	0.97	51.90	49.73	78.65	56.89
56	4-Methyldithiole-3-thione	21.95	4.37	0.67	4.41	0.86	–	–	–	–
57	2,2-Dimethyl-1,3-dithiane	22.14	–	–	–	–	28.23	24.38	41.97	33.73
58	2-Ethenyl-1,3-dithiane	22.29	–	–	–	–	0.14	0.09	0.15	0.31
59	1-Propene-1-thiol	22.30	–	–	–	–	0.61	0.48	0.67	0.64
60	N,N′-Dimethyldithiooxamide	22.55	–	–	–	–	0.10	–	–	–
61	4,5-Dimethylthiazole	22.89	–	–	–	–	0.58	0.42	0.57	0.66
62	Methyl allylthioacetate	23.12	0.19	0.08	0.81	0.40	0.09	0.09	0.16	0.21
63	2,2-Dipropyl-N-ethylpiperidine	23.66	8.47	3.22	14.19	8.92	4.56	4.80	10.11	8.76
64	Allyl dithiopropanoate	23.83	–	–	–	–	0.10	0.13	0.18	0.13
65	Methyl(E)-1-propenyl sulfide	24.26	4.94	1.77	11.50	5.39	1.46	2.01	3.15	3.09
66	4-Methylthiothiophen-2(5H)-one	24.95	–	–	–	–	0.78	0.55	0.75	0.73
67	Tetrahydro-2H-1,3-oxazine-2-thione	25.14	–	–	–	–	0.17	0.25	0.34	0.21
68	Dimethyl tetrasulfide	25.63	–	–	0.12	–	0.10	0.14	0.22	0.19
69	Z-4-Dodecenol	25.80	–	–	–	–	0.02	0.03	0.05	0.05
70	1-(Propylsulfanylmethylsulfanyl)propane	25.92	–	–	–	–	0.07	0.10	0.17	0.07
71	Dimethyl pentasulfide	26.69	1.00	0.50	1.55	2.01	0.11	0.15	0.24	0.21
72	2-Butyl-1,3-dioxolane	26.95	0.58	0.32	1.00	–	0.01	0.02	0.02	0.03
73	2-Propyl-1,3-dioxolane	27.22	–	0.11	0.51	–	–	–	–	–

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Table 1 (*continued*)

			Nectar				Flower			
n°	Compound identified	Rt	OP	MSL 1	MSL 2	MSL 3	OP	MSL 1	MSL 2	MSL 3
74	2-Heptyl-1,3-dioxolane	27.42	–	0.29	1.27	–	–	–	–	–
75	Tris(methylthio) ethene	27.82	–	–	–	–	0.03	0.02	0.03	0.05
76	1-(propylsulfanylmethylsulfanyl)propane	28.19	0.18	0.07	0.46	0.12	0.21	0.38	0.93	0.59
77	1-(Isopropylthio)pentane	28.33	–	–	–	–	0.04	0.05	0.11	0.09
78	NN	29.14	0.06	0.12	0.13	–	0.03	0.03	0.04	0.05
79	Propyl octanoate	29.30	0.19	–	1.11	–	–	0.03	0.05	0.03
80	5-Methoxy thiazole	30.66	–	–	0.18	0.07	0.19	0.16	0.30	0.34
81	Dipropyl trisulfide	31.29	2.00	0.26	2.37	1.08	4.15	4.49	7.68	7.89
82	Cis-3,5-diethyl 1,2,4-trithiolane	31.72	–	–	3.31	0.81	4.57	0.45	0.64	0.60
83	TRANS-3,5-diethyl 1,2,4-trithiolane	32.02	–	–	1.24	0.16	2.77	2.57	2.86	3.17
84	3-Ethynylaniline	32.26	–	–	–	–	0.03	0.06	0.03	0.05
85	2-Tridecanol	33.41	–	–	–	–	0.01	0.03	0.02	0.03
86	2-Furandithiocarboxylic acid propyl ester	33.60	–	–	–	–	0.03	–	0.02	–
87	(Z)-7-hexadecenal	33.74	–	–	–	–	0.02	0.04	0.03	0.17
88	Pyrazolo[5,1-c][1,2,4]benzotriazin-8-ol	34.22	–	–	–	–	0.11	0.11	0.22	0.19
89	2-Isopropyl-2-methyl-1,3-oxathiane	34.76	–	–	–	–	0.17	0.33	0.57	0.40
90	2-Furandithiocarboxylic acid propyl ester	34.87	–	–	–	–	0.06	0.03	0.05	0.06
91	Thiazolidine	35.14	–	–	–	–	0.14	0.39	0.87	0.49
92	2,2-Bis(methylthio) propane	35.41	–	–	–	–	0.15	0.22	0.30	0.27
93	NN	36.00	–	0.06	0.12	0.12	–	–	–	–
94	2,4-Dimethyl thiazole	36.12	–	–	–	–	0.05	0.09	0.23	0.22
95	1,1′-Thiobis[3-(methylthio) propane	37.42	–	–	–	–	–	0.07	0.16	0.24
96	Nonadecane	37.81	–	–	–	–	0.02	0.02	0.02	0.03
97	NN	38.22	–	–	–	–	–	–	0.02	0.03
98	3,5-Dimethyl isothiazole	38.65	–	–	–	–	0.10	0.16	0.66	0.33
99	2,4-Thiazolidinedione	40.93	–	–	–	–	0.37	0.42	0.62	0.45
100	2,7-Dimethyl thiazolo[5,4-f]quinoline	41.10	–	–	–	–	0.18	0.20	0.62	0.44
101	2,4-Dihydro-4-methyl 3H-1,2,4-triazole-3-thione	41.52	–	–	–	–	0.14	0.20	0.23	0.20
102	3H-1,2,4-triazole-3-thione,2,4-dihydro-4-methyl	42.00	–	–	–	–	0.07	0.09	0.08	0.07
103	4-Methyl-1,2,4-triazolidine-3,5-dithione	42.38	–	–	–	–	0.16	0.19	0.22	0.18
104	3-Methylrhodanine	42.45	–	–	–	–	0.27	0.36	0.41	0.28
105	2,5-Dimethyl thiazole	42.79	–	–	–	–	0.04	0.03	0.09	0.06
106	7-Methyl thieno[3,2-b]pyridine	43.65	–	–	–	–	0.02	0.05	0.11	0.07
107	3H-naphtol[2,3-c]-1,2-dithiol-3-one	44.42	–	–	–	–	0.04	0.03	0.03	0.08
108	3,4-Dimethy isothiazole	45.15	–	–	–	–	0.08	0.08	0.19	0.19

the sample matrix; hence depended on the type of stationary phase, the polarity and thickness of the fiber. In this study four different fibers of PDMS, PDMS/DVB, CAR/PDMS and DVB/CAR/PDMS were evaluated to determine which fibers most effectively extracted volatile compounds from fresh flowers and nectar of different onion cultivars. Fig. 1 shows the total ion current (TIC) of four fibers extraction volatile compounds from fresh onion flowers. In agreement with [11,28,29] most of sulfides are identified by using the four fibers, but the number of identified compounds is more with Carboxen/PDMS and PDMS/DVB. As seen from the total ion strength of Fig. 1, CAR/PDMS fiber has much better extraction efficiency than the other fibers. The peak area of the most target compounds was higher when they were extracted by the CAR/PDMS fiber than that of the other fibers at equal extraction times, thus the CAR/PDMS fiber was selected as optimal.

3.1.3. Extraction temperature

HS-SPME efficiency is strongly influenced by extraction temperatures as the partition coefficients are temperature-dependent; the higher extraction temperature, the larger the partition coefficients of the analytes between the gas phase and the sample matrix, but the smaller the partition coefficients between the fiber coating and gas phase. Nectar and its components can already be subject to conversions at temperatures a little higher than room temperature. This may lead to uncontrolled changes in the matrix composition; temperatures higher than 40 °C are not advisable. Considering temperature conditions of the normal growing conditions of flowers, and the average temperature during November (30 °C), the influence of temperature of extraction was examined at, 25 °C, 30 °C, and 40 °C. The best results concerning extraction efficiency and reproducibility were obtained at 30 °C (data not shown). As a result, extraction temperature of 30 °C was selected as optimal, since this profile corresponds to the aroma perceived at natural atmospheric conditions in November.

3.1.4. Extraction time

The time required to reach the equilibrium is the optimal sampling time. To check the course of the microextraction of the studied samples, time profiles in the range of 1–60 min were constructed. Extraction amounts increased over time and an extraction balance was obtained after 20 min. Further exposure up to 30 min did not show great increase in the response. On the basis of these results, 30 °C and 30 min, with a CAR/DVB fiber were chosen as the optimum extraction conditions.

3.1.5. Repeatability and reproducibility

In order to determine the repeatability (within day precision) of the method, replicate analysis were carried out on the same day ($n = 6$). In all cases, the precision, expressed as relative standard deviation (RSD) was lower or equal to 10%. The reproducibility (between-day precision) was also tested over 3 days by performing six repeated analyses each day, the RSDs values being lower or equal to 12%.

3.2. Volatile composition of onion lines

Once the optimal conditions were set, the characterization of the different cultivars under study was performed using SPME–GC–MS and statistical analysis. Using the developed methodology, we carried out the analysis of nectar and flower onion samples. The calibration graph for the internal standard for the recommended procedure was linear with a correlation coefficient of 0.998 at levels near the detection limits up to at least 100 μg g^{-1}. The GC–MS data showed differences in the profiles of compounds between flowers and nectar and also among the four lines studied. In flower samples we observed the presence of more than

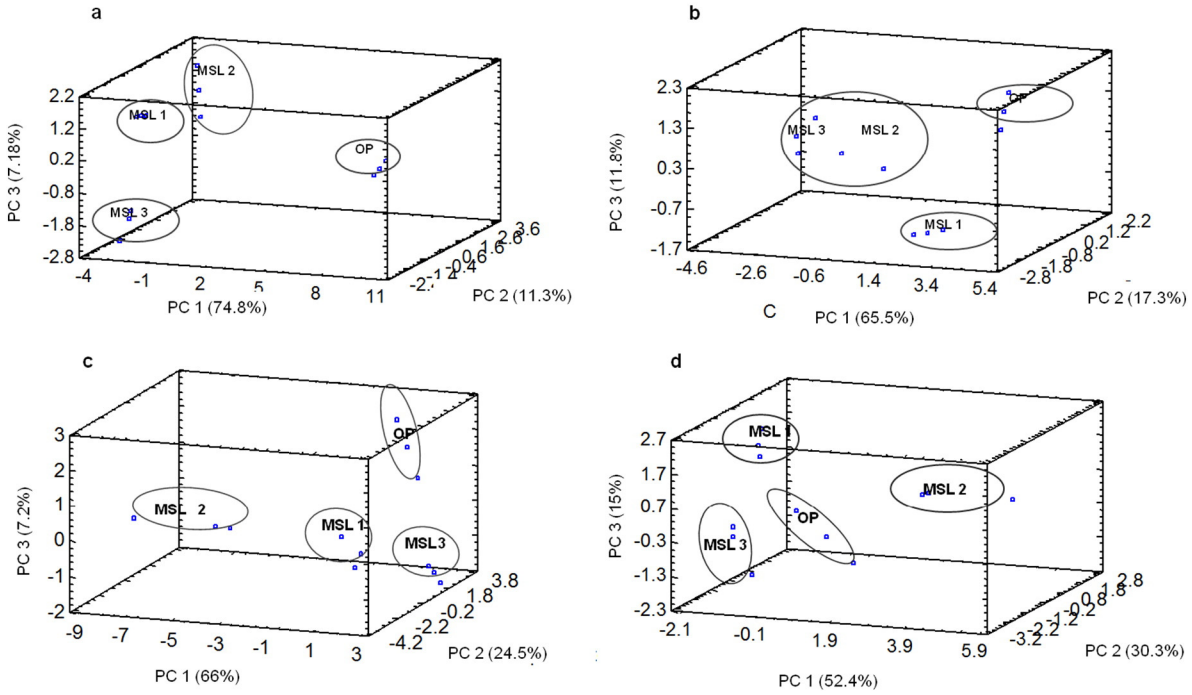

Fig. 2. Principal component analysis (PCA) plot of four onion lines calculated on the basis of their (a) total volatile compounds from flowers; (b) repellent compounds from flowers; (c) total volatile compounds from nectar; (d) repellent compounds from nectar.

100 compounds, of which more than 90 were identified, while in nectar only 34 compounds were tentatively identified (Table 1).

Differences in total ion current (TIC) chromatographic profiles were observed when comparing flower samples from different onion cultivars. From Table 1, it can be seen that 96 volatile compounds were identified in flower and nectar onion samples, including esters, alcohols, alkenes, sulfides, heterocycles, carboxylic acids, ketones, and aldehydes. Sulfur compounds were the most common chemical class. The predominant volatile compound was dipropyl disulfide, followed by 1,3-dithiane, 2,2-dimethyl, dimethyl trisulfide, piperidine 4-methyl-3-pentenal, methyl(E)-1-propenyl sulfide, dipropyl trisulfide, and 3,4-dimethylthiophene.

There were marked differences in volatile sulfur compounds among examined onion plants. Variation among onion plants in their volatile sulfur compounds and their compositions has been reported, 1-propenyl and methyl groups are commonly found in onion, scallion, shallot, leek, and chive [33]. In our study, methyl and propyl groups were the dominant compounds observed in onion hybrids. Sulfides, including dimethyl disulfide, dimethyl trisulfide, dimethyl tetrasulfide and methylthio sulfides isolated from studied samples arise from the degradation of amino acids containing sulfur. Among these; cysteine and methionine are the largest sulfur-containing amino acids in onion. According to Kubec et al., (1999) [34] pyruvic acid can easily decarboxylate to acetaldehyde, by whose aldolization 2-methyl-2-butenal is generated. Furthermore, compounds such as 2-methyl-2-pentenal, probably arise through the aldolization of acetaldehyde and propionaldehyde. We found that 4-methyl-3-pentenal was one of the most predominant volatile compounds, probably due to the same reaction of the pyruvic acid. These carbonyls are proposed to participate significantly in the formation of pyridines found in model systems. Their condensation with ammonia or primary amines (including amino acids) leads to the complex mixture of various alkyl-substituted pyridines.

Comparing VOCs emitted from nectar and flowers, we found that most of these compounds are in lower concentration in nectar and many of the volatile compounds of flowers were not found in nectar. Only a small number of compounds were found only in nectar, such as methyl sulfide or 2,4-nonadiyne. Other VOCs like dioxolanes were at higher concentration in nectar probably due to their low volatility and high solubility in water. On the other hand, also higher concentrations of alkenyl-sulfides were generally found in nectar.

Principal component analysis (PCA) and canonical discriminant analysis (CDA) were applied to highlight the data structure and to find the relationships between VOCs and onion MSL. From a statistical calculation based on SPME–GC profiles, the principal component analysis (PCA) applied shows that analyzed lines are separated in four groups in both kind of samples, nectar and flowers. PCA permitted a reduction of all VOC found in onion to three principal components. These three PCs were extracted explaining 93% of the total variance of flower samples. The first principal component (PC 1) represented about 74.8%, and the next PCs, 11.3%, and 7.1%, respectively. PCA results of VOCs content marked difference within MS lines and between them and the OP line (Fig. 2), distributing flower samples into groups represented their type. Therefore, the complete volatile profile emitted by flowers would be affecting honeybee preference. Similar results were obtained for nectar samples (Fig. 2).

Table 2
Standarized coefficient for discriminant functions obtained from onion flowers lines. Variance explained, Wilks Lambda and probability values.

Functions	1	2
Explained variance percentual	99.18	0.82
Canonical correlation	1.00	1.00
Wilks Lambda	$3.629\ 10^{-32}$	$7.47\ 10^{-19}$
Probability value	0.000	0.000
dimethylamine	237,329	−41,909.6
1-Propanethiol	155,639	59,227.1
2-Methyl 2,3-pentanediol	71,697.3	−55,037.3
Allyl mercaptan	−3491.44	2406.73
2,3-Dimethyl oxirane	−133,314	23,186.4
1-Propene 1-thiol	−461,133	39,073.7
Methyl allylthioacetate	−60,862.6	155,272
2,4-Thiazolidinedione	249,455	−87,741.4
4-Methyl-1,2,4-triazolidine-3,5-dithione	−386,213	−70,504.7

It is noteworthy that alkyl-sulfide compounds occur at higher amounts in the MSL compared to the OP line. Also alkyl-substituted piperidines and dioxolanes were also higher for male sterile onions. Derivates of these compounds have been reported as insect repellents, even as insecticide [35–39]. Thus, PCA was also performed on the basis of these "repellent" compounds (Fig. 2). PCA allowed a reduction of all "*repellent*" compounds to three principal components. The total variance (94.7%) of flower samples was explained with these three PCs. The first principal component (PC 1) represented almost 65%, the second PC (PC 2), 17%, and PC 3, 11.8%. This analysis shows that MSL2 and MSL3 could be grouped in the same cluster, while "*repellent*" compounds of nectar samples shows differences within MS lines and between them and the OP line (Fig. 2).

A correlation study was performed (Pearson's test, 95% confidence level) in order to select the appropriate variables of onion flowers to canonical discriminant analysis (CDA). It was observed that 17 were independent of each other. Nine variables were able to discriminate the samples: dimethylamine, 1-propanethiol, 2,3-pentanediol 2-methyl, allyl mercaptan, oxirane 2,3-dimethyl, 1-propene 1-thiol, methyl allylthioacetate, 2,4-thiazolidinedione and 4-methyl-1,2,4-triazolidine-3,5-dithione ($p < 0.05$). These compounds were selected as predictor variables; the discriminant analysis provided 2 discriminant functions that accounted jointly for 100% of the total variance, with $p < 0.05$ and statistical significance at 95% confidence level. Function 1 account for 99.18% of the total variance, and function 2, accounted 0.82%. Both functions showed Wilks' Lambda values of $3.62 \cdot 10^{-32}$ and $7.47 \cdot 10^{-19}$, respectively, indicating a satisfactory discrimination (Table 2).

The scatter plot of the samples against two first discrimination functions indicates that the 4 lines of onion are separated (Fig. 3). The group centroid is the mean value of the discriminant score for a given category of the dependent variable. There are as many centroids as there are groups or categories. The recognition ability for three classes of MSL and the OP onion by means of CDA was highly satisfactory. This model showed a discriminant efficiency of 100% among the different types of onion lines.

On the other hand, in the analysis of nectar samples, 16 variables were observed to be independent of each other. Among these, twelve play the most important role in the discrimination of these samples. Using a stepwise selection algorithm (forward selection), we found that 7 variables were significant predictors of the different lines (Table 3). The 3 discriminating functions with P-values less than 0.05 are statistically significant at the 95% confidence level. Function 1 explains 70.27% of the total variance, function 2 and 3 explain 26.97% and 2.75%, respectively. The most influencing parameters are identified based on standardized canonical coefficients. In the case of nectar samples, variability intragroups is smaller than flower samples, even though according to the classification parameters, the obtained model could be successfully used in order to distinguish MSL from OP onion nectar and also MSL among them.

Table 3
Standarized coefficient for discriminant functions obtained from nectar of onion lines. Variance explained, Wilks Lambda and probability values.

Functions	1	2	3
Explained variance percentual	70.27	26.97	2.75
Canonical correlation	0.99995	0.99985	0.99875
Wilks Lambda	$6.335\ 10^{-11}$	$6.422\ 10^{-7}$	0.0025
Probability value	0.000	0.000	0.000
2-Methyl 2-propanamide	13.93	−16.72	0.37
2-Methyl thirane	23.54	−18.33	5.58
Dimethyl disulfide	−12.15	19.57	−3.03
2-Propyl 1,3-dioxolane	23.17	3.78	3.45
4-Methyl dithiole-3-thione	−5.1	−2.43	1.11
2-Methyl 2-pentenal	−16.24	−4.19	−5.66
2-Butyl 1,3-dioxolane	−25.77	21.52	−0.99

When groups are overlapped in the multidimensional space, they make Wilks' Lambda values close to 1. As the groups are separated, the intergroup variability will increase, and intragroup variability will gradually become comparatively smaller relative to the total variability, reducing the value of this statistic. Therefore values close to 1 indicate a great similarity between groups, while values close to 0 indicate a difference between them. Wilkis' Lambda values for flower samples were smaller than nectar samples, indicating a higher separation among onion lines and lower intergroup variability (replicates) (Fig. 3).

It is worth mentioning that the compounds suitable for CDA analysis are different in nectar and flower. Nonetheless, the analyzed volatile compounds provided enough information to differentiate OP and MSL as well as among male sterile lines, not only from nectar but also from flowers samples.

3.3. Foraging rates and impact of bee pollination on seed production

Our observations of pollinator behavior showed that the onion lines we analyzed attract very different numbers of bee visitors. All MSL had lower number of visits than the OP line. In fact, MSL 1 had the lowest foraging population, which was 6-fold lower than those observed in the OP line (Table 4). *Apis mellifera* L. activity was very low at 09.00 h when air temperature was 25 °C for all the onion lines. In male sterile lines the number of visits was between 0.11 and 0.29 visits/umbel/min, while for the open pollinated cultivar was 0.67 visits/umbel/min. The foraging population increased thereafter, and maximum abundance was observed between 12.00 and 15.00 h when the air temperature ranged between 30 and 35 °C and solar radiation was 1298 μmol/m^2/seg. The number of visits/umbel/min at that time was 0.16 and 0.43; while for the open pollinated cultivar was 0.94 visits/umbel/min (Table 4). A positive correlation between bee abundance with air temperature and solar radiation was found (data not shown). These results are in concordance with those reported by Abrol (2010). As reported, different cultivars differ greatly in their attractiveness to pollinating insects; flower visitation

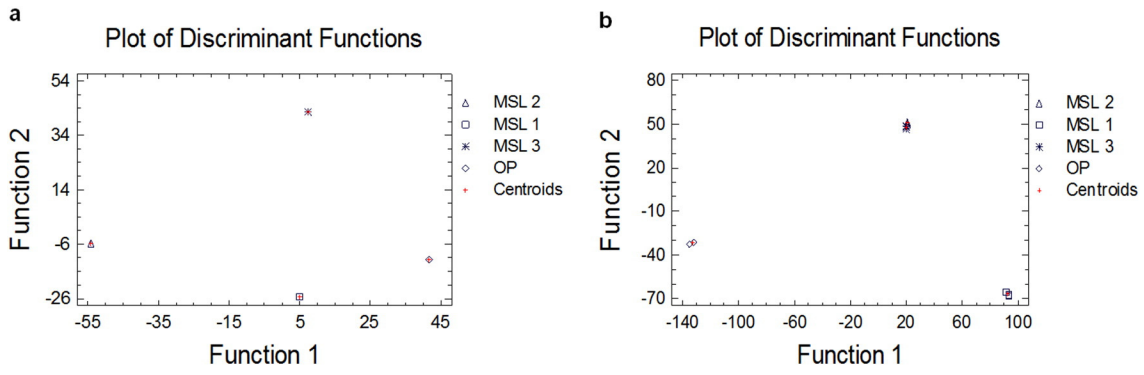

Fig. 3. Canonical discriminant analysis (CDA) of four onion lines according to the VOCs emitted from their (a) flowers (b) nectar. Scatter plot representing the projection of the point of each sample on the plane formed by the first two discriminant functions.

Table 4
Number of honeybee visits during day hours at 50% of blooming and seed yield per inflorescence in different onion lines.

Line	Hour of observation			Seed yield
	9:00 AM	12:00 AM	15:00 PM	
Valcatorce INTA (OP)	0.67 ± 0.07 a	0.94 ± 0.08 a	0.93 ± 0.10 a	2.98 ± 0.53 a
MSL 1	0.11 ± 0.05 b	0.16 ± 0.07 c	0.19 ± 0.05 c	0.90 ± 0.42 c
MSL 2	0.29 ± 0.05 b	0.43 ± 0.07 b	0.42 ± 0.08 b	2.01 ± 0.20 b
MSL 3	0.23 ± 0.04 b	0.35 ± 0.09 bc	0.34 ± 0.03 bc	1.30 ± 0.06 bc

OP: open pollinated line. MSL: male sterile line. Values represent mean ± SD of 3 determinations. Number of visits during day is expressed as visits of honeybees/umbel/minute. Seed yield is expressed as g/umbel. Values in the same column with different letters present significant differences $P < 0.05$.

rates differ at different times of the day depending upon atmospheric conditions, availability of nectar, pollen and bee species involved.

The pollination effectiveness was determined on the basis of the weight of seeds obtained from inflorescences. A great difference was found between the yield of hybrids and OP seeds, Valcatorce INTA had 3-fold higher amount of seed than the MSL with lower yield. There were also seed yield differences among MSL. Frequency of honeybee visits and seed yield were correlated ($r = 0.93$) (data not shown). These results are in concordance with those reported by Wilkaniec et al. [40]. On the other hand, it has to be stated that the results obtained in the studied lines under a cage were consistent with data obtained for the same hybrids under field conditions ($r = 0.94$) (data not shown).

3.4. Relation of VOCs to pollination and MSL seed production

There is a clear relationship between the volatile profile and pollinator foraging behavior for each onion line. The most visited onion line was the one showing the most complex volatile profile and the least visited lines contained more of the putatively repellent compounds. Nevertheless, there was not a direct correlation between VOCs profile and pollination or seed yield. This could be due to the fact that MSL 1 showed much smaller umbels than the other lines, which could have affected bees foraging behavior, and data obtained from this line affects the correlation between variables (Fig. 4). Therefore, excluding MSL 1 data, a positive correlation was found between these variables (Fig. 4). In addition to that, it is worth remarking that a PCA made on the basis of floral putatively repellent compounds (Fig. 2) shows that MSL2 and MSL3 could be grouped in the same cluster, in concordance with those observed in bee foraging rates. Considering that MSL showed greater amounts of deterrent compounds, the difference in these specific volatile compounds of MSL could be related to the fact that MSL were the least attractive lines.

4. Conclusions

The results obtained demonstrate that SPME–GSMS is a fast and useful method for the analysis of volatile compounds emitted from onion plants, allowing a rapid screening of the aroma emitted from flowers and nectar of different cultivars or lines. A total of 96 compounds were identified from the different onion lines studied.

The experiments performed in this study clearly showed that MSL have different profiles than OP onions. Moreover, this study proved that the combination of chemical information and statistical analysis is able to differentiate the studied onion male sterile lines from the open pollinated onion as well as MSL among themselves.

We did not observe a direct correlation between VOCs and pollination or seed yield. However, honeybee preference trials suggest that OP variety is more attractive than F1 hybrids. Male sterile lines had putatively repellent compounds such as alkyl-sulfides. Indeed, a negative relationship between the amount of "*repellent*" compounds and bee pollination was found. It is known that other factors, such as nectar quantity and nectar quality determine bee foraging behavior. Among such factors, variable proportions of sugars, amino acids, proteins, lipids, phenols, or alkaloids could give it a particular taste and smell that may be essential for the maintenance of pollinators. Further studies are being carried out in our laboratory that will provide more information regarding the relationship between nectar chemical composition and seed yield. Nonetheless, the markedly qualitative and quantitative analytical differences in the volatile profile of male sterile and open pollinated lines found in this study contribute to the understanding of the factors that affect onion pollination for hybrid seed production.

Acknowledgments

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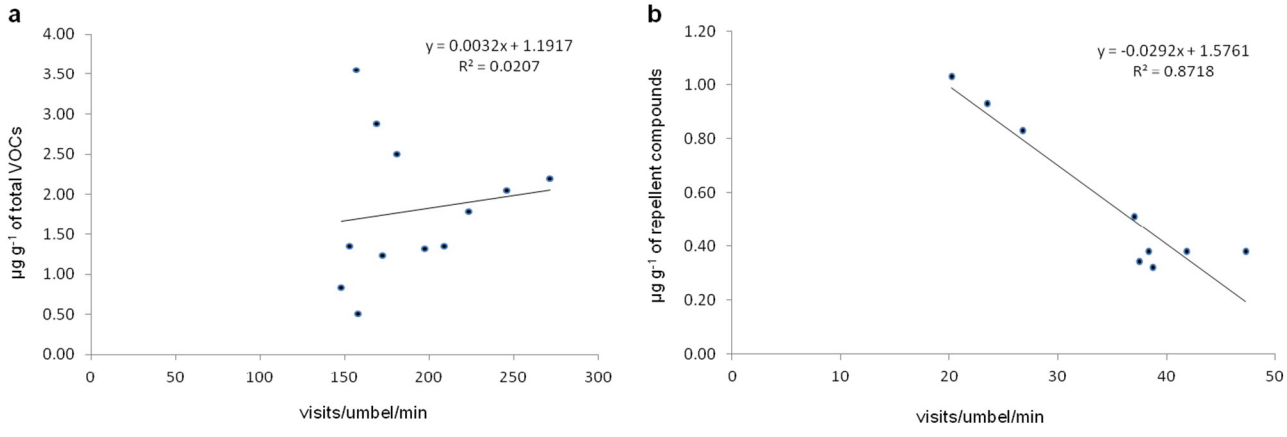

Fig. 4. Correlation between honeybee visits/umbel/min and (a) concentration of total VOCs (b) concentration of repellent compounds emitted from flowers of the different onion lines.

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