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Full Length Article

First Look at Olive Floral Volatiles: Identification and Discrimination among Mediterranean and Chinese Taxa

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Abstract

Although *Olea europaea* was generally considered as the anemophilous species, it was reported that olive flowers could attract pollinators during blossom. Yet, olive floral volatiles have not been studied to provide further phytochemical evidences. In this paper, headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry was used to determine the volatile organic compounds (VOCs) of two Chinese (Ezhi, Chenggu) and two Mediterranean (Hojiblanca, Koroneiki) olive flowers. A total of 52 VOCs were identified, and 20 of them were marked as insect attractants in previous literature. (Z)-8-heptadecene, an uncommon and odorless floral VOC, was the major metabolite (41.5%~50.7%) in olive. Moreover, Chinese taxa emitted more esters and alcohols, which processed fresh/green/grassy scent; Mediterranean cultivars emitted more terpenoids (fruity/citrus/floral odor) and aliphathics (fusel-like scent). According to the PCA and PLS-DA, *cis*-jasmon, *cis*-3-hexen-1-ol, 3-methyl-4-penten-1-ol, heptadecane, pentadecane and 9-octadecyne contributed significantly to Chinese and Mediterranean taxon separation. In conclusion, the four olive varieties can be distinguished from each other on the basis of their floral VOCs. For the first time, our study reported the volatile metabolites of olive flowers. Our findings match the description of pollinators attraction in fields, and in a broad context, may bring a wider vision on the biodiversity and cultivar certification in *O. europaea*.

Keywords: Olive flowers; Volatile organic compounds; Secondary metabolites; HS-SPME-GC-MS

Introduction

Floral fragrance compounds are secondary metabolites released by plant flowers (Fan *et al*. 2019). They mainly consist of abundant low molecular weight volatiles, including terpenes, benzenoid aromatics, and fatty acid derivatives (Dudareva *et al*. 2013; Shi *et al*. 2019). The ubiquity and significance of floral volatile organic compounds (VOCs) has been vocalized by phytochemists and entomologist (Pichersky and Gershenzon 2002; Dudareva *et al*. 2013; Yang *et al.* 2018). Undoubtedly, the diversity of flora VOCs suggests that plant flowers are subtle communicators interacting with their environment. Apart from attracting specific pollinators to ensure reproductive success (Raguso 2008; Schiestl 2015; Borghi *et al.* 2017), many flower VOCs can serve protective functions against colonizing microorganisms (Huang *et al.* 2011; Ortega *et al*. 2016; Shi *et al*. 2019) and florivores (Junker *et al.* 2011). Moreover, VOCs emitted from pollinated flowers can deter pollinators and direct them to as yet unpollinated counterparts (Schiestl and Ayasse 2001).

Generally, bee activity, though recorded occasionally in field, is not regarded as the main polling momentum for olive (*Olea europaea* L.) trees (Morettini 1972; Griggs *et al*. 1975). However, recent studies indicate that the honey bee is the active and efficient pollen collector of olive inflorescence in both wild and orchard (Canale and Loni 2010; Aronne *et al*. 2012; Giovanetti 2018; Carlos *et al*. 2019). Yet, as an important ornamental trait (Shi *et al.* 2019), flower scent is rarely reported in anemophilous species. So, do the volatile metabolites in olive flowers include pollinator attractants? The literatures concerning composition, biosynthesis and function of olive VOCs are many, but they focus mostly on olive oils and few on leaves (Kiritsakis 1988; Campeol *et al*. 2001; Flamin *et al*. 2003). Thus, no phytochemical information is available regarding the compounds of olive inflorescence scent; and the question above still remains unanswered.

As a star tree species in Mediterranean countries, olive was first introduced and propagated by Chinese government in large-scale at 1964 (Xu and Han 1965; Xu 1981). Now, the prosperous olive industries can be seen in Gansu, Sichuan, Yunnan and Shaanxi provinces (Rao *et al*. 2019). A few domestic olive varieties (*O. europaea* L. cv. Ezhi-8, *O. europaea* L. cv. Chenggu-32, etc.) were bred during the half century domestication, and ideally, Chinese olive varieties are less sensitive to abiotic and biotic stresses. Previous comparative studies between domestic and foreign olive cultivar mainly focused on phenology, fruit and oil quality, etc. (Kong *et al*. 2016; Zhang *et al*. 2016; Han *et al*. 2017). The flower fragrance and VOCs have not yet been mentioned.

Investigations of flower aroma were essentially encouraged by the rapid development of modern analytical method and equipment (Surburg *et al*. 1993). This present study was aimed to identify and discriminate floral VOCs in two Chinese and two Mediterranean olive varieties, using head-space solid-phase micro-extraction and gas chromatography-mass spectrometry (HS-SPME-GC-MS). Key VOCs in four taxa were screened through the multivariate statistical analysis, which was usually used in omics. In this paper, two basic questions are addressed: (1) What are the fractions of olive floral VOCs? (2) What is the difference between Chinese and Mediterranean varieties? The obtained results will fill the research gap in olive inflorescence VOCs, as well as may explain the bee activities during blossom. Further, this study could provide further knowledge for understanding the biodiversity, origin and cultivar certification in olives.

Materials and Methods

Plant materials

Two Chinese [*O. europaea* L. cv. Ezhi-8 (Ezhi), *O. europaea* L. cv. Chenggu-32 (Chenggu)] and two Mediterranean olive varieties [*O. europaea* L. cv. Hojiblanca (Hojiblanca), *O. europaea* L. cv. Koroneiki (Koroneiki)] were studied in the garden of Longnan Xiangyu Olive Development Co., Ltd, in Longnan, Gansu, China (105°00'48"~105°00'54"E, 33°20'09"~33°20'15"N). The site has a dry-warm valley climate (annual average temperature, 14.9°C; sunshine duration, 1872 h; annual rainfall, 400~900 mm; frost-free period, 120~284 days; data from *www.weather.com.cn*).

Experimental design

This study was conducted with one-factor (olive taxon) experimental design. From May 5^{th}~10^{th}, each taxon was sampled by three different trees with an approximately same height and crown size. Ten branches with flowering inflorescences attached were randomly collected per tree at 9:00~10:00 am. The branch cuts were covered by wet towels and sealed in plastic bags separately. Within 6 h, the fresh samples were transported by flight in a dry cooler (4±1°C) to the laboratory in Nanjing, Jiangsu, China.

HS-SPME extraction

Branches with inflorescences from four taxa were placed into deionized water separately once the sample arrived at the laboratory. And HS-SPME extraction was carried out on the following day (at 8:00~9:00 am). The 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME portable sampler (Supelco, Bellefonte, Pennsylvania, USA). A 3.0 g of newly bloomed inflorescences were randomly collected and placed into a 20 mL clean capped vial. To absorb VOCs of olive flowers, the SPME sampler was inserted into the vial headspace. The extraction was equilibrated for 30 min at 60±1°C. After the extraction, the portable SPME samplers were stored in dry ice before GC-MS analysis. All measurements were triplicated.

GC-MS analysis

In a random fashion, flower VOCs in the SPME sampler were injected into a TRACE DSQ GC-MS (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The GC injector port temperature was set at 250°C in non-split mode for 1 min. An Agilent DB-5ms silica capillary column (30 m×0.33 mm×0.25 μm, 5% phenylmethyl siloxane; Agilent Technologies Inc., Santa Clara, California, USA) was used to separate different volatile compounds. The carrier gas was helium at a constant flow rate of 1 mL/min. The GC oven temperature program was set as follows: (1) initially 50°C for 1 min, (2) gradually increasing at a rate of 6°C/min to 120°C, which was held for 1 min, (3) increase at a rate of 4°C/min to 140°C, with no hold, (4) final increase at a rate of 12°C/min to 250°C, and hold for 3 min. The temperature of the transfer line and ion source was both set as 250°C. Electron ionization was carried out with 70 eV of ionization energy. The mass spectra of eluted compounds were recorded over an m/z range of 33 to 450. The linear retention indices (LRI) of the volatile compounds were calculated using an alkane series standard (C7~C30) (Sigma, St. Louis, Missouri, USA) under the same conditions. The mass spectra peaks were compared using X-caliber 3.1 (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) with reference to the National Institute of Standards and Technology (NIST) 12th database. The identification the floral VOCs was then made basing on the spectra similarity and LRI. An ion current peak area normalization method was used to calculate the relative amount of each component.

Statistical analysis

The relative amount of floral VOCs was expressed as the

mean ± standard deviation (%) of three independent experiments. Among four olive taxa, one-way analysis of variance (ANOVA) was performed using SPSS 19.0 (IBM Corp., Armonk, NY, USA) followed by Duncan's multiple range test. The principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA) was performed by SIMCA 13.0 software (Umetrics, Umeå, Sweden). Differential accumulated metabolites (DAMs) between each two olive taxa were identified if variable important in projection (VIP)>1.0, t-test $p<0.05$, and fold change (FC) >1.50 or <0.67. Origin 2017 (OriginLab Corp., Northampton, Massachusetts, USA) was used to process PCA biplot, Venn diagram, histogram and heatmap with hierarchical cluster analysis (HCA), Pearson's correlation coefficient analysis. The odor characteristic of each volatile compound was obtained from Pherobase (*www.pherobase.com*) and the "Good Scents" company network database (www.thegoodscentscompany.com).

Results

Identification and comparison of VOCs

After the GC-MS data was acquired, parallelism of the biological replications in groups was checked using Pearson correlation analysis (Fig. S1). Identification (Table 1; Fig. S2) and classification (Fig. 1) of the floral VOCs were reported. The compounds released from flowers were significantly affected by olive taxa ($p<0.001$). A total of 52 VOCs were identified from four varieties, and only 28 common compounds were found, which exhibited a great diversity in olive floral VOCs. Categorical analysis indicated that the main fraction of flower VOCs were different (Fig. 1).

Concretely, the most abundant chemical component shared by four taxa was the odorless (Z)-8-heptadecene (constitutes 42.0, 43.6, 41.5 and 50.7% for Ezhi, Chenggu, Hojiblanca and Koroneiki, respectively). Other primary aliphatics also included heptadecane, pentadecane, 9-Octadecyne, (Z)-7-hexadecene and (Z)-4-Tetradecene (13.9% of total for Ezhi, 15.1% for Chenggu, 27.4% for Hojiblanca, 31.9% for Koroneiki). The rest volatiles were not identical among taxa. For example, *cis*-3-hexenyl acetate, *cis*-3-hexen-1-ol and (E)-butanoic acid, 3-hexenyl ester with fruity and green aroma were largely released in Chinese (constituting 16.8% and 17.2% for Ezhi and Chenggu, respectively) than in Mediterranean (1.2% and 0.7% for Hojiblanca and Koroneiki, respectively) olive flowers. Furthermore, as we can see in Table 1, a few olive floral volatiles (classified as terpenes and aliphatics) were found to process roles as insect semiochemicals in previous studies.

Data (Fig. 1) showed that Chinese olive flowers emitted abundant esters, alcohols and alkanes (total sum of 78.2 and 77.7% for Ezhi and Chenggu respectively). For

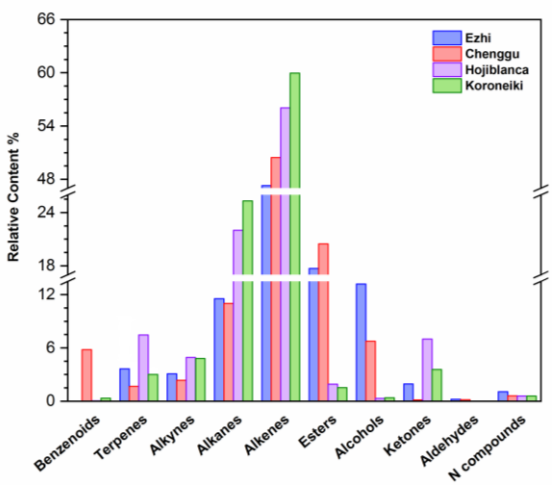


Fig. 1: Categorical discrimination of the volatile organic compounds (VOCs) present in the flowers of two Chinese (Ezhi and Chenggu) and two Mediterranean (Hojiblanca and Koroneiki) olive cultivars. Compounds of different chemical classes among taxa were shown

Mediterranean taxa, the major metabolites were alkanes and alkenes (78.1 and 85.3% for Hojiblanca and Koroneiki respectively). Interestingly, benzenoids were predominantly emitted in Chenggu (5.8%), while no benzenoid was detected in Ezhi.

PCA and HCA based on the GC-MS data

To determine the contribution of 52 compounds identified to the floral scent variation, PCA was performed (Fig. 2a). The first principal component (PC 1) and the second principal component (PC 2) contribution reached 78.3% in total. All taxa were distributed clearly in four quadrants. *Cis*-3-hexenyl acetate, heptadecane, pentadecane and *cis*-jasmon had high contributions on PC 1, while (Z)-8-heptadecene, benzene, 1,3,5-trimethoxy-, (E)-4,8-dimethylnona-1,3,7-triene, and *cis*-jasmon contributed significantly to PC 2. The contents of cis-3-hexenyl acetate and benzene, 1,3,5-trimethoxy- in floral VOCs positively corresponded to the identity of Chenggu. 4-penten-1-ol, 3-methyl-, *cis*-3-hexen-1-ol, *cis*-3-hexenyl 2-methylbutanoate and butanoic acid, (E)-3-hexenyl ester, were highly correlated to with Ezhi. Compounds with highly negative scores on PC 1 (*cis*-jasmon) and PC 2 ((E)-4,8-dimethylnona-1,3,7-triene) were related to the discrimination of Hojiblanca. Koroneiki's identity associated greatly to compounds of (Z)-8-heptadecene, heptadecane and pentadecane.

HCA was conducted to further interpret the data. The biological replications from two Chinese and two Mediterranean olive varieties were clearly clustered accordingly (Fig. 2b), which showed the good stability and reliability of the GC-MS results. Generally, most esters and

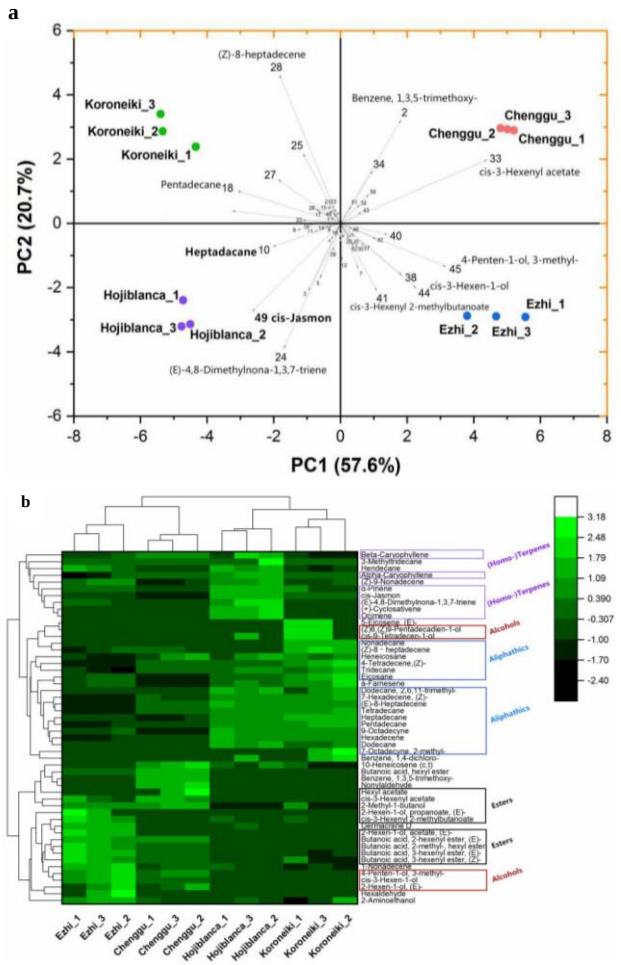
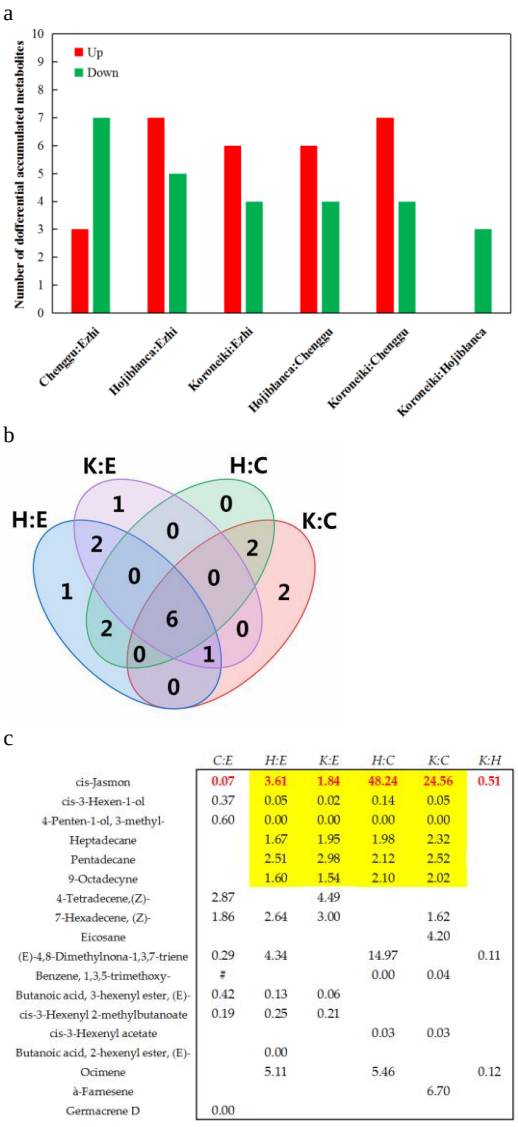


Fig. 2: Multivariate statistical analysis of the volatile organic compounds (VOCs) present in the flowers of two Chinese (Ezhi and Chenggu) and two Mediterranean (Hojiblanca and Koroneiki) olive cultivars. (a) PCA biplot based on the volatile compounds of four olive taxa. Numbers correspond to those in Table 1. (b) Clustering of the 52 VOCs detected in four olive taxa. Light green indicates low expression metabolite and dark green indicates high expression metabolite

some alcohols were clustered together under the Chinese taxa (Ezhi and Chenggu), whereas terpenes and aliphathics were grouped under the two Mediterranean olive varieties (Hojiblanca and Koroneiki).

Determination of key VOCs through DAMs analysis

Under the PLS-DA model, DAMs between each two olive taxa were identified as key VOCs if variable important in projection (VIP)>1.0 with *p*-value<0.05 in the t-test and fold change (FC) >1.5 or <0.6. Compared with Ezhi, a total of 12 and 10 DAMs were identified for Hojiblanca and Koroneiki, respectively (Fig. 3a). There were 10 DAMs identified between Chenggu and Ezhi. Relative to Chenggu, a total of 10 and 11 DAMs were identified for Hojiblanca and



c

	C:E	H:E	K:E	H:C	K:C	K:H
cis-Jasmon	0.07	3.61	1.84	48.24	24.56	0.51
cis-3-Hexen-1-ol	0.37	0.05	0.02	0.14	0.05	
4-Penten-1-ol, 3-methyl-	0.60	0.00	0.00	0.00	0.00	
Heptadecane		1.67	1.95	1.98	2.32	
Pentadecane		2.51	2.98	2.12	2.52	
9-Octadecyne		1.60	1.54	2.10	2.02	
4-Tetradecene,(Z)-	2.87		4.49			
7-Hexadecene, (Z)-	1.86	2.64	3.00		1.62	
Eicosane					4.20	
(E)-4,8-Dimethylnona-1,3,7-triene	0.29	4.34		14.97		0.11
Benzene, 1,3,5-trimethoxy-	#			0.00	0.04	
Butanoic acid, 3-hexenyl ester, (E)-	0.42	0.13	0.06			
cis-3-Hexenyl 2-methylbutanoate	0.19	0.25	0.21			
cis-3-Hexenyl acetate				0.03	0.03	
Butanoic acid, 2-hexenyl ester, (E)-		0.00				
Ocimene		5.11		5.46		0.12
à-Farnesene					6.70	
Germacrene D	0.00					

Fig. 3: Determination of key VOCs present in the flowers of two Chinese (Ezhi and Chenggu) and two Mediterranean (Hojiblanca and Koroneiki) olive cultivars using differential accumulated metabolites (DAMs) analysis. (a) The number of up-regulated (red bars) and down-regulated (green bars) DAMs. (b) Venn diagram indicating the similarities and differences in VOCS among different taxa. The numbers in the overlapping areas indicate the compounds shared by the corresponding taxa. (c) 18 key VOCs identified through differential metabolites analysis under partial least squares-discriminate analysis (PLS-DA) model. Fold changes (FCs) were marked in box. #indicate the relative content of benzene, 1,3,5-trimethoxy- was 0.00% in Ezhi. Red colour represents key VOCs identified to discriminate all four olive taxa. Yellow background highlights key VOCs to discriminate Chinese and Mediterranean olive cultivars. E-Ezhi, C-Chenggu, H-Hojiblanca, K-Koroneiki.

Koroneiki, respectively, while there were 3 DAMs (all down-regulated) identified for Koroneiki compared with Hojiblanca. In general, more up-regulated DAMs were

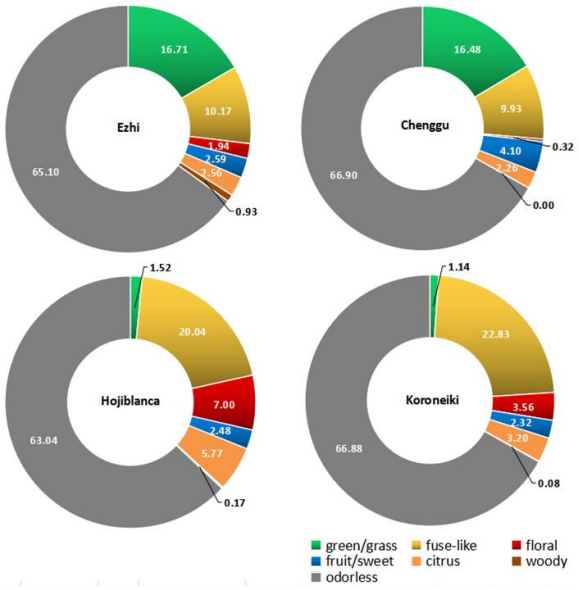


Fig. 4: Proportions of odor compounds in two Chinese (Ezhi and Chenggu) and two Mediterranean (Hojiblanca and Koroneiki) olive cultivars

noted in Mediterranean than in Chinese taxa. For Venn diagram, there were 6 common DAMs identified among taxa (Fig. 3b). Eighteen DAMs were identified through differential metabolites analysis under PLS-DA model (Fig. 3c). *Cis*-jasmon (jasmine-like scent) was a key VOC to discriminate all four olive taxa. Six VOCs (*cis*-jasmon, *cis*-3-hexen-1-ol, 3-methyl-4-penten-1-ol, heptadecane, pentadecane and 9-octadecyne) were crucial in distinguishing Chinese olives from Mediterranean ones.

Discussion

Although *O. europaea* flowers attract honey bees (Canale and Loni 2010; Aronne *et al.* 2012; Giovanetti 2018; Carlos *et al.* 2019), previous study barely discussed olive floral VOCs. During the pre-investigation in olive groves of China, the light fragrance could be sensed by sniffing close to the inflorescence. So in this paper, the question of whether olive flowers emit scent is firstly asked. Further, this study is the first to compare the differences in floral VOCs between two Chinese and two Mediterranean olive cultivars. Moreover, as a mainstream method, chromatogram combined with solid-phase micro-extraction and headspace analysis have been widely performed to determine the VOCs of some aromatic plants (Dötterl *et al.* 2005; Rusanov and Kovacheva 2011; Cai *et al.* 2014; Li *et al.* 2016). The PLS-DA has been always used in many omics researches to identify differential metabolites, proteins or genes. Also, the key VOCs to discriminate different plant taxa could be identified by PLS-DA in many plant species (Shi *et al.* 2019), such as in teas (Zhu *et al.* 2017), apples (Giannetti *et al.* 2017), and *Lycoris* (Shi *et al.* 2019). Our study successfully identified the VOC profiles of four olive taxa and distinguished domestic varieties from Mediterranean ones.

Floral perfume is a satisfactory feature for ornamental and olfactory markets. The scent emitted by flowers mainly depends on the contents and proportions from a range of VOCs and their mixtures, which is largely influenced by the genetic characteristics of the taxa (Shi *et al.* 2019). Fragrant compounds of different plants species varied, even different varieties of *Syringa oblata* had distinct floral VOCs (Li *et al.* 2006). But the major VOCs of aromatic flowers are mostly terpenoids (*e.g.* ocimene in *Lycoris* (Shi *et al.* 2019) or benzenoids (*e.g.* benzyl alcohol and phenylethanol in *Malus* (Li 2012; Fan *et al.* 2019)). Most VOCs emitted from olive flowers are odorless. Our results indicated odorless (Z)-8-heptadecene was the most abundant (41.5~50.7%) VOC in all four olive taxa. However, fragrant benzenoids (0.05~5.81%) and terpenoids (2.3~7.2%) were also presented (Table 1 and Fig. 1). In fact, 63.0~66.9% of the total identified floral VOCs were odorless (Fig. 4), which explained the light fragrance in olive flowers. The odor characteristics of other aromatic volatiles of the olive flowers were also illustrated in the pie chart (Fig. 4). The fragrance types of in all the taxa were different. VOCs with green and herbal odor were the more abundant in Chinese olive varieties (16.5~16.7%) than in Mediterranean ones (1.1~1.5%). Grass odor comes from C6/C9 aldehydes and C6 alcohols (Qian *et al.* 2019). However, in olive they were mostly attributed to the *cis*-3-hexen-1-ol and its acetate. Except odorless VOCs, scent compounds with unpleasant fusel-like odor (predominately alkanes) were the most abundant in Mediterranean olive flowers, reaching up to 20.0~22.8% (Fig. 4). Yet, VOCs with refreshing odor, including floral and citrus (mostly attributed to jasmone and some mono-terpenes), were found much higher in Mediterranean olives than in Chinese counterparts. However, the overall scent of olive flower cannot be described only by odor characteristic fractions. Sensory evaluation integrated with E-nose analysis could be further performed.

As confirmed by statistics, the four cultivars can be distinguished from each other on the basis of their floral volatile compositions. Apparent chromatogram peaks at RT 14.22 (Butanoic acid, 3-hexenyl ester, (E)-, RC 3.34%) and 15.52 (*cis*-3-Hexenyl 2-methylbutanoate, RC 3.12%) were seen in Ezhi only (Fig. S2). Moreover, the two corresponding substances were identified as DAMs of Ezhi VOCs compared with Chenggu, Hojiblanca and Koroneiki in PLS-DA (Fig. 3c). Therefore, they could be the key VOCs to discriminate Ezhi from other three olive taxa. Using the same analytic method, 1,3,5-trimethoxy-benzene (RT 20.53, RC 5.81%) was uniquely emitted in Chenggu; ocimene (RT 10.76, RC 2.79%) and (E)-4,8-dimethylnona-1,3,7-triene (RT 12.43, RC 7.34%) were the specific VOCs emitted in Hojiblanca compared with other taxa. Evidences showed that ocimene may be described as a general pollinator attractant (Farré-Armengol *et al.* 2013). In

Table 1: Volatile organic compounds (VOCs) in the flowers of two Chinese and two Mediterranean olive cultivars

Peak No.	Retention Time/min	Volatile Compound	CAS No.	Odor Characteristic	Relative Content/%				P value
					Chinese Variety		Mediterranean Variety		
					Ezhi	Chenggu	Hojiblanca	Koroneiki	
		Benzenoids							
1	10.05	Benzene,1,4-dichloro-	106-46-7	camphor	—	—	0.05±0.08	0.09±0.08	0.519
2	20.53	Benzene,1,3,5-trimethoxy-	621-23-8	—	—	5.81±0.31 Aa	—	0.23±0.05 Bb	< 0.001
		Terpenes							
3	10.76	Ocimene*(Shi *et al*. 2019)	13877-91-3	citrus, floral, grassy	0.55±0.12 Bb	0.51±0.04 Bb	2.79±0.24 Aa	0.32±0.06 Bb	< 0.001
4	19.64	(+)-Cyclosativene	22469-52-9	—	—	—	0.15±0.01	—	—
5	19.84	α-Pinene *(Miller and Rabaglia 2009)	80-56-8	fruity, sweet, pine	0.50±0.04 b	—	1.09±0.13 a	0.27±0.47 b	0.031
6	20.85	Beta-Caryophyllene*(Flint *et al*. 1979)	87-44-5	spicy, citrus, woody, mild syringa	1.01±0.08 b	1.31±0.04 a	1.37±0.28 a	0.91±0.06 b	0.014
7	21.01	Germacrene D*(Mozuraitis *et al*. 2002)	37839-63-7	earthy, wood, spice	0.93±0.01 Aa	—	0.17±0.01 Bb	0.08±0.07 Bc	< 0.001
8	21.50	Alpha-Caryophyllene	6753-98-6	spicy, citrus, woody, mild syringa	0.10±0.10 Bb	0.18±0.00 ABb	0.30±0.02 Aa	0.18±0.01ABb	0.006
9	22.26	à-Farnesene*(Sutherland and Hutchins 1972)	502-61-4	citrus, lavender, neroli, green	0.91±0.09 Bc	0.27±0.08 Cd	1.31±0.15 ABb	1.78±0.37 Aa	< 0.001
		Alkynes							
10	25.02	9-Octadecyne	35365-59-4	—	3.08±0.45 Bb	2.34±0.10 Bc	4.93±0.51 Aa	4.73±0.08 Aa	< 0.001
11	25.25	7-Octadecyne,2-methyl-	35354-38-2	—	—	—	—	0.08±0.07	—
		Alkanes							
12	11.99	Hendecane	1120-21-4	fusel-like, fruity, sweet	0.50±0.17 Aa	0.26±0.03 ABb	0.53±0.07 Aa	0.22±0.07 Bb	0.009
13	14.58	Dodecane	112-40-3	fusel-like	—	—	0.39±0.05	0.26±0.08	0.078
14	16.89	Dodecane,2,6,11-trimethyl-	31295-56-4	—	—	—	0.16±0.03	0.11±0.10	0.463
15	17.46	Tridecane*(Witte *et al*. 2007)	629-50-5	fruity, fusel-like	0.11±0.11 Bb	0.29±0.00 ABa	0.32±0.02 Aa	0.35±0.07 Aa	0.009
16	19.47	3-Methyltridecane	6418-41-3	—	—	—	0.04±0.08	—	—
17	20.17	Tetradecane*(Benítez *et al*. 2017)	629-59-4	mild, herbal, sweet, fusel-like	0.39±0.00 Cc	0.59±0.00 Bb	0.77±0.01 Aa	0.77±0.08 Aa	< 0.001
18	22.07	Pentadecane*(Murali-Baskaran *et al*. 2018)	629-62-9	mild green, fusel-like	2.90±0.10 Cc	3.44±0.07 Cc	7.29±0.44 Bb	8.65±0.49 Aa	< 0.001
19	23.53	Hexadecene	544-76-3	fusel-like, fruity, sweet	0.31±0.02 Bb	0.34±0.01 Bb	0.79±0.05 Aa	0.74±0.02 Aa	< 0.001
20	24.75	Heptadecane*(Murali-Baskaran *et al*. 2018)	629-78-7	fusel-like	5.96±0.48 Cc	5.03±0.26 Cd	9.96±0.41 Bb	11.64±0.40 Aa	< 0.001
21	25.79	Nonadecane*(McDonough *et al*. 1986)	629-92-5	fusel-like	—	—	—	0.20±0.01	—
22	26.75	Eicosane*(Mitra *et al*. 2017)	112-95-8	fruity, sweet	0.94±0.07 Bb	0.42±0.07 Cc	1.14±0.09 Bb	1.74±0.26 Aa	< 0.001
23	28.48	Heneicosane*(Mendki *et al*. 2000)	629-94-7	—	0.44±0.04 b	0.66±0.11 a	0.62±0.12 a	0.65±0.03 a	0.039
		Alkenes							
24	12.43	(E)-4,8-Dimethylnona-1,3,7-triene	19945-61-0	—	1.69±0.17 Bb	0.49±0.02 Bb	7.34±1.60 Aa	0.81±0.08 Bb	< 0.001
25	20.00	4-Tetradecene,(Z)-	41446-65-5	—	0.60±0.03 Cc	1.73±0.04 Bb	1.55±0.07 Bb	2.70±0.61 Aa	< 0.001
26	21.76	(E)-8-Heptadecene	2579-04-6	—	—	0.28±0.01 Bb	0.49±0.04 Aa	0.45±0.02 Aa	< 0.001
27	23.27	7-Hexadecene, (Z)-*(Silva *et al*. 2018)	35507-09-6	—	1.40±0.16 Cd	2.60±0.04 Bc	3.70±0.30 Ab	4.21±0.20 Aa	< 0.001
28	24.53	(Z)-8-heptadecene*(Bohman *et al*. 2020)	16369-12-3	—	42.03±1.88 Bb	43.56±0.43 Bb	41.50±0.36 Bb	50.70±1.25 Aa	< 0.001
29	26.54	(Z)-9-Nonadecene*(McDonough *et al*. 1986)	51865-02-2	—	0.31±0.03 Ab	0.15±0.03 Cd	0.36±0.01 Aa	0.22±0.02 Bc	< 0.001
30	26.67	1-Nonadecene	18435-45-5	—	0.40±0.05 Aa	0.17±0.03 Bb	0.14±0.02 Bb	0.04±0.06 Bc	< 0.001
31	27.55	5-Eicosene,(E)-	74685-30-6	—	—	—	—	0.07±0.06	—
32	28.37	10-Heneicosene *(Pennanech *et al*. 1995)	95008-11-0	—	0.86±0.02 Bb	1.48±0.22 Aa	0.98±0.21 Bb	0.75±0.15 Bb	0.004
		Esters							
33	9.71	*cis*-3-Hexenyl acetate*(James 2003)	3681-71-8	fruity, banana, apple	7.87±4.72 ABb	13.77±3.10 Aa	0.47±0.13 Bc	0.39±0.54 Bc	0.001
34	9.83	Hexyl acetate*(Millar *et al*. 1997)	142-92-7	fruity, spicy, herbal, sweet wine	—	2.22±2.20	—	—	—
35	9.92	2-Hexen-1-ol,acetate,(E)-	2497-18-9	—	0.25±0.03	—	—	—	—
36	12.18	2-Hexen-1-ol,propanoate,(E)-	53398-80-4	—	0.11±0.11	—	—	—	—
37	13.05	Butanoic acid,3-hexenyl ester, (Z)-	16491-36-4	fresh fruit, faint creamy	0.71±0.19 Aa	0.34±0.02 ABb	0.20±0.04 Bb	0.17±0.29 Bb	0.018
38	14.22	Butanoic acid,3-hexenyl ester,(E)-	53398-84-8	—	3.34±0.38 Aa	1.40±0.21 Bb	0.42±0.11 Cc	0.19±0.19 Cc	< 0.001
39	14.36	Butanoic acid,hexyl ester	2639-63-6	fruity, sweet	0.23±0.01 Bb	0.83±0.02 Aa	0.05±0.09 Cc	0.05±0.09 Cc	< 0.001
40	14.45	Butanoic acid,2-hexenyl ester,(E)-	53398-83-7	—	1.19±0.19 a	0.88±0.05 b	—	—	0.049
41	15.52	*cis*-3-Hexenyl 2-methylbutanoate	53398-85-9	green apple, black pepper-like	3.12±0.71 Aa	0.59±0.05 Bb	0.77±0.24 Bb	0.66±0.74 Bb	0.001
42	15.67	Butanoic acid,2-methyl-,hexyl ester	10032-15-2	—	0.89±0.21 Aa	0.47±0.03 ABb	—	0.07±0.13 Bc	0.001
		Alcohols							
43	4.22	2-Methyl-1-butanol	137-32-6	roasted, fruity, alcoholic, malt	0.23±0.02 ab	0.31±0.00 a	—	0.08±0.14 b	0.046
44	6.29	*cis*-3-Hexen-1-ol*(Ruther *et al*. 2002)	928-96-1	grassy, herbal, leaf	5.56±1.65 Aa	2.03±0.19 Bb	0.28±0.13 Bc	0.10±0.08 Bc	< 0.001
45	6.53	4-Penten-1-ol,3-methyl-	51174-44-8	—	7.24±1.59 a	4.32±0.61 b	—	—	0.041
46	6.81	2-Hexen-1-ol,(E)-	928-95-0	green fruit	0.16±0.16	0.09±0.09	—	—	0.545
47	25.14	(Z)6,(Z)9-Pentadecadien-1-ol	77899-11-7	—	—	—	—	0.09±0.08	—
48	25.39	*cis*-9-Tetradecen-1-ol	35153-15-2	—	—	—	0.04±0.06	0.10±0.09	0.366
		Ketones							
49	20.33	*cis*-Jasmon*(Birkett *et al*. 2000)	488-10-8	floral, jasmine-like	1.94±0.21 Cc	0.15±0.03 Dd	7.00±0.39 Aa	3.56±0.27 Bb	< 0.001
		Aldehydes							
50	5.19	Hexaldehyde	66-25-1	—	0.22±0.22	—	—	—	—
51	12.13	Nonylaldehyde	124-19-6	floral, waxy, melon, soapy	—	0.18±0.03	—	—	—
		N compounds							
52	2.62	2-Aminoethanol	141-43-5	ammoniacal	1.06±0.18	0.59±0.19	0.58±0.17	0.57±0.49	0.187

Note: The odor characteristic of each VOC was obtained from "Pherobase" (www.pherobase.com) and "Good Scents" company network database (www.thegoodscentscompany.com). Different low-case letters within the same VOC item represent significance among taxa with $p < 0.05$, and upper-case represent significance with $p<0.01$. * represents attractants (verified or potential) for insects reported in the literatures

addition, the terpenoids have strong antioxidant activity (Xu and Yu 2015). They protect plants against peroxidation and also play a key role in plant defense mechanisms (Loreto and Velikova 2001). (E)-4,8-dimethylnona-1,3,7-triene is formed by oxidative degradation of the sesquiterpene (E)-nerolidol (Ament *et al*. 2006). Interestingly, Hojiblanca emitted more *cis*-jasmon than other olive taxa (Table 1). Since *cis*-jasmon (related to plant defense) induces the production of volatile compounds, including the monoterpene ocimene (Birkett *et al*. 2000) and (E)-4,8-dimethylnona-1,3,7-triene (Ament *et al*. 2006), it is likely that the related metabolic pathway is up-regulated in Hojiblanca compared with other three olive taxa.

Among four olive taxa, the Chinese varieties were clustered together, while the two Mediterranean varieties were clustered together (Fig. 2b). These findings were likely. What is worth noticing is that the contents of *cis*-3-hexen-1-ol and 3-methyl-4-penten-1-ol were relatively high in Chinese taxa, while Mediterranean varieties emitted more *cis*-jasmon (Fig. 3c). The three key VOCs are all fatty acid derivatives, which arise from linoleic or linolenic (Dudareva *et al*. 2013) and share the same lipoxygenase (LOX) pathway; 13-hydroperoxy intermediate. Jasmonates (methyl jasmonate, jasmonic acid, jasmon, etc.) are formed through

allene oxide cyclase, allene oxide synthase, and β-oxidation pathway (Song *et al*. 2005; Dudareva *et al*. 2013), while in contrast, C6 and C9 aldehydes/alcohols (including *cis*-3-hexen-1-ol) are formed via another LOX branch (through the alcohol dehydrogenases pathway) (Gigot *et al*. 2010; Dudareva *et al*. 2013). Since the relative content of jasmon and ocimene involving plant defense of Hojiblanca inflorescence were the most prominent, further research questions could be asked. For example: (1) Might Hojiblanca flowers receive more visits from honey bees, which would cause the chain of reactions in plant defense? (2) If yes, what the mechanism would be?

To answer the questions above, we may first discuss the role of (Z)-8-heptadecene, which is an uncommon floral VOC and is usually identified as a minor VOC if present (Azuma *et al*. 1997; Peter *et al.* 2003). It was detected as the major VOC in *Litsea cubeba* (Asakawa *et al*. 2017), and in *Trimenia moorei* (Peter *et al*. 2003). Yet, the biological and ecological function remained unknown. Interestingly, Bohman *et al*. (2020) identified 8-heptadecene and pentadecane (which were majorly detected in Mediterranean olive flowers than in Chinese ones) as the electrophysiologically active compounds in both *Ophrys insectifera* and its *Argogorytes* pollinators. Moreover, long-chain hydrocarbons in other *Ophrys* species were identified as pollinator attractants (Schlüter *et al*. 2011; Sedeek *et al*. 2016). And these specific semiochemicals may be formed from the same carboxylic acid precursors, after elongation and decarbonylation, or possibly by decarbonylation without elongation (Bohman *et al*. 2020). Yet, the putative role of sex pheromone mimicry of (Z)-8-heptadecene should be tested further in the field. Actually, 20 compounds (74.6~87.3% of total VOCs) identified in olive flowers were identified or potential insect attractants, and most of them were terpenes, alkanes and alkenes, which again match the description of pollinators (mostly honey bees) attraction in fields (Canale and Loni 2010; Aronne *et al*. 2012; Giovanetti 2018; Carlos *et al*. 2019) and our own observation in olive groves. As most members of the *Oleaceae* are entomophilous, perfumes for attraction could be a relic character.

Although the odor characteristics of olive VOCs were provided, the question of "how olive flowers smell like" still could not be answered. The sense of plant aroma is pretty objective (Fan *et al*. 2018). So, an accurate approach to evaluate plant scent is a problem for researchers (Dudareva *et al*. 2004). The sensory evaluation and gas chromatography analysis could be combined to conduct the olive floral research (*e.g*. in *Malus* taxa (Fan *et al*. 2019) and some commercial cut flowers (Aros *et al*. 2020)). This study also has limitations in determining methods. First, the VOCs extraction was performed on the *in vitro* flowers, the VOCs proportion and content may be affected. Second, no chemical compound standard was used to perform accurate quantitative analysis. However, since all experimental conditions (*i.e*. sampling, equilibrium temperature, scent extraction and GC-MS procedures, etc.), and since GC injection orders of samples were random, the present study still can provide the peer researchers with data of some reference value.

Conclusion

For the first time, olive floral VOCs were explored in this study. Abundant 52 volatiles were identified by HS-SPME-GC-MS in four olive varieties. Among them, less than 40% compounds were found to process fragrant odor, which explained the light scent of the small white flowers. Moreover, 20 compounds of olive VOCs were reported by previous researchers as insect attractants. All the studied taxa were distinguished based on their floral VOCs via PCA, PLS-DA, and olive cultivars from the same origin were clustered together through HCA. As the products from different LOX pathway chains, green leave volatiles (*e.g*. 3-hexen-1-ol) with fresh/grassy odor were much more in Chinese olive flowers than in Mediterranean ones, while the two Mediterranean taxa contained more fractions of hydrocarbon and jasmone with floral/fruit/fusel-like scent. Our findings support that olive is not an anemophilous species, which makes a further step on bringing a wider vision on the origin, biodiversity and cultivar certification in *O. europaea*.

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Author Contributions

Conceptualization, JZ; methodology, ZZ and MZ; software, MZ and LZ; validation, ZW and JZ; formal analysis, ZZ; investigation, MZ and LZ; resources, JZ; data curation, ZW, MZ and LZ; writing—original draft preparation, ZZ; writing—review and editing, ZZ, ZW and JZ; visualization, ZZ; supervision, JZ and ZW; project administration, JZ; funding acquisition, ZW and ZZ.

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