Full Length Article

Changes in Volatiles of Olive Tree *Olea europaea* According to Season and Foliar Fertilization

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Abstract

In the present study, four foliar fertilizers (FF1, FF2, FF3 and FF4) were separately sprayed on Chemlali olive trees at different moments of the vegetative cycle. FF1 (rich in nitrogen) was applied during the vegetation stage (Last January-February) at a dose of 5 L/ha per spray (three sprays per season). FF2 (rich in boron, magnesium and sulphur), FF3 (rich in phosphorus and potassium) and FF4 (rich in phosphorus and calcium) were applied respectively during the stages of flowering (Last March-April), fruit growth (July-August) and ripening (October-November), at a dose of 3 L/ha per spray (three sprays per season for each fertilizer).The volatile fraction was analysed by GC-MS, 46 volatile compounds were identified and their amount were expressed as relative abundance (%). In a general view, the most abundant volatiles in Chemlali olive leaves across the vegetative cycle were (*E*)-2-hexenal, nonanal, (*E*)-β-damascenone, 3-ethenyl pyridine and β-caryophyllene. The levels of these main compounds and the general composition of the volatile fraction varied significantly through season.The volatile levels were mainly affected by the two foliar fertilizers enriched with nitrogen and Boron respectively. The most affected volatiles were (*E*)-2-hexenal, nonanal, 3-ethenyl pyridine, (*E,E*)-α-farnesene, and (*E*)-nerolidol. Less impact was noticed after the use of the other foliar fertilizers.Our study is the first investigation bringing data about the variation of leaf volatile profile of Chemlali cultivar across a vegetative cycle and showing the impact of nutrient foliar sprays on olive leaf volatiles. © 2017 Friends Science Publishers

Keywords: Olive leaves; Foliar sprays; Nutrients; Vegetative cycle; Volatile compounds

Introduction

Olive cultivation is the most important agriculture activity in Tunisia. About 80 million trees, dominated by Chemlali cultivar, are counted in this country and planted in an area of 1.803.300 hectares (International Olive Council, 2012; 2016). Olive cultivation and olive oil industry lead to many solid by-products such as leaves and branches. Olive leaves represent about 10% of the total weight dedicated to olive oil extraction and 25% of the total weight of by-products after olive tree pruning (Talhaoui *et al.*, 2015). Nowadays, this by-product is thrown away which represents a potential environment damage.

Endowed with interesting biological activities, many studies focused on valorising olive leaves in food industry as functional food or as source of nutraceuticals (Herrero *et al.*, 2011; Alba *et al.*, 2015). In fact, volatiles were considered as a main compound among the olive leaf fractions (Rodrıguez-Perez *et al.*, 2017). Recent studies reported the interesting antioxidant and microbiological activities of olive leaf volatiles (Brahmi *et al.*, 2012; 2015).

Olive leaf volatiles consists of different metabolites produced via several pathways, mainly the lipoxygenase pathway which transforms the linoleic and linolenic fatty acids into C_6 aldehydes, alcohols and their esters (Angerosa, 2002; Scala *et al.*, 2013). Indeed, the plant volatiles were considered as a plant language which reflected the plant physiological status in response to the surrounding environment (Blande *et al.*, 2014). Many studies were conducted to assess the impact of biotic and abiotic factors on olive leaf volatiles. In a previous study, Flamini *et al.* (2003) showed that aldehyde and terpene compositions changed significantly according to season in the leaves of an Italian olive cultivar. In addition to season impact, Campeol *et al.* (2001; 2003) described the varietal effect on olive leaf volatiles for three Italian cultivars: Leccino, Frantoio and Cipressino, and proposed the analysis of leaf volatiles as a tool to discriminate between olive varieties. on the other hand, Saidana *et al.* (2015) focused on the impact of the edaphoclimatic conditions on Chemlali cultivar in Tunisia and reported difference between samples regarding leaf volatiles. Besides, many studies reported the impact of biotic factors, such as fly attack, on olive leaf volatiles (Malheiro *et al.*, 2015; 2016). Nevertheless, the impact of agronomic practices, such as foliar fertilization, on olive leaf volatiles was not deeply investigated.

The mineral nutrition can occur through the external leaf surface either through the cuticle (solutes) or through stomata (gases and solutes) (Eichert and Fernández, 2012). This led to the development of foliar fertilization as a compliment for soil fertilization for crop plants (Mengel, 2002). Indeed, the foliar fertilization was employed in several fruit trees and showed improvement of pomegranate quality (Khorsandi *et al.*, 2009), enhancement of apple yield production (Balan and Vamasescu, 2015), and improved the tolerance of *Citrus macrophylla* (L.) to drought conditions (Gimeno *et al.*, 2014). As for olive cultivation in Tunisia, a recent study estimated that 30% of farmers applied foliar fertilizers (Larbi *et al.*, 2016). Apart from their impact on olive oil yield and quality, many studies assessed the impact of foliar fertilization on several physiologic parameters in olive leaves, such as foliar nutrient status, leaf pigment concentration (Chatzistathis *et al*., 2017) and phenolics (Ben Abdeljelil *et al*., 2017). However, the impact of fertilization on the olive leaf volatiles was not described in the literature and to the best of our knowledge no study has focused on the impact of foliar fertilization on Chemlali olive leaves.

Hence, to contribute to a better understanding of the olive leaf volatiles, our study aims to assess the impact of season and foliar nutrition on the leaf volatiles of the Tunisian olive cultivar Chemlali.

Materials and Methods

Field Study and Sampling

The experimental field study was conducted in 2013 in an orchard situated in the Region of Monastir (on the Mid-eastern Coast of Tunisia, 35°40'N, 10°40'E). The chosen geographical site ensured that the experimental field study was far from industrial and urban emissions and discharges. All the olive trees in the field belonged to Chemlali variety and were 25 years old. There was no implanted irrigation system in the field. The physicochemical characteristics of the soil at this site were as follows: sand: 690 g kg^{-1}; clay: 140 g kg^{-1}; silt: 170 g kg^{-1}; pH: 8; Electrical conductivity: 0.82 mΩcm^{-1}; organic C: 8.7 g kg^{-1}; N: 7.3 g kg^{-1}; Olsen P: 5 mg kg^{-1}. The monthly variations in temperature and rainfall during the study period are shown in Fig. 1.

Foliar fertilizers were employed at different stages of the vegetative cycle of olive trees. The compositions of the different fertilizer solutions and their mineral concentrations are detailed in Table 1. The spray was always conducted early in the morning. The experimental trees were arranged in a randomized block design with three blocks and four treatments

F1: consisted of olive trees exposed to a foliar fertilizer rich in nitrogen (FF1) and sprayed 3 times, at 10 days intervals, during the vegetation stage (last January-February 2013). FF1 was sprayed at 5 L/ha. A sample of olive leaves (S1) was conducted two weeks after the last fertilizer spraying, in March 2013.

F2: consisted of trees exposed to a foliar fertilizer rich in boron, magnesium, sulphur and manganese (FF2) and sprayed 3 times, at 10 days intervals, during the flowering stage (last March-April). FF2 was sprayed at 3 L/ha. A sample of olive leaves (S2) was conducted two weeks after the last fertilizer spraying, in April 2013.

F3: consisted of trees exposed to a foliar fertilizer rich in phosphor and potassium (FF3) and sprayed 3 times, at 10 days intervals, during the stage of fruit growth (July-August). FF3 was sprayed at 3L/ha. A sample of olive leaves (S3) was conducted two weeks after the last fertilizer spraying, in August 2013.

F4: consisted of trees exposed to a foliar fertilizer rich in phosphor and calcium (FF4) and sprayed 3 times, at 10 days intervals, during the ripening stage of olive fruits (October-November). FF4 was sprayed at 3 L/ha. A sample of olive leaves (S4) was conducted two weeks after the last fertilizer spraying, in November 2013.

C: Control trees: No foliar fertilizer was sprayed in this block of trees. Leaf samples were taken from this block two weeks after the fertilizer spraying: (C1), (C2), (C3) and (C4) samples were collected in the same sampling campaign of (S1), (S2), (S3) and (S4), respectively.

In every sampling, homogenous and not wounded leaves were carefully collected early in the morning from all sides of olive trees. The samples were immediately transferred to the laboratory and roughly rinsed with ultrapure water and air dried for one hour.

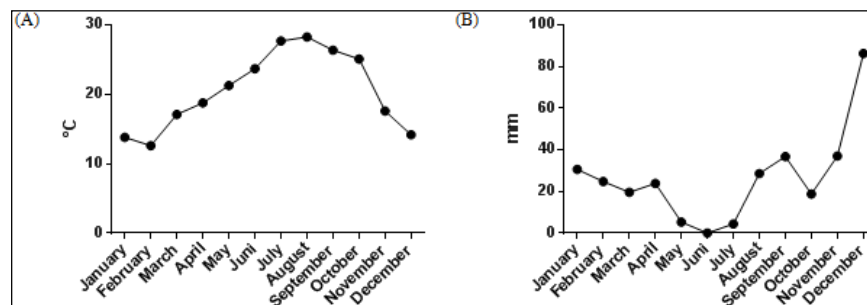### Volatile Compound Extraction and Identification

A sample of 100 g of fresh olive leaves (S1, C1, S2, C2, S3, C3, S4 and C4) was transferred in a round-bottom flask containing 1 L of ultrapure water. The extraction of volatile compounds was carried out by hydrodistillation in a Clevenger-type apparatus during 4 h (Clevenger, 1928). Volatile compounds were trapped in 2 mL of hexane and conserved at -20°C in amber glass vials hermetically closed until analysis.

The GC analyses were accomplished using a HP-5890 Series II instrument with dual FID detector and equipped with DB-WAX and DB-5 capillary columns (30 m x 0.25 mm, 0.25 μm film thickness), working with the following temperature program: 60°C to 240°C at 3°C/min. Injector and detector temperatures were set at 220°C. The carrier gas was helium (2 mL/min) with a split ratio of 30:1. The identification of the components was performed, for the both columns, by comparison of their retention times with those of pure authentic samples and by mean of their linear retention indices (lri) relative to the series of *n*-hydrocarbons.

GC-MS analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm; coating thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. The injector and transfer line temperatures were set at 220 and 240°C

Table 1: Fertilizer solution compositions and their mineral concentrations (g/l)

Foliar fertilizers	N	P_2O_5	K_2O	MgO	SO_3	CaO	B	Cu	Fe	Mn	Mo	Zn
FF1	355						0.215	0.085	0.500	0.530	0.02	0.410
FF2				50	111		27			10		
FF3		240	318				8					
FF4		60				186						11

Fig. 1: Monthly variations in temperature (A) and rainfall (B) during the study period

respectively. The oven temperature was programmed from 60°C to 240°C at 3°C/min. Helium was used at 1 mL/min with a split ratio of 30:1. Identification of the constituents was based on comparison of their retention times with those of authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons, and on computer matching against commercial (NIST, 2014; Adams, 2007) and home-made library mass spectra built up from pure substances and components of known oils and MS literature data (Adams, 2007). The levels of the identified volatiles were expressed relative abundance (%).

Statistical Analysis

Values are expressed as mean ± standard deviation (SD) of three measurements. Analysis of variance (ANOVA) and post hoc tukey test were performed in order to examine mean differences between controls across the vegetative cycle. Test student was performed to examine mean differences between the treatment and the corresponding control. Significant differences were considered at $P<0.05$. High significant differences were considered at $P<0.01$. Statistical tests were performed using SPSS Release 11.0 for Windows.

Results

Effect of Season on Volatile Compounds in Olive Leaves

The volatile fraction of fresh olive leaves was analysed by GC-MS, and the constituent levels are presented in Table 2. Throughout seasons, the main volatiles of the fresh leaves of Chemlali were: (*E*)-2-hexenal, ranging from 23.33 to 7.17%; nonanal, ranging from 12.6 to 6.4%; (*E*)-β-damascenone, ranging from 11.90 to 7.37%; 3-ethenyl pyridine, ranging from 14 to 1.3%; and β-caryophyllene

ranging from 9.33 to 4.40%. Nevertheless, other constituents reached considerable levels at some moments of the vegetative cycle. In particular, the apocarotene (*E*)-β-damascone and the aldehyde (*E*)-2-decenal were among the main compounds in C1 and C2, but their levels decreased later. The oxygenated sesquiterpene (*E*)-nerolidol was among the major compounds in C3 and in the same sample the aromatic amine 3-ethenyl pyridine reached its lowest level. Methyl salicylate touched its highest level in C4, joining here the group of the main volatiles in this fraction.

It is also worth noticing that in the present study the sesquiterpene hydrocarbons sharply increased in August (10.2%). The oxygenated monoterpenes were detected in controls starting from August and the oxygenated sesquiterpenes decreased sharply in November, reaching 5.83% of the total volatile fraction. The apocarotenes remained in the range of 21.73-21.77% in March, April and August and then they slightly decreased in November. On another hand, the identified volatiles in leaves increased from 19 compounds in March (C1) to 22 in April (C2) then reached 34 and 35 in August (C3) and November (C4), respectively.

Effect of Foliar Fertilization on Volatile Compounds in Olive Leaves

The identified leaf volatiles and their levels are reported in Table 2. In the volatile fraction of the fresh leaves S1, sampled from the first fertilization treatment block F1, 21 constituents were identified and represented 96.23% of the total volatile fraction.

The sesquiterpenes (*E,E*)-α-farnesene and humulane-1,6-dien-3-ol appeared in S1, while they were absent in C1. The aromatic amine 3-ethenyl pyridine level in S1 was significantly higher than in C1 ($P<0.01$). On the other hand, the second major constituent in C1 volatile fraction,

Table 2: Changes in the levels of volatile compounds in olive leaves according tofoliar fertilization and season

Sampling period		March 2013		April 2013		August 2013		November 2013	
Volatile compound	LRI	C1	S1	C2	S2	C3	S3	C4	S4
(Z)-2-hexenal	842	1.47 ± 0.32	1.07 ±0.21	1.33 ± 0.29	2.03 ± 0.11 ^{x}	1.57 ± 0.65	2.67 ± 0.99	0.80 ± 0.20	0.77 ± 0.06
(E)-2-hexenal	856	23.33 ± 2.055 (c)** (a,b)*	25.90 ± 10.97	16.27 ± 3.29 (a,e)*	28.03± 2.59 ^{xx}	14.87 ± 3.09 (b,f)*	22.20 ± 2.15 ^{x}	7.17 ± 1.44 (c)** (e,f)*	11.27 ± 1.33 ^{x}
n-nonane	900	-	-	-	-	1.03 ± 0.06	1.30 ± 0.10 ^{x}	-	-
Heptanal	901	-	-	-	-	-	-	0.97 ± 0.23	0.73 ± 0.32
Benzaldehyde	962	1.27 ± 0.23 (a,c)**	1.83 ± 0.21 ^{x}	2.50 ± 0.52 (a,d)**	1.80 ± 0.20	0.90 ± 0.20 (d,f)**	1.40 ± 0.35	2.63 ± 0.32 (c,f)**	2.87 ± 0.42
3-ethenyl pyridine	968	4.90 ± 1.55 (a)** (c)*	12 ± 0.95 ^{xx}	14 ± 1.73 (a,d)** (e)*	5.43 ± 0.45 ^{xx}	1.30 ± 0.26 (d,f)**	1.13 ± 0.91	9.73 ± 1.46 (f)** (c,e)*	10.23 ± 1.05
(E,Z)-2,4-heptadienal	1001	-	-	-	-	2.90 ± 0.46	2.00 ± 0.95	2.53 ± 0.93	1.50 ± 0.26
Octanal	1002	-	-	-	-	0.63 ± 0.15	-	0.83 ± 0.11	0.63 ± 0.23
(E,E)-2,4-heptadienal	1012	-	-	-	-	0.57 ± 0.11	-	0.97 ± 0.31	-
Phenylacetaldehyde	1045	-	-	1.03 ± 0.11	-	-	-	2.63 ± 1.11	2.30 ± 0.40
1-octanol	1071	2.47 ± 0.11	1.60 ± 0.46	3.13±0.32 (d)*	2.40 ± 0.36	2.13 ± 0.29 (d)*	0.90 ± 0.53 ^{x}	2.80 ± 0.36	2.27 ± 0.21
Linalool	1101	-	-	-	-	1.60 ± 0.70	0.97 ± 0.11	1.40 ± 0.40	0.87 ± 0.31
Nonanal	1104	12.33±1.33 (b)**	6.20 ± 0.75 ^{xx}	11.23±0.45 (d)**	9.77 ± 1.93	6.40±1.23 (b,d,f)**	6.97 ± 1.10	12.67 ± 0.32 (f)**	13.17 ± 3.07
α-terpineol	1191	-	-	-	-	0.50 ± 0.17	-	-	-
methyl salicylate	1192	-	-	-	1.13 ± 0.15	1.80± 0.36 (f**)	1.37 ± 0.40	4.60± 0.46 (f**)	2 ± 0.79 ^{x}
Decanal	1206	1.93 ± 0.29	1.33 ± 0.40	1.40 ± 0.53	1.57 ± 0.40	1.47 ± 0.15	0.97 ± 0.32	1.87 ± 0.40	1.33 ± 0.21
β-cyclocitral	1222	-	-	-	-	0.77 ± 0.06	-	0.70	-
(E)-2-decenal	1263	4.13±0.23 (b,c)**	2.70 ± 0.26 ^{xx}	4.77±0.31 (d,e)**	3.90 ± 0.20 ^{x}	2.13±0.38 (b,d)**	1.67 ± 0.15	2.93 ± 0.29 (c,e)**	2.90 ± 0.40
theaspirane I	1298	2.50±0.50 (b,c)**	1.17 ± 0.31 ^{x}	1.70 ± 0.20	1.43 ± 0.59	0.83 ± 0.11 (b)**	1.20 ± 0.17 ^{x}	1.07 ± 0.40 (c)**	1.13 ± 0.25
4-vinylguaiacol	1313	-	-	-	-	2.07 ± 0.65	-	-	-
theaspirane II	1315	2.47 ± 0.40	1.97 ± 0.15	2.53 ± 0.46	2.07 ± 0.31	2.30	1.43 ± 0.55	2.90 ± 0.10	3.70 ± 0.46
(E,E)-2,4-decadienal	1316	-	-	-	-	-	-	0.80	-
Eugenol	1358	-	-	-	-	-	-	1.10 ± 0.17	1.13 ± 0.40
(E)-2-undecenal	1364	-	-	-	-	-	-	-	1.30 ± 0.46
methyl 4-formylbenzoate	1365	-	-	-	-	1.50 ± 0.26	-	1.00 ± 0.17	-
(E)-β-damascenone	1382	10.07±1.16 (c)** (a)*	7.47 ± 0.51 ^{x}	7.77±0.21 (d)** (a)*	9.13 ± 5.17	11.90±0.44 (d,f)**	8.70 ± 0.53 ^{xx}	7.37 ± 0.71 (c,f)**	7.70 ± 1.50
dihydro-γ-ionone	1396	2.27 ± 0.25	0.97±0.06 ^{xx}	2.77 ± 0.32	1.67 ± 0.42 ^{x}	2.60 ± 0.62	2.70 ± 0.72	2.07 ± 0.55	1.90 ± 1.13
n-tetradecane	1400	-	-	-	-	1.10 ± 0.36	1.23 ± 0.42	-	-
(E)-β-damascone	1412	4.67±0.91 (b,c)**	3.37 ± 1.05	5.43±0.38 (d,e)**	4.57 ± 0.64	1.77±0.60 (b,d)**	2.60 ± 0.85	2.50 ± 0.17 (c,e)**	2.33 ± 0.25
β-caryophyllene	1419	5.07±0.87 (b)**	5.03 ± 0.25	4.77±0.64 (d)**	5.47 ± 0.72	9.33±1.45 (b,d,f)**	6.97 ± 2.04	4.40 ± 0.72 (f)**	5.40 ± 0.79 ^{x}
(Z)-geranylacetone(syn. nerylacetone)	1436	-	-	-	-	1 ± 0.14	-	0.87 ± 0.15	-
(E)-geranylacetone	1455	-	-	-	-	1.10 ± 0.44	1.50 ± 0.36	1.00 ± 0.30	-
(E)-β-ionone	1487	-	-	1.05 ± 0.07	-	0.97 ± 0.25	1.30 ± 0.10	-	-
n-pentadecane	1500	-	-	-	-	0.87 ± 0.23	-	0.97 ± 0.25	-
(E,E)-α-farnesene	1508	-	7.13 ± 6.96	-	-	0.87 ± 0.11	1.93 ± 1.02	1.43 ± 0.67	1.73 ± 0.67
ethyl 4-ethoxybenzoate	1530	-	-	-	-	-	-	-	1.33 ± 0.40
Liguloxide	1532	2.27±0.25(b,c)*	2.07 ± 0.51	1.33 ± 0.32	1.70 ± 0.56	1.20 ± 0.52 (b)*	3.03 ± 1.05	1.10 ± 0.36 (c)*	1.63 ± 0.40
5-methylpentadecane	1550	-	-	-	-	-	-	0.97 ± 0.06	1.17 ± 0.23
epi-ligulyl oxide	1551	-	-	-	-	0.83 ± 0.35	0.90 ± 0.44	0.97 ± 0.06	1.33 ± 0.32
(E)-nerolidol	1564	3.67 ± 0.35	4.00 ± 0.85	2.83 ± 0.15	5.23 ± 0.25 ^{xx}	5.33 ± 2.44 (f)*	6.17 ± 0.67	1.27 ± 0.46 (f)*	2.57 ± 0.76
(Z)-3-hexenyl benzoate	1570	-	-	-	-	-	-	-	-
caryophyllene oxide	1582	2.73 ± 0.55	2.13 ± 0.06	1.90 ± 0.17	2.77 ± 1.00	3.67 ± 1.91	1.93 ± 0.29	2.50 ± 1.23	5.67 ± 0.15
n-hexadecane	1600	3.07±1.10 (c)*	1.43 ± 0.49	2.03 ± 0.35	2.20 ± 1.11	1.80 ± 0.26	3.20 ± 1.21	1.43 ± 0.31 (c)*	1.43 ± 0.25
humulene epoxide II	1607	-	-	-	-	-	-	-	1.27 ± 0.55
humulane-1,6-dien-3-ol	1615	-	1.80 ± 0.36	1.25 ± 0.49	-	-	-	-	-
selin-11-en-4-α-ol	1655	2.27 ± 0.81	2.60 ± 0.61	3.23 ± 0.21	2.20 ± 0.53	-	-	-	-
Oxygenated monoterpenes		-	0.23 ± 0.40	-	-	2.10 ± 0.87	0.97 ± 0.11	1.80 ± 1.06	0.87 ± 0.32
Sesquiterpene hydrocarbons		5.07 ± 0.87	12.17 ± 7.16	4.77 ± 0.64	5.47 ± 0.72	10.20 ± 1.39	8.900 ± 1.77	5.83 ± 1.27	7.13 ± 1.35
Oxygenated sesquiterpenes		12.10 ± 3.25	13.20 ± 2.61	10.47 ± 1.10	12.30 ± 3.12	11.03 ± 5.05	12.70 ± 2.46	5.83 ± 1.86	12.47 ± 0.29
Apocarotenes		21.97 ± 0.93	15.33 ± 2.48	21.73 ± 2.66	18.87 ± 4.72	21.77 ± 1.66	19.87 ± 2.06	18.80 ± 1.55	16.77 ± 2.10
Phenylpropanoids		-	-	-	-	-	-	1.100 ± 0.17	1.133 ± 0.40
Non-terpene derivatives		54.90 ± 1.15	55.30 ± 9.88	56.80 ± 1.91	57.83 ± 2.76	47.13 ± 5.95	49.50 ± 7.02	58.93 ± 4.74	57.73 ± 4.23
Total identified		97.07 ± 1.66	96.23 ± 2.89	93.77 ± 0.67	94.47 ± 0.51	92.23 ± 1.71	91.93 ± 1.02	92.30 ± 1.75	96.10 ± 2.34

C1, C2, C3 and C4: Control olive leaf samples; S1, S2, S3 and S4: leaf samples from olive trees fertilized with F1, F2, F3 and F4 foliar sprays, respectively. Results are expressed as means ± standard errors (n=3). LRI: Linear retention indice (DB-5 capillary column)
a, b, c, d: Control Values in the same row with the same letters showed statistically significant differences according to one way ANOVA analysis. The symbol "*" referred to (P<0.05) and the symbol "**" referred to (P<0.01)
x, xx. : Sample Value showing statically significant difference with the control, according to test student. The symbol "x" referred to (P<0,05) and the symbol "xx" referred to (P<0.01)

nonanal, decreased significantly in S1 (P<0.01) compared to C1. Moreover, another aldehyde, (E)-2-decenal decreased significantly from 4.13% in C1 volatile fraction to 2.70% in S1 (P<0.01). Also, the apocarotene (E)-β-damascenone decreased significantly to 7.47% (P<0.05). Similarly, dihydro-γ-ionone dropped from 2.27% in C1 to 0.97% in S1 (P<0.01). A minor constituent, benzaldehyde, rose significantly from 1.27% in C1 to 1.83% in S1 (P<0.05). In an overall view, total sesquiterpene hydrocarbons increased from 5.07% in C1 to 12.17% in S1 and apocarotenes decreased from 21.97% in C1 to 15.33% in S1.

The volatile fraction of S2, corresponding to the block

of olive trees (F2) sprayed with a foliar fertilizer (FF2) enriched with boron, magnesium, manganese and sulfur, included 20 constituents corresponding to 94.47% of the total volatile fraction. The main volatile group in S2 was partially different compared to C2. The aldehyde (*E*)-2-hexenal increased drastically in S2 compared to C2 (P<0.01).

In the volatile fraction of S3, corresponding to the block of olive trees (F3) sprayed with the foliar fertilizer (FF3) enriched with phosphorous and potassium, 26 volatile constituents were identified, accounting for 91.93% of the total volatiles. The most abundant volatile compounds were the same compared to C3. The level of (*E*)-2-hexenal increased significantly in this volatile fraction compared to C3 (P<0.05), whilst the percentage of (*E*)-β-damascenone decreased significantly compared to this control sample (P<0.01). We noticed also the absence of several minor compounds in S3 detected in C3: methyl 4-formylbenzoate, *n*-pentadecane, 4-vinylguaiacol, (*E,E*)-2,4-heptadienal, octanal, β-cyclocitral, α-terpineol and (*Z*)-geranylacetone. Besides, theaspirane II was detected in S3 volatile fraction, while it was absent in C3.

In the volatile fraction of S4, corresponding to the block of olive trees (F4) sprayed with a foliar fertilizer (FF4) enriched with phosphorous and calcium, 32 constituents were identified, representing 96.10% of the total volatiles. The major components of this volatile fraction were slightly different compared to C4: methyl salicylate decreased significantly to 2% and was ranked out of this pool in S4. We noticed also an increase of the levels of (*E*)-2-hexenal (11.27%) and β-caryophyllene (5.40%) in S4 compared to C4 (P<0.05). Many minor constituents were absent in S4, while they were identified in C4: methyl 4-formylbenzoate, *n*-pentadecane, (*E,E*)-2,4-heptadienal, (*E,E*)-2,4-decadienal, (*E*)-geranylacetone, (*Z*)-geranylacetone. Besides, some other volatile compounds were present in S4 and absent in C4: ethyl 4-ethoxybenzoate, (*E*)-2-undecenal and humulene epoxide II.

Discussion

The volatile compounds in olive leaves are produced by many biochemical pathways: the polysaccharide metabolism, the fatty acid metabolism, the conversion of the amino acids and mainly the lipoxygenase pathway (Angerosa, 2002; Scala *et al.*, 2013). Consequently, the above described qualitative and semi-quantitative variations of leaf volatiles reflected a season-dependant variability of these pathways involved in volatile compound biosynthesis.

The most abundant volatile constituent identified in the present study was (*E*)-2-hexenal. This C_6 aldehydes is a leaf volatile synthesised after the oxidation of the linolenic acid by the enzyme 13-lipoxygenase, followed by the activity of the hydroperoxide lyase associated with an isomerase activity (Hassan *et al.*, 2015). We noticed that the highest levels of (*E*)-2-hexenal were detected during the less rainy

months, March and April (Fig. 1). This means that the biosynthesis of (*E*)-2-hexenal in our field study was probably stimulated by water scarcity which represented an abiotic stress. Similarly, Sofo *et al.* (2004) reported increased lipoxygenase activity in olive leaves during drought conditions. Many other studies associated the biosynthesis of the leaf volatiles with biotic and abiotic stress, such as the induction of (*Z*)-3-hexenol in rice as a strategy against insect herbivores (Obara *et al.*, 2002) and the induction of C_6 aldehydes in *Citrus* leaves after exposition to Jasmonic and Salicylic acids (Asai *et al.*, 2016).

It is also worth noticing that alcohols were sharply less abundant than aldehydes in the different volatile fractions through the vegetative cycle. Campeol *et al.* (2003) reported that aldehydes in fresh olive leave from three Italian cultivars (Leccino, Frantoio and Cipressino), were the most abundant volatiles regardless the sample time. Moreover, they reported that (*E*)-2-hexenal, nonanal, 3-ethenyl pyridine, (*E*)-2-decenal, (*E*)-β-damascenone and (*E*)-β-damascone were the main constituents of the volatile fraction of the leaves belonging to Cipressino cultivar and sampled in July. This volatile profile is quite similar to the leaf volatile fraction of Chemlali sampled in April C2. Nevertheless, the levels of (*E*)-2-hexenal in the Italian study increased from July to November, which was not in concordance with our results. On the other hand, Brahmi *et al.* (2012) reported that the total alcohols surpassed the total aldehydes in fresh olive leaves belonging to the Tunisian cultivars Chemlali, Chemchali and Neb Jmel. Regarding Chemlali leaves, these authors observed that (*E*)-3-hexenol represented 16% of the volatile fraction, nonanal reached 6.4% and (*E*)-2-hexenal was not detected. In the same sense, Brahmi *et al.* (2015) spotted that alcohols represented 39.5% of the volatile fraction extracted from dried leaves belonging to the Tunisian olive cultivar Chetoui. This study reported that aldehydes represented 19.1% and (E)-2-hexenal accounted only for 1.1% of the volatiles. The differences between our results and those of the above mentioned studies conducted on the Tunisian cultivars are probably due to the employment of different extraction methods. In fact, the hydro-distillate in our study was trapped in hexane then conserved in -20°C. In the two previous studies of Brahmi *et al.*(2012; 2015), the hydro-distillate was trapped in diethyl ether, dried over anhydrous sodium sulphate, evaporated and concentrated under a gentle stream of nitrogen and stored at 4°C until analysis.

The aromatic amine 3-ethenyl pyridine was among the major abundant volatiles in our samples and it varied significantly through the vegetative cycle. The presence of important amounts of this compound has been previously reported in the volatile fractions of olive leaves belonging to many cultivars. However, its occurrence was not deeply discussed. 3-ethenyl pyridine is a product of the Maillard-type reaction. Briefly, the Maillard reaction consists in the sequence of reactions occurring at high temperatures and that start with carbonyl-amine condensation reaction between an

amine and a reducing sugar to form finally aroma compounds and other kinds of macromolecules (Parker, 2015). In our case, this reaction may have occurred because of the thermal disintegration of amino acids and sugar during hydrodistillation, which led to the formation of 3-ethenyl pyridine. Consequently, the level of 3-ethenyl pyridine may reflect the levels of peptide and sugar in olive leaves. Many field studies conducted on different olive cultivars reported that sugar amounts in leaves dropped during summer (Oddo *et al.*, 2002; Proietti and Famiani, 2002). Hence we can consider that the sharp decrease of 3-ethenyl pyridine in August was due to an eventual decrease in carbohydrate level in Chemlali leaves. Indeed, further investigations are required to assess the links between the level of peptides, sugar and the occurrence of aromatic amine in leaf volatiles.

Our findings suggest that, in general, the leaf volatile profile of Chemlali cultivar is dominated by (*E*)-2-hexenal, nonanal, (*E*)-β-damascenone, 3-ethenyl pyridine and β-caryophyllene through the season, at the considered moments. Nevertheless the season impacts the levels of these compounds and the richness of the volatile profile with minor components.

It is almost possible to claim that no previous study assessed the effect of foliar fertilization on the olive leaf volatiles. Indeed, few data describing the effect of fertilizers on olive leaves are shown in literature. According to a recent study (Toker and Yavuz, 2015), it was shown that boron supply increased the amount of (*E*)-2-hexenal in olive oil. The same finding was noticed for the sesquiterpene (*E*)-nerolidol (P<0.01) in S2 sample (March) compared to C2. On the contrary, the aromatic amine 3-ethenyl pyridine considerably decreased in S2 compared to C2 (P<0.01). In fact, boron supply was shown to decrease the carbohydrate levels in olive leaves and olive oil (Saadati *et al.*, 2013; Liakopoulos *et al.*, 2005). Hence a possible inhibition of the Maillard reaction, due to the decrease of sugar amounts in leaves may explain the decrease of 3-ethenyl pyridine in S2. On the other hand, the sesquiterpene humulane-1,6-dien-3-ol, the aldehyde phenylacetaldehyde and the apocarotene (*E*)-β-ionone were not detected in S2, while they were minor constituents of C2. Otherwise, methyl salicylate appeared in S2 as a minor constituent while it was absent in C2.

Our results provided clear evidences that foliar fertilizers affected the volatile fraction of olive leaves. These modifications in olive leaf volatiles may affect the biological activities of olive leaf extracts and the interaction with the surrounding environment. Moreover the employment of volatile fraction analysis to distinguish between varieties or between geographic locations may be affected by the use of fertilizers. Further investigations are required to assess these impacts.

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