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# Deciphering How Olive Volatiles and Fatty Acids Shape *Bactrocera oleae* (Rosii) Oviposition Preference Using Multivariate Regression Models

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## ABSTRACT

The olive fly (*Bactrocera oleae* (Rossi)), the major pest of olives, primarily recognises visual, olfactory, acoustic, gustatory and tactile signals that regulate its behavioural activity, courtship and reproductive success, as well as the search for ideal conditions for its offspring. Recent studies highlight the importance of chemical compounds present in olive fruits, particularly volatile organic compounds (VOCs), in these processes. This paper aims to further explore the relationships between *B. oleae* and specific chemical traits in fresh sound fruits that might trigger and enhance infestations, namely VOCs, their precursors (fatty acids) and lipidic antioxidants (tocopherols). The study has been performed within the framework of a table olive cross-breeding program in order to develop predictive models to identify genotypes less susceptible to olive fly infestation. Significant differences in chemical traits were observed among the genotypes studied, highlighting their role in oviposition preference of the olive fly. 'Hojiblanca' and 'Kalamon', known for their lower susceptibility, stood out for their high concentrations of D-limonene among the 33 identified VOCs and low saturated fatty acid content. Specific VOCs like α-pinene, copaene, nonanal and o-xylene, along with some minor fatty acids, were key predictors for developing multivariate models that estimate susceptibility to olive fly oviposition.

## 1 | Introduction

All plant organs release volatile organic compounds (VOCs), low molecular weight molecules with high vapour pressures that can easily evaporate at ambient temperatures (Dudareva et al. 2013). VOCs play an important role in the plant kingdom, facilitating the interaction between plants and their surrounding environment, triggering communication, reproduction or defence mechanisms. Diverse biosynthetic pathways produce these VOCs which include a wide diversity of chemical classes, such as aldehydes, esters, alcohols, ketones, terpenes and carboxylic acids (Possell and Loreto 2013). Some factors such as biotic and abiotic stresses, environmental conditions of the year, genetics and age of the plant can modify the diversity and amount of VOCs released (Ninkovic et al. 2021; Possell and Loreto 2013).

*Bactrocera oleae* (Rossi) is the main pest of olive trees, threatening global olive production, with losses estimated at up to 15%

worldwide (Malheiro et al. 2015a). Oviposition in the fruit and the development of larvae inside negatively affects the quality of table olives and olive oil, leading to their rejection by the industry (Daane and Johnson 2010). The behaviour of the olive fly responds to visual, olfactory, acoustic, gustatory and tactile signals (Giunti et al. 2020; Gonçalves et al. 2012; Rebora et al. 2020; Terzidou et al. 2022). The size, firmness and colour of the olive fruit are considered the most decisive physical characteristics in the preference of the olive fly for oviposition (Gonçalves et al. 2012; González-Fernández et al. 2023; Quesada-Moraga et al. 2018). However, although *Olea europaea* L. does not stand out as a species that releases large amounts of VOCs (Baratella et al. 2012), the olive fly responds to them using its olfactory sensilla to identify optimal oviposition sites and feeding conditions that provide the best conditions for their offspring (Liscia et al. 2013).

The profile of olive fruit VOCs can vary significantly among genotypes, agronomic conditions and stages of ripening (Greco et al. 2022). Some classes of VOCs exhibit duality in fly behaviour, with some compounds acting as attractants and others as repellents. The VOCs most commonly identified as attractive to olive fly oviposition belong to the terpenes (α-copaene), aromatic hydrocarbons (toluene and ethylbenzene), ketones (6-methyl-5-heptene-2-one) and aldehydes (nonanal) classes (Bononi and Tateo 2017; de Alfonso et al. 2014; Gerofotis et al. 2013; Giunti et al. 2020; Malheiro, Casal, Cunha, et al. 2015; Malheiro et al. 2016; Scarpati et al. 1993). On the contrary, some aldehydes (hexanal and (E)-2-hexenal), terpenes (D-limonene) and aromatic hydrocarbons (o-xylene) act as repellents for this insect (Giunti et al. 2020; Lo Scalzo et al. 1994; Scarpati et al. 1993). Most of these VOCs are mainly produced by the oxidation of fatty acids (FA) through the lipoxygenase (LOX) pathway. The latter has been suggested to play a crucial role in genotype selection by *B. oleae*, although, to our knowledge, only a low content of polyunsaturated fatty acids (PUFA) has been found to favour olive fly attack (Malheiro et al. 2015b). There is still little literature on how the composition of FAs might influence the preference of the olive fly or if the lipidic antioxidant pool, particularly tocopherols, is implicated. Most studies to date have focused on how the attack of *B. oleae* modifies the composition of these compounds, affecting the quality of olive oil (Brkić Bubola et al. 2018) but not on how the composition of sound olive fruits triggers the infestation process, specifically in table olives.

This study was performed in the context of the table olive breeding program at the University of Seville (US). Understanding the mechanisms of the resistant response to the olive fly of certain genotypes is crucial to identifying the key traits that may be used as selection criteria, thereby increasing the effectiveness of olive cross-breeding programs. In a previous work predictive models that included physical traits and phenolic composition of olive fruits in four genotypes were developed to explain the oviposition preference of *B. oleae* (González-Fernández et al. 2023). The hypothesis of the present work is that olive fruit VOCs may also be used as predictors of the olive fly behaviour due to their attraction and repellent actions. In this sense, the present work aims to analyse the potential of new predictive models based on VOCs, FA and tocopherols, which will allow the selection of genotypes that are less attractive to the olive fly based on the most decisive traits.

## 2 | Materials and Methods

### 2.1 | Sampling and Olive Fly Preference Trial

The study was carried out in 2022 in a 7-year field trial located in Morón de la Frontera (37°11′ N, latitude 5°28′ W and altitude of 136 m, Seville, Spain) using fruits from two advanced selections of the University of Seville olive breeding program, US-06-1388 and US-06-194, and two traditional cultivars, one from Spain, 'Hojiblanca', and another from Greece, 'Kalamon'. The trees were planted in a 7 × 5 m layout under irrigation conditions (around 120 mm of total water applied) and a north–south orientation. No pesticide treatments were applied to avoid interference with the trial. On September 16th, fruits were hand-picked from three trees per genotype and with the same maturity index 1 (yellowish green epidermis). This is the ripening stage required for green dressing, Spanish style, or black dressing, California style (Rallo et al. 2018). To obtain a representative sample for each genotype, 4.5 kg of olives per tree were randomly harvested at a height of 1.5 m from the ground in all directions of the canopy. The samples were kept at 4°C during transport and were subsequently used to analyse fruit composition in terms of VOCs, FAs and tocopherols and to evaluate olive fly preference using an oviposition bioassay previously published (González-Fernández et al. 2023). Chemical analyses were performed in the shortest period possible upon arrival at the laboratory. The fruits used for the oviposition assay were stored in airtight bags at the same temperature for the entire 10 days of the assay.

The oviposition bioassay was carried out under controlled conditions and results were reported in (González-Fernández et al. 2023) (no additional bioassay was carried out). Three independent insect-rearing cages per genotype were randomly placed in a growth chamber; each cage contained fly adults (second-generation) fed *ad libitum* and a set of sound fruits. Every 24 h, the initial set was replaced by a fresh set on 10 consecutive days. The behaviour of the olive fly was assessed by the percentage of total infestation, the number of punctures/fruit and punctures/infested fruit and the percentage of offspring out of the total number of oviposition. The genotype US-06-194 was the most susceptible to olive fly oviposition, US-06-1388 was intermediate, and 'Hojiblanca' and 'Kalamon' were the least susceptible (González-Fernández et al. 2023). Observations in the field trial confirmed these results, including the year before and after the oviposition trial.

### 2.2 | Olive Volatile Organic Compounds Characterisation

The organic compounds were analysed using headspace solid phase microextraction (HS-SPME) and gas chromatography coupled to mass spectrometry (GC/MS). Briefly, two fruits, weighing approximately 3 g each, were used per repetition of each genotype and placed in a 50 mL glass vial. Then, 5 μL of internal standard (4-methyl-2-pentanol) (Sigma-Aldrich,

St. Louis, MO, USA) was added at a 0.125 mg/mL concentration. The flask was placed in a water bath at 25°C for 5 min to release the volatile compounds. Subsequently, maintaining the same temperature and stirring conditions, the SPME fibre (divinylbenzene/carbonex/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm) (Supelco, Bellefonte, PA, USA) was exposed for 30 min to adsorb the volatile compounds. The thermal extraction of the compounds from the fibre was carried out in the injection port of the chromatograph at 220°C for 1 min. After this, the fibre remained in the injector for 10 min to be cleaned and conditioned.

A Shimadzu GC-2010 Plus chromatograph equipped with a Shimadzu GC/MS-QP2010 SE mass spectrometer with a TRB-5MS (30 m × 0.25 mm × 0.25 μm) column (Teknokroma, Barcelona, Spain) was used. The injector operated at 220°C with manual injection in splitless mode, using helium 5.0 0 (Linde, Lisbon, Portugal) as carrier gas with a linear velocity of 30 cm/s and a flow rate of 24.4 mL/min. The oven was programmed at 40°C for 1 min, increasing by 2°C/min until it reached 220°C, and maintained at 250°C, with an energy of 70 eV and a current of 0.1 kV, for ionisation. All the mass spectra were obtained by electron ionisation in the m/z 35–500 range. The compounds were identified by comparing the mass spectra and Kovats indices in the NIST 69, PubChem and ChemSpider databases. The compounds' areas were determined by integrating the total ion chromatogram (TIC). Semi-quantification of the volatile compounds was based on the relative area of each peak, which was converted into mass equivalents based on the internal standard added. Each sample was analysed in triplicate.

### 2.3 | Fatty Acids and Tocopherol Characterisation

The lipids were extracted from the pulp, using petroleum ether with 0.01% BHT (2,6-di-tert-butyl-4-methylphenol, Sigma) by Soxhlet extraction for 8 h. After solvent removal under vacuum (rotary evaporator RE300DB, Stuart, Stone, United Kingdom) the extracted lipids were stored at −20°C until analysis.

A portion of the extracted lipids was used for the fatty acid analysis after cold alkaline methylation with 2 M methanolic potassium hydroxide solution (COI/T.20/Doc. No 33/Rev. 1) and separation by gas chromatography with flame ionisation detection on a Select FAME column (50 m × 0.25 μm, Agilent, USA). The results are expressed in relative percentage of chromatographed fatty acid areas after calibration of the detector response with a certified reference material (TraceCERT, Supelco CRM47885, Merck Life Science, Portugal).

A second portion was used for α-, β- and γ-tocopherols evaluation by high-performance liquid chromatography (HPLC) following the methodology outlined in ISO 9936 (2016) with minor modifications. Quantification was supported by individual calibration curves using authentic standards (Sigma, Barcelona, Spain) and tocol as an internal standard (Matreya Inc., Pleasant Gap, PA, USA). The extracted lipids and IS were diluted in n-hexane and injected into a normal phase silica column (SupelcosilTM LC-SI; 7.5 cm × 3 mm; 3 μm, Supelco, Bellefonte, PA, USA), with isocratic elution of 2.5% (v/v) 1,4-dioxane in n-hexane, at 23°C, and fluorescence detection (Jasco HPLC system, Japan). The concentrations were expressed as milligrams per kilogram (mg/kg) of extracted lipids.

### 2.4 | Statistical Analysis

One-way analysis of variance (ANOVA) and a mean comparison test (Tukey, $p < 0.05$) across four genotypes were performed for all chemical compounds analysed using Statgraphic Centurion Version 18.1.14. When appropriate, the variables were transformed using the Box–Cox transformation (Box and Cox 1964) or the square root to normalise and homogenise the variances. Multiple linear regression models (MLRMs) were developed using the oviposition preference bioassay data. The most influential independent variables (VOC's, FA and tocopherols) were selected by applying the simulated annealing (SA) variable selection. Each model is based on an independent variable subset, ranging from 2 to 10 (based on the degrees of freedom) according to the experimental dataset. The selection considers the independent variable sets that give the best prediction performance for leave-one-out cross-validation (LOO-CV) variant, that is, the minimum root mean square error (RMSE) and the maximum correlation coefficient (R). It is important to note that some independent variables may be used in multiple models. For each established model, collinearity of the selected variables was evaluated based on the R-Pearson correlation coefficient. Additionally, multicollinearity was evaluated by calculating the variable inflation factor (VIF). MLRMs were developed using the open-source statistical packages available in R Studio version 2021.09.0, also known as the 'Ghost Orchid' release (077589bc, 2021-09-20), at a significance level of 5%.

## 3 | Results

### 3.1 | Volatile Organic Compounds (VOCs)

As detailed in Table 1, a total of 33 VOCs were identified from nine different chemical classes: alcohols (1), aldehydes (4), aromatic hydrocarbons (4), carboxylic esters (2), esters (1), hydrocarbons (6), ketones (2), terpenes (12) and other compounds (1). VOC profiles varied qualitatively according to genotype, with different genotypes exhibiting distinct numbers of identified VOCs. Statistically significant differences were also observed.

In terms of qualitative differences, 25 VOCs were identified in US-06-1388, 21 in US-06-194, 23 in 'Hojiblanca' and 19 in 'Kalamon'. Of the total number of VOCs identified and semi-quantified, 12 compounds were detected in all genotypes: 1-dodecanol, decanal, heptanal, nonanal, decane, dodecane, tetradecane, 2,2,4,4,6,8,8-heptamethylnonane, 2,2,4,6,6-pentamethylheptane, linalool, terpinolene and D-limonene. Among the others, four VOCs (tridecane, copaene, α-citral and γ-terpinene) were only released by US-06-1388, two VOCs (m-xylene and lauryl acetate) and (pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl isobutyl ester and α-terpineol) by US-06-194 and 'Hojiblanca', respectively, and 1-ethoxyoctane by 'Kalamon'. Furthermore, hexanal and

**TABLE 1** | Volatile organic compounds of four genotypes (mean values ± standard deviation, $n = 3$; μg/kg of fresh healthy fruit).

| | US-06-1388 | US-06-194 | 'Hojiblanca' | 'Kalamon' | *p* |
|---|---|---|---|---|---|
| Alcohols | | | | | |
| 1-Dodecanol | $0.3 \pm 0.3^{b}$ | $0.1 \pm 0.1^{b}$ | $1.2 \pm 0.8^{a}$ | $1.1 \pm 0.1^{a}$ | 0.032 |
| Aldehydes | | | | | |
| Decanal | $0.08 \pm 0.06^{b}$ | $0.04 \pm 0.01^{b}$ | $0.45 \pm 0.14^{a}$ | $0.31 \pm 0.06^{a}$ | < 0.001 |
| Hexanal | n.d. | $0.08 \pm 0.01^{b}$ | $0.55 \pm 0.20^{a}$ | $0.81 \pm 0.11^{a}$ | < 0.002 |
| Heptanal | $0.15 \pm 0.13^{b}$ | $0.08 \pm 0.01^{b}$ | $0.74 \pm 0.20^{a}$ | $0.80 \pm 0.01^{a}$ | < 0.001 |
| Nonanal | $0.31 \pm 0.22^{b}$ | $0.14 \pm 0.04^{b}$ | $1.62 \pm 0.26^{a}$ | $1.56 \pm 0.27^{a}$ | < 0.001 |
| Aromatic hydrocarbons | | | | | |
| m-Cymene | 1.0 ± 1.2 | n.d. | 1.8 ± 0.4 | n.d. | 0.296 |
| m-Xylene | n.d. | 0.30 ± 0.07 | n.d. | n.d. | |
| o-Xylene | $0.6 \pm 0.4^{b}$ | n.d. | $3.6 \pm 0.7^{a}$ | $3.6 \pm 0.5^{a}$ | < 0.001 |
| p-Cymene | n.d. | $0.18 \pm 0.09^{b}$ | n.d. | $1.69 \pm 0.74^{a}$ | 0.037 |
| Carboxylic esters | | | | | |
| Lauryl acetate | n.d. | 0.03 ± 0.00 | n.d. | n.d. | |
| 2-Ethyl-3-hydroxyhexyl 2 methylpropanoate | 0.1 ± 0.1 | n.d. | 0.4 ± 0.2 | 0.4 ± 0.1 | 0.079 |
| Esters | | | | | |
| Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester | n.d. | n.d. | 0.45 ± 0.08 | n.d. | |
| Hydrocarbons | | | | | |
| Decane | $0.59 \pm 0.63^{b}$ | $0.18 \pm 0.04^{b}$ | $1.70 \pm 0.29^{a}$ | $2.10 \pm 0.37^{a}$ | 0.001 |
| Dodecane | $0.05 \pm 0.04^{c}$ | $0.02 \pm 0.00^{c}$ | $0.19 \pm 0.01^{b}$ | $0.26 \pm 0.03^{a}$ | < 0.001 |
| Tetradecane | $0.04 \pm 0.01^{b}$ | $0.04 \pm 0.02^{b}$ | $0.34 \pm 0.14^{a}$ | $0.32 \pm 0.04^{a}$ | < 0.001 |
| Tridecane | 0.04 ± 0.04 | n.d. | n.d. | n.d. | |
| 2,2,4,4,6,8,8-Heptamethylnonane | $0.09 \pm 0.09^{b}$ | $0.04 \pm 0.01^{b}$ | $0.42 \pm 0.13^{a}$ | $0.36 \pm 0.09^{a}$ | 0.002 |
| 2,2,4,6,6-Pentamethylheptane | $0.45 \pm 0.50^{a,b}$ | $0.12 \pm 0.06^{b}$ | $1.18 \pm 0.05^{a}$ | $1.07 \pm 0.45^{a}$ | 0.015 |
| Ketones | | | | | |
| 2-Hexanone | n.d. | $0.15 \pm 0.01^{b}$ | $1.28 \pm 0.36^{a}$ | $1.44 \pm 0.55^{a}$ | < 0.000 |
| 6-Methyl-5-heptene-2-one | $0.05 \pm 0.01^{b}$ | n.d. | $0.49 \pm 0.12^{a}$ | n.d. | 0.025 |
| Terpenes | | | | | |
| Copaene | 0.3 ± 0.3 | n.d. | n.d. | n.d. | |
| Dihydromyrcenol | $0.3 \pm 0.2^{b}$ | $0.1 \pm 0.1^{b}$ | $1.3 \pm 0.3^{a}$ | n.d. | < 0.001 |
| Fenchol | $0.06 \pm 0.07^{b}$ | $0.01 \pm 0.00^{b}$ | $0.22 \pm 0.03^{a}$ | n.d. | < 0.001 |
| Levomenthol | 0.04 ± 0.03 | 0.02 ± 0.01 | n.d. | 0.14 ± 0.05 | 0,052 |
| Linalool | $0.07 \pm 0.07^{b}$ | $0.03 \pm 0.00^{b}$ | $0.24 \pm 0.01^{a}$ | $0.26 \pm 0.05^{a}$ | < 0.001 |
| Terpinolene | $0.13 \pm 0.13^{b}$ | $0.03 \pm 0.00^{b}$ | $0.45 \pm 0.13^{a}$ | $0.24 \pm 0.05^{a,b}$ | 0.004 |
| D-Limonene | $2.3 \pm 2.3^{a,b}$ | $0.5 \pm 0.4^{b}$ | $5.8 \pm 1.4^{a}$ | $3.7 \pm 1.6^{a,b}$ | 0.018 |
| α-Citral | 0.06 ± 0.06 | n.d. | n.d. | n.d. | |
| α-Muurolene | 0.07 ± 0.08 | n.d. | 0.19 ± 0.05 | n.d. | 0.098 |

(Continues)

**TABLE 1** | (Continued)

| | US-06-1388 | US-06-194 | 'Hojiblanca' | 'Kalamon' | *p* |
|---|---|---|---|---|---|
| α-Pinene | 0.22 ± 0.25 | 0.08 ± 0.03 | n.d. | n.d. | 0.433 |
| α-Terpineol | n.d. | n.d. | 0.15 ± 0.01 | n.d. | |
| γ-Terpinene | 0.2 ± 0.2 | n.d. | n.d. | n.d. | |
| Others | | | | | |
| 1-Ethoxyoctane | n.d. | n.d. | n.d. | 0.9 ± 0.2 | |
| Σ Alcohols | 0.3 ± 0.3$^{b}$ | 0.1 ± 0.1$^{b}$ | 1.2 ± 0.8$^{a}$ | 1.1 ± 0.1$^{a}$ | 0.032 |
| Σ Aldehydes | 0.54 ± 0.42$^{b}$ | 0.33 ± 0.07$^{b}$ | 3.37 ± 0.35$^{a}$ | 3.48 ± 0.43$^{a}$ | < 0.001 |
| Σ Aromatic hydrocarbons | 1.5 ± 1.6$^{b}$ | 0.5 ± 0.2$^{b}$ | 5.4 ± 1.1$^{a}$ | 5.3 ± 1.3$^{a}$ | 0.001 |
| Σ Carboxylic esters | 0.12 ± 0.15$^{a,b}$ | 0.03 ± 0.00$^{b}$ | 0.40 ± 0.16$^{a}$ | 0.43 ± 0.13$^{a}$ | 0.011 |
| Σ Esters | n.d. | n.d. | 0.45 ± 0.08 | n.d. | |
| Σ Hydrocarbons | 1.26 ± 1.31$^{b}$ | 0.40 ± 0.01$^{b}$ | 3.81 ± 0.23$^{a}$ | 4.11 ± 0.94$^{a}$ | 0.001 |
| Σ Ketones | 0.05 ± 0.01$^{c}$ | 0.15 ± 0.01$^{b}$ | 1.77 ± 0.32$^{a}$ | 1.44 ± 0.55$^{a}$ | < 0.001 |
| Σ Terpenes | 3.7 ± 3.6$^{a,b}$ | 0.8 ± 0.4$^{b}$ | 8.3 ± 1.3$^{a}$ | 4.4 ± 1.6$^{a,b}$ | 0.016 |
| Σ Others | n.d. | n.d. | n.d. | 0.9 ± 0.2 | |
| Σ Total Volatile Compounds | 7.5 ± 7.4$^{b}$ | 2.3 ± 0.6$^{b}$ | 24.7 ± 2.2$^{a}$ | 21.2 ± 5.0$^{a}$ | < 0.001 |

*Note:* Different letters (a,b,c) in the same row mean significant statistical differences (Tukey, *p*-value < 0.05) among the genotypes evaluated for each chemical trait.

2-hexanone were absent only in US-06-1388, o-xylene and 2-ethyl-3-hydroxyhexyl 2-methylpropanoate in US-06-194, levomenthol in 'Hojiblanca' and dihydromyrcenol and fenchol in 'Kalamon'.

Quantitatively, total VOC ranged from 2.3 μg/kg in US-06-194 to more than 20 μg/kg in 'Hojiblanca' and 'Kalamon'. The latter showed the highest values of all VOC class content, and US-06-1388 and US-06-194 showed the lowest ones. Terpenes, aromatic hydrocarbons, hydrocarbons and aldehydes were the main chemical classes quantified. Among the most abundant VOCs identified, only five of them were present in all genotypes studied, namely: 1-dodecanol, nonanal, decane, 2,2,4,6,6-pentamethylheptane and D-limonene. D-limonene was the most abundant VOC in all genotypes studied, with the lowest value in US-06-194 (0.5 μg/kg) and the highest in 'Hojiblanca' (5.8 μg/kg).

## 3.2 | Fatty Acids (FA) and Tocopherols

The FA composition varied across genotypes, with significant differences in most cases, as shown in Table 2. The relative abundance of SFA ranges from 15.9% in 'Kalamon' to 21.8% in US-06-1388. 'Hojiblanca' and US-06-194 displayed intermediate levels. Palmitic acid was the most abundant SFA. Among the four genotypes, 'Kalamon' exhibited the lowest relative levels of the identified SFAs, except for stearic acid and, by inheritance, the highest amount of unsaturated FA.

Monounsaturated fatty acids (MUFA) were the most abundant FA, mainly represented by oleic acid followed by palmitoleic acid. MUFA values ranged from 68.0% in US-06-1388 to 73.2% in 'Hojiblanca'. Likewise, the genotypes US-06-194 and 'Kalamon' showed intermediate values. In terms of polyunsaturated fatty acids (PUFA), the values ranged from 8.2% in 'Hojiblanca' to 12.9% in 'Kalamon'. The other two genotypes showed similar values, close to 10.0%. Linoleic acid was the most abundant PUFA and followed a similar trend.

Statistical analysis also revealed significant differences between genotypes regarding tocopherols composition (Table 2). 'Kalamon' (434.7 mg/kg) and US-06-194 (303.7 mg/kg) showed the lowest values of total tocopherols, while US-06-1388 and 'Hojiblanca' showed the highest (close to 700.0 mg/kg). The contents of α-tocopherol (major compound) and β-tocopherol displayed a similar trend. Concerning γ-tocopherol, the highest value was also recorded in US-06-1388 (21.8 mg/kg), compared to the lower values observed in the other genotypes (≤ 5.6 mg/kg).

## 3.3 | Relationship Between VOCs, FA, Tocopherols and *B. oleae* Preference

An overview of the chemical traits that could potentially explain the olive fly behaviour, including their positive or negative contribution (according to the coefficient sign), is provided in Table 3. For each MLRM established (training procedure) based on selected subsets of combinations of FA and tocopherols, or VOCs alone, the correlation coefficients (*R*), as well as the root mean square errors (RMSE), are provided.

For VOCs, no single compound was included in all models, but three models included o-xylene and α-terpineol, two models included α-pinene, m-cymene, γ-terpinene, terpinolene, nonanal, fenchol and decanal, and one model included copaene, pentanoic acid, lauryl acetate, 6-methyl-5-hepten-2-one, 1-dodecanol,

**TABLE 2** | FA, expressed as relative percentage among the FA (%), and tocopherols, expressed in mg/kg of extracted pulp lipids (mean values ± standard deviation of three independent samples) of four genotypes.

| | US-06-1388 | US-06-194 | 'Hojiblanca' | 'Kalamon' | *p* |
|---|---|---|---|---|---|
| Saturated fatty acids (SFA) (%) | | | | | |
| Myristic ($C_{14:0}$) | $0.03 \pm 0.0^{a}$ | $0.03 \pm 0.0^{a}$ | $0.02 \pm 0.0^{b}$ | $0.02 \pm 0.0^{b}$ | < 0.001 |
| Palmitic ($C_{16:0}$) | $19.1 \pm 0.4^{a}$ | $15.8 \pm 0.7^{b}$ | $15.4 \pm 0.2^{b}$ | $13.5 \pm 0.4^{c}$ | < 0.001 |
| Margaric ($C_{17:0}$) | $0.18 \pm 0.02$ | $0.19 \pm 0.00$ | $0.18 \pm 0.00$ | $0.16 \pm 0.00$ | 0.057 |
| Stearic ($C_{18:0}$) | $1.65 \pm 0.00^{d}$ | $3.03 \pm 0.01^{a}$ | $2.10 \pm 0.01^{b}$ | $1.71 \pm 0.04^{c}$ | < 0.001 |
| Arachidic ($C_{20:0}$) | $0.47 \pm 0.01^{c}$ | $0.55 \pm 0.00^{a}$ | $0.51 \pm 0.01^{b}$ | $0.35 \pm 0.01^{d}$ | < 0.001 |
| Behenic ($C_{22:0}$) | $0.16 \pm 0.01^{a}$ | $0.15 \pm 0.00^{a}$ | $0.16 \pm 0.01^{a}$ | $0.11 \pm 0.00^{b}$ | < 0.001 |
| Lignoceric ($C_{24:0}$) | $0.14 \pm 0.01^{a}$ | $0.12 \pm 0.02^{a}$ | $0.13 \pm 0.02^{a}$ | $0.08 \pm 0.00^{b}$ | 0.002 |
| Σ SFA | $21.8 \pm 0.4^{a}$ | $19.8 \pm 0.7^{b}$ | $18.6 \pm 0.2^{c}$ | $15.9 \pm 0.4^{d}$ | < 0.001 |
| Monounsaturated fatty acids (MUFA) (%) | | | | | |
| Palmitoleic ($C_{16:1}$) | $2.3 \pm 0.1^{a}$ | $1.2 \pm 0.0^{b}$ | $1.1 \pm 0.0^{b}$ | $1.1 \pm 0.1^{b}$ | < 0.001 |
| Margaroleic ($C_{17:1}$) | $0.43 \pm 0.02^{a}$ | $0.28 \pm 0.00^{c}$ | $0.31 \pm 0.00^{c}$ | $0.38 \pm 0.01^{b}$ | < 0.001 |
| Oleic ($C_{18:1}$) | $64.9 \pm 0.3^{c}$ | $67.8 \pm 0.8^{b}$ | $71.4 \pm 0.3^{a}$ | $69.3 \pm 1.7^{a,b}$ | < 0.001 |
| Eicosenoic ($C_{20:1}$) | $0.37 \pm 0.01$ | $0.26 \pm 0.01$ | $0.31 \pm 0.09$ | $0.37 \pm 0.01$ | 0.058 |
| Σ MUFA | $68.0 \pm 0.3^{c}$ | $69.5 \pm 0.9^{b,c}$ | $73.2 \pm 0.2^{a}$ | $71.1 \pm 1.7^{a,b}$ | < 0.001 |
| Polyunsaturated fatty acids (PUFA) (%) | | | | | |
| Linoleic ($C_{18:2}$) | $8.45 \pm 0.58^{b}$ | $9.33 \pm 0.13^{b}$ | $6.55 \pm 0.02^{c}$ | $11.64 \pm 1.26^{a}$ | < 0.001 |
| Linolenic ($C_{18:3}$) | $1.72 \pm 0.01^{a}$ | $1.25 \pm 0.00^{b}$ | $1.66 \pm 0.00^{a}$ | $1.24 \pm 0.04^{b}$ | < 0.001 |
| Σ PUFA | $10.17 \pm 0.59^{b}$ | $10.57 \pm 0.13^{b}$ | $8.21 \pm 0.02^{c}$ | $12.88 \pm 1.28^{a}$ | < 0.001 |
| Tocopherols (mg/kg) | | | | | |
| α-Tocopherol | $671.4 \pm 58.0^{a}$ | $294.7 \pm 6.9^{b}$ | $673.7 \pm 13.8^{a}$ | $426.5 \pm 87.0^{b}$ | < 0.001 |
| β-Tocopherol | $8.7 \pm 0.6^{a}$ | $3.4 \pm 0.1^{b}$ | $8.0 \pm 0.2^{a}$ | $4.3 \pm 0.7^{b}$ | < 0.001 |
| γ-Tocopherol | $21.8 \pm 0.9^{a}$ | $5.6 \pm 0.2^{b}$ | $5.1 \pm 0.1^{b,c}$ | $3.9 \pm 0.4^{c}$ | < 0.001 |
| Σ Total tocopherols | $701.8 \pm 58.1^{a}$ | $303.7 \pm 7.2^{b}$ | $686.7 \pm 14.0^{a}$ | $434.7 \pm 88.0^{b}$ | < 0.001 |

*Note:* Different letters (a,b,c,d) in the same row mean significant statistical differences (Tukey, $p$-value < 0.05) among the genotypes evaluated for each chemical trait.

dodecane and tridecane. For FA and tocopherols, all regression models included linolenic and palmitoleic acids and β-tocopherol; margaric acid and α-tocopherol were included in three models; myristic, stearic and oleic acids in two; arachidic, eicosenoic and margaroleic acids and γ-tocopherol in one. The MLRM established based on VOC content satisfactorily predicted (LOO-CV) olive fly preference variables ($0.937 \leq R_{\text{LOO-CV}} \leq 0.992$), whereas those based on FA and tocopherols were less satisfactory ($0.833 \leq R_{\text{LOO-CV}} \leq 0.955$) (Figure 1).

It should be noticed that the independent variables included in each MLRM, which were selected by the SA algorithm, show, in general, low to moderate collinearity with R-Pearson correlation coefficients lower than 0.7. However, according to the VIF values, which were in general greater than 10, a high multicollinearity was observed and thus some precaution should be used when using the MLRM coefficients to explain the possible role of each variable on olive fly preferences. Nevertheless, it should be noticed that multicollinearity is often exhibited by data from observational experiments and although some predictor variables are correlated among themselves, it does not, in general, inhibit the capability to obtain a good fit nor does it tend to affect inferences about mean responses or predictions of new observations (Neter et al. 1996).

## 4 | Discussion

Female olive fly is more attracted to large fruits that are greener rather than dark coloration, as well as spherical and firm fruits (Malheiro et al. 2015b; Rizzo et al. 2012), although preferences for small fruits have also been observed in some cases (Quesada-Moraga et al. 2018). Differences in susceptibility between cultivars have also been related to the chemical composition of the fruit, such as moisture, oil content or phenolic compounds. In addition to these traits, olive fruits release a variety of VOCs which are involved in plant–host interactions, as occurs in other species (Bruce 2015). However, this relationship is highly

**TABLE 3** | MLRMs to estimate (training) the attack preference behaviour of *B. oleae* in the oviposition preference trial. FA (%), tocopherols (mg/kg) and VOCs (μg/kg).

| Independent parameters | MLRM equations |
|---|---|
| FAs and tocopherols | Total infestation (%) = −1880 (± 285) + 2970 (± 1310) × [Myristic] + 60 (± 25) × [Palmitoleic] − 10,200 (± 1610) × [Margaric] + 3400 (± 510) × [Linolenic] + 767 (± 138) × [Arachidic] + 2.47 (± 0.40) × [α-tocopherol] − 523 (± 80) × [β-tocopherol] $R$ = 0.972; RMSE = 6.39 |
| VOCs | Total infestation (%) = −11.9 (± 0.9) + 82.0 (± 1.9) × [o-Xylene] + 933 (± 15) × [α-Pinene] − 462 (± 10) × [m-Cymene] + 1369 (± 36) × [γ-Terpinene] + 522 (±16) × [Terpinolene] − 247 (± 5) × [Nonanal] + 5055 (± 105) × [α-Terpineol] $R$ = 0.999; RMSE = 0.777 |
| FAs and tocopherols | Punctures/fruit = −6030 (± 505) + 563 (± 46) × [Palmitoleic] − 26,500 (± 1840) × [Margaric] + 328 (± 26) × [Stearic] + 122 (± 29) × [Oleic] + 9210 (± 622) × [Linolenic] − 329 (± 53) × [Eicosenic] + 6.34 (± 0.47) × [α-tocopherol] − 1350 (± 94) × [β-tocopherol] $R$ = 0.996; RMSE = 6.61 |
| VOCs | Punctures/fruit = 170 (± 3) − 114 (± 3) × [o-Xylene] + 10,671 (± 181) × [Fenchol] − 23,117 (± 401) × [α-Terpineol] + 900 (± 27) × [Decanal] − 2409 (± 42) × [Copaene] + 2044 (± 59) × [Pentanoic acid] − 10,979 (± 223) × [Lauryl acetate] $R$ = 0.999; RMSE = 1.888 |
| FAs and tocopherols | Punctures/infested fruit = −28.7 (± 4.0) + 2.66 (± 0.36) × [Palmitoleic] − 139 (± 14) × [Margaric] + 1.93 (± 0.20) × [Stearic] + 0.056 (± 0.023) × [Oleic] + 46.5 (± 4.8) × [Linolenic] + 0.030 (± 0.004) × [α-tocopherol] − 6.66 (± 0.72) × [β-tocopherol] $R$ = 0.993; RMSE = 0.052 |
| VOCs | Puncture/infested fruit = 0.323 (± 0.023) + 5.453 (± 0.232) × [6-Methyl-5-heptene-2-one] − 5.118 (± 0.076) × [m-Cymene] − 2.559 (± 0.062) × [Nonanal] + 82.5 (± 1.2) × [Fenchol] − 89.8 (± 1.7) × [α-Terpineol] + 12.72 (± 0.29) × [Decanal] + 0.951 (± 0.023) × [1-Dodecanol] $R$ = 0.999; RMSE = 0.0105 |
| FAs and tocopherols | Offspring (%) = 704 (± 128) + 3249 (± 886) × [Myristic] − 242 (± 39) × [Palmitoleic] − 225 (± 80) × [Margaroleic] − 519 (± 100) × [Linolenic] + 45.6 (± 9.2) × [β-tocopherol] + 18.0 (± 2.9) × [γ-tocopherol] $R$ = 0.969; RMSE = 4.70 |
| VOCs | Offspring (%) = 100 (± 3) + 18.7 (± 2.3) × [o-Xylene] − 518 (± 33) × [α-Pinene] + 6090 (± 286) × [γ-Terpinene] − 124 (± 10) × [Terpinolene] − 419 (± 29) × [Dodecane] − 26,605 (± 1243) × [Tridecane] $R$ = 0.997; RMSE = 1.54 |

complex and not fully explored. In this context, researchers have focused on elucidating how the composition of VOCs, FA and tocopherols might be implicated in the olive fly attractiveness and/or repellency capacity (Giunti et al. 2020; Greco et al. 2022; Kokkari et al. 2021; Malheiro, Casal, Cunha, et al. 2015; Vitanović et al. 2024).

In this work, when examining the VOCs profile of the fruits of the four genotypes studied, a total of 33 VOCs were identified. Although there are not many studies on VOCs in fresh olive fruits, most of these compounds were reported in previous works (Giunti et al. 2020; Kokkari et al. 2021; Malheiro, Casal, Cunha, et al. 2015) or in studies where the VOCs profile was evaluated in processed fruits (Cascos et al. 2023; Collin et al. 2008) as well as in olive oil (Athanasiadis et al. 2022). In contrast, some compounds have not been previously identified in olive fruits to the authors' best knowledge, namely, lauryl acetate or α-citral, which were only identified in a single genotype and, moreover, in low amounts. Focusing on the VOCs profile of genotypes studied in previous works, differences are observed both at the qualitative and quantitative levels of the identified compounds (Bononi and Tateo 2017; Kokkari et al. 2021; Malheiro et al. 2015b; Vitanović et al. 2024) which could be explained by the effect of cultivar, agronomic practices, fruit ripening stage, location and climatic conditions, combined with the characteristics of the analytical methods used in each case (Greco et al. 2022). The total number of VOCs identified per genotype was quite similar between the least susceptible genotypes to the olive fly and the most susceptible genotype. This suggests that the total number of VOCs is not implicated in the fruit–host interaction. On the other hand, highly significant differences were observed in the total amounts of VOCs, with the genotypes 'Kalamon' and 'Hojiblanca' showing the highest concentrations of total VOCs released under the analytical method conditions, while the lowest value (approximately 1/10th of the former) was found in US-06-194. However, previous works have shown a higher abundance of total volatile compounds in the most susceptible genotypes (Malheiro, Casal, Cunha, et al. 2015; Vitanović et al. 2024). This again suggests that the total amount of VOCs per se does not seem to be a determinant of the olive fly preference either. As for individual

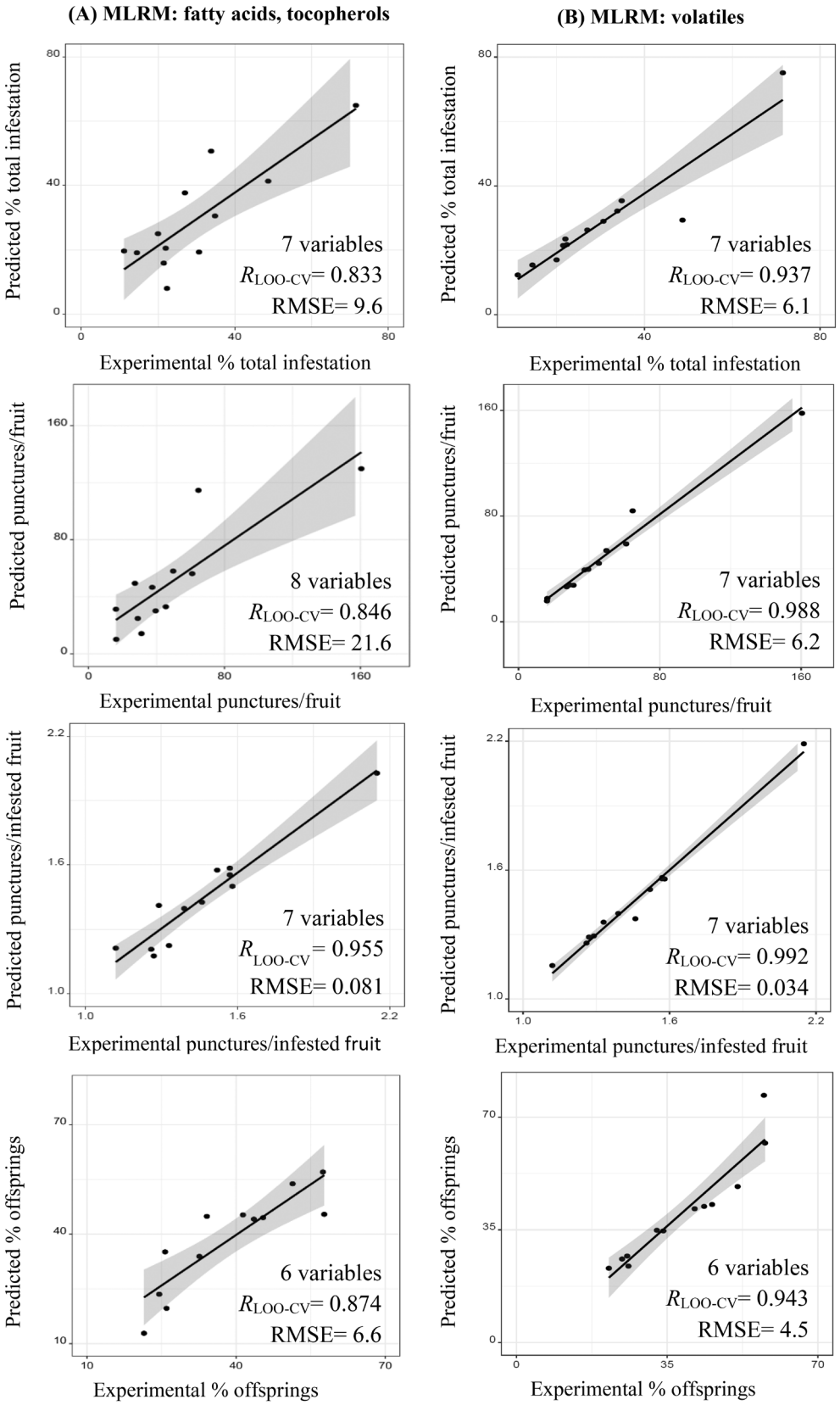


**FIGURE 1** | MLRMs' predictive performances (LOO-CV) highlighting the behaviour of *B. oleae*. Models based on FAs and tocopherol (A), and VOCs (B). Variable selection performed by applying the SA algorithm.

VOCs, the most abundant classes of VOCs identified in these olive fruits were also identified in other studies, most notably terpenes, hydrocarbons and aromatic hydrocarbons, which have been mainly related to repellent effects on various insects, including *B. oleae* (Bononi and Tateo 2017; Giunti et al. 2020; Kokkari et al. 2021; Scarpati et al. 1993; Vitanović et al. 2024).

The terpene D-Limonene, the most abundant VOC in the genotypes studied, is known to exert a repellent action, in agreement with other works (Giunti et al. 2020).

The predictive models developed based on the VOCs showed a good fit to the oviposition data ($R_{LOO\text{-}CV}$ values between 0.937 and 0.992), superior to the other models found in this work and previously. They provided further insight into how these compounds may influence the attraction and/or repulsion of the olive fly. These effects appear to result from interactions between multiple compounds, as no single compound fully explains the oviposition preference of *B. oleae* on its own. Of the VOCs selected by MLRM, several have been previously referred to *B. oleae*, for their attractant (nonanal) (Kokkari et al. 2021) or repellent (o-xylene) (Scarpati et al. 1993) action. Other studies have highlighted specific compounds, such as copaene (de Alfonso et al. 2014; Malheiro, Casal, Cunha, et al. 2015) and α-pinene (Kokkari et al. 2021; Scarpati et al. 1993), as being associated with the behaviour of *B. oleae*. These compounds were selected by the MLRMs but detected in the genotypes evaluated in low content. However, information is lacking on other compounds identified by the models. The action of these compounds may vary depending on the insect species; for example, 1-dodecanol has been described as a repellent for *Spodoptera exigua* (Chen et al. 2024), but as an attractant for *Anopheles gambiae* (Mweresa et al. 2016).

The most abundant fatty acids in olive oil, namely oleic acid, and the two predominant PUFAs, linoleic and linolenic acid, are involved in forming volatile compounds by the LOX pathway (Sánchez and Salas 2000). Fatty acids, the precursors of VOCs (El Hadi et al. 2013), showed a considerable variation among genotypes, agreeing with a previous work that has shown that the fatty acid profile is strongly influenced by cultivar, clearly being the main source of variability (León et al. 2004). Focusing on the main classes of fatty acids, the most tolerant genotypes, 'Kalamon' and 'Hojiblanca', showed lower SFAs and higher MUFA contents. On the other hand, the role of PUFA was uncertain since the two most tolerant genotypes showed the lowest and highest contents, respectively, so no clear relationship was observed with olive fly preference. Previous studies highlighted a negative correlation between PUFA content and the influence of olive fly oviposition as well as better larval development inside the fruit (Malheiro et al. 2015b), matching the results observed in 'Kalamon' but not with 'Hojiblanca'. As previously mentioned, information on the influence of fatty acids on olive fly preference is limited and contradictory: some studies indicate that some particular fatty acids have a deterrent effect, while others suggest an attractive effect. In this sense, palmitic, linolenic and lignoceric acids were negatively correlated to oviposition (Gonçalves et al. 2012). The results of the present study agree with the latter findings in the case of 'Hojiblanca', but disagree for 'Kalamon' since it was not susceptible and recorded the lowest content. This contradiction between the most tolerant genotypes once again shows that the content of these compounds is not sufficient to explain the behaviour of the fly. Regarding the fatty acid profile of the most susceptible genotype, significant differences were only found in the contents of myristic, stearic and arachidic acids, all minor SFAs which were notably higher. These results suggest that these specific SFAs might play a role in *B. oleae*. However, to date, no studies in the literature have explored this relationship in the context of the olive fly.

The MLRMs developed based on the contents of FAs and tocopherols to predict the behaviour of the olive fly also obtained satisfactory fits, but their quantitative performance was lower compared to VOCs. The linolenic acid was selected for all models. This PUFA has been negatively related to the oviposition of this pest (Gonçalves et al. 2012). The models also identified the above-mentioned SFA, namely myristic (in predicted % total infestation and predicted % offspring), stearic (in predicted punctures/fruit and predicted punctures/infested fruit), and arachidic acids (in predicted % total infestation). This finding suggests that fatty acids may play a crucial role in *B. oleae* preference supporting the idea of other authors (Malheiro et al. 2015b). The same can be considered for tocopherols, whose relationship to olive fly behaviour has not been previously explored. Despite the absence of a consistent pattern linking higher or lower tocopherol content to greater or lesser susceptibility to olive fly attacks, β-tocopherol was identified in all models. Similarly, α-tocopherol was included in all models except for the one predicting the percentage of offspring, while γ-tocopherol was exclusively identified in the latter model.

In this study, the development of predictive models based on VOCs, or FAs and tocopherols, supports the perspectives of other authors who highlight the importance of integrated knowledge, as no single compound provides a clear indication of a genotype's susceptibility to a pest. In this context, although the reliability of the models may change with fruit ripening or the year as the profiles of the compounds analysed may also change, the developed models show predictive capacity in explaining olive fly behaviour during oviposition.

## 5 | Conclusion

The oviposition behaviour of *B. oleae* in olive fruit is influenced by the VOCs, FAs and tocopherols, particularly the former. More than the amount and diversity of chemical compounds identified on the fruits of the genotypes studied, specific VOCs with previously demonstrated attraction action, such as α-pinene, copaene and nonanal, or with repellent action such as o-xylene, D-limonene and other compounds with unexplored contributions to olive fly, and their interactions, seem to be the most relevant in predicting olive fly behaviour. The same occurs with specific FAs such as stearic, myristic and arachidic acids, which have attractive actions, and linolenic acid, which has proven repellent action. As for tocopherols, a clear trend has not been observed, but β-tocopherol was identified in all models developed. Predictive models using olive fruit VOCs better fit olive fly oviposition data than those based on fatty acids and tocopherols, proving to be the most effective in explaining fly attraction or repellency.

### Author Contributions

**Antonio González-Fernández:** investigation, formal analysis, writing – original draft. **Pilar Rallo:** conceptualization, funding acquisition, writing – review and editing. **António M. Peres:** software, formal

analysis, writing – review and editing. **Nuno Rodrigues and Susana Casal:** methodology, formal analysis, writing – review and editing. **Jose Alberto Pereira and Ana Morales-Sillero:** conceptualization, supervision, writing – review and editing.


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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that support the findings of this study are openly available in [idUS, Depósito de Investigación Universidad de Sevilla] at https://doi.org/10.12795/11441/176414.

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