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Developing Predictive Models under Controlled Conditions for the Selection of New Genotypes That Are Less Susceptible to *Bactrocera oleae* (Rossi) in Table Olive (*Olea europaea* L.) Breeding Programs

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Abstract: *Bactrocera oleae* (Rossi), the olive fly, represent an important biotic factor in olive groves (*Olea europaea* L.) causing significant production losses. Ensuring effective management of this pest is of paramount importance to safeguard and uphold the quality and quantity of table olive production. The University of Seville's (US) table olive breeding program has focused its attention on finding new cultivars that exhibit reduced susceptibility to the olive fly. This study attempted to develop predictive models to enable the selection of new genotypes that are less susceptible to the olive fly based on fresh fruit traits. An extensive analysis of fruit physical (weight, size, symmetry, color, and texture) and chemical traits (moisture, oil content, and phenolic compounds) was conducted to evaluate the fly's preference in oviposition bioassays (multiple choice and no choice) among four genotypes (US-06-1388, US-06-194, 'Hojiblanca', and 'Kalamon'), under controlled conditions. The oviposition bioassays revealed the higher susceptibility of genotype US-06-194 and the lower susceptibility of 'Kalamon'. The predictive models incorporated physical traits such as, fruit weight, longitudinal diameter, symmetry, CIELAB color attributes (L*, a*, and b*), and compression hardness, as well as chemical traits such as moisture, and the contents of demethyloleuropein, oleuropein, rutin, and verbascoside. These traits consistently predicted the preference of *B. oleae* for certain fruits.

Keywords: olive fruit; selection tools; olive fly; oviposition preference; bioassay

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1. Introduction

The olive tree (*Olea europaea* L.), which has been cultivated in the Mediterranean basin for centuries, has undergone significant changes in its cultivation practices since the late 20th century, moving towards intensive and technologically advanced growing systems [1,2].

Approximately 10% of the world's olive production is used for table olives. Over the past three decades, their global production has tripled, accounting for about 3.2 million tons, 90% of which is obtained in Mediterranean countries such as Spain, Egypt, Turkey, Algeria, Greece, and Morocco [3]. However, current table olive production still relies on traditional genotypes that were selected many centuries ago, and most of these genotypes are not well-suited for modern mechanized plantations [2]. Moreover, new challenges have emerged, including the demand for organic products, high-quality in terms of taste and health benefits, sustainable production methods, and resilience to climate change. To

address these challenges, plant breeding emerges as a promising strategy for developing new table olive genotypes in this complex scenario.

Although olive breeding programs are relatively limited compared to other crops, some of them are currently focused on developing new cultivars for table olive production [4]. These programs aim to create genotypes that are well adapted to intensive mechanized planting systems [5,6] while considering specific quality traits as selection criteria [7]. Olive fruit appearance, including size, shape, color, and absence of damage, is crucial for producing a high-quality final product. Additionally, tolerance to biotic and abiotic stresses, such as the olive fly (*Bactrocera oleae*, Rossi), plays a significant role in the selection process. The olive fly is considered the most destructive pest, causing substantial damage to the fruit. This damage leads to pulp consumption, which can account for 2–20% of the fruit, rendering the fruit unsuitable for table olive production. According to estimates, the olive fly is responsible for annual losses of 5–15% in both the quantity and quality of olive production worldwide [8]. However, so far, breeding programs have not given much attention to this particular biotic stress.

Bactrocera oleae is a polyvoltine dipteran that undergoes several generations per year (2–5 in Europe). The females lay their eggs beneath the epicarp of the fruit, and the emerging larvae feed on the fruit pulp, causing significant damage. The larvae complete their life cycle inside the olives, and when they are ready to pupate, they either exit the fruit to pupate in the soil or pupate inside the fruit, with the adults later creating an exit hole. Infested fruit exhibits brown spots after oviposition, which are more noticeable in the green maturity stage. The fruit also has a soft texture and deformities due to larval galleries and mutilations [9].

Significant variations in the susceptibility of different olive genotypes to the olive fly suggest a complex interaction of various traits that need further exploration [10]. However, studies in this regard are limited and have primarily focused on fruits intended for olive oil production, harvested after veraison. The physical traits of the fruit may play a role in the susceptibility of olive trees to the olive fly. Females appear to be more attracted to large and spherical fruit, although preferences for small fruit were also observed. The olive fly also seems to prefer less mature fruit with green-yellowish skin color, as dark coloration confuses them during drupe recognition [11]. Additionally, the olive fly prefers fruit with high skin firmness but low skin elasticity [9,12]. Differences in the chemical composition of the fruit, such as water, oil, and phenolic compounds, may also influence the fruit's susceptibility [13]. The last compounds may be crucial in safeguarding fruits against pathogens and insects [14].

In 2003, the University of Sevilla in Spain initiated a table olive cross-breeding program that is currently evaluating 30 advanced selections in various field trials, and three new genotypes are in the process of being registered. One of the program's objectives is to search for resistance to the olive fly. Understanding the factors contributing to the varying susceptibility among the different genotypes is crucial for developing an effective selection tool to be used in breeding programs.

In order to explore and develop a predictive model for selecting new, less susceptible genotypes to the olive fly, a comprehensive analysis of physical and chemical traits was conducted on the table olive genotypes. The results of this analysis were compared with the findings from two preference trials of olive fly among the genotypes under controlled conditions.

2. Materials and Methods

2.1. Plant Material

The fruit used in the present work was obtained from a field trial of the University of Sevilla (US) table olive breeding program, located in Morón de la Frontera (37°11′ N, latitude of 5°28′ W, and altitude of 136 m, Sevilla, Spain). The trees were seven years old and were cultivated under the same conditions following the integrated production guidelines and under irrigation conditions (around 120 mm of total water applied). The study included

two traditional olive cultivars, 'Hojiblanca' and 'Kalamon', and two advanced selections of the above-mentioned breeding program, US-06-1388 and US-06-194. The genotype US-06-1388 originated through natural pollination of 'Toffahi' trees, while US-06-194 was the result of a controlled cross between 'Manzanilla de Sevilla' and 'Hojiblanca'. Both genotypes stand out for key characteristics, including substantial fruit weight (6 g for US-06-1388 and 5 g for US-06-194), a high pulp-to-pit ratio of 7 in both, well-suited tree architecture for mechanical harvesting, early onset of fruit production, and high productivity. According to [10], cv. 'Hojiblanca' is susceptible and cv. 'Kalamon' presents high tolerance to the olive fly. There is no prior evidence regarding the susceptibility of the other two genotypes.

On 16 September 2022 (DOY 259), seven trees of each genotype were selected. Olive fruits were collected at a height of 1.5 m, in all directions. The fruits had a maturity index 1, corresponding to the green maturation stage (US-06-1388: 156 DAF; US-06-194: 147 DAF; 'Hojiblanca' and 'Kalamon': 137 DAF) and were examined for various physical characteristics (including fruit weight, pulp to pit ratio, longitudinal and equatorial diameters, symmetry, color, compression hardness, texture, and skin firmness) as well as chemical traits (such as moisture, oil content, and phenolic compounds) as outlined in Sections 2.2 and 2.3. Subsequently, the collected fruits were utilized for the development of oviposition bioassays (Section 2.4). The average yield for the four genotypes was as follows: 49.2 $\pm$ 3.9 kg/tree (US-06-1388), 19.5 $\pm$ 0.9 kg/tree (US-06-194), 41.9 $\pm$ 3.7 kg/tree ('Hojiblanca'), and 24.2 $\pm$ 4.1 kg/tree ('Kalamon'). For each tree and sample, 4.5 kg of fruits were randomly harvested, transported, and stored under cold conditions in the laboratory.

2.2. Fruit Physical Traits

The fruit weight, an average of 100 fruits, was measured in a precision balance (Cobos JT 1200-C). The pulp-to-pit ratio (fresh weight) was estimated using a 50-fruit sample by calculating the difference between fruit and pit weights. Maximum longitudinal and equatorial diameters (mm) were measured individually in 30 fruits using a Digital Vernier caliper (Altraco Inc., Sausalito, CA, USA). Fruit symmetry was classified into three qualitative categories (1: symmetrical; 2: slightly asymmetrical; and 3: asymmetrical) based on the position of maximum asymmetry in a sample of 30 fruits [15].

The fruit color attributes CIELAB were determined with a Minolta CM-700d (Konica Minolta Inc., Tokyo, Japan) spectrophotometer, where L* denotes the lightness, a* denotes the color axis from green to red, and b* denotes the color axis from blue to yellow. Three measurements were taken in the equatorial zone of 30 fruits.

The mechanical properties of the fruit were assessed using a TA.TX2 texture analyzers (Stable Micro Systems, Godalming, UK) connected to a computer and fitted with different load cells. Two types of tests were conducted using different probes: compression hardness (Ø = 20 mm; 30 kg load cell) (N), and texture, shear compression hardness with a Kramer cell (HDP/KS10; 50 kg load) (N/g fresh fruit weight). Compression hardness test (10 measurements; 1 fruit per measured) was performed to achieve a 6% deformation of the fruit with a flat steel plate. Lastly, texture test (10 measurements; 3 fruits per measurement) was applied to achieve a simulation of the destructive chewing process of pitted olives with a cross-head speed of 200 mm min^{-1}. Fruit skin firmness (10 fruits), expressed in Newtons, was measured using a penetrometer (FT 011) (Ø = 2 mm).

2.3. Fruit Chemical Traits

To determine the moisture (%), 70 g of fruits were weighed and oven-dried at 105 °C until a constant weight was attained. In fresh and dry samples, the fruit oil content (%) was determined using nuclear magnetic resonance on a Maran Ultra spectrometer (Oxford Instruments, Abingdon, Oxfordshire, UK).

The extraction and analyses of phenolic compounds were made according to [16]. Longitudinal and thin pieces of mesocarp tissue were cut from 20 fruits and kept at 4 °C for 72 h in dimethyl sulfoxide (6 mL g^{-1} of fruit), with syringic acid (24 mg mL^{-1}) used as

internal standard. The supernatants obtained were filtered through a 0.45 µm mesh nylon and kept at −20 °C until HPLC analysis.

Phenolic compounds (µg/g fresh fruit weight) were analyzed on a Beckman Coulter liquid chromatography system (Beckman coulter, Brea, California, USA) equipped with a System Gold 168 photodiode array detector, a solvent module 126, an autosampler module 508 and a Waters column heater module. All the analyses were made in a Superspher RP 18 column (4.6 mm i.d. × 250 mm, particle size 4 µm: Dr Maisch GmbH, Ammerbuch, Germany, EU) at flow rate of 1 mL min^{-1}, with a temperature of 35 °C. The identification of compounds was first made based on their UV–vis spectra, and later was confirmed by HPLC/ESI-qTOF-HRMS [17].

2.4. Olive Fly Preference Trials

For these trials, second-generation olive fly adults were reared on 'Gordal Sevillana' fruit. Infested 'Gordal Sevillana' olive fruits were collected at the same grove, located in Osuna (Andalucia, Spain), transported to the laboratory and placed in plastic boxes (0.04 m^3), and maintained under controlled conditions in a growth chamber with the following specifications: 26 ± 1 °C, relative humidity of 70 ± 10%, with a photoperiod of 16L:8D. Pupae were transferred to insect-rearing cage (0.03 m^3) and adults that emerged were separated daily into new insect-rearing cages and fed ad libitum (honey solution (10% *w*/*v*), with an artificial diet (sucrose and yeast extract at a ratio of 4:1 and water). All of them were maintained in the same conditions. When they reached 14 days of age, healthy olives of the same cultivar were provided to them for oviposition, thus obtaining a new generation of individuals.

The possible preference of olive fly for certain genotypes was evaluated in these trials using insect-rearing cages (0.03 m^3) containing 20 adult flies with a sex ratio of 1:1. The flies were 14 days old to ensure female fertility. Before exposing the olives, the flies were placed in separate cages without any fruit for 24 h. Two different types of bioassays were conducted under controlled conditions, as follows:

(i) Multiple-choice oviposition. For this assay, seven independent cages were used (n = 7) and randomly placed at different locations within the growth chamber. Each cage contained 60 healthy olives from the four genotypes (US-06-1388, US-06-194, 'Hojiblanca', Kalamon'; 15 fruits from each genotype) and 20 adult flies with a sex ratio of 1:1. After 24 h, the initial set of fruits was replaced with a fresh set, and this cycle continued for ten consecutive days. In total, 1050 fruits per genotype were assessed, providing ample opportunities for olive fly to interact with the available fruit resources.

(ii) No-choice oviposition. The methodology, parameters, and timeframe employed in this assay were similar to those of the multiple-choice assay. Here, a set of 60 olives from a single genotype was placed inside each cage. Three independent cages were used for each genotype (n = 3) and distributed arbitrarily throughout the growth chamber, yielding an evaluation of 1800 fruits per genotype.

For both assays, every 24 h, the fruit was inspected using a binocular stereomicroscope (Zeiss Stemi DV4) (Carl Zeiss AG, Oberkochen, Alemania, EU) to quantify the number of ovipositions present. Subsequently, the oviposited fruits continued to be kept under conditions described above for a month to collect and record the number of adults that emerged. The following parameters were taken into consideration: total infestation (%); punctures/fruit; punctures/infested fruit; and offspring (%).

2.5. Statistical Analyses

Data were analyzed with the Statgraphic Centurion 18 Version 18.1.14. An analysis of variance (ANOVA) and a mean comparison test (Tukey, $p \leq 0.05$) were conducted among genotypes. When necessary, the variables were transformed using the Box–Cox transformation [18] to normalize and homogenize the variances. Pearson's correlation coefficients were calculated for every possible pair of evaluated traits Principal Component

Analysis (PCA) was also performed for the multiple-choice assays. On the other hand, Multiple Linear Regression Models (MLRMs) were developed using data obtained for the one-choice assays, performed under controlled conditions. For the latter, the simulated annealing (SA) algorithm, a meta-heuristic variable selection algorithm, was applied to identify the most statistically influential independent variables. The MLRMs incorporate coefficients for each independent variable, which are optimized aiming to minimize the sum of squared errors. These errors represent the difference between the experimental value of the dependent variable and the MLRM-calculated value. N models are established, each based on subsets of independent variables ranging from 2 to the number of experimental assays minus 2 (i.e., degrees of freedom). The selection of independent variables for each MLRM is performed using the SA algorithm. This selection process considers sets of independent variables that yield the best predictive performance, as indicated by the minimum root mean square error (RMSE) and maximum correlation coefficient (R), for the leave-one-out cross-validation (LOO-CV) variant. Thus, varying numbers and types of independent variables can be chosen to explain different dependent variables. It is worth noting that some of these independent variables may be used across different models. PCA and MLRMs were conducted using the R statistical program, specifically the open-source packages available in RStudio version 2021.09.0, also known as the "Ghost Orchid" Release (077589bc, 2021-09-20), at a 5% significance level.

3. Results

3.1. Fruit Physical and Chemical Traits

Table 1 shows the average values of the physical characteristics, while Table 2 reports the evaluated chemical traits, both for the fresh fruits of the four examined genotypes during the multiple-choice and no-choice bioassays. As can be inferred, the statistical analysis revealed significant differences among genotypes for the majority of the characteristics.

Table 1. Evaluation of physical traits (mean values ± standard errors) on fresh fruit of four genotypes (US-06-1388, US-06-194, 'Hojiblanca', and 'Kalamon').

			Olive Genotype		*p*-Value
Physical Traits (*n* = 7)	**US-06-1388**	**US-06-194**	**'Hojiblanca'**	**'Kalamon'**	
Weight (g)	4.07 ± 0.04 b	4.84 ± 0.09 a	3.59 ± 0.12 c	3.05 ± 0.02 d	<0.001
Pulp-to-pit ratio	5.6 ± 0.2 b	6.1 ± 0.1 a	5.5 ± 0.1 b	5.1 ± 0.1 c	<0.001
Longitudinal diameter (mm)	22.2 ± 0.2 b	23.6 ± 0.2 a	22.0 ± 0.5 b	24.1 ± 0.2 a	0.0002
Equatorial diameter (mm)	18.2 ± 0.3 b	20.1 ± 0.2 a	17.0 ± 0.3 c	14.8 ± 0.2 d	<0.001
Symmetry	2.80 ± 0.05 a	1.78 ± 0.07 b	1.34 ± 0.10 c	2.70 ± 0.04 a	<0.001
L*	66.7 ± 0.3 a	58.3 ± 0.7 b	52.4 ± 0.4 c	53.1 ± 0.4 c	<0.001
a*	−9.8 ± 0.1 a	−11.2 ± 0.1 c	−11.7 ± 0.1 d	−10.4 ± 0.1 b	<0.001
b*	38.0 ± 0.3 ab	39.9 ± 0.3 a	37.1 ± 0.4 b	33.3 ± 1.2 c	<0.001
Compression hardness (N)	78.0 ± 2.0 a	76.7 ± 4.6 a	79.2 ± 2.6 a	47.9 ± 7.9 b	<0.001
Texture (N/g)	56.5 ± 1.5 ab	47.9 ± 0.8 c	62.5 ± 0.9 a	50.4 ± 2.5 bc	<0.001
Skin firmness (N)	11.2 ± 0.3 ab	11.6 ± 0.4 ab	12.8 ± 0.4 a	10.0 ± 0.8 b	0.008

Different letters in the same row mean significant statistical differences (Tukey, *p*-value < 0.05) among the genotypes evaluated for each physical trait.

Table 2. Evaluation of chemical traits (mean values ± standard errors) on fresh fruit of four genotypes (US-06-1388, US-06-194, 'Hojiblanca', and 'Kalamon').

			Olive Genotype		*p*-Value
Chemical Traits (*n* = 3)	**US-06-1388**	**US-06-194**	**'Hojiblanca'**	**'Kalamon'**	
Oil Content (% f.w.)	4.3 ± 0.2 c	12.0 ± 0.4 a	7.1 ± 0.6 b	12.8 ± 0.2 a	<0.001
Oil Content (% d.w.)	13.8 ± 0.8 c	32.1 ± 0.3 a	20.2 ± 1.2 b	31.7 ± 0.7 a	<0.001
Moisture (%)	68.9 ± 0.2 a	62.4 ± 1.4 bc	64.9 ± 0.9 b	59.5 ± 0.2 c	<0.001
Total phenolic content (μg/g)	26,942 ± 426 c	32,822 ± 928 b	30,531 ± 1348 bc	43,601 ± 1887 a	<0.001

Table 2. *Cont.*

			Olive Genotype		*p*-Value
Chemical Traits (*n* = 3)	**US-06-1388**	**US-06-194**	**'Hojiblanca'**	**'Kalamon'**	
Oleuropein (μg/g)	22,829 ± 369 b	26,172 ± 783 b	23,964 ± 1045 b	33,527 ± 1419 a	<0.001
Ligstroside (μg/g)	1012 ± 43 b	845 ± 126 b	1304 ± 90 b	7103 ± 583 a	<0.001
Hydroxytyrosol-glucoside (μg/g)	1751 ± 49 b	2669 ± 86 a	1093 ± 74 c	676 ± 50 d	<0.001
Verbascoside (μg/g)	11.8 ± 5.0 c	1407 ± 273 a	2007 ± 310 a	174.3 ± 71.6 b	<0.001
Rutin (μg/g)	408 ± 42 c	451 ± 24 bc	1523 ± 31 a	992 ± 267 ab	0.002
L-7-G (μg/g)	299 ± 41 a	541 ± 47 a	218 ± 19 a	280 ± 120 a	0.073
Demethylligstroside (μg/g)	207 ± 114 a	212 ± 158 a	210 ± 102 a	477 ± 60 a	0.427
Tyrosol-glucoside (μg/g)	298.8 ± 4.2 a	286.4 ± 8.8 a	64.6 ± 2.8 b	241.6 ± 29.3 a	<0.001
FDAO (μg/g)	61.5 ± 9.0 ab	144.7 ± 3.1 a	113.8 ± 51.0 ab	17.5 ± 3.6 b	0.044
A-7-G (μg/g)	34.9 ± 5.2 b	45.5 ± 5.6 b	80.9 ± 0.3 a	45.2 ± 1.4 b	<0.001
OleA (μg/g)	17.0 ± 4.0 a	26.3 ± 4.7 a	15.6 ± 5.9 a	33.4 ± 18.0 a	0.546
LA (μg/g)	3.6 ± 0.3 a	6.0 ± 0.1 a	30.4 ± 5.5 a	19.6 ± 14.2 a	0.116
Demethyloleuropein (μg/g)	8.8 ± 6.6 a	15.5 ± 0.7 a	7.9 ± 0.3 a	14.6 ± 2.0 a	0.342

Different letters in the same row mean significant statistical differences (Tukey, *p*-value < 0.05) among the genotypes evaluated for each chemical trait; f.w.: fresh weight; d.w.: dry weight; L-7-G: Luteolin-7-glucoside; FDAO: dialdehydic form of decarboxymethyl oleuropein aglycon; A-7-G: Apigenin-7-glucoside; OleA: Oleuropein aglycones; LA: Ligstroside aglycones.

The genotypes examined differed in fruit size and morphology. The fruit weight (g) ranged from 3.0 to 4.8, while the pulp-to-pit ratio varied between 5.1 and 6.1. Among the genotypes, US-06-194 had high values for both longitudinal and equatorial diameters, and, in line with these highest values, it also showed the highest fruit weight. Other genotypes (e.g., cv. 'Kalamon') displayed high longitudinal diameter values but relatively low equatorial diameter values showing an "elongated" fruit shape. 'Hojiblanca' and US-06-194 fruit had symmetrical values close to 2, indicating slight asymmetry. The fruit of 'Kalamon' and US-06-1388 were nearly asymmetric, with symmetry values of 2.7 and 2.8, respectively. Regarding color traits, among the four genotypes analyzed, the olives of US-06-1388 had the lightest color with an L* value of 66.7. Additionally, these olives exhibited a less pronounced green hue, as evidenced by their a* mean value of −9.8. Conversely, genotype US-06-194 displayed higher positive b* values (39.9), resulting in a more yellowish appearance of the fruit. All texture tests revealed that 'Hojiblanca' had the highest values across all traits. In contrast, 'Kalamon' displayed the lowest values for compression hardness (47.9 N) and skin firmness (10.0 N). Genotypes US-06-1388 and US-06-194 had intermediate values, although genotype US-06-194 exhibited the lowest texture value when using the Kramer probe (47.9 N/g).

Regarding the chemical traits (Table 2), it was observed that the 'Kalamon' and US-06-194 genotypes exhibited the highest oil content in terms of fresh weight (f.w.: 12.8%, 12.0%) and dry weight (d.w.: 31.7%, 32.1%), respectively. Conversely, genotype US-06-1388 displayed the lowest oil content (4.3% and 13.8% for f.w. and d.w., respectively) but stood out for having the highest moisture percentage (68.9%). The total phenolic content ranged from 26,942 μg/g (US-06-1388) to 43,601 μg/g ('Kalamon'). US-06-194 and 'Hojiblanca' showed intermediate values of 32,822 μg/g and 30,531 μg/g, respectively. Among the individual phenols, significant differences were found among genotypes for most of them, except for demethyloleuropein, demethylligstroside, oleuropein aglycones (OleA), ligstroside aglycones (LA), and luteolin-7-glucoside (L-7-G). The most abundant phenols found in the olive fruit were oleuropein, hydroxytyrosol-glucoside, ligstroside, verbascoside, and rutin. The content of oleuropein ranged from 22,829 μg/g (US-06-1388) to 33,527 μg/g ('Kalamon'), while hydroxytyrosol-glucoside ranged from 676 μg/g in 'Kalamon' to 2669 μg/g in US-06-194. Ligstroside content was notably high in the cv. 'Kalamon' (approximately 7000 μg/g) compared to the others (around 1000 μg/g). As for verbascoside, US-06-1388 had the lowest value (11.8 μg/g), significantly lower than 'Kalamon' (174 μg/g), and the other two genotypes (US-06-194 and 'Hojiblanca'), whose values were recorded within the range of 1500–2000 μg/g. Rutin was also present in significant

amounts in the fruit, with the highest value in cv. 'Hojiblanca' (1523 μg/g) and the lowest in genotype US-06-1388 (408 μg/g).

3.2. Multiple-Choice Oviposition Bioassay

During the multiple-choice oviposition bioassay, four genotypes were provided simultaneously to olive fly females, in order to assess their comparative attractiveness for oviposition. Significant differences were observed among the genotypes (Table 3). Notably, the 'Kalamon' fruits exhibited fewer punctures from olive fly, resulting in the least amount of damage recorded. Over a 10-day period, genotype US-06-194 was identified as the preferred choice for oviposition, with a total of 52.0% of fruits being infested. In comparison, 'Kalamon', 'Hojiblanca', and US-06-1388 accounted for 18.8%, 31.7%, and 33.1% of the fruits infested, respectively. Furthermore, genotype US-06-194 demonstrated an average of 1.05 punctures/fruit. When considering only the infested fruit, this percentage increased to 1.9. The punctures/fruit and punctures/infested fruit on 'Hojiblanca', 'Kalamon', and US-06-1388 were similar among them and lower than those observed for genotype US-06-194.

Table 3. Olive fly responses in the multiple-choice oviposition bioassay over 10 consecutive days (mean values ± standard errors, n = 7) on fresh fruit of four genotypes (US-06-1388, US-06-194, 'Hojiblanca', and 'Kalamon').

	Olive Genotype				*p*-Value
Multiple-Choice Oviposition Assays	**US-06-1388**	**US-06-194**	**'Hojiblanca'**	**'Kalamon'**	
Total infestation (%)	33.1 ± 6.6 ab	52.0 ± 7.0 a	31.7 ± 7.6 ab	18.8 ± 5.5 b	0.020
Punctures/fruit	0.51 ± 0.11 b	1.05 ± 0.15 a	0.42 ± 0.11 b	0.26 ± 0.08 b	0.001
Punctures/infested fruit	1.4 ± 0.1 b	1.9 ± 0.1 a	1.2 ± 0.1 b	1.4 ± 0.1 b	<0.001
Offspring (%)	41.7 ± 7.5 a	22.1 ± 3.6 a	36.4 ± 12.0 a	20.0 ± 3.2 a	0.205

Different letters mean significant statistical differences (Tukey, $p \leq 0.05$) among the genotypes evaluated for each parameter.

Interestingly, 'Hojiblanca' exhibited the highest percentage of offspring, although no significant differences were observed among the different genotypes. Specifically, US-06-1388 had the highest percentage of offspring (41.7%), while the lowest percentages were found in US-06-194 (22.1%) and 'Kalamon' (20.0%). This study also revealed significant correlations among the variables under investigation. Notably, a strong correlation was observed between total infestation (%) and the punctures/fruit (R-Pearson = 0.95).

In Figure 1, the PCA-3D biplot successfully showed the ability to distinguish genotypes through an unsupervised approach, based on the outcomes of the multiple-choice bioassay (i.e., total infestation (%), punctures/fruit, punctures/infested fruit, and offspring (%)). This analysis confirmed that the preference behavior of *B. oleae* is influenced by the individual genotypes, as previously indicated in Table 3. Indeed, the behavior of *B. oleae* can be effectively differentiated among the four genotypes studied using the four associated variables. By visualizing the PCA-3D biplot, where the first three principal components (PCs) account for 99.5% of the data's variability, it becomes evident that genotypes US-06-194 and 'Kalamon' stand out distinctly from the other two. The group centroid of genotype US-06-194 is situated within the positive region of the three principal components, whereas the centroid of 'Kalamon' is positioned in the negative region. Among these genotypes, the fruit preference of the olive fly follows a specific pattern. Genotype US-06-194 attracts the highest preference from the olive fly, followed by US-06-1388 and 'Hojiblanca'. Finally, the PCA highlighted the factors that contributed to the distinction between genotype US-06-194 and 'Kalamon', in terms of the olive fly's preference. Specifically, the variables that played a major role were the total infestation (%), punctures/fruit, and punctures/infested fruit. On the contrary, when distinguishing between genotypes US-06-1388 and 'Hojiblanca' from the other two genotypes, the variable that had the greatest impact was the percentage of offspring.

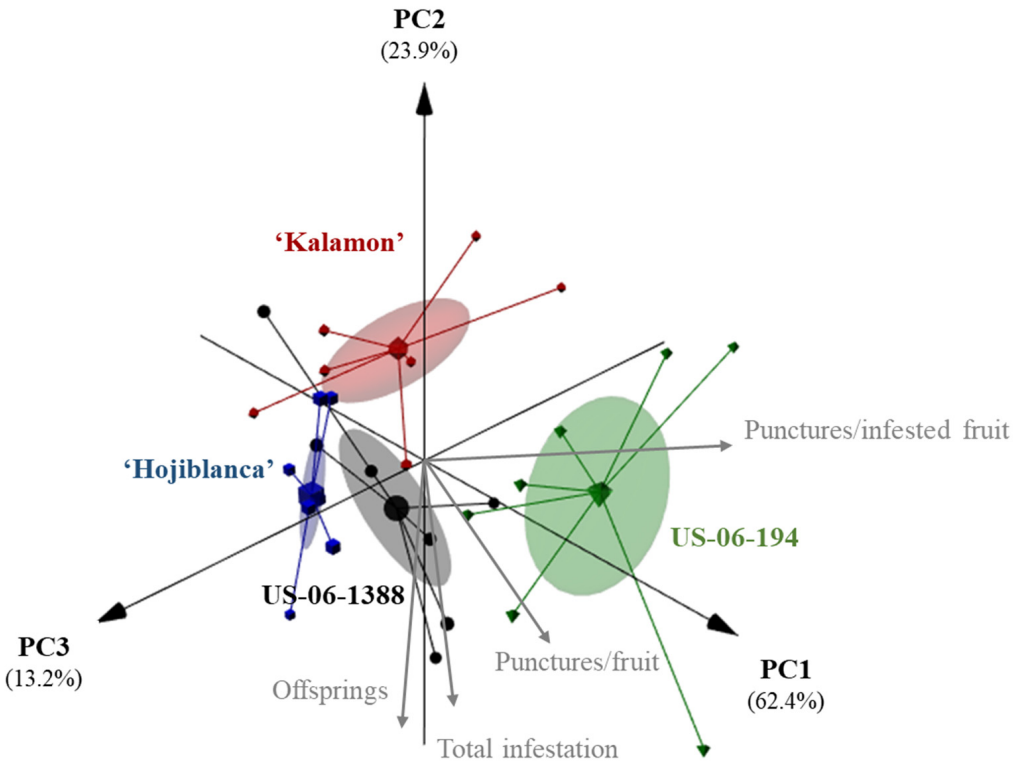

Figure 1. Principal component analysis (3D biplot) based on the behavior of *B. oleae* (Rossi) in a multiple-choice bioassay of oviposition with four genotypes (PC: principal component function).

3.3. No-Choice Oviposition Bioassay

In the no-choice oviposition bioassay, female olive flies were subjected to a single olive genotype, eliminating the possibility of making a true choice. The findings revealed that olive fly did not demonstrate any distinct behavior towards specific genotypes, and there were no significant differences observed. However, a pattern emerged indicating a slight preference of olive fly towards certain genotypes (as depicted in Table 4). 'Hojiblanca' fruit experienced fewer infestations by olive fly, resulting in the least amount of recorded damage. On the contrary, the genotype US-06-194 was identified as the preferred option for oviposition by the fly. Furthermore, it exhibited 0.81 punctures/fruit, which increased to 1.8 when considering only the infested fruit. The punctures/fruit and punctures/infested fruit were lower for 'Hojiblanca', 'Kalamon', and US-06-1388. Regarding offspring (%), 'Kalamon' and 'Hojiblanca' displayed the lowest values, although without significant differences among the others.

Table 4. Olive fly responses in the no-choice oviposition bioassay for 10 consecutive days (mean values ± standard errors, $n = 3$), on fresh fruit of four genotypes (US-06-1388, US-06-194, 'Hojiblanca', and 'Kalamon').

	Olive Genotype				*p*-Value
No-Choice Oviposition Biassays	**US-06-1388**	**US-06-194**	**'Hojiblanca'**	**'Kalamon'**	
Total infestation (%)	32.7 ± 9.9	40.3 ± 15.7	20.6 ± 5.6	25.8 ± 4.0	0.550
Punctures/fruit	0.47 ± 0.16	0.81 ± 0.40	0.28 ± 0.07	0.37 ± 0.07	0.464
Punctures/infested fruit	1.3 ± 0.1	1.8 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	0.173
Offspring (%)	41.9 ± 9.5	47.7 ± 7.1	33.3 ± 4.5	30.9 ± 7.4	0.396

Finally, predictive MLRMs were developed to provide insights into the possible selection of plant material less susceptible to olive fly, considering the various interactions that influence their choice for a specific genotype. These models utilize the data obtained

from bioassays conducted under controlled conditions for each genotype. It should be observed these models, as well as the trends observed, are valid for the specific controlled conditions studied.

Table 5 provides an overview of the physical and chemical traits of the fruit that could potentially explain the olive fly behavior, as well as their positive or negative contribution (according to the coefficient sign) to describe the observed preference behavior. The models utilized the key physical and chemical traits selected as the most relevant by the SA algorithm to ensure a satisfactory fit and accurate prediction (LOO-CV procedure) of the variable related to the behavior of the olive fly. The predictive models primarily incorporated physical traits such as fruit weight, longitudinal diameter, symmetry, CIELAB color attributes (L*, a*, and b*), and compression hardness. Among the chemical traits investigated, moisture, demethyloleuropein, oleuropein, rutin, and verbascoside were consistently chosen to predict the preference response of *B. oleae* towards the fruit.

Table 5. MLRMs for estimating (training) the attack preference behavior of *B. oleae* in no-choice oviposition bioassays.

Traits	Equations
Physical	Total infestation (%) = 1234.0 ($\pm$328.0) − 1.2 ($\pm$0.6) × [compression hardness] + 11.9 ($\pm$3.5) × [longitudinal diameter] + 50.8 ($\pm$14.8) × [symmetry] − 19.6 ($\pm$3.9) × [L*] + 136.0 ($\pm$27.0) × [a*] + 30.0 ($\pm$5.0) × [b*] R = 0.95; RMSE = 7.95
Chemical	Total infestation (%) = 460.0 ($\pm$64.0) − 4.8 ($\pm$0.8) × [moisture] − 1.3 ($\pm$0.3) × [demethyl oleuropein] + 40 × 10^{-3} ($\pm$8x10^{-3}) × [demethyl ligstroside] − 38 × 10^{-4} ($\pm$6 × 10^{-4}) × [oleuropein] + 24 × 10^{-3} ($\pm$3 × 10^{-3}) × [rutin] R = 0.98; RMSE = 4.77
Physical	Punctures/fruit = 2077.0 ($\pm$393.0) + 151.0 ($\pm$46.0) × [skin firmness] − 2.6 ($\pm$0.6) × [compression hardness] + 37.0 ($\pm$4.0) × [longitudinal diameter] + 103.0 ($\pm$15.0) × [symmetry] − 40.0 ($\pm$4.0) × [L*] + 286.0 ($\pm$29.0) × [a*] + 62.0 ($\pm$6.0) × [b*] R = 0.99; RMSE = 8.07
Chemical	Punctures/fruit = 1940.0 ($\pm$125.0) − 21.0 ($\pm$1.0) × [moisture] − 0.3 ($\pm$11.0) × [tyrosol glucoside] − 3.1 ($\pm$0.4) × [demethyl oleuropein] − 14 × 10^{-3} ($\pm$1 × 10^{-3}) × [oleuropein] − 26 × 10^{-3} ($\pm$4 × 10^{-3}) × [verbascoside] − 9 × 10^{-2} ($\pm$2 × 10^{-2}) × [rutin] R = 0.99; RMSE = 6.91
Physical	Punctures/infested fruit = 22.0 ($\pm$5.0) − 13 × 10^{-3} ($\pm$6 × 10^{-3}) × [compression hardness] − 2 × 10^{-2} ($\pm$1 × 10^{-2}) × [texture] + 0.4 ($\pm$0.2) × [weight] − 20 × 10^{-2} ($\pm$5 × 10^{-2}) × [L*] + 1.5 ($\pm$0.4) × [a*] + 0.2 ($\pm$0.1) × [b*] R = 0.97; RMSE = 0.09
Chemical	Punctures/infested fruit = 11.1 ($\pm$0.5) − 141 × 10^{-3} ($\pm$7 × 10^{-3}) × [moisture] − 13 × 10^{-3} ($\pm$2 × 10^{-3}) × [demethyl oleuropein] − 16 × 10^{-5} ($\pm$1 × 10^{-5}) × [ligstroside] − 36 × 10^{-4} ($\pm$8 × 10^{-4}) × [OleA] − 37 × 10^{-5} ($\pm$3 × 10^{-5}) × [verbascoside] − 31 × 10^{-6} ($\pm$5 × 10^{-6}) × [rutin] + 12 × 10^{-3} ($\pm$2 × 10^{-3}) × [A-7-G] R = 0.99; RMSE = 0.04
Physical	Offspring (%) = −594.0 ($\pm$102.0) + 1.1 ($\pm$0.3) × [compression hardness] + 16.0 ($\pm$3.2) × [longitudinal diameter] − 12.5 ($\pm$2.8) × [equatorial diameter] + 27.7 ($\pm$4.4) × [symmetry] + 9.1 ($\pm$2.1) × [b*] R = 0.97; RMSE = 4.58
Chemical	Offspring (%) = 40.0 ($\pm$6.0) − 14.0 ($\pm$2.0) × [oil content f.w.] + 6.2 ($\pm$0.9) × [oil content d.w.] − 1.4 ($\pm$0.2) × [demethyl oleuropein] + 0.6 ($\pm$0.1) × [OleA] + 9 × 10^{-3} ($\pm$2 × 10^{-3}) × [verbascoside] − 0.6 ($\pm$0.1) × [A-7-G] R = 0.99; RMSE = 3.13

The predictive performance of the developed MLRMs was assessed and the $R_{\text{LOO-CV}}$ and RMSE values are shown in Figure 2. The results showed that the MLRMs were able to satisfactorily predict the four variables related to the olive fly's preference for the fruit ($0.793 \leq R_{\text{LOO-CV}} \leq 0.945$).

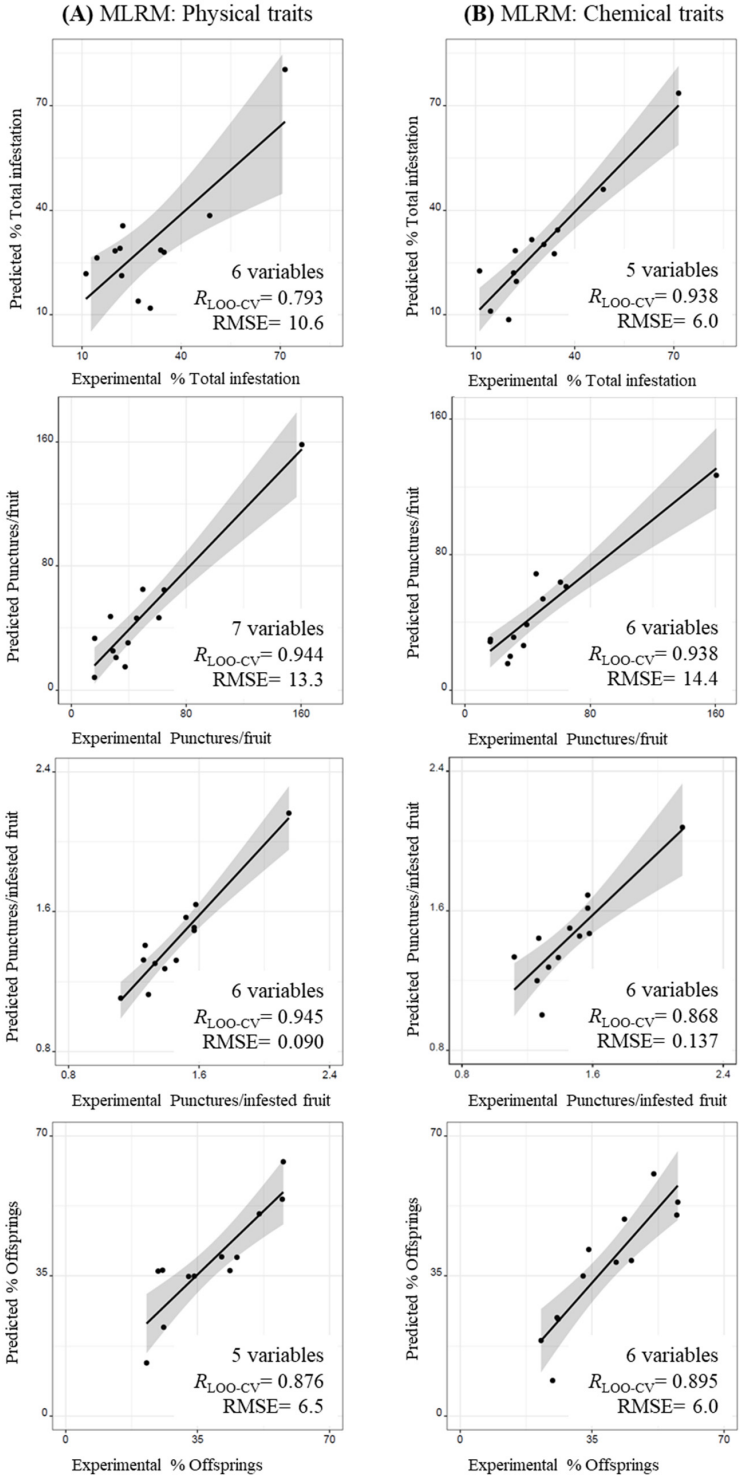

Figure 2. MLRM's predictive performances highlighting the behavior of *B. oleae* (Rossi) in no-choice oviposition bioassays, conducted under controlled conditions. (**A**) model based on physical traits of fruit and (**B**) model based on chemical traits of fruit.

4. Discussion

Understanding the factors that determine the preference of the olive fly for certain genotypes is essential to develop efficient selection tools to identify the most promising progenies and accelerate the breeding process. This goal was considered in the present work within the framework of the University of Sevilla's table olive breeding program.

Based on previous research findings [10,13,19–21], certain genotypes have a higher likelihood of being chosen by female olive fly for oviposition. Both multiple-choice and no-choice oviposition bioassays provided valuable insights (Tables 3 and 4), enabling the identification of the preferred genotypes. The behavior of *B. oleae* in the multiple-choice oviposition bioassay led to the unsupervised differentiation of the studied genotypes, as indicated by the PCA results (Figure 1). The results from the no-choice oviposition bioassay (Table 4) showed a similar trend, albeit without significant differences among the genotypes. This observation could be attributed to the olive fly's adaptation to its host, whereby even the less attractive genotypes still received egg-laying by adult female *B. oleae* due to the absence of alternative choices. These findings align with previous studies on the behavior of *B. oleae* towards commercial genotypes such as 'Hojiblanca' and 'Kalamon' [10,21], where significant differences compared to other genotypes were observed, with 'Kalamon' being less susceptible.

The choice of the olive fly in terms of oviposition may depend on various factors, including visual, olfactory, and tactile aspects characterizing the fruit, as well as phenological state, agronomic practices, and environmental conditions [12]. The predictive models developed in this study identified the most relevant physical and chemical traits of the fruit, displaying a good fit for the data. Olive fly behavior, measured by the total infestation (%), punctures/fruit, and punctures/infested fruit, showed positive associations (positive coefficients in the MLRM developed, as shown in Table 5) with fruit weight, longitudinal diameter, symmetry, a* and b* color attributes, and skin firmness. Conversely, negative relationships were found with L* color attribute, compression hardness, and texture, as can be inferred by the negative sign of the respective coefficients in the MLRM listed in Table 5. These relationships align with entomological reasoning and are consistent with other studies. Adequate fruit size, higher weight, and greener colors (a*) ensure conditions for larval development inside the fruit [22], providing more protection within the pulp. However, in certain cases, flies exhibit a preference for smaller-sized olives [10]. Similarly, our findings indicate a preference for more symmetric fruit, which is consistent with the findings of [23]. At first sight, these results seem to be in conflict with our breeding goals, since an adequate fruit size is critical for the table olive industry. Nonetheless, all the genotypes included in this study are suitable for table olive processing and, even 'Kalamon' and 'Hojiblanca', which exhibited relatively lower fruit size, are among the most important table olive varieties worldwide [15]. Furthermore, the predictive model developed would be very useful for the fast selection of less susceptible genotypes in early selection stages after a first screening for minimum fruit size and/or pulp-to-pit ratio. The large variability observed in progenies for most of the attributes considered by the model ensures the possibility of finding genotypes that combine both tolerance to olive fly and suitability for table olive processing. Moreover, genotypes with a high tolerance to olive fly according to the predictive model but insufficient fruit size could be used as progenitors in new crosses.

The fruit texture may hinder olive fly oviposition, leading to the fly's rejection of olives as a suitable host. Previous observations by [12] noted that females preferred ovipositing their eggs on fruit with higher PuS (puncture curve slope), representing the fruit's resistance to deformation. However, our results contradict this, as the olive fly demonstrated a preference for ovipositing on fruit with lower compression hardness and shear-compression force (texture) values, while exhibiting higher skin firmness. The positive relationship between the percentage of offspring and the hardness fruit suggests that successful larval development might be attributed to fruit with higher compression hardness.

Moreover, the predictive models also indicated negative relationships between olive fly oviposition and the contents of water and phenolic compounds (demethyl oleuropein, demethyl ligstroside, oleuropein, rutin, tyrosol glucoside, verbascoside, ligstroside, OleA). However, the percentage of offspring did not correlate with moisture levels. Excessive fruit moisture and high contents of phenolic compounds can negatively impact larval development and serve as plant defense mechanisms against pathogens and insects [21]. Additionally, it is noteworthy that fruit with high concentrations of oleuropein, such as

'Kalamon', are not preferred by *B. oleae* for oviposition. This finding is consistent with [24] but contradicts [13]. It appears that the olive fly's immature stage development is inhibited by oleuropein, particularly during the early stages of ripening. High concentrations of oleuropein may contribute to protecting the drupe from pests, as suggested by [25]. In the study conducted by [26], it was found that the larvae of olive fly, which feed on green olives, exhibit an overexpression of distinct genes. Other phenols also influenced the olive fly's choice, including demethyloleuropein, ligstroside aglycones, and oleuropein aglycones, although no significant differences were found among the studied genotypes. Additionally, the high level of fruit infestation in the US-06-194 genotype cannot be solely attributed to the content of oleuropein and other phenolic compounds, suggesting that multiple factors influence olive fly preference for different olive genotypes. The interaction of phenolic compounds with β-glucoside, an enzyme that induces the cleavage of oleuropein, was reported as a defense mechanism against phytophagous insects [9]. However, the differences in susceptibility to *B. oleae* among genotypes may also result from a combination of other chemical compounds such as volatiles [9] and fatty acids [12], which warrant further exploration.

5. Conclusions

The genotype US-06-194 stood out for being the most susceptible to the olive fruit fly, while 'Kalamon' was the least susceptible. This fact could be attributed in part to a higher oleuropein content, a lower compression hardness, and a lower symmetry of 'Kalamon' fruits compared to the rest of the genotypes studied. The lower susceptibility of the genotypes cannot be attributed solely to a set of physical and chemical fruit parameters; there are multiple factors that influence olive fly preference, which should be interpreted collectively. The proposed predictive models can be a useful tool for the selection of new genotypes that are less susceptible to olive fly infestation and can be an important strategy for table olive breeding programs. Predictive models based on fruit traits have shown a satisfactory fit and accurate prediction of the behavior of the olive fly in the no-choice oviposition bioassay. The consistently selected physical and chemical traits were compression hardness, longitudinal diameter, symmetry, and CIELAB color attributes (L*, a*, and b*), moisture, demethyloleuropein, oleuropein, rutin, and verbascoside. This selection tool, based on the above-mentioned traits, can contribute to significant resource savings in breeding programs by allowing for the quick, accurate, and cost-effective screening of the progenies under study.

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