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# Biochar-Derived Smoke Waters Affect *Bactrocera oleae* Behavior and Control the Olive Fruit Fly under Field Conditions

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**Abstract:** *Bactrocera oleae* is the key pest of olive production. Several attempts have been carried out over time to control it using biological solutions but with results rarely comparable to those obtained with chemical applications. The purpose of this work was to identify and test new compounds from samples of various Smoke Waters (SWs) for their effect on the fly, and given their low impact on the environment. SWs obtained from different feedstocks were used in in vitro and open field applications. SWs were shown to alter *B. oleae* fitness, acting on its microbiome, particularly on the presence and activity of the primary endosymbiont "*Ca.* Erwinia dacicola", and also to affect the behavior of the adult flies, altering the attractiveness of the drupes susceptible to attack. The effects recorded were concentration-dependent and varied among repulsion, up to 87% towards females, indecision, up to 70% towards males, and attraction, comparable to fresh green olives, based on the starting materials. These responses were confirmed in electroantennography trials and during two-years of field trials carried out in South and Central Italy. Gas Chromatography–Mass Spectrometry highlighted the presence of compounds such as guaiacol and hydroquinone as potentially important for the observed activity. Principal Component Analyses confirmed the proximity among SWs obtained from similar feedstocks. In controlled conditions, females appear to be more sensitive to the SW treatments. Field trials have shown how the effects of SWs can lead depression of infestation levels obtainable with other well-known compounds, such as kaolin clay and isopropyl-myristate (repulsive), or pheromones (attractive).

**Keywords:** fly microbiome; natural compounds; VOCs; biocontrol; *Candidatus* Erwinia dacicola

## 1. Introduction

The olive fruit fly *Bactrocera oleae* (Diptera: Tephritidae) (Rossi) (OLF) is the key pest in olive cultivation, since it can cause a significant loss of production [1,2]. The damage to the fruit [3] decreases quantity and quality, interfering with the biochemical drupe composition [4–6]. OLF infestation depends on cultivar [7,8] and landscape [9], and is influenced by the size of the drupes, the ripening period, the hardness of the epicarp,

the quality of chemicals inside the drupes, the harvesting method and by the presence of substances on the surface of fruits (e.g., kaolin, copper, honeydew or bird feces) [10–12].

The best known and most widely used strategies to control OLF are based on toxic bait and chemical pesticides [13,14]. Low-impact alternatives concern mass trapping techniques [15,16], and incompatible- and sterile-insect techniques; both have been investigated and used, but not always with appreciable effectiveness [17,18]. Over nearly 100 years, considerable efforts have been made to manage the olive fruit fly in southern Europe by numerous known natural enemies with results that are not always satisfactory [19–21].

The most important and abundant species in the OLF microbiome is the obligate bacterial endosymbiont "*Candidatus* Erwinia dacicola" [18,22–24]. These beneficial bacteria are harbored inside a cephalic organ, the oesophageal bulb or pharyngeal bulb, connected to the pharynx, in which the symbionts multiply rapidly, forming masses [25,26]. In a recent study it was demonstrated that "*Ca*. Erwinia dacicola" presence is crucial for OLF instar development in the drupes [27]. It is also confirmed by the high larval mortality in OLF rearings using diets supplemented with antibiotics due to the symbioticide effects [18,27,28], comparable to what is obtained with copper exposure [25]. These results point to the endosymbiont as a possible target for the control of the host, using compounds that directly act on it, altering its activity or abundance.

Biochar, a solid material obtained from the thermochemical conversion of biomass [29], is produced by pyrolysis at high temperatures (250 to >1000 °C) [30] under oxygen limited conditions. Several by-products are obtained during the pyrolysis process, such as bio-oils, volatile organic compounds (VOCs), and smoke aerosol with different particle sizes [31,32] with several interesting properties useful for conventional as well as organic farming [33]. In particular, smoke-derived compounds can stimulate the germination of seeds [34] of both wild and cultivated plants thanks to the activity of several combustion products including "karrikins" [35–38]. The most abundant and biostimulant among these compounds is the karrikinolide (KAR1), isolated and characterized for the first time from smoke waters (SWs) [39]. A recent study was conducted to assess the effect of SW on OLF, reporting a strong repulsive effect toward the adult fly during in vitro experiments [40]. In this context, the aim of the present work was to study the effects of SWs obtained from the pyrolysis of five organic feedstocks on OLF fitness, behavior and pest control under field conditions. The SWs produced under laboratory conditions by pyrolysis were characterized by gas chromatography-mass spectrometry (GC-MS) prior to use. The SW effect on the behavior and microbiome of OLF adults was assessed combining ingestion and topical bioassay with olfactometric and electroantennography (EAG) tests. The EAG is an electro-physiological technique that targets the olfactory responses towards volatile compounds. The technique is based on the measurement of the stimulus-dependent variation of the membrane electric potential (voltage) elicited by nerve cells that are sensitive to a certain stimulus. The EAG response represents the sum of the depolarization potentials of the membranes of the antennal olfactory neurons induced by the tested stimulus [41]. The higher the recorded depolarization is, the higher the antennal sensibility towards the tested treatment until saturation levels are reached. These responses can vary depending on the nature and number of stimuli and temperature, humidity, species, sex and the physiological conditions of the insects, such as if they are virgin or mated. SWs were previously tested separately basing on the starting raw materials, then using mixtures of SWs that exerted similar responses in the preliminary tests. SW treatment impacts were also evaluated at field scale at two study sites and compared with commercial products commonly used to control the OLF. Specific hypotheses were:

(i) ingestion of SWs can increase mortality and reduce the fitness of OLF;
(ii) SWs alter the OLF microbiome reducing the "*Ca*. Erwinia dacicola" titer;
(iii) exposure to SWs can hamper the ability of adult OLF to locate the position of fresh green olives in the lab; and
(iv) SW application can reduce the damage to olive production caused by the female OLF.

## 2. Materials and Methods

### *2.1. SWs Production*

The SWs used for the trials were obtained from five organic feedstocks: cellulose from filter paper (hereinafter referred to as CELL), sawdust of *Fagus sylvatica* L. (WOOD), *Zea mays* L. dried and finely ground (MAIZE), *Medicago sativa* L. hay (αα) and olive mill waste (Olive Waste) at two temperatures of pyrolysis: 300 °C and 500 °C [40]. From the original feedstocks, a total of ten SW types were thus obtained and labeled with a unique code using the name of the feedstock and the applied pyrolysis temperature. In addition, two blends were produced, the first one by mixing SWs made from olive mill waste at 300 and 500 °C in equal parts and named "MIX1". The second blend was produced by mixing all SWs in equal parts with the exception of those from Olive Waste (MIX2). All substances were stored at −20 °C until use.

### *2.2. SWs Chemical Characterization*

A gas chromatography-mass spectrometry (GC-MS) analysis was performed on the SWs, with and without derivatization with tetramethylsilane (TMS), to analyze both the neutral organic compounds and also the more polar compounds in the GC-MS [42,43].

GC-MS was performed using an Agilent 6890 GC connected to a 5973 mass selective detector (MSD) operating in the electron impact (EI, 70 eV) mode. The samples were injected in split mode (1:10) and separation was achieved using a 50 m × 0.2 mm, 0.32 µm, DB-5 ms (J&W Scientific, Folson, CA, USA) column with ultrahigh-purity (UHP) helium as the carrier gas (1 mL/min). The initial oven temperature was set to 40 °C and held for 1 min before increasing at 5 °C/min to 280 °C, and held for 10 min (inlet temperature, 280 °C; transfer line, 280 °C). The ion source was set at 200 °C, and the spectrometer was set to record from $m/z$ 45 to 500. Chromatogram peaks were putatively identified by comparison of electron mass spectra to matches (>95% similarity index (SI)) with the National Institute of Standards and Technology (NIST) 2011 library of mass spectra.

### *2.3. OLF Collection*

Infested olives were picked up from branches of pesticide-free olives *cv.* Leccino located in Parco Gussone of Portici (Naples–Italy), UTM: 445093,878 E; 4518644,785 N 33T, and stored in plastic boxes, with the aim of collecting from them wild new-emerged adult OLF for use in the laboratory assays. In addition, some branches were protected with dry straw enclosed in an entomological fabric sleeve, that slowed the maturation of the drupes for months, allowing us a constant availability of fresh green drupes all through the experimental period each year.

### *2.4. OLF Ingestion Bioassay and Effect on Microbiome*

Ingestion bioassays were carried out to assess the effect of SWs and pure karrikinolide (K) on the fitness of the exposed adult OLF. All the diets used in the bioassays were prepared by mixing the compounds or solution (0.5 mg) with sucrose (300 mg). 150 µL of distilled water was added to each diet in a Petri dish (3 cm ø) to obtain a uniform mixture of the diet components. Subsequently, the water was removed by evaporation in a controlled environment. Positive and negative control diets were made of sucrose only (CONT) and sucrose supplemented with the antibiotic Piperacillin (Sigma-Aldrich, S. Louis, MO, USA) (ANT) at a dose of 100 µg/mL. For the K ingestion assays, Karrikinolide 0.0001% ($v/v$) was added to the sucrose. For the SW ingestion assays we used MIX1 and MIX2. All the tests were conducted with four biological replications.

Olives with OLF oviposition signs were harvested every two weeks, stored in plastic boxes (35 × 25 × 15 cm) with ventilation holes on the top, with a layer of absorbent paper on the bottom, and placed in a climatic chamber at 24 ± 2 °C, 60 ± 10% Relative Humidity (RH) and 12:12 light/dark ratio. The OLF pupae were collected every two days and stored in smaller plastic containers (20 cm ø × 15 cm height) with pressure caps equipped with ventilation holes and stored in the same chamber. Once they emerged, the adults were

immediately collected with an aspirator in 15 specimens, separated by sex, in the same cylindrical plastic boxes. Each group was fed ad libitum for 14 days following the trials with the specifically intended diet. Each group's water supply was assured by cotton balls placed on the top of each cage, daily soaked. Mortality was recorded on the 14th day of each treatment for both sexes. The flies fed with the same diet were grouped in a variable number of specimens of both sexes' groups, depending on how many were still alive. Fresh green olives were exposed for three days (one per each female) in each cage, then the olives were replaced with the same amount of unripe fruits for another three days. All the flies were finally collected and then stored in 90% alcohol at −18 °C. All the diets were weighed before and at the end of the experiment, and food consumption was related to OLF mortality. The mean number of stings per exposed drupe/female was calculated. Half of the fruits with oviposition signs were dissected to evaluate the ratio of sterile stings to the number of laid eggs. Pre-imaginal and pupal mortality, sex ratio and new emerging adults were computed from the remaining half of exposed drupes, stored in a climatic chamber in the same conditions.

At the end of the ingestion assays, molecular analysis of the effects on the OLF microbiome were completed to evaluate the actual alteration in the "*Ca*. Erwinia dacicola" titer in the exposed flies. For the molecular data, four females from each group of each biological replication of the ingestion trials were used as a pool. "*Ca*. Erwinia dacicola" titer was assessed through absolute quantification after Real-Time qPCR analyses on the DNA extracted from the Oesophageal Bulbs (OB) and intestine (Ads) of the treated flies, using a slightly adapted high-salt protocol.

This protocol is focused on precipitation, so it does not need any specific kit, except for the TNES buffer, which is made of 2.5 mL 1 M Tris–HCl pH = 7.5; 4 mL 5M NaCl; 10 mL EDTA; 5 mL SDS 0.5% and water up to 50 mL. The frozen samples were quickly washed in Sodium Hypochlorite 5% and rewashed twice in distilled water, then left drying on sterile blotting paper, under laminar flow hood. Such treated samples were then dissected with a sterile scalpel separating the heads and the abdomens from the thoraxes. Each dissected part was disposed in a single Eppendorf safe-lock 1.5 mL tube, adding 35 µL of a solution of K Proteinase 20 mg/mL (Thermo Fisher Scientific) and 600 µL of TNES; then the content of each tube was smashed with a sterile pestle and the tubes were placed for 3 h at 55 °C. After that step, 166.7 µL NaCl 6M was added to each tube, which was then agitated by inversion 5 times in a row and centrifuged at 13,300 rpm per 7 min at room temperature (20 °C ± 2°C). The supernatant was gently recovered and placed in a new Eppendorf safe-lock 1.5 mL tube, adding an equal volume of Ethanol 100% (~600–700 µL) previously stored under ice for at least 30 min. The tubes were agitated by inversion 15 times and centrifuged in a cooled centrifuge (4 °C) at 13,300 rpm per 18 min. The ethanol was then discarded and replaced with 450 µL of cool Ethanol 70%, after other 5 agitations by inversion, paying attention to not discard the DNA also in each step. In the last step, 450 µL of room temperature Ethanol 70% were used and eventually discarded leaving the tubes open and in ice to allow the last Ethanol residues to evaporate. This final step had a variable duration, depending on the lab temperatures, with a minimum period of 25–30 min. The DNA pellet was re-suspended in 20 µL of distilled sterile water. The same extraction protocol was completed on at least 15 wild and non-treated adult OLF to obtain a standard curve to be compared with treated OLF. The subsequent step implied the cloning of the 16s region of the "*Ca.* Erwinia dacicola" rDNA on *Escherichia coli* vectors, using the TOPO® TA Cloning® Kit (Life Technologies-Thermo Fisher Scientific) and the same two primers were then used in the Real-Time qPCRs: EdEnRev [18] and EdF1 [24] to generate an amplicon of 90 base pairs (bp). The cloned, amplified and purified 16S region of the "*Ca.* Erwinia dacicola" rDNA portions (1 µL) was used for the Real-Time qPCRs, in three replicates, also adding 10 µL of SYBR® Green PCR 2X Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µL of each primer (10 ng) and water up to 20 µL in each plate well. A standard calibration curve was obtained using scalar concentration dilutions of the cloned 16S rDNA region. All the Real-Time qPCRs results here shown were completed on

a StepOne™ Real-Time PCR System (Applied Biosystems) with a first Hold step (10 min at 95 °C), then 40 PCR cycles (15 s at 95 °C and 60 °C for 1 min, each), a Melt Curve step and the final cooling step. The analyses were performed at the Institute for Research on Insect Biology (France) and the Tremblay Laboratory of the Department of Agriculture of the University of Naples "Federico II" (Italy).

All the retrieved Threshold Cycles (CT) were then analyzed with the formula:

$$\text{Number of copies} = \frac{X_{\text{ng}} \times 6.0221 \times 10^{23}{}_{\text{molecules/mole}}}{(N \times 660\ {}_{\text{g/mole}} \times 1 \times 10^{9}{}_{\text{ng/g}})}$$

where $X$ is the amount of DNA and $N$ is the number of base pairs. Using the CTs computed with the standard curve, it was possible to get the actual quantities of the 16S region of the "*Ca.* Erwinia dacicola" rDNA still presents in the specimens after each treatment and thus the amounts of the bacterium itself, by extension.

### *2.5. SWs Topic Bioassay*

Topic assays were carried out to check the possible effects elicited by SWs by contact on adult OLF. Drops of 1 µL of both MIX1 and MIX2 were used in the topic bioassays. A single drop was put on the thorax of a newly emerged wild adult of *B. oleae*. These specimens were separated by sex and collected in single-sex groups of 15 individuals each. Each group was fed with water and sucrose ad libitum and placed in optimal conditions (24 ± 2 °C, 60 ± 10% RH e 12:12 light/dark ratio). The same volume of drops of distilled water were-was used as control tests and seven biological replications were carried out for each assay. Mortality and behavioral data were collected each hour for the first 12 h, then every 12 h until the end of the second day of the assay, daily until the end of the week and, eventually, weekly until the specimens died.

### *2.6. SWs Olfactometry Trials*

The olfactometry trials were designed to assess the SWs effect on OLF behavior. Tests were carried out in a glass Y olfactometer with a constant flow of pure air and stable humidity level. Based on preliminary tests, 12 fresh green olives were the minimum amount that elicited attractiveness on at least 90% of adult OLF, so the same amount (OLIVES) was used for the controls, against clean air or 12 other olives combined with the compounds for the assays. All the SWs, including MIX1 and MIX2, were used at two concentrations (100% and 10%) to evaluate the repellent activity. Trials were carried out in an air-conditioned room (24 ± 2 °C; 60 ± 10% RH) on newly emerged wild adult OLF only during the hours between 09:00 and 15:00, for which the positive biorhythm of the fly is known under lab conditions [44]. 5 mL of a single SW were used on imbibing filter paper (Whatman, No. 4) of 9 $cm^2$ to evaluate the ability of the flies to locate the olives. The papers were placed in one of the two branches of the olfactometer with 12 fresh green olives. Overall, 30 males and 30 females were used for each trial.

Only one adult at a time was inserted in the Y from the bottom; then, it had 5 min to choose and walk across one arm of the olfactometer. If it could not select a clear direction in one of the two arms, the behavior was registered as "indecision". The three possible answers were collected, and all the data were analyzed through a $\chi 2$ test ($p$ value = 0.05) using the data obtained in both the controls as expected values. Between each trial, to reach the system's efficiency and to limit technical errors or interference by residues, the glass Y olfactometer was disassembled, cleaned, re-assembled inverted, and left subject to a clean airflow for 5 min.

### *2.7. OLF Electroantennography*

EAG tests were carried out to evaluate the intensity of the response to the exposure of the adult OLF antennae to a gradient (5, 10 and 15 µL) of each of the ten SW types. The SWs were dropped on a filter paper (Whatman, No. 1) square (1 $cm^2$), then introduced in

a Pasteur pipette (15 cm length) and used three times maximum, then the pipettes with the square paper inside were stored at −20 °C at the end of each trial. The EAG test was conducted with both adult males and females ($n$ = 5 + 5) for each SW, following techniques used in previous works [45–47]. Within both capillaries of the system, there was a Kaissling salt solution and a silver electrode. The purified and humidified air flow (500 mL/min) was constantly blown on the antenna through a glass tube (8 mm ø) whose extremity was placed 1 cm away. The extremity of the pipette containing the stimulus to be tested was inserted in a small hole (4 mm ø), 9 cm away from the end of the glass tube. Antennal stimulation was performed, blowing 2.5 $cm^3$ vapor present in the pipette in 0.5 s. The antenna-generated signal was sent to an amplifier IDAC-4 (Syntech, Hilversum, Netherlands) and visualized on a monitor using EAG PRO program (Syntech, Hilversum, Netherlands). The interval between two subsequent stimuli was 1 min. At the beginning of the series, at the end and every three trials, a control stimulus made of mineral oil and filter + water was applied to consider possible solvent effects and background noise. A standard reference stimulus (10 µL of a solution of (Z)-3-hexen-1-ol 1 µg/µL) was used to correct the potential reduction of the antennal sensibility during the tests. The amplitude of the EAG responses to the stimulus was expressed in millivolts (mV) and corrected and normalized using the already used formula [48]:

$$Sx(\text{corr.}) = \frac{Sx(\text{registr.}) \times (\text{Ref. (Is. } - 1) + \text{Ref. (Is } + 1)}{\text{Ref. (x } - 1) + \text{Ref. (x } + 1)}$$

where: $Sx$ (corr.) = response to the stimulus $x$ (mV) corrected with the decrease of the antennal sensitivity during the experiment; $Sx$ (registr.) = response to the stimulus $x$ (mV) recorded during the trial; Ref. (Is − 1) = response to the reference stimulus (mV) that comes before the first stimulus; Ref. (Is + 1) = response to the reference stimulus (mV) that follows the first stimulus; Ref. (x−1) = response to the reference stimulus (mV) that comes before the stimulus $x$; Ref. (x + 1) = response to the reference stimulus (mV) that follows the stimulus $x$.

### *2.8. SWs Effect on OLF under Field Conditions*

In 2019 and 2020, field tests were conducted using SWs in two 0.6 hectares of commercial pesticide-free olive orchards in Central and Southern Italy. The first orchard was located in Crotone, in the Calabria region (UTM: 681039 E; 4323690 N 33S), at 84 m asl, with a west-north-west slope. It was composed of 15-year-old olive trees cv. Frantoio. The second site was located in Pozzilli, Isernia province, in Molise Region (UTM: 424663 E; 4594475 N 33T), at 703 m asl, and was composed of 30-year-old olive trees cv. Leccino. The shape of the fields was rectangular, North-South oriented. Both areas were divided into homogenous clusters of 3 trees of similar size and the assignation of each set was determined randomly (Supplementary Figure S1a,b). Hereafter the two locations are identified as Crotone and Isernia, respectively. The experiment included MIX1 and MIX2. For each substance, three tubes (120 mm × 27 mm ø) containing 15 mL of the substances were randomly placed at regular distances from each other, around each canopy of the tree. Each tube when open was covered with a layer of Parafilm® M and closed with their screw cap appropriately modified with a single hole in the center to limit the quick evaporation of the mixtures from the tubes. Two negative controls were carried out using spray application of Kaolin (Surround WP-Novasource, Phoenix, AZ, USA) (CAO) and arranging tubes with 15 mL of isopropyl-myristate (PA ≥ 98%, Merck KGaA, Darmstadt, Germany) (MIRI), using the same criteria applied for SW dispensers. CAO and MIRI are well known to exert visual-tactile and olfactory repulsiveness [49]. As positive control we used chromotropic traps (*Dacus* Track plus –Serbios, Badia Polesine (RO), Italy) placed in the center of each randomly distributed cluster, both activated with the specific attractive OLF pheromone and 15 g of ammonium bicarbonate (Farmalabor) (YT + attr.) or not (YT). The tests were carried out during the OLF field trophic and reproductive activity period, with green olives susceptible to the attack still on the trees. The data were collected monthly from

August (moment of application of the treatments) to November. The numbers of catches are expressed as mean values; the males and females found on the chromotropic traps were computed together since no significat differences between the two sexes were recorded. At the end of the trial, 30 olives per cluster were randomly collected to evaluate the damage to the crop production.

### *2.9. Statistics and Data Analysis*

The data obtained during the chemical characterization were finally analyzed using the freely available web tool ClustVis with a Cluster Analysis with Euclidean distance and Ward method together with a Principal Component Analysis (PCA) with a standardization (autoscaling) and the Singular Value Decomposition (SVD). All the molecular and biological data observed during and at the end of the ingestion bioassays, the EAG data and the open field trials data were elaborated and analyzed with a one-way ANOVA and the Student–Newman–Keuls post-hoc test. $\chi^2$ tests were carried out for the olfactometry trials, using the results recorded in the controls as the expected ones.

## 3. Results

### *3.1. SWs Chemical Characterization*

The starting SWs, MIX1 and MIX2, were fully characterized throughout gas-chromatographic characterizations that highlighted some differences among their organic compound profiles (Figure 1). The differences among the profiles were smaller after derivatization with the silyating reagent for the more polar compounds (Supplementary Figure S2). A total of 37 main volatile and 16 non-volatile compounds were detected in >1% abundance. In the dendrogram, the closer SWs obtained with the clustering of the profiles are the Olive Waste 300 and MIX1, as also shown by the subsequent PCAs (Figure 1), both before and after the derivatization with TMS (Figures 1 and S2). Phenol,2-methoxy-, also known as "guaiacol", is among the most abundant molecules identified in all the SWs. Another characteristic molecule widely present is furfural. CELL SWs are close to each other in the clustering and far from all the other ones in the PCA. CELL 300 was the richest in VOCs, but only CELL 500 and WOOD 300 had hydroquinone. In the CELL 500 profile, Ethanone,1-(2-furanyl)- and Phenol,2-methoxy-4-methyl- are below 1% abundance, while they are present in the Olive Waste SWs and MIX1. The two CELL SWs are the only ones that showed the presence of maltol, also found in MIX2 (that contains both CELL SWs) where it is also more concentrated. The higher distance among the VOCs in the clustering is between cyclopentanone and methyl-pyrazine, which is present only in αα 300 and αα 500. After the derivatization, the only non-volatile compounds found in Olive Waste 500_TMS were Silane, [1,2-phenylenebis/oxy)]bis[trimethyl- and Benzaldehyde, 3-methoxy-4-[trimethylsilyl)oxy]-. Although αα 500 was the poorest SW in VOCs, as also confirmed by the distances in PCA, it was among the four richest in non-volatile compounds, along with CELL 500_TMS, MAIZE 500_TMS and CELL 300_TMS. The most extended interval in PCA, in this case, was between αα 500_TMS and WOOD 500_TMS.

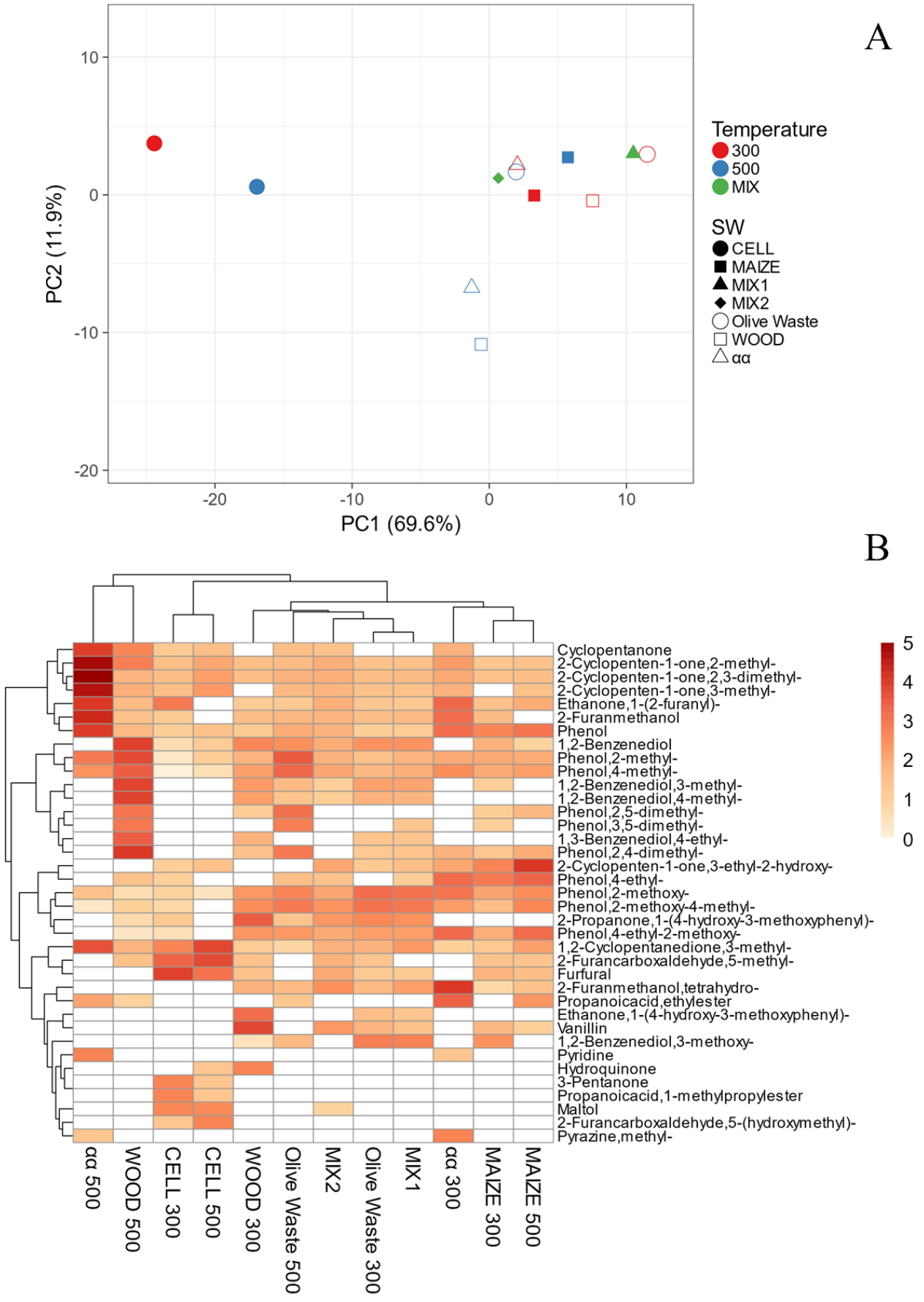


**Figure 1.** PCA analysis (**A**) and heat-map (**B**) of the relative abundance of the different compounds

identified in the individually produced SWs and their mixtures analyzed by GC −MS. αα 300 = SW obtained from alfalfa at 300 °C; αα 500 = SW obtained from alfalfa at 500 °C; MAIZE 500 = SW obtained from corn at 500 °C; MAIZE 300 = SW obtained from corn 300 °C; WOOD 500 = SW obtained from wood at 500 °C; WOOD 300 = SW obtained from wood at 300 °C; CELL 500 = SW obtained from cellulose at 500 °C; CELL 300 = SW obtained from cellulose at 300 °C; Olive Waste 500 = SW obtained from olive mill waste 500 °C; Olive Waste 300 = SW obtained from olive mill waste 300 °C; MIX2 = Mixture in equal parts of the SWs obtained from cellulose, wood, corn and alfalfa at both 300 and 500 °C; MIX1 = Mixture in equal parts of the SWs obtained from olive mill waste at both 300 and 500 °C.

### *3.2. OLF Ingestion Bioassay and Effect on Microbiome*

All the treatments elicited alterations in reproductive activity and fitness (Supplementary Figure S3). The number of the surviving specimens after 14 days of treatment highlighted a higher sensitivity of the females to the oral administration of the added diets. The recorded mortality was similar for each treatment, except for the negative control corresponding to sucrose and Piperacillin (ANT) used to purge the OLFs from their microbiome. The recorded diet consumption was similar among the trials, with the highest mean value observed with the addition of Karrikinolide (K). Generally, the number of stings was higher during the second exposure of the unripe olives. In particular, K lowered the laid eggs and increased, at the same time, the number of sterile stings. MIX2 limited the laid eggs, the overall number of stings and the number of sterile ones similarly to ANT. MIX1 altered the fitness of the exposed adult OLF less, with similar data to the positive control. The number of specimens that did not complete the whole life cycle and the number of the first generation emerged adults were variable among the treatments (Supplementary Figure S4). MIX1 led to higher mortality and the number of new adults was lower than for the CONT assays. In any case, with the MIX2 and K treatments, the pre-imaginal stage dead specimens were always more than the emerged adults. From the few eggs laid after the ANT exposition, no hatchings were recorded. The weighted averages of not emerged and emerged adults recorded in CONT trials were significantly higher than the other tests. The treatments did not alter the sex ratio of the adults (1:1) (Supplementary Figure S4).

The use of Karrikinolide and MIX2 strongly limited the proliferation of "*Ca.* Erwinia dacicola" in the Oesophageal Bulbs. Compared to the positive control, MIX1 increased the mean number of copies in both the OBs (1801) and Abdomens (0.1267). MIX2 led to 0.109 copies in the Abdomens, almost half of the control (0.0196). Mean data highlighted differences among treatments and between them, but with high variability related to the beginning and conclusion moments of each biological replication (Supplementary Table S1).

### *3.3. SWs Topic Bioassays*

The topical applications of the SW mixtures did not alter the behavior or movement of the treated OLFs during the whole test period (data not reported). Mortality rates were also comparable with what was recorded in the control (Figure 2).

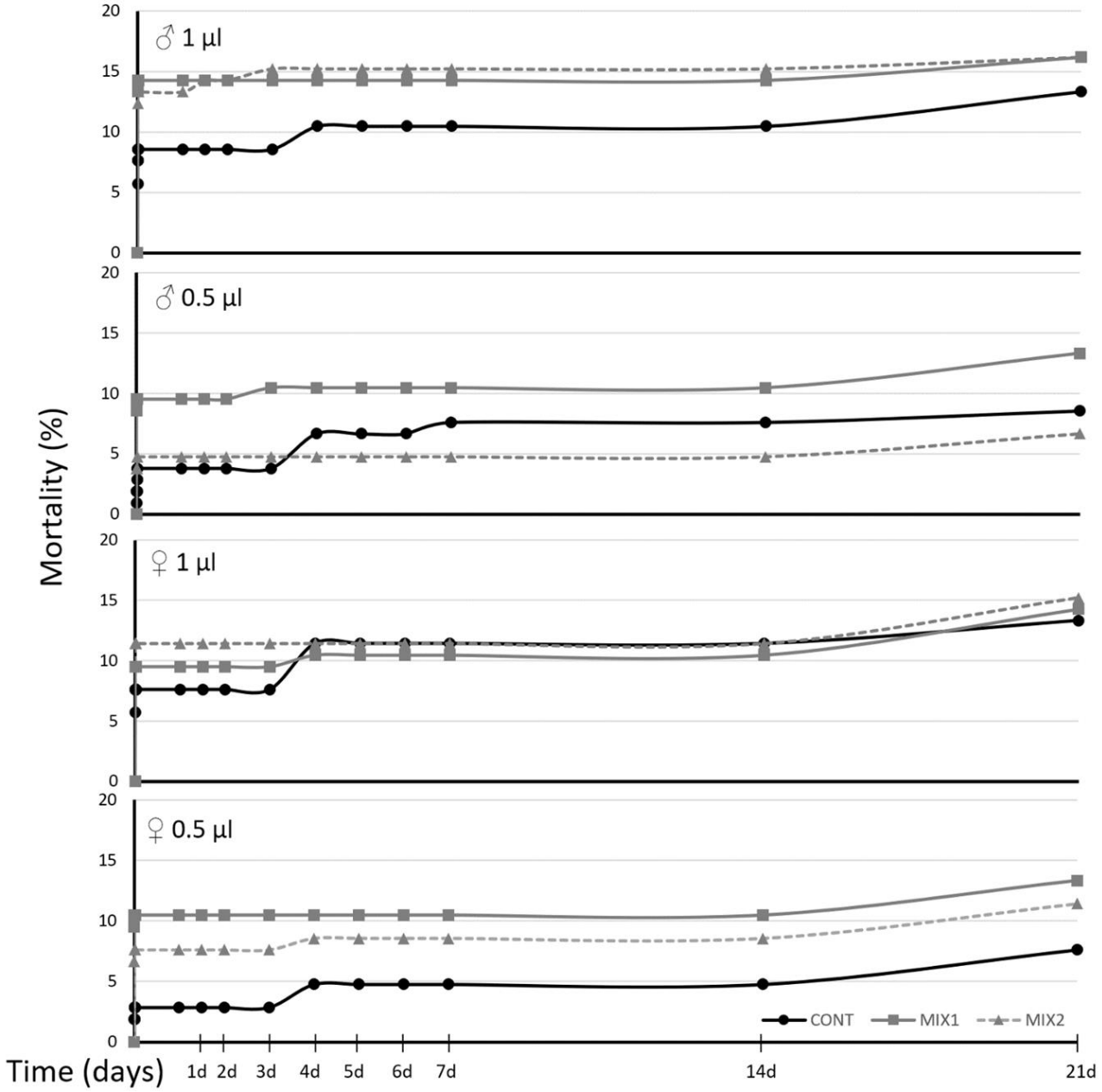


**Figure 2.** Mortality (%) recorded during the 7 replications of topic bioassays on adult OLF. Cont. = Control made with water drops; MIX2 = drop of mixture in equal parts of the SWs obtained from cellulose, wood, corn and alfalfa at both 300 °C and 500 °C; MIX1 = drop of mixture in equal parts of the SWs obtained from olive mill waste at both 300 °C and 500 °C.

### *3.4. SWs Olfactometry Trials*

The assays carried out with the ten SWs highlighted a concentration-dependent response (Figure 3). Neutral effects were recorded with the tested adults exposed to the αα SWs (300 and 500 °C) and MAIZE 300, while MAIZE 500 elicited more of a repellent effect (Supplementary Figure S5). The remaining SWs were repulsive for at least 50% of the tested OLFs. Olive mill waste SWs showed direct concentration-dependent effects, but the more robust response was the attractiveness. Both the olive mill derived SWs elicited attraction from at least 30% of males and females, when diluted at 10%. Olive mill SW made at 300°C and not diluted was attractive for 60% and 53% of males and females, respectively, while the Olive mill SW made at 500 °C was attractive for about 50% of both OLF sexes. All the response distributions were significantly different from the control Air-Olives, as

regards repellency, indecision and attractiveness. Non-diluted MIX2 was strongly repellent (Figure 3) towards males (73%) and females (86%). None of the females have ever chosen that arm in the tests, with a distribution of the responses significantly different of all the three controls ($p$-value < 0.005). MIX1 elicited strong preponderant indecision (70% and 56% of the tested males and females, respectively) when diluted to 10%. MIX1 resulted in attraction at both the concentrations tested, when offered in contraposition to clean air. The distribution of the recorded responses was comparable to the control Air-Olives and small indecision (13%) was registered with the females exposed to MIX1 10%. When the mixture was exposed in combination with olives, the distribution was again statistically similar to the control Air-Olives. The distribution of the responses was balanced when MIX1 was offered as an alternative to real olives. It elicited 16% and 20% indecision for males and females when tested diluted to 10%. The same parameter increased to 23% for the males but decreased to 6% for the females when the mixture was undiluted; in that case, the distribution of the responses was less different than the Air-Air control ($p$-value < 0.05) and similar to the Olives-Olives control. The combination of MIX1 + real olives as an alternative to other similar olives caused indecision (20% for males when diluted to 10%), also being significantly different from all the controls ($p$ values < 0.005). The indecision was halved for the females, leading to a similar distribution as with Olives-Olives control. The records were statistically comparable to the Air-Air control for both sexes and the Olive-Olive control also for the females when the mixture was tested at 100%.

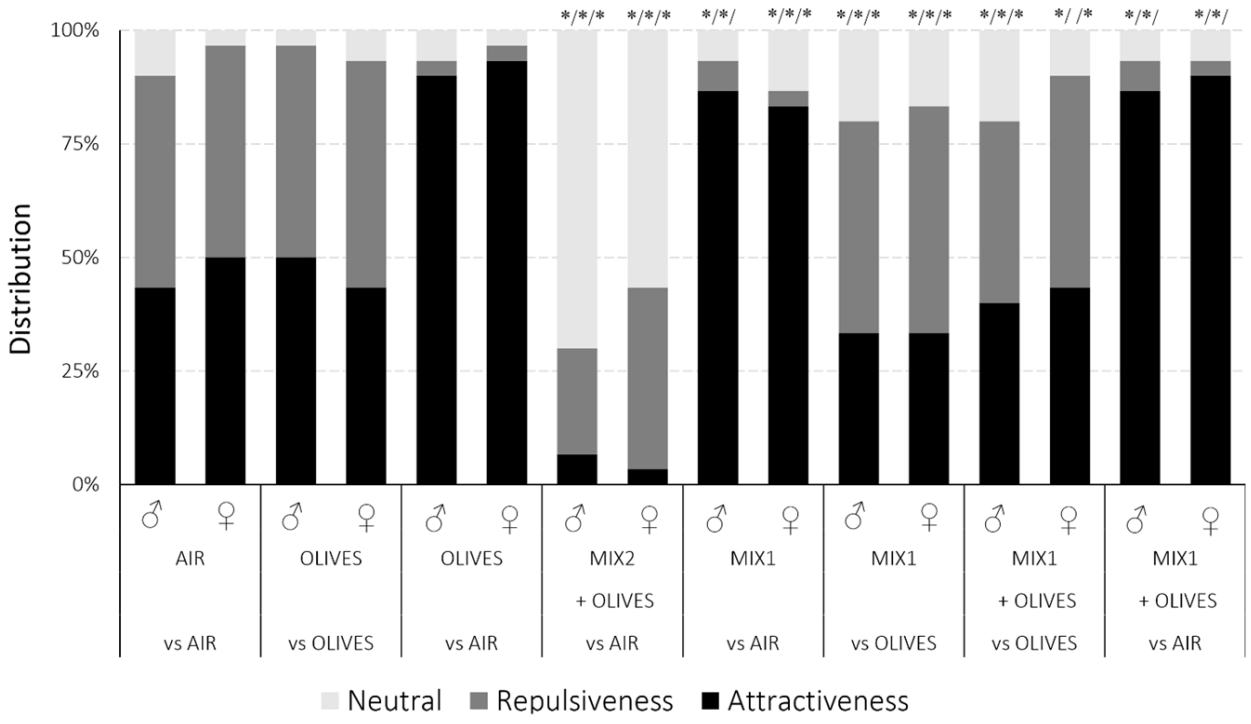


**Figure 3.** Distribution (%) of the responses among attractiveness (black), repulsiveness (grey) and neutral (light grey) of the male (♂) and female (♀) adult OLF in olfactometry tests with different SWs at 10% concentration. AIR vs. AIR = Control with both the lateral chambers left empty; OLIVES vs. OLIVES = Control with 12 olives (attractiveness) in each lateral chamber. AIR vs. OLIVES = Control with 12 olives, attractiveness, in contraposition with clean air; MIX2 + OLIVES vs. AIR = 150 µL of Mixture in equal parts of the SWs obtained from cellulose, wood, corn and alfalfa at both 300 and 500 °C offered in combination with 12 olives (attractiveness) and in contraposition to clean air; MIX1 vs. AIR = Mixture in equal parts of the SWs obtained from olive mill waste at both 300 and 500 °C offered in contraposition to clean air; MIX1 vs. OLIVES = Mixture in equal parts of the SWs obtained from olive mill waste at both 300 and 500 °C offered in contraposition to 12 olives (attractiveness);

MIX1 + OLIVES vs. OLIVES = Mixture in equal parts of the SWs obtained from olive mill waste at both 300 and 500 °C offered in combination with 12 olives (attractiveness) and in contraposition to other 12 olives (attractiveness); MIX1 + OLIVES vs. AIR = Mixture in equal parts of the SWs obtained from olive mill waste at both 300 and 500 °C offered in combination with 12 olives (attractiveness) and in contraposition to clean air. χ2 tests were carried out using the results recorded in the control tests as expected ones and are displayed, in order, referred to controls: */ AIR vs. AIR, /*/ OLIVES vs. OLIVES and /* AIR vs. OLIVES. * = significance ($p$ value < 0.005); empty space = without significance.

### 3.5. OLF Electroantennography

The intensity of the responses (Figure 4) was dose-dependent for both the sexes and almost all the SWs tested. The mean highest response was recorded with 15 µL of αα 500 for both sexes. MAIZE 500 elicited a stronger reactivity on the males' antennae when tested with 5 µL than 10 µL: 0.892 and 0.802 mV, respectively. The lower activity with the females was recorded with 5 µL of MAIZE 500 (0.338 mV) and 10 µL of the same test elicited the lower one for males (0.802 mV). The most significant differences were recorded for females with MAIZE 300 (15 µL) tests and MAIZE 500 (5 µL). In the EAG tests performed with the CELL SWs, there were no significant differences for females within the minimum and maximum average values recorded of 0.632 and 1.605 mV. The largest gap among the males' records was between the doses at 15 µL and 5 µL of CELL 300. Both sexes recorded the highest response with 15 µL of αα 500.

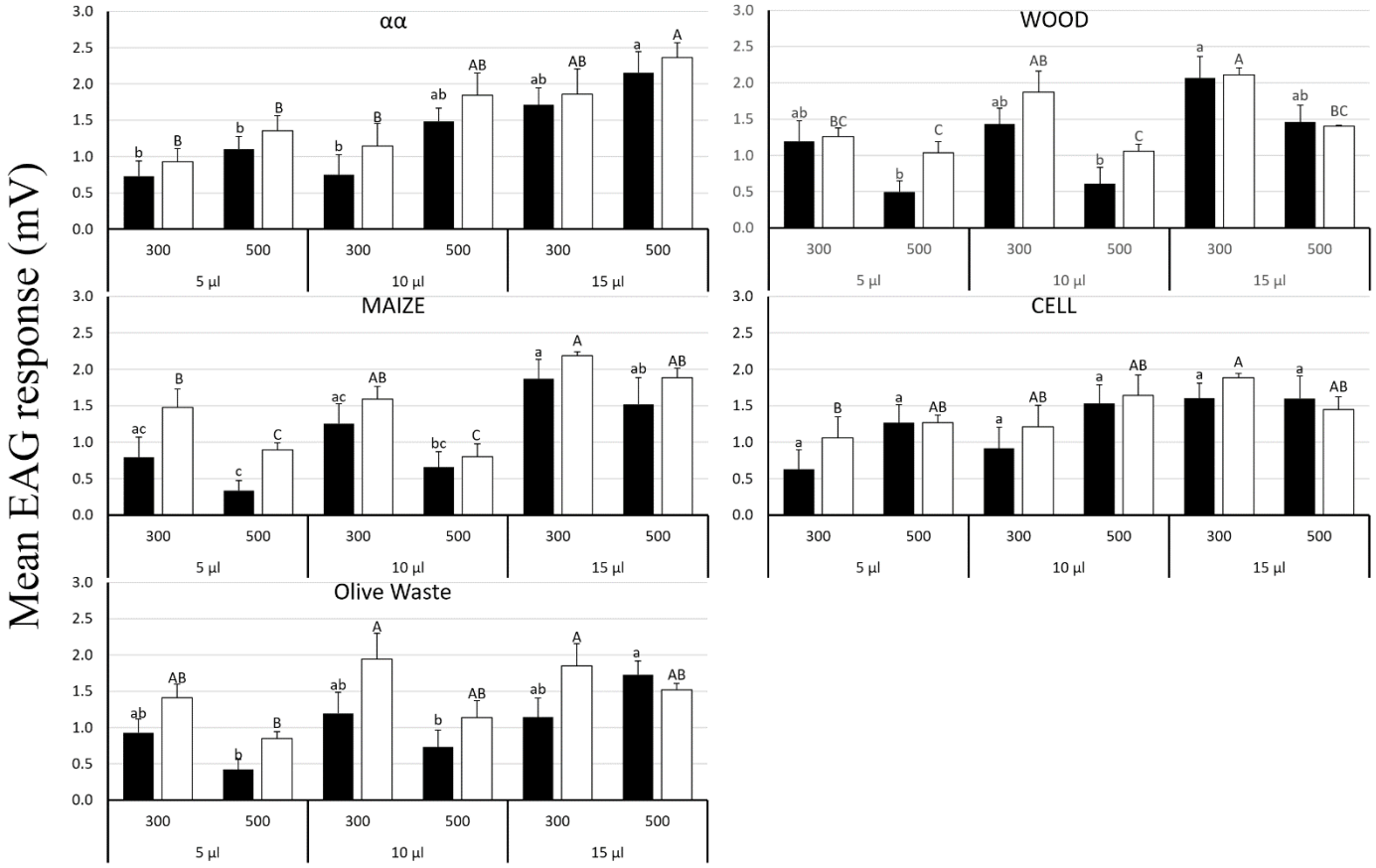


**Figure 4.** Mean EAG response (mV) recorded with female (black) and male (white) of two-week-old OLF to application of 5 µL, 10 µL or 15 µL SW as stimuli. Abbreviations as indicated in Figure 1. Different letters mean significance (ANOVA test, Post-hoc Student–Newman–Keuls test). Uppercase and lowercase letters refer to males and females, respectively.

### *3.6. SWs Effect on OLF under Field Conditions*

The mean catches in the MIX2 clusters were consistently low in Crotone, with the highest value of 9.6 specimens found at the harvest of the fruits. MIX1 led to a mean number of catches always more elevated than all the other clusters, all through the period of OLF activity, with mean values even higher than in both the controls (YT and YT + attr.). In Isernia, the catches were lower until October (Figure 5). On that second field, the MIX1 treatment resulted in an intermediate number of mean catches between the two controls all through the trial. MIX2 gave a strong repellency, comparable with the results obtained with kaolin clay and myristate. The highest mean number of catches obtained with MIX2 (4.6) was at the harvest of the olives (November), and was lower than those obtained in the CAO (8.0) and MIRI (7.8) clusters at the same date of collection.

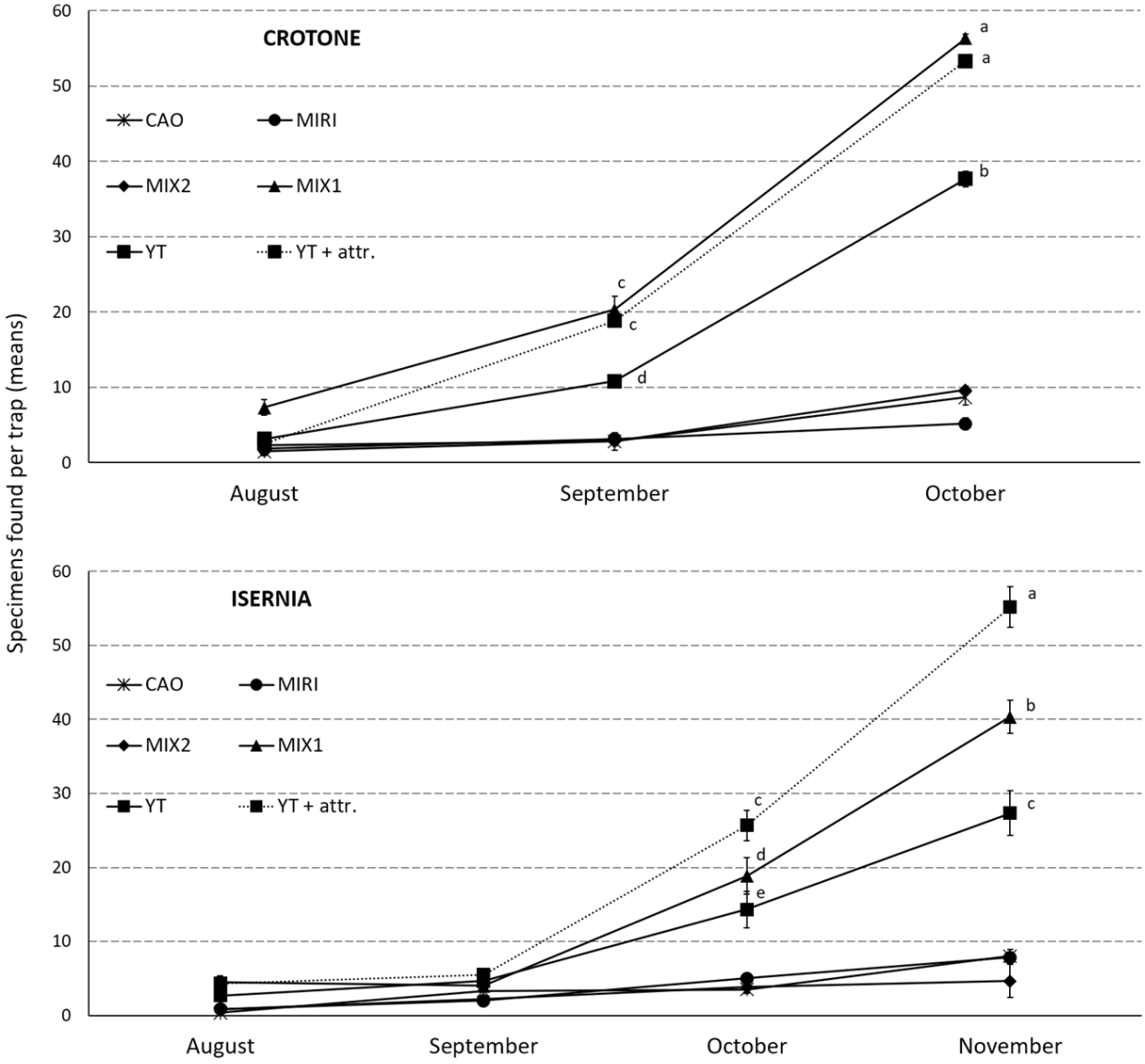


**Figure 5.** Catches (average ± standard deviation) of adult OLF recorded in Crotone (upper graph) and Isernia (lower graph) during 2019–2020. CAO = Kaolin clay sprayed on the crowns; MIRI = Isopropyl-Myristate, 15 mL per dispenser; MIX2 = Mixture in equal parts of the SWs obtained from cellulose, wood, corn and alfalfa at both 300 and 500 °C, 15 mL per dispenser; MIX1 = Mixture in equal parts of the SWs obtained from olive mill waste at both 300 and 500 °C; 15 mL per dispenser; YT = control (Yellow trap); YT + attr. = control (Yellow trap), panel + food bait and pheromone. Different letters mean significance, one-way ANOVA test, Post-hoc Student–Newman–Keuls test.

The number of stings per drupe recorded at the harvest time (November) had similar values on the two sites in correspondence of the same treatment (Figure 6). MIX2 limited the number of stings similarly to CAO and MIRI, leading to the lowest percentage of damage in Isernia and Crotone, respectively. MIX2 also limited the drupe damage to 73.3% (Crotone) and 90% (Isernia). The ratio between sterile stings and eggs laid was 0.9:1 in Isernia and 1.38:1 in Crotone. This was also the only case in which the sterile stings were more than the eggs. Notably, 100% of the collected drupes showed at least 1 sting with MIX1, as well as in the control clusters. These three treatments also led to the highest number of laid eggs on both sites. Similar statistical differences are also recorded for sterile stings.

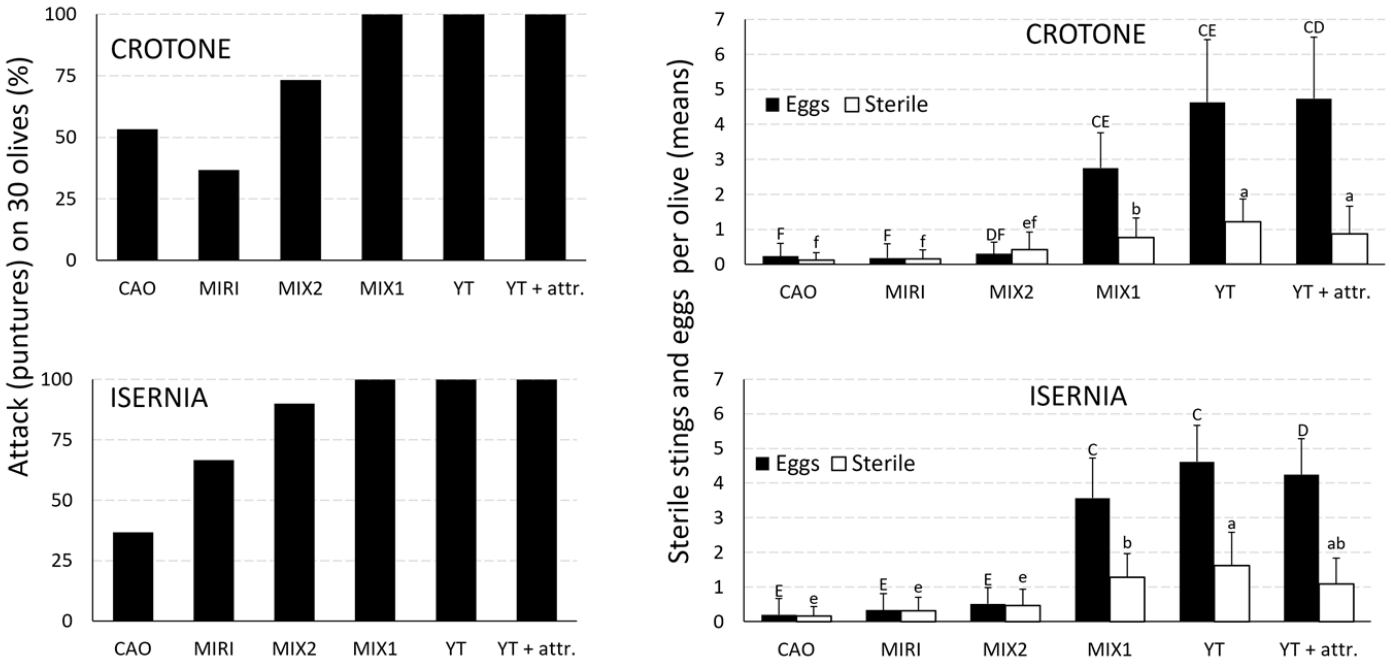


**Figure 6.** Mean number of stings with (black) and without (white) laid eggs of OLF recorded on the drupes at the harvest in the clusters of the field trials in Crotone (Calabria–Italy) (upper graphs) and in Pozzilli (Isernia, Molise–Italy) (lower graphs) and, left to each graph, percentage of oviposition stings on 30 random collected olives. Abbreviations as indicated in Figure 5. Different letters mean significance, one-way ANOVA test, Post-hoc Student–Newman–Keuls test. Uppercase and lowercase letters are exclusively referred to laid eggs and sterile stings respectively.

## 4. Discussion

In our study, SWs obtained from different feedstocks elicited specific effects on OLF microbiome, behavior and selection capacity. The negative effect of SWs, with the exception of that derived from Olive waste, also reduced the damage caused by OLF in field trials, suggesting a strong potential application for pest management.

All tests were conducted on adults of *B. oleae* collected from wild populations to simulate effects on the insect's microbiota similar to those of the open field. Our results showed that SW treatments alter the perception and development of OLF by limiting the capacity to identify mature olives and harming the development of subsequent generations. In particular, the MIX2, which is composed of SWs from various organic origins (cellulose, sawdust, corn and alfalfa) and has a complex but relatively balanced chemical composition of each of its constituent compounds, had a strong repulsive effect and reduced the fitness of the exposed OLFs. Similar results due to effects attributable to antibiotic activity, with symbioticidal activity, have been described for some essential oils obtained from *Thymbra spicata* L. and *Pimpinella anisum* L. [50], suggesting SW activity on the olive fruit fly. The high mortality recorded in several studies after the oral administration of the antibiotic confirm the direct positive relationship between the OLF and its symbiont [27,51,52].

Molecular assay showed a significant reduction of the symbiont "*Ca.* Erwinia dacicola" after Karrikinolide application. On the other hand, our tests exclude any topical SWs' effect. This suggests they may have a significantly low toxicological profile, unlike other natural extracts active against OLF, that are recorded in the bibliography as lethal at low concentrations [53,54].

In vitro assays showed variable effects between the different products, according to the concentration, as previously known [55]. The SWs obtained from cellulose pyrolysis exerted a strong repellency if used undiluted, but indecision and even attractiveness increased with the 10% dilution (Figure 3, Supplementary Figure S5). A similar dose-dependent trend for females was recorded with WOOD 500 and MAIZE 500. Differences among the responses depending on sex, age and mating status of the insects are considered as a common situation for Tephritids, particularly for *B. oleae* [56,57], which has a distinctive inversion for sex attraction compared to the other Tephritids [55]. Less concentrated SWs resulted in increased indecision, except for $\alpha\alpha$ 500 and $\alpha\alpha$ 300, which exerted repellency. The MIX2 almost totally reverted the attractiveness of olives when offered along with them. The strong repulsiveness exerted on the females by MIX2 at high concentration was replaced by indecision when the mixture was diluted to 10%. The response distribution for MIX1 produced from Olive waste is interesting since it attracts OLF as much as drupes would. Olive waste SWs and MIX1 are the only ones to show attractiveness for both adult OLF sexes in a dose-dependent manner. The attractiveness of MIX1 is the strongest for both sexes when offered alone or with fresh green olives and in opposition to clean air.

The EAG tests allowed us to check the electrophysiological alterations due to the olfactory stimulation [58,59]. In particular, it was highlighted that the responses differ depending on the raw materials keeping in mind that similar EAG responses may indicate opposite behaviors. The responses were dose-dependent, with differences between the sexes, despite the number of sensilla on the antennae and maxillary palps being similar in males and females [58,60]. In the literature, similar dose-dependent results were obtained at low concentrations of essential oils extracted from various cultivar olive leaves [61]. The GC-MS analysis of the SWs are consistent with the olfactometry tests. Lower distances on PCA among the VOCs' compositions have been recorded between Olive Waste 300 and MIX1, which also elicited similar responses in the olfactometry tests (Figure 3, Supplementary Figure S5). The most abundant compounds (revealed by the peak areas) that play a major role in the control behavior identified in the selected smoke waters are 5-methyl-2-furancarboxaldehyde, guaiacol and furfural. The 5-methyl-2-furancarboxaldehyde was often identified as a VOC involved in the fermentation process [62]; it is present in several plant species and its extracts and smokes are obtained during their combustion [63–67]. This compound is characterized by a roasted-smoked flavor [68] and can also be found in some extracts that could act with larvicide activity [69]. Guaiacol is probably responsible for the strong and persistent smoked smell that characterizes these waters; it is sometimes produced by insects' own gut microbiota [70] and can directly modify the behavior of the exposed adults and younger stages [71]. At the same time, the presence of furfural is interesting because this aldehyde has an LD50 of 400–500 mg/kg in mice, is irritating by contact [72] but can also inhibit mushrooms' tyrosinase activity [73] and can help in finding the appropriate oviposition sites when detected [74]. WOOD 500 expresses the highest repellency, particularly against females. At the same time, $\alpha\alpha$ 500 elicits the highest indecision; it is characterized by a simple chromatographic profile, with few significant peaks and its unicity is confirmed by the distance shown on PCA. The presence of hydroquinone and maltol could be strictly linked with the higher repellency elicited by CELL 300, CELL 500 and MIX2 [75,76].

The contrasting activities of MIX1 (attractive) and MIX2 (repulsive) reported during in vitro assays were confirmed during the two-year trials under field conditions. The two MIXs reached similar effectiveness as the already known and sold solutions for olive defense such as Mass Trapping, McPhail traps, kaolin clay organophosphates and the Attract and Kill techniques [11,14,77]. The distance and the different pedoclimatic conditions of the

two sites have allowed us to contain the effects of variability due to external factors, which can alter the speed of development of the populations [78,79]. The records of the two summer/autumnal consecutive seasons gave similar results for both the sites. The treatment effectiveness against OLF is greater with higher population rates and can exceed what we had found in the two-years trials [80]. The repellency of MIX2 was comparable to that of kaolin clay [81] and myristate; it is already known as a vegetal VOC strongly active on OLF [49]. In contrast, MIX1 showed a higher attractiveness than the controls, but was not significant on both sites. Interestingly, the attractiveness of the MIX1 is exclusively due to molecules derived from the host plant, therefore of vegetal derivation, and absent in the sexual interactions between males and females [58,82]. The actual damage to the production in terms of the number of stings per drupe was strongly lowered with MIX2 treatment, underlining a drastic and significant reduction of OLF activity, similar to what was obtained with kaolin clay and copper [83,84]. Based on the data obtained, it is possible to conclude that SWs could provide some control over OLF populations under field conditions by acting on the target and its microbiome, as well as on the fly's behavior, with potential future use as attractant (MIX1) or repellent (MIX2). Further studies can be useful to allow the development of applications of these active substances and their strategies and technical requirements, aligned to the indications and suggestions of the Integrated Pest Management approach.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12112834/s1, Supplementary Figure S1a: Cluster disposition during the trials carried out in Crotone (Calabria – Italy) during 2020. Supplementary Figure S1b: Cluster disposition during the trials carried out in Pozzilli (Isernia, Molise – Italy) during 2020. Supplementary Figure S2: CA analysis (A) and heat-map (B) of relative abundance of the different compounds identified in the SWs and their mixtures analyzed by GC-MS after the derivatization with tetramethylsilane (_TMS). Supplementary Figure S3: Number of laying stings made by adult OLF and recorded after the ingestion bioassays. Supplementary Figure S4: Number of recorded specimens in the first OLF generation after the ingestion bioassays. Supplementary Table S1: Number of copies of "*Ca*. Erwinia dacicola" recorded with Real-Time qPCRs on OLF Oesophageal bulbs (BULBS) and abdomens (ABDOMENS) after ingestion assays. Supplementary Figure S5: Distribution (%) of the answers among attractiveness (black), repulsiveness (grey) and neutral (light grey) of the males (♂) and females (♀) OLF adults in olfactometry tests with the ten SWs presented, at 10% concentration.

**Author Contributions:** G.J.: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Resources, Data Curation, Writing–Original Draft, Visualization; S.L.: Conceptualization, Methodology, Validation, Resources, Writing–Review and Editing, Supervision; G.B.: Conceptualization, Methodology, Validation, Resources, Writing–Review and Editing; G.F.: Investigation, Resources; S.G.G.: Investigation, Formal analysis, Resources, Visualization, Supervision; M.P.: Investigation, Formal analysis, Resources, Visualization; D.G.: Investigation, Resources, Writing–Review and Editing, Supervision; A.B.: Investigation, Resources, Writing–Review and Editing; F.V.: Conceptualization, Methodology, Validation, Resources, Writing–Review and Editing, Supervision. All authors have read and agreed to the published version of the manuscript.

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