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Contact Toxicity and Ovideterrent Activity of Three Essential Oil-Based Nano-Emulsions against the Olive Fruit Fly *Bactrocera oleae*

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Abstract: The control strategies for the olive crop key pest, *Bactrocera oleae*, involve synthetic chemical insecticides and few eco-sustainable alternatives, such as ovideterrents and lures. In the last few decades, the interest concerning the formulation of botanical based biopesticides increased, but little research investigated the suitability of these approaches for *B. oleae* control. This research aimed to investigate the residual contact toxicity and the oviposition deterrence of three essential oil (EO)-based nano-emulsions (*Pimpinella anisum*, *Foeniculum vulgare*, *Mentha* × *piperita*) against *B. oleae* adult flies. All the nano-emulsions possessed optimal physical characteristics, with droplets dimensions ranging from 115 to 152 nm and low PDI values (<0.2), even after 1 year of storage. Although no notable residual contact toxicity was noted, all the tested formulations reduced the number of oviposition puncture in no-choice tests (percent repellence: mint < fennel < anise). In choice trials, olives treated with fennel and anise EO-formulations at the highest concentration (7.5%, 75 g of EO/L) were less attractive respect to control fruits and a significant reduction of olive punctures was recorded. Nano-biopesticides are promising eco-friendly tools to integrate *B. oleae* pest management programs and to reduce the use of harmful conventional active ingredients.

Keywords: nano-insecticide; botanical; repellence; Tephritidae; IPM

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1. Introduction

The olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is the worldwide key pest of olive crop; this species belongs to the Dacinae sub-family, is strictly monophagous and can develop exclusively inside the drupes of *Olea* species in field. The flies of the Dacinae sub-family are generally present in the tropical regions, while *B. oleae* is also abundant in the northern Mediterranean, causing severe yield reduction [1]. In this area, the olive fruit fly population increases during summer until the harvest season, producing from 2 to 7 generations per year [2].

The control of this pest relies on different approaches, including: (*i*) chromotropic and chemotropic traps, (*ii*) sex pheromones, (*iii*) attract and kill baits, although (*iv*) chemical control remains the main strategy against this pest [3]. Due to the high problems related to development of resistance and environmental hazard [4], innovative eco-friendly approaches have been proposed and investigated. Oviposition deterrents are one of the innovative methodologies, which has been largely used in the last decade to protect olive production from *B. oleae* infestation [1]. Indeed, the susceptibility to insect-pest infestation results primary from the ovipositional preferences of mated females and from the attractiveness of the oviposition substrates [5,6]. The most diffused oviposition deterrents are kaolin and copper compounds, allowing high-quality oil production but causing moderate harm toward non-target predators [7].

Botanical insecticides have been widely investigated as alternative pest management tools to conventional pesticides because they are considered safe and environmentally friendly [8], but little information is available about their effectiveness and suitability in real field conditions [9]. Plant secondary metabolites can have excellent bioactivity against insect pests, acting as toxicants, deterrents, repellents or growth regulators [10,11]. Essential oils (EOs) are botanical extracts with acknowledged biological activity against several arthropod pest species [11–14], which can be used to develop novel nano-pesticides with prolonged persistence in field conditions [15].

In this scenario, this study aimed to develop stable EO-based nano-emulsions and to test their residual toxicity and oviposition deterrence against the olive fruit fly under laboratory conditions, simulating *B. oleae* field treatments during the late season. Indeed, few efforts have been carried out to investigate the bioactivity of EOs, and botanical extract in general, against *B. oleae*. Furthermore, the toxicity of EOs against the olive fruit fly has been tested mainly through ingestion toxicity trials, by formulating protein-based baits [16–18] and also by contact toxicity [19]. Repellence or oviposition deterrence of these botanicals have been neglected despite olive yield losses are directly linked to female oviposition and to larval growth inside the drupae; thus, research about the oviposition preferences and the semiochemicals involved in oviposition sites selection is crucial [20–22].

2. Materials and Methods

2.1. Plant Material

Olives (cv. Carolea) were collected from an organic olive grove placed in southern Italy, near the town of Delianuova (38°1458.0″ N; 15°5509.8″ E), in October and November 2020 from irrigated 20-year-old olive trees. Plants from which the olives were harvested were not treated with any pesticides for over a year before the experiments. Olives were manually collected, stored inside aerated PVC containers, and transferred to the laboratory within 5–3 h. First, the ripening stage (0–7) of the fruits was assessed using the maturity index (MI), considering the color of olive skin and pulp [23]. Ripe fruits with red-black skin (MI $\geq$ 3) were discarded, as well as completely unripe fruits (MI = 0). Fruit with soft pulp and less than 50% of the skin turned red-black are the most suitable for *B. oleae* oviposition [24], thus these fruits were used for further experiments. Mechanically damaged and *B. oleae* infested fruits were also removed, and intact and completely undamaged olives were selected. Drupes were stored for maximum 48 h at 5 °C and then used in the trials.

2.2. Bactrocera oleae Mass-Rearing

Bactrocera oleae was reared as described by Canale et al. [25]. The original annual colony was obtained from pupae and larvae collected from olives in a local pressing mill in Delianuova (RC, Italy), from late September to November. Pupae (ca. 500) were placed in plastic containers with vermiculite to avoid excessive moisture. Then, the pupae were transferred to rearing cages BugDorm-6S610® (MegaView Science Education Services Co., Ltd., Taichung, Taiwan) under controlled conditions (22 ± 1 °C, 50 ± 5% RH, natural photoperiod). Emerged adults were fed with a dry diet of yeast extract and sucrose (1:5 *w:w*), while water was provided ad libitum with soaked cotton wicks connected to a plastic container.

2.3. GC-MS Analysis of EOs

Commercial EOs of *Pimpinella anisum* L. (Apiaceae) (anise), *Foeniculum vulgare* Miller (Apiaceae) (fennel) and *Mentha* × *piperita* L. (Lamiaceae) (mint) were purchased from Esperis S.p.A. (Milan, Italy).

The chemical characterization of the purchased EOs was attained following the methods described by Giunti et al. [26]. Briefly, a Thermo Fisher TRACE 1300 GC with a MEGA-5 capillary column (30 m × 0.25 mm; coating thickness = 0.25 µm) and a Thermo Fisher ISQ LT mass detector (ionization mode: EI; scan time: 1.00 s; scan mass range: 30–300 *m/z*) were used setting injector and transfer line at 250 and 240 °C, respectively, and

a temperature ramp from 60 to 240 °C at 3 °C min^{-1} (carrier gas: He 1 mL min^{-1}). The pure EOs were diluted (1:10 *v:v*) in hexane (95%, Sigma Aldrich, Munich, Germany) and 0.2 µL were injected at a split ratio of 1:30. The identification of peaks was made using computer matching against the commercial libraries (NIST 05, Wiley FFNSC and ADAMS) and by comparing linear retention indices (LRI), calculated using a commercial series of *n*-hydrocarbons (C7-C30 saturated alkanes standard mixture, Supelco®, Bellefonte, PA, USA) [27], with those of pure/known substances and the MS literature data [28–32].

2.4. Nano-Emulsion Formulation and Physical Characterization

EO-based oil in water (O/W) nano-emulsions were obtained following the methodologies described by Giunti et al. [26]. Nano-emulsions were obtained by self-emulsification processes followed by a short sonication (UP200ST ultrasonic immersion homogenizer, Hielsher©, Teltow, Germany). First, the oily phase (30 g EO + 10 g Tween 80®, Sigma Aldrich, Munich, Germany) was mixed, then the aqueous phase (160 mL of bi-distilled water) was added, and last the obtained coarse emulsion (15% EO, 5% Tween 80 and 80% water) was sonicated for 5 min at 100 W power.

The nano-emulsions were stored at 25 ± 0.5 °C in an airtight stainless-steel bottle and used for the Dynamic Light Scattering (DLS) analysis after 24 h and for the bioassays within the following 7 days. Furthermore, 100 mL of the developed nano-emulsions were separately stored at 25 ± 0.5 °C in a similar airtight stainless-steel bottle and used to determine the stability of the formulations over time, performing additional DLS analysis after 1 year of storage. The physical characteristics (i.e., dimension, homogeneity and surface charge of the micelles) were determined using a DLS instrument Zetasizer Nano® (Malvern Panalytical Ltd., Malvern, UK). To avoid multiple scattering effects, the developed nano-emulsions were diluted (1:200 *v:v*) in bi-distilled water prior to the analyses. The instrument provided the droplets dimension, expressed in terms of average size (nm) and polydispersity index (PDI) and surface charge, as zeta potential (ζ) values (mV) and conductivity (mS/cm). Three samples were analyzed as replicates.

2.5. Residual Contact Toxicity

The bioactivity of EO-based nano-formulations was tested according to the fruit-deep technique. This method aims to determine the toxicity of the formulations against adult *B. oleae*, simulating a field treatment applied directly on the canopies of olive trees. In this case, the adult olive fruit flies may subsequently come in contact with the treated vegetation and fruit, thus to be affected by the residues of the treatment causing acute toxicity.

Sequential dilutions of the nano-emulsions were therefore prepared, considering 7.5% of active ingredient (75 g/L) the maximum tested EO concentration, because preliminary investigations highlighted damages towards plant tissues when higher application rates were used. Five serial dilutions of EO-based nano-emulsions were thus prepared for bioassays (7.5, 5.0, 3.75, 2.5 and 1.25% of EO in water). The diluted formulations maintained the same physical properties (e.g., droplet dimension) of the original nano-emulsions, because the addition of bi-distilled water does not interfere with the stability nor the characteristics of the micelles. Intact olives selected according to the previous criteria were then singly immersed in diluted nano-emulsions for 15 s and subsequently allowed to dry on a glass Petri dish (9 cm) for about 30 min at room temperature.

Ten olives treated with the same concentration of the same nano-emulsion were then placed inside transparent PP arena (length × width: 20 cm × 20 cm; height: 5 cm), closed by a nylon mesh lid to allow ventilation. In addition, a water dispenser and *B. oleae* adult diet were placed inside the arena. Lastly, 20 *B. oleae* sexually mature adults (10 males and 10 females, 18–25 days old) were gently transferred in the arena. Both females and males tend to spend time on the olives, the former looking for oviposition sites, the latter looking for viable mating sites. Residual contact toxicity was evaluated by determining the number of dead adult *B. oleae* after 96 h of exposure to treated olives. Flies were considered dead if they did not move or if they were unable to walk or fly without being externally

stimulated. The negative control was performed using olives immersed in double-distilled water. Four replicates were then prepared for each essential oil and each dilution, as well as for the control.

2.6. Oviposition Deterrence

2.6.1. No-Choice Bioassays

The no-choice oviposition tests were planned as described for the residual contact toxicity bioassays. The same EO concentrations were applied (7.5, 5.0, 3.75, 2.5 and 1.25% of EO in water), and the same arena set up was used (i.e., 10 treated olives for 10 sexually mature *B. oleae* females and 10 males). Olives immersed in double-distilled water were used as control. For each treatment (i.e., different doses of each essential oil and control) four replicates were provided.

Flies were left inside the arenas for 48 h in order to allow the egg laying; then the specimens were removed from the cages and the oviposition punctures (i.e., the signs left on the fruit skin by the insertion of the female fly ovipositor in the fruit pulp) were counted. Olives were observed using a stereomicroscope Stemi 2000-C (Zeiss, Jena, Germany).

The number of oviposition punctures was used to determine the percent repellency (PR) for every replicate. For every replicate the PR was calculated as follows:

$$PR = [(\text{No. ovipositions control} - \text{No. ovipositions treatment})/\text{No. ovipositions control}] \times 100$$

2.6.2. Choice Bioassays

The evaluation of the ovideterrence of the EO nano-emulsions was also carried out under choice conditions (i.e., choice test). The tests were carried out using the same arenas described above, and provided with food and water. The total number of olives exposed to flies per replicate was then the same as the no-choice trials (n = 10), but EO-treated (n = 5) and untreated/control (n = 5) olive fruits were placed at the two opposite sides of the above-described arena to allow *B. oleae* flies to choose the more suitable oviposition sites. Similar to no-choice trials, olives were collected after 48 h and the number of oviposition punctures were counted. The number of oviposition punctures was used to determine the percent repellency (PR) for every nano-emulsion and concentration tested according to the formula reported above.

Based on the results from the previous experimentation, three concentrations (7.5, 3.75 and 1.25%) were tested for each nano-formulation and three replicates were arranged. PR values for every replicate were calculated with the above-described formula.

2.7. Data Analysis

All the data were analyzed using SPSS® 20 and SAS JMP® 11 software. The characteristics of the developed nano-emulsions were compared by non-parametrical tests, the Mann–Whitney U test (i.e., fixed factor: storage time) and the Kruskal–Wallis H test followed by Dunn's post hoc test (i.e., fixed factor: EO plant source or storage time* EO plant source).

Bioassay results about the residual contact toxicity (i.e., mortality) and the oviposition deterrence (i.e., PR values) were analyzed using a series of Kruskal–Wallis H tests followed by Dunn's post hoc test, using as fixed factor: (*i*) the treatment (i.e., the EO plant source), (*ii*) the EO concentration, and (*iii*) their interaction (i.e., EO plant source*EO concentration). Mortality data from residual contact toxicity trials were corrected using the Abbott formula [33]. Furthermore, binary data from choice tests were also analyzed using the Chi square test to highlight statistical differences within the two cues confronted in the trials by comparing the number of oviposition punctures on treated olives vs. control (i.e., untreated) olives in every specific choice trial.

The PR values of no-choice ovideterrence trials were subjected to probit analysis to calculate the median repellence concentration (RC_{50}, the EO concentration needed to halve the number of oviposition punctures on the olive fruits) and the 95% fiducial limits (FL). Pearson's goodness-of-fit test was used to confirm that the analyzed data fitted with the

probit model. RC_{50} values were considered statistically different if the respective fiducial limits did not overlap.

3. Results

3.1. Characterization of Essential Oils and Nano-Emulsions

The GC-MS analysis allowed us to identify over the 98% of all the components of the three tested EOs. Anise EO mostly constituted of *E*-anethole (82.51%), as well as fennel EO (43.81%), which also presented some other abundant components (i.e., limonene 23.98% and fenchone 10.14%) (Tables S1 and S2). Instead, mint EO contained a high amount of menthol (36.72%), menthone (18.23%) and iso-menthone (13.56%) (Table S3).

The physical features of the developed nano-formulations showed that all the EO-based nano-emulsions had nano-metrical droplet size and an optimal homogeneity, represented by the low PDI values. The droplets were negatively charged, and conductivity of the formulation was low, highlighting the absence of dissolving/solubilizing components (Table 1).

Table 1. Physical characterization of the essential oil-based nano-emulsions. Values are means (± standard error) of three replicates. Different letters indicate significant differences among the values of the same parameter within the same column (Dunn's post hoc test, $p < 0.05$).

EO [1]	Storage Time	Dimension (nm)	PDI [2]	Zeta Potential (mV)	Conductivity (mS/cm)
Anise	24 h	133.7 ± 0.9 ab	0.193 ± 0.003 a	−23.8 ± 0.1 ab	0.008 ± 0.000 b
	1 year	141.7 ± 0.3 ab	0.132 ± 0.008 b	−29.3 ± 2.9 a	0.010 ± 0.004 ab
Fennell	24 h	116.5 ± 0.5 b	0.156 ± 0.003 ab	−14.4 ± 0.3 b	0.012 ± 0.005 ab
	1 year	151.9 ± 0.6 a	0.187 ± 0.006 a	−13.2 ± 0.9 b	0.020 ±0.002 ab
Mint	24 h	122.4 ± 0.5 ab	0.183 ± 0.006 ab	−20.3 ± 0.5 ab	0.025 ± 0.000 a
	1 year	130.3 ± 0.3 ab	0.150 ± 0.002 ab	−15.4 ± 0.2 ab	0.019 ± 0.000 ab

[1] EO = essential oil; [2] PDI = Polydispersity Index.

The EO plant source resulted in slight differences in the nano-emulsion features; while the droplet dimension and the PDI were quite similar for all the tested formulations ($H_{2,15} = 3.80$; $p = 0.150$ and $H_{2,15} = 0.80$; $p = 0.672$, respectively), the zeta potential and conductivity were affected by the EO used ($H_{2,15} = 15.16$; $p = 0.0005$ and $H_{2,15} = 8.87$; $p = 0.012$). In detail, conductivity was significantly different just among the anise and the mint EO-based formulations ($Z_{1,11} = 2.81$; $p = 0.005$), whereas significant differences of the zeta potentials were observed among all the nano-emulsions by the Dunn's post hoc tests for all pairs (fennel < mint < anise).

In contrast, storage time did not cause relevant changes in the developed nano-emulsions. Indeed, among the four selected physical characteristics just droplet dimension statistically differed between all the freshly prepared and stored nano-emulsions ($U_{1,16} = 7.75$; $p = 0.005$), while the other three parameters did not change consistently with storage time in all the developed formulations (PDI: $U_{1,16} = 2.83$; $p = 0.093$; zeta potential: $U_{1,16} = 0.05$; $p = 0.825$; conductivity: $U_{1,16} = 0.05$; $p = 0.825$).

Lastly, when considering the interaction of the plant source and the storage time, significant differences were recorded for all the physical parameters considered (droplet dimension: $H_{5,12} = 16.61$; $p = 0.005$; PDI: $H_{5,12} = 15.15$; $p = 0.010$; zeta potential: $H_{5,12} = 16.25$; $p = 0.006$; conductivity: $H_{5,12} = 11.93$; $p = 0.036$) (Table 1).

3.2. Residual Contact Toxicity

The residual toxicity of the tested EO-based nano-emulsions was extremely low (≤5%, Table 2). In the control trials no mortality was recorded, thus mortality data were not corrected with the Abbott formula. No significant difference was accounted for among the mortality caused by the three nano-emulsions tested ($H_{2,57} = 1.13$; $p = 0.568$), while the EO concentration has a significative impact on toxicity ($H_{4,55} = 13.86$; $p = 0.008$),

since the two lowest dosages caused no mortality. In contrast, considering the interaction of the two factors no significant differences among the treatments were highlighted ($H_{14,45}$ = 17.39; p = 0.236).

Table 2. Residual contact toxicity of essential oil-based nano-emulsions against *B. oleae* adult flies. Mean mortality values were calculated with four replicates (n = 20). Different letters indicate significant differences among the values in the same column (Dunn's test, $p < 0.05$).

EO [1] Dose (%)	Mortality (%) ± SE [2]		
	Anise	Fennel	Mint
7.50	1.67 ± 0.33 a	5.00 ± 0.58 a	3.33 ± 0.33 a
5.00	1.67 ± 0.33 a	3.33 ± 0.33 b	3.33 ± 0.67 ab
3.75	0.00 ± 0.00 b	0.00 ± 0.00 c	1.67 ± 0.33 b
2.50	0.00 ± 0.00 b	0.00 ± 0.00 c	0.00 ± 0.00 c
1.25	0.00 ± 0.00 b	0.00 ± 0.00 c	0.00 ± 0.00 c

[1] EO = essential oil; [2] SE = standard error.

3.3. Oviposition Deterrence

In no-choice trials the number of oviposition punctures was significantly affected by the tested formulations ($H_{3,596}$ = 153.41; $p < 0.0001$), the EO concentration ($H_{4,595}$ = 105.62; $p < 0.0001$) and their interaction ($H_{19,580}$ = 297.50; $p < 0.0001$) (Figure 1). In detail, the three EO-based nano-emulsions significantly reduced the number of *B. oleae* oviposition behavior compared to control, and anise and fennel EOs were more repellent than mint EO ($H_{2,447}$ = 47.93; $p < 0.0001$). The oviposition deterrence of all the EO nano-formulations fitted the probit model (Anise: χ^2_3 = 2.06; p = 0.56; Fennel: χ^2_3 = 1.14; p = 0.77; Mint: χ^2_3 = 1.13; p = 0.77), and the RC_{50} values for anise [3.25% (FL = 2.06–4.87)] and fennel [3.47% (FL = 2.20–5.10)] nano-emulsions were significantly lower compared to mint EO-based formulation [7.95% (FL = 5.29–10.91)].

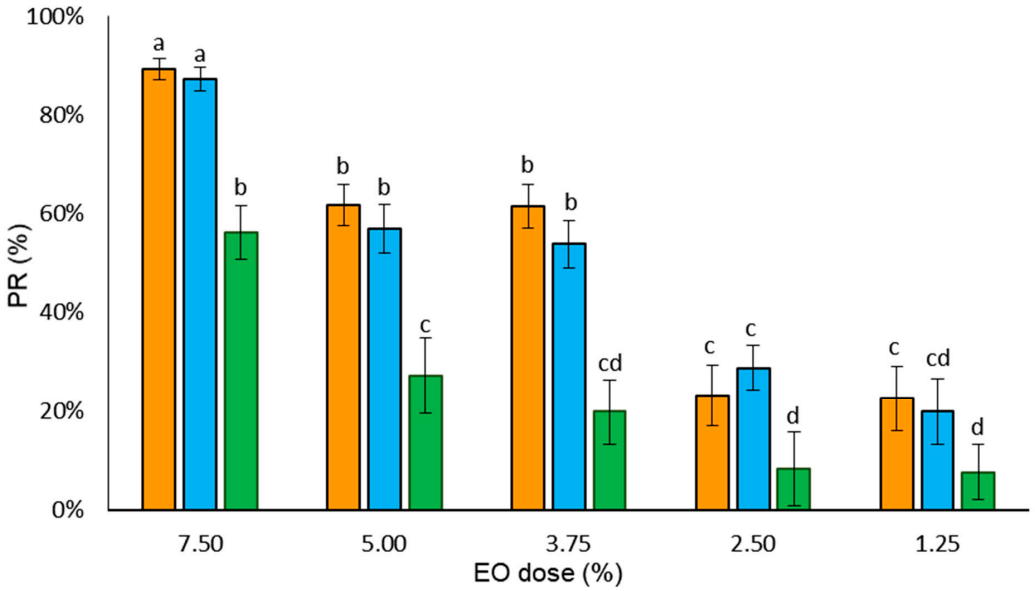

Figure 1. Oviposition deterrence (mean percent repellency ± SE) of the essential oil-based nano-emulsions toward *B. oleae* females in no-choice trials. Four replicates (n = 10) were provided for every EO dose. Orange bars = anise EO; blue bars = fennels EO; green bars = mint EO. Different letters indicate significant differences among the PR values (Dunn's test, $p < 0.05$).

In choice trials, fennel nano-emulsion significantly reduced *B. oleae* oviposition at the highest concentration (χ^2_2 = 6.39; p = 0.011), whereas anise-based one significantly repelled female flies both at 7.5% (χ^2_2 = 6,16; p = 0.013) and 3.75% (χ^2_2 = 3,87; p = 0.049) (Figure 2). In

contrast, mint formulation had not significant effect on the number of oviposition punctures in the olive fruit.

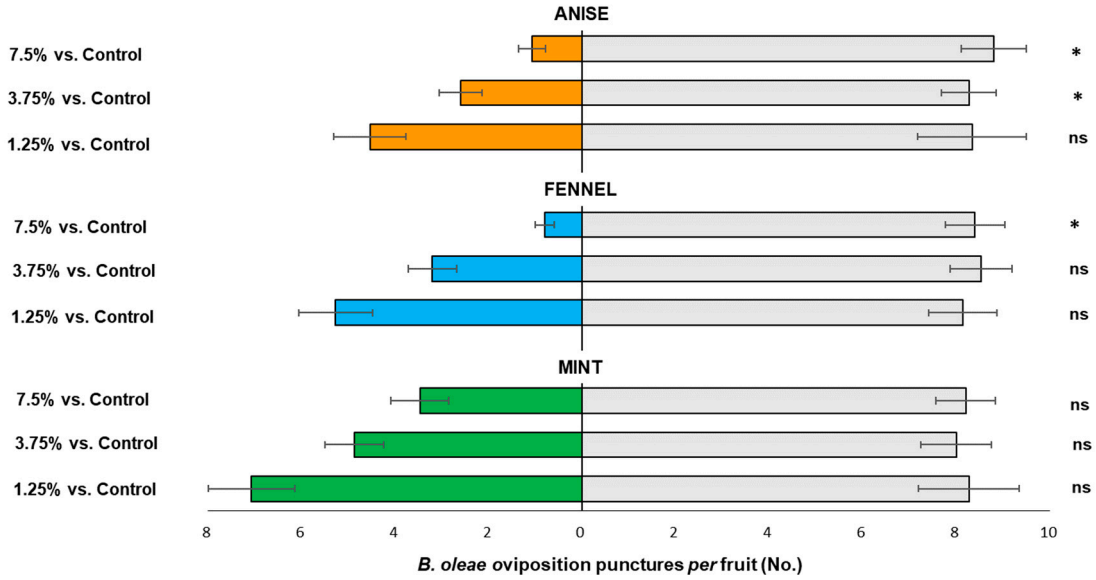

Figure 2. Oviposition behavior (mean oviposition puncture ± SE) of *B. oleae* females in choice trials with essential oil-treated (colored bars) versus control olives (gray bars). Three replicates (n = 10) were provided for every EO dose. Asterisks indicate significant differences between EO and control (Chi square test, $p < 0.05$). ns = not significant.

Significant differences in the PR values were attributable to the tested EO-based nanoemulsions ($H_{2,24} = 7.27$; $p = 0.026$), the EO concentration ($H_{2,24} = 16.89$; $p = 0.0002$) and their interaction ($H_{8,18} = 25.05$; $p = 0.002$) (Figure 3). In detail, according to Dunn's post hoc test, the ovideterrence abilities of anise and mint formulations significantly differed from each other ($Z_{1,17} = -2.55$; $p = 0.032$).

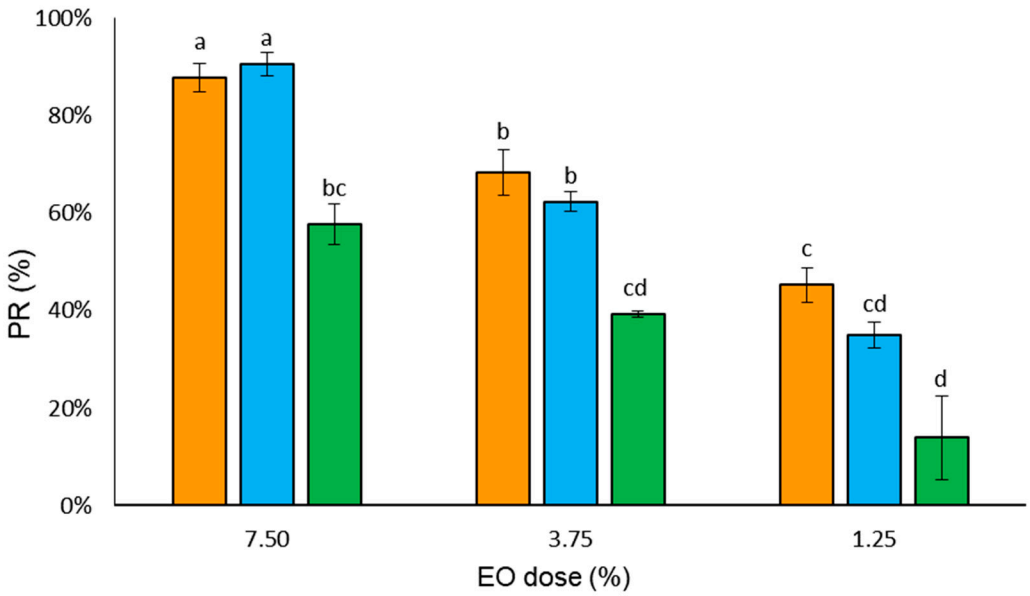

Figure 3. Oviposition deterrence (mean percent repellency ± SE) of the essential oil-based nanoemulsions toward *B. oleae* females in choice trials. Three replicates (n = 10) were provided for every EO dose. Orange bars = anise EO; blue bars = fennels EO; green bars = mint EO. Different letters indicate significant differences among the PR values (Dunn's test, $p < 0.05$).

4. Discussion

To the best of our knowledge, this study proved the repellency of two EO-based nano-emulsions toward the key pest species, *Bactrocera oleae,* for the first time, and determined the residual contact toxicity of the formulations against adult flies. To date, the control strategies against *B. oleae* in field conditions mainly rely on lure and kill and mass-trapping techniques. In this scenario, EOs have been previously proposed as active ingredients for attractive protein baits for *B. oleae,* since they have proven to be effective against a wide range of pest species [10,34,35]. As an example, the oral toxicity against adult *B. oleae* of two EOs from Apiaceae family, anise *P. anisum* and *Trachyspermum ammi* (L.) Sprague, showed dose-dependent mortality and LC_{50} values of 633 ppm and 771 ppm, respectively, when incorporated into protein baits [18]. Similarly, the LC_{50} values of the *Carlina acaulis* EO and its main compound, carlina oxide, were 706 ppm and 1052 ppm, respectively, in ingestion toxicity trials against olive fruit fly adults [16]. The effectiveness of attract and kill lures should also been tested in field conditions, to understand the durability of the baits. The incorporation of EOs of *Lavandula angustifolia* Miller and *Hyptis suaveolens* (L.) Poiteau in protein baits can cause more than 60% of flies mortality at a concentration of 1.75% (*w:v*) in semi-field trials, suggesting that this kind of technique can be effective also in real conditions [17].

In contrast, no information was available on the contact toxicity of EOs against *B. oleae* adults. Nevertheless, the topical toxicity of mint and fennel EOs was assessed against *Musca domestica* L. (Diptera: Muscidae) adult flies, highlighting moderate toxicity against this pest species [36,37]. The contact toxicity of EOs also seems to play a far less considerable role against this target species. Indeed, results from residual contact toxicity tests demonstrated that the developed EO-based nano-emulsions could not cause acute mortality in *B. oleae* adult flies, while other biological control tools, as entomopathogenic fungi, can be effective contact insecticides [38]. Furthermore, the use of oviposition deterrents against the olive fruit fly increased in the last few decades, considering the need to develop new effective, as well as sustainable, pest-management programs. Nowadays, kaolin and other ovideterrents, such as copper compounds, are commonly used for *B. oleae* control and EOs could be administered with the same technologies used for these substances or could be mixed with them to improve crop protection during the late season. Few efforts have been made to shed light on the repellent activity of EOs against dipteran crop pests, but peppermint EO was particularly effective to repel *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) flies, maintaining 100% of the repellency for 6 days post-application [39].

EOs and their components can be also perceived by *B. oleae* adult antennae [16] and can be attractive or repellent to several insect species [14]. A deep knowledge about the impact of EOs and their constituents on the behavioral responses of target insects is fundamental to understand the suitability of these substances for IPM programs. In detail, for lure and kill baits repellent active ingredients may alter the effectiveness of the developed tools by deterring insect feeding [16]. In contrast, this feature can be exploited if the active ingredients are used as ovideterrents against adult *B. oleae* females. Nevertheless, the high volatility of EOs can reduce the durability of EO-based repellents under field conditions [13,40], but their adequate formulation can overcome this negative characteristic. Therefore, the design of stable and more durable formulations based on EOs is fundamental to reduce the number of applications required to cover the oviposition period of the olive fruit fly under field conditions and to improve the persistence of the active ingredients.

In the last few decades, nanotechnologies have also been proposed for the pharmaceutical and plant protection industry to develop more effective and stable bioinsecticide [15,41]. The EO-based nano-emulsions used in the present study proved effective ovideterrence and showed optimal physical characteristics, which remained quite stable after 1 year of storage. As previously reported by Campolo et al. [12], the EO source and its composition impacted deeply on the quality of the formulations, and the above-presented results confirm this aspect. In this study, the droplet dimension increased in all the nano-formulations during the storage, although a significant modification occurred just for the

fennel EO-based formulation. On the other hand, concerning the other physical parameters, the different nano-emulsions did not show analogous trends after storage, with values sometimes increasing and sometimes decreasing. However, only minor significant differences were recorded among the various nano-formulations and the characteristics of all EO-based nano-emulsions were excellent compared to the literature even after 1 year of storage [41,42].

The repellent activity of different kind of EO nano-pesticides has been investigated against several target pests and a few research studies focused on the bioactivity of the same EOs selected for the present experimental set up [43,44]. As an example, EO-based gels proved to be highly repellent against the confused flour beetle, *Tribolium confusum* du Val (Coleoptera: Tenebrionidae) and the most repellent formulation among the tested ones was the gel containing anise EO nano-emulsion (RC_{50} = 0.033 mg of EO) [45]. Similarly, in the present study, the best ovideterrent toward *B. oleae* was the nano-emulsion produced from anise EO. This EO is rich in *E*-anethole, similarly to fennel EO which, indeed, evoked similar behavioral responses in *B. oleae* females. *E*-anethole is the main constituent of anise EO, which caused *B. oleae* complete mortality when included in protein baits at 0.5% *w/v* concentration [18]. Both fennel and anise EO-based nano-emulsions significantly reduced the number of ovipositions on treated olives compared to control at 7.5% in choice trials. In contrast, the nano-formulation containing mint EO did not significantly reduce the number of oviposition punctures on fruit skin, suggesting that the EO composition is a key factor to determine target insect responses. Moreover, repellence of a given EO can differ considering different target species; fennel EO nano-emulsion proved lower repellence than mint formulation toward the lesser grain borer, *Rhyzopertha dominica* F. (Coleoptera: Bostrichidae), one of the most destructive pests of stored grains, seeds and legumes [46]. Furthermore, this research highlighted that the durability of the repellent activity of the EO-based nano-emulsions could decrease because of the onset of natural physiological processes, such as habituation to adverse cues [46]. Thus, the suitability of novel approaches, including biopesticides, should be addressed carefully in real conditions to understand the actual effectiveness of the proposed techniques and the persistence of the treatments.

Among the promising aspects related to the development of EO-based biopesticides, the safety of these active ingredients is a key factor. Since ancient times, many EOs have been used in the pharmacopeia; at present, EOs are largely employed by the food and pharmaceutical industries as food additives, food preservatives, and/or natural flavors and fragrances [47]. Therefore, it has been generally acknowledged that EOs are harmless for mammalians and are considered safe for humans [48]. For this reason, EO-based biopesticides can also be used close to crop harvest, in contrast to conventional pesticides which can have long residual times and require protracted safety intervals from treatment to harvest (i.e., over 3 weeks for some neonicotinoids and organophosphate) [49]. This characteristic is fundamental for the development of innovative successful insecticides, since the timely response to sudden unexpected crop damages by pest populations is a key factor in crop protection.

5. Conclusions

The developed EO-based nano-emulsions showed optimal physical characteristics and a considerable ovideterrent activity against *B. oleae* females in no-choice and choice trials. The use of botanical-based repellent could be considered an integrative eco-friendly tool to reduce the damages caused by the olive fruit fly to olive production. The development of EO-based formulations containing high concentrations of active ingredients (i.e., 15% of EO) is essential to translate the huge amount of literature about EO insecticidal activity into practical applications and actual products for crop protection. The integration of different approaches, including the use of botanicals and natural active ingredients, can support eco-sustainable control programs and help to reduce the application of synthetic chemical insecticides, guaranteeing safe pesticide-free products on the market.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae8030240/s1, Table S1: GC-MS analysis of the anise (*Pimpinella anisum*) essential oil, Table S2: GC-MS analysis of the fennel (*Foeniculum vulgare*) essential oil, Table S3: GC-MS analysis of the mint (*Mentha* × *piperita*) essential oil.

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