Bioactivity of essential oil-based nano-biopesticides toward *Rhyzopertha dominica* (Coleoptera: Bostrichidae)

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Highlights

Three essential oil (EO)-based nano-emulsions were developed and characterized.
Repellence of EO-based nano-emulsions was verified against the lesser grain borer.
Habituation in *Rhyzopertha dominica* adults after repeated exposure was evaluated.
The decline of *R. dominica* responsiveness was attributable to learning process.
Habituation could alter the repellent ability of EO-based formulations.

Abstract

Essential oils (EOs) are promising active ingredients to produce biopesticides, although their physicochemical characteristics are a critical issue to develop commercial formulates. Repellent and toxic activity of EOs against crop and stored product pests has been widely investigated in the last decades; however, no information is available on the occurrence of adverse undesirable behavioral responses (i.e. habituation) toward these repellents in target pest species. In this study, stable EO-based nano-emulsions from commercial fennel (*Foeniculum vulgare*), mint (*Mentha x piperita*) and sweet orange (*Citrus sinensis*) EOs were developed, and their repellence was tested against a major stored product pest, the lesser grain borer *Rhyzopetha dominica*. Besides, the occurrence of habituation in *R. dominica* adults following successive exposure to the repellent formulations was evaluated, considering the main characteristics of this kind of non-associative learning. Nanometric droplet size was achieved for all developed nano-emulsions (<200 nm). All the tested EO-based nano-formulations were repellent to *R. dominica,* whereas *F. vulgare*-based nano-emulsions triggered lower repellence both in area choice and arena bioassays. The occurrence of habituation was validated for the strongest repellents, *M. piperita* and *C. sinensis* formulations and the decline of *R. dominica* responses was frequency-dependent. Furthermore, insects completely recovered their responsiveness toward the biopesticides just after 24 h from the end of exposure. The decline of the responsiveness in *R. dominica* adults was attributable to real learning process, since motor and sensory fatigue were excluded by testing stimulus specificity and dishabituation. The results demonstrated that habituation could occur for repellent EO-based formulations, thus this behavioral process can reduce the effectiveness of these kind of treatments against *R. dominica* and should be considered to articulate adequate IPM programs against stored product pests.

Keywords: Botanical active substance; Habituation; Non-associative learning; Repellence; Stored product pest

1. Introduction

Research and development of bio-insecticides and plant-borne active ingredients are key factors to improve the available techniques for Integrated Pest Management (IPM) programs. Crop and stored product protection mainly relies on application of synthetic insecticides (Boyer et al., 2012), although several concerns are related to their widespread application, covering environmental risks (e.g., ozone layer depletion, bioaccumulation) (Ristaino and Thomas, 1997; Serça et al., 1998) and ecological implications (e.g., resistance, toxicity against non-target organisms) (Schlipalius et al., 2002; Xiao et al., 2016). Thus, an increasing interest is rising about eco-friendly alternatives, with particular reference to botanical extracts and essential oils (EOs) (Campolo et al., 2018; Isman, 2020a; Pavela and Benelli, 2016). EOs are secondary metabolites synthetized by many plant species as indirect plant defense, that can exhibit toxic and/or repellent activity toward several insect pests (Isman, 2000b; Pavela, 2015; Regnault-Roger et al., 2012). Overall, EOs are recognized to be promising active ingredients for biopesticides, but EO-based pesticides may present some concerns related to their physicochemical characteristics.

Apart from the complexities about the standardization of the chemical composition (Do et al., 2015; Thompson et al., 2003), the high volatility, the scarce water solubility and the rapid degradation of the EOs are true obstacles to their application under real conditions (Moretti et al., 2002). Recently, nanotechnologies have been proposed to overcome these problematics, increasing the stability and solubility of EOs (Kah et al., 2013) by encapsulating them inside nanoparticles thus, extending the durability of EO-based insecticides (Campolo et al., 2017; Werdin González et al., 2014) or formulating them as nano-emulsions (Giunti et al., 2019; Golden et al., 2018; Pavoni et al., 2019). EOs are recognized to act as strong repellents against several pests (Campolo et al., 2018; Nerio et al., 2010), but the high volatility of the active ingredients can complicate their use as plant protectants. Furthermore, when the repellent effect of EOs toward pests is investigated, the majority of researches do not calculate RD_{50} values (i.e. the dose required to repel half the members of a tested population after a specified test duration) and the repellence is assessed after short periods (2−4 h), missing to evaluate persistence (Campolo et al., 2018).

Furthermore, another important critical issue about repellent and feeding deterrent compounds is the alteration of pest behavioral responsiveness, which can reduce the treatment efficacy. Indeed, when animals experience repeatedly an adverse stimulus which did not cause direct negative effects, they can often decline their responses toward the cue, causing “habituation” (Thompson and Spencer, 1966). This process has been investigated for relatively short time period (e.g. hours) for simple behaviors, like shock responses, escape and proboscis extension (Braun and Bicker, 1992; Engel and Wu, 2009; Rankin and Broster, 1992), but also for its role driving intra- and interspecific ecological relationships, like predation (Deecke et al., 2002) and conspecific alarm signaling (Dong and Clayton, 2009; Owen and Perrill, 1998). Nevertheless, very limited information is available about habituation occurrence in insect pests caused by commercial repellents or potential natural deterrents. Habituation is a kind of non-associative learning occurring in animals, characterized by specific features involving neurological and molecular mechanisms (Rankin et al., 2009). The main characteristics are the decline of the responses after repeated distinct exposure to a given cue, followed by the “spontaneous recovery” of the responsiveness when the cue is no longer offered to the animal. Furthermore, several characteristics can be used to distinguish the occurrence of real learning process from motor or sensory fatigue (i.e., the response decline due to the saturation of the structure recognizing chemical, visual or acoustic stimuli).

The lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), is a cosmopolitan pest of stored cereals, which can also survive on a wide range of materials like legumes, stored pharmaceuticals, leather stuffing, mud plaster, packaging materials made from wood, paper, bound books, and cork. The adults and the larvae feed on the germ and the endosperm of kernels, boring inside the caryopses. Adults are the most noxious instar, since they are quite voracious and their lifespan can vary from 25 to 65 days depending on environmental conditions (Edde, 2012). *Rhyzopertha dominica* adults disperse from existing infestations to invade clean resources usually during the first weeks of storage (Campbell and Arbogast, 2004) and they generally locate and reach the new food sources in flight by using volatile chemicals emitted by host-foodstuff (Ahmad et al., 2013; Barrer, 1983; Edde, 2012). Repellent dispenser may alter the orientation of *R. dominica* adults, since very few chemical insecticides can be directly used on stored products (Boyer et al., 2012). On the other hand, some interesting results have been reported for EO-repellence toward this pest. In particular, the EOs from *Pistacia lentiscus* (Anacardiaceae) presented low RD_{50} values toward *R. dominica* in area choice bioassays (0.01 $\mu L/cm^2$) (Bougherra et al., 2015), while *Laurus nobilis* (Lauraceae) EO produced different repellence effects depending on the geographical origin of the plant material (RD_{50} = 0.013 and 0.036 $\mu L/cm^2$) (Mediouni Ben Jemâa et al., 2012).

This study aimed to develop stable EO-based nano-emulsions and to test their repellence and their ability to induce habituation in *R. dominica* adults. The EOs were selected according to the results of previous trials highlighting good repellent activity toward stored product pests and according to their availability at reasonable cost on the market (Campolo et al., 2018). Thus, fennel (*Foeniculum vulgare*), mint (*Mentha x piperita*) and sweet orange (*Citrus sinensis*) were selected. First the bioactivity of the experimental nano-emulsions as repellent was assessed in area choice bioassay, a classic method to quantify repellence (Giunti et al., 2019; Nerio et al., 2010). Then, the repellent activity was tested in arena, recording the residence durations of insects on treated rice kernels, to also assess the reliability of residence duration as parameter to evaluate repellence. Lastly, the characteristics of habituation of *R. dominica* adults toward the best repellents were tested. Results from repellence trials demonstrated that EO-based nano-formulations can be effective against *R. dominica* adults, although their behavioral responses may be altered by habituation occurrence, which can reduce the efficacy of treatments in real conditions.

2. Material and methods

2.1. Insect colonies and rearing conditions

The original strain of *R. dominica* was collected from infested wheat provided in February 2019 by a local mill (Melito Porto Salvo, Reggio Calabria, Italy). *Rhyzopertha dominica* was reared on wheat for several generations under controlled conditions at the Department of Agriculture, University *Mediterranea* of Reggio Calabria (Reggio Calabria, Italy). The rearing conditions were: 28 ± 1 °C, 50 ± 5% R.H., with a photoperiod of 12 h:12 h (L:D). To obtain insect specimens of the same age, about 300 unsexed adult beetles were placed inside 5 L glass jars containing 500 g of uninfested wheat. After 5 days, adult insects were removed, and their progeny was used for the tests. Insects were collected daily from rearings using a 450-μm sieve (Technotest; Modena, Italy) and a mouth aspirator. For all the bioassays, 5–10-day-old *R. dominica* adults were used.

2.2. GC–MS chemical characterization of the essential oils

Commercial sweet orange (SO) essential oil extracted from citrus peel using cold pressing technique from fruit grown in southern Italy, pesticide-free certified, was kindly provided by Capua SRL, Campo Calabro Italy. Fennel (F) and mint (M) essential oils were extracted at the Department of Agriculture, University *Mediterranea* of Reggio Calabria by a stem distillation unit (Albrigi Luigi, Stallavena, Italy) from fresh plants locally organic cultivated. GC–MS analyses were performed with a Thermo Fisher TRACE 1300 gas chromatograph equipped with a MEGA-5 capillary column (30 m x 0.25 mm; coating thickness= 0.25 μm) and a Thermo Fisher ISQ LT ion trap mass detector (emission current: 10 microamps; count threshold: 1 count; multiplier offset: 0 V; scan time: 1.00 s; prescan ionization time: 100 microseconds; scan mass range: 30−300 *m/z*; ionization mode: EI). The analytical conditions were as follow: injector and transfer line temperature at 250 and 240 °C, respectively; oven temperature programmed from 60 to 240 °C at 3 °C min^{-1}; carrier gas, helium at 1 mL min^{-1}; injection, 0.2 μL (10 % hexane solution); split ratio, 1:30. Chemicals were identified by the comparison of retention times (RT) and linear retention indices (LRI) with those of pure chemicals and of a homemade library built from pure substances, components and the MS literature data (Adams, 1995; Davies, 1990; Jennings, 1980; Masada, 1976; Stenhagen et al., 1974), and on computer matching against some commercial libraries (NIST 05, Wiley FFNSC and ADAMS). LRI was calculated by comparing the retention times of the compounds to those of a standard mixture of alkanes (C7-C30 saturated alkanes standard mixture, Supelco®, Bellefonte, PA, USA) (Van Den Dool and Kratz, 1963), which was analyzed by GC–MS set at the identical conditions of the essential oils.

2.3. Nano-emulsion formulation and characterization

The EO nano-emulsions were prepared following the method by Campolo et al., 2020a. The spontaneous emulsification process, which occurs when an organic phase and an aqueous phase are mixed, was exploited for the first step in nano-emulsion preparation. Firstly, a mixture of EO and Tween 80® [Polyoxyethylene (20) sorbitan monooleate, Sigma Aldrich, Munich, Germany] was gently stirred for at least 30 min and then double-distilled water was added to this mixture and stirred for 2 h. The obtained emulsion was composed by 5% Tween 80®, 15 % EO and 80 % water. To improve the characteristics of the nano-emulsions, sonication at 100 W power was applied to the coarse emulsions for 5 min using an UP200ST ultrasonic immersion homogenizer (Hielsher©, Teltow, Germany). After sonication the nano-emulsions were stored at 25 ± 0.5 °C in an airtight glass bottle, analyzed with a Dynamic Light Scattering (DLS) instrument (Zetasizer Nano, Malvern®) after 24 h and used for the bioassays within the following 5 days. DLS analyses was used to determine the droplet surface charge at

25 °C, indicated by the zeta potential (ζ) values, and the droplets dimension, expressed in terms of Z-average size (d) and polydispersity index (PDI) of the nano-emulsions.

2.4. Area choice bioassays with *nano-emulsions*

The classic method for the assessment of the repellent activity of chemicals toward stored product pests, the area choice bioassay in Petri dish, was adopted as described by Giunti et al. (2019). Briefly, insects were placed inside a glass Petri dish (9 cm diameter) covered with a filter paper (9 cm diameter, Whatman n°1) split in two identical halves, one treated with the putative repellent and the other one untreated. Here, half filter paper disks were treated with the nano-emulsions at 1.50, 0.75, 0.37, 0.19 or 0.09 mg of EO/cm^2, while the other half paper disks were soaked just with Tween 80® water-solution, which is not repellent for *R. dominica*. Both half disks were dried under a cold fan and then placed inside a glass Petri dish closed with a nylon mesh (0.05 mm) to avoid the odor saturation inside the testing arena during the 24 h duration of the trials. Negative controls, consisting in both half filter paper disks treated with Tween 80® water-solution (0.50 mg/cm^2), were also set up to evaluate eventual position effects. The arenas were maintained at 25 ± 1 °C, 50 ± 5% R.H., under constant light condition. Insects were used only once. Repellence (i.e. the number of insects in the two halves) was recorded after 2, 4 and 24 h of exposure. The percent repellence (PR) of EO nano-emulsions was calculated, for every considered time, by the formula: $PR(\%) = [(Nc - Nt)/(Nc + Nt)] \times 100$ where Nc is the number of insects in the control half paper and Nt the number of insects in the treated one. A Completely Randomized Design (CRD) was adopted and for every dose, 9 replicates (i.e. 10 unsexed *R. dominica* adults for replicate) were obtained.

2.5. Residence on food source treated with nano-emulsions

Repellent activity of nano-emulsions can alter the resident duration of beetle on food sources, encouraging the insects to leave the foraging area. To test the effect of EO nano-emulsions on the residence duration on food, a Petri dish arena was designed (Fig. 1). The arena consisted in a 11 cm diameter filter paper (Whatman n°1) whose central area (5 cm diameter) was treated with nano-emulsion (1.50 mg of EO/cm^2, i.e., the highest tested concentration in area choice bioassays) or with a Tween 80® water-solution (0.50 mg/cm^2) as control. When the paper disk was dry, the treated area was covered with a single layer of rice grains (about 2 g). Then, a fine nylon mesh (0.1 mm) was posed over the paper disk, to avoid insects to contact either the food or the EOs directly and the arena was closed with a glass lid, to prevent insect escape. Adults of *R. dominica,* starved for 12 h, were individually released in the center of the arena, where food and repellents are present, and observed to record residence duration (i.e. time spent in the central area with food). Insects remaining still for 2 min or more from the release were discarded from further analyses. The arenas were maintained at 25 ± 1 °C, 50 ± 5% R.H., under constant light condition. After the bioassays, tested *R. dominica* were chilled at −20 °C for 30 s and sexed by squeezing method (Sinclair, 1981). For every treatment, residence durations of 200 insects, tested only once, were recorded and used for statistical analyses.

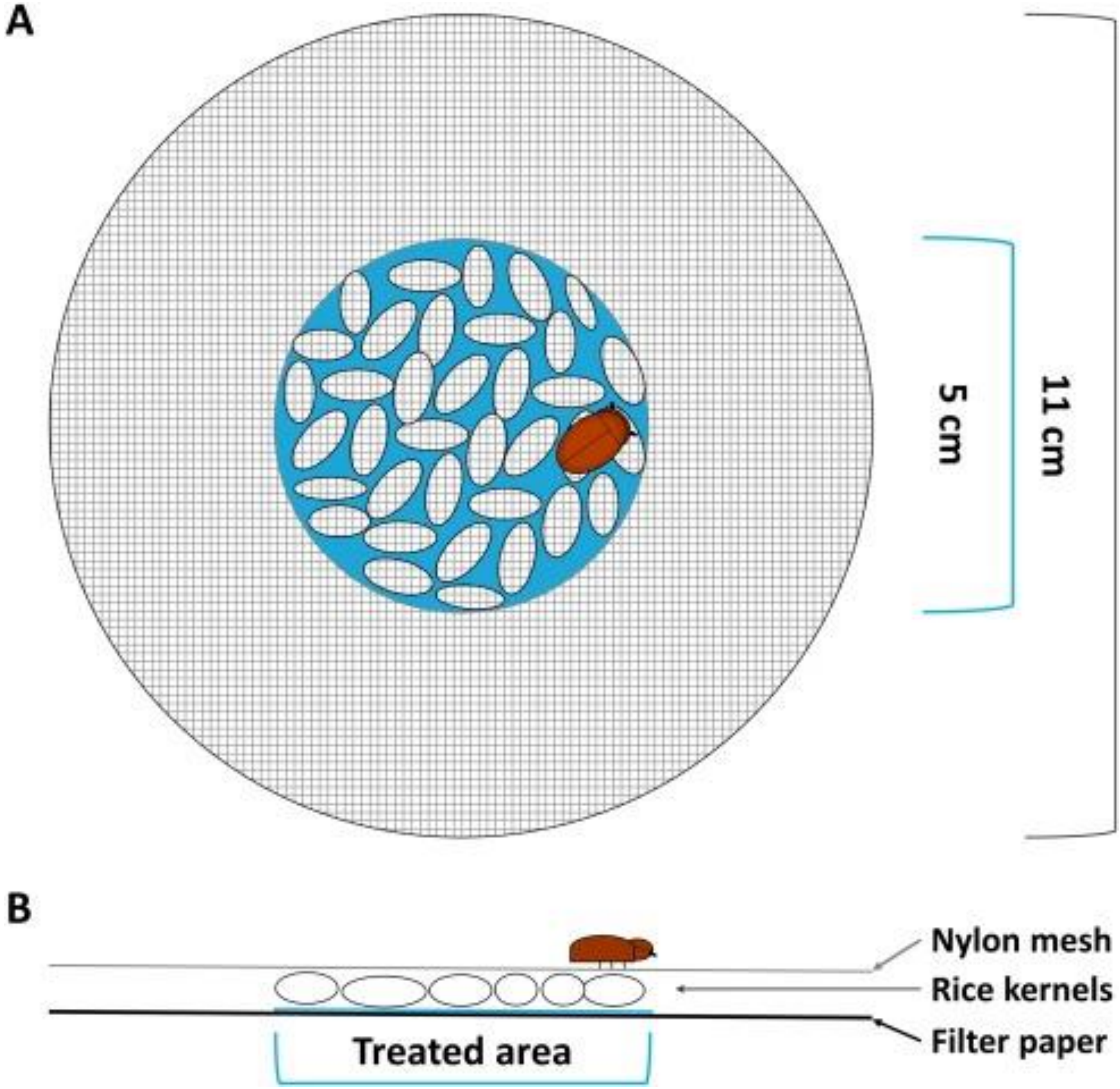

Fig. 1. Design of the Petri dish arena used to test *R. dominica* residence durations on food: top (A) and side (B) view.

2.6. Habituation characteristics for repellence of selected nano-emulsions

Habituation was evaluated for SO and M-based nano-emulsions, since these EOs presented the highest repellent activity toward *R. dominica*. In detail, the following characteristics were evaluated: (*i*) the decline in responsiveness over a series (i.e., five, labelled from I to V) of consecutive training sessions administered at different training intervals (i.e. three frequencies); (*ii*) the spontaneous full or partial recover of the responsiveness in two different testing sessions; (*iii*) the stimulus specificity of the declined responses; (*iv*) the occurrence of dishabituation (i.e., recover in the responsiveness) after the administration of a novel stimulus within the same stimulus modality of the habituated cue.

All the bioassays were conducted using the same arena and the same conditions described to test the effect of repellent nano-emulsions on residence durations and the same concentration of repellent cues. First, the increase in the intensity of the residence durations of beetle on food, over a series of consecutive exposure to treated forage patches was assessed. In detail, *R. dominica* adults, starved for 12 h, were trained on 5 (I–V) subsequent training sessions with food and repellent (released into an arena, allowed to leave, and re-collected), using three different training frequencies, 15 min, 30 min and 60 min between every training session. *Rhyzopertha dominica* adults were maintained in glass vials without food between the training sessions, as well as after the conclusion of training. Furthermore, the recovery of the response after training was determined by testing the behavioral responses of trained beetles when re-tested after 24 h and 48 h from the last training session. Insects were randomly assigned to 24 h or 48 h groups. For each EO and every training frequency, 100 insects were tested, and they were used only once. Concurrently, the residence durations of untrained *R. dominica* adults, with no previous experience of repellent, were used as control for training effectiveness. Thus, 100 untrained insects were tested for each testing interval (24 h and 48 h).

Then, stimulus specificity was tested. Stimulus specificity implies that, within the same stimulus modality, the behavioral response should have some specificity; this characteristic is a crucial aspect of habituation useful to distinguish it from sensory adaptation or motor fatigue (which should generalize across a wide range of stimuli). To test this characteristic, a novel stimulus was presented to trained *R. dominica* after the fourth training session, and the responses to the novel stimulus should not be altered by training with a different one. For insects trained with SO as novel stimuli F and M nano-emulsions were used, while for M-trained beetles, the stimulus specificity using F and SO formulations was tested. For each novel stimulus and every training frequency, 100 insects were tested. Residence durations displayed by insects which experienced the stimulus without training or after four training sessions with a different repellent were recorded and then compared to evaluate stimulus specificity/generalization.

Lastly, dishabituation was evaluated. This phenomenon involves that the presentation of a different stimulus should result in a partial recover of the behavioral response to the original stimulus. Usually, only strong stimuli are used to produce dishabituation. Here, a strong (M or SO) and a weak (F) novel cues were used to induce dishabituation. Therefore, after having been exposed to a novel stimulus (i.e. the dishabituating stimulus), as described above, *R. dominica* adults were again exposed to the original habituated stimulus. To test the occurrence of dishabituation, the behavioral responses from the fifth (V) training sessions of insects which experienced or not the dishabituating stimulus were compared, to highlight any partial recover of the repellence attributable to dishabituation. 100 insect specimens were used for every dishabituation stimulus/training frequency combination, and their residence durations during the training sessions were recorded.

2.7. Data analyses

Statistics were carried out using JMP® 11 software. In area choice assays, PR data calculated after 2 h, 4 h and 24 h were subjected to probit analysis in order to calculate the median repellent dose (RD_{50}) and 90 % repellent dose (RD_{90}) of the tested EO-formulations. Values were considered significantly different if their 95 % fiducial limits did not overlap. Since data did not meet the ANOVA assumptions (normality and homoscedasticity) when subjected to Shapiro-Wilk and Levene's test respectively, a non-parametric model, the Kruskal-Wallis H test followed by Dunn's post hoc test, was used.

Residence durations were analyzed using a Generalized Linear Mixed Model (GLMM): $y = X\beta + Zu + \varepsilon$ where y is the vector of the observations (time spent on the cue), X is the fixed effect matrix, β is the vector of fixed effects (e.g. treatment, sex, etc.), Z is the random effect matrix, u is the vector of random effects (i.e. individual) and ε is the vector of the residual effects. For residence durations recorded in repellence trials the fixed effects used were "treatment" and "sex". For each EO-based nano-emulsion, the residence durations from habituation trials were subjected to several GLMM: a) to verify the frequency-dependent decrease in the response, the values recorded in the different training sessions were analyzed (fixed factors: session, frequency, sex); b) to evaluate the frequency-dependent recovery of the response, the values recorded in the different testing sessions were compared (fixed factors: phase, frequency, sex); c) to assess the recovery occurrence and magnitude, the residence durations from the testing phases were compared with those of last training session (GLMM: fixed factors: phase, frequency, sex) and those of naïve insects (GLMM: fixed factors: experience, frequency, sex). To confirm the occurrence of non-associative learning over fatigue processes, the resident durations of the novel stimuli were compared to those of naïve insects for the same stimuli and to expected residence durations from the last training session (V) with GLMMs (fixed factors: experience, frequency, sex), to check for stimulus specificity. Lastly, for dishabituation tests, the data from last training session (V) of insects exposed to dishabituating stimulus or not were analyzed also with a GLMM (fixed factors: experience, frequency, sex). The values were analyzed for both sexes together and for each sex separately.

3. Results

3.1. GC–MS chemical characterization of essential oil

Volatile Organic Compounds identified from GC–MS analyses are listed in **Supplementary Table S1-S3.** The SO essential oil (29 chemical constituents) was almost entirely composed by *R*-limonene (93.25 %), followed by *β*-myrcene (3.37 %) and *α*-pinene (1.14 %). The M essential oil presented 95 identified compounds, mainly oxygenated monoterpenes, including menthol (36.72 %), menthone (18.23 %), isomenthone (13.56 %), menthyl acetate (5.79 %) and eucalyptol (4.73 %). Lastly, the F essential oil (90 compounds identified) was constituted principally phenylpropanoids, namely by (*E*)-anethole (43.81 %) and estragole (3.15 %), and monoterpenes, like limonene (23.98 %), fenchone (10.14 %), α-phellandrene (3.33 %).

3.2. Nano-emulsion formulation and characterization

All the nano-emulsions showed an average size of the droplets within the nanometer range (Table 1). The nano-emulsions had few or no aggregates (<4.5 %) and the formulations presented good size-homogeneity, as evidenced by the low values of the polydispersity index (0.12–0.22). The surface charge (ζ Potential) of the droplets, always negative, ranged from -18.4 to -27.7 mV.

Table 1. Physical characterization (mean ± SE, n = 3) of EO nano-emulsions analyzed with a DLS instrument.

Essential oil	**Size (nm)**	**PDI**	**ζ Potential (mV)**	**Conductivity (mS/cm)**
Sweet Orange	131.4 ± 0.29	0.118 ± 0.002	−27.7 ± 0.18	0.007 ± 0.002
Mint	168.8 ± 0.40	0.146 ± 0.007	−18.4 ± 0.76	0.004 ± 0.00004
Fennel	143.8 ± 0.38	0.223 ± 0.007	−16.5 ± 0.35	0.011 ± 0.00007

PDI = Polydispersity Index.

3.3. Repellent activity of EO nano-emulsions

In area choice bioassays, a good repellent activity was noted for all the tested formulations, but the repellence slightly decreased after 24 h of exposure (Table 2). Statistical differences in the PR values were highlighted just after 2 h of exposure; the highest repellent activity was recorded for the M nano-emulsion, followed by SO one, whereas the F nano-formulation exhibited the lowest repellence toward *R. dominica* adults ($H_{2,132} = 38.40$; $P < 0.0001$). After

24h the PR values (average ± SE) for SO and M nano-emulsions at the highest tested concentration were similar, 84.44 ± 6.48 % and 88.89 ± 4.84 % respectively, while the PR value for F nano-formulation was significantly lower (60.00 ± 7.45 %) ($H_{2,132} = 23.83$; $P < 0.0001$) (**Supplementary Figure S4**).

Table 2. Repellent doses (RD) calculated through Probit analysis for essential oil-based nano-emulsions in area choice bioassays with *R. dominica* adults. RD values are expressed as mg of EO/cm^2.

Time of exposure	Empty Cell	**Fennel**	**Mint**	**Sweet Orange**
2 h	**RD_{50} (LB -UP)**	0.96 (-0.30 -2.23)	0.08 (0.02 -0.14)	0.17 (-1.06 -1.40)
	RD_{90} (LB -UB)	2.08 (0.89 -3.27)	0.90 (-1.07 -2.87)	1.25 (-0.91 -3.40)
	χ^2_3	0.052	0.322	0.256
	P	0.98	0.95	0.96
4 h	**RD_{50} (LB -UP)**	1.09 (-0.27 -2.45)	0.09 (-1.41 -1.59)	0.24 (-0.93 -1.40)
	RD_{90} (LB -UB)	2.18 (0.96 -3.40)	1.07 (-0.96 -3.10)	1.35 (-0.94 -3.62)
	χ^2_3	0.086	0.327	0.350
	P	0.98	0.95	0.95
24 h	**RD_{50} (LB -UP)**	1.15 (-0.31 -2.61)	0.13 (-1.17 -1.41)	0.45 (-0.44 -1.33)
	RD_{90} (LB -UB)	2.26 (0.99 -3.53)	1.21 (-0.91 -3.34)	1.39 (0.51 -2.27)
	χ^2_3	0.101	0.336	0.482
	P	0.97	0.95	0.92

χ^2 and P values refer to Pearson's Goodness of Fit Test.
LB = lower bound; UB = Upper bound.

Residence durations from repellence trials showed similar results to area choice bioassays. No statistical differences were highlighted for sex ($F_{1,792} = 0.016$; $P = 0.90$) nor for treatment*sex interaction ($F_{3,792} = 0.18$; $P = 0.91$). In contrast, statistical differences were revealed among the different treatments ($F_{3,792} = 279.35$; $P < 0.0001$) since shorter residence durations were reported for the strongest repellents (Fig. 2). Furthermore, the behavioral responses of male ($F_{3,393} = 146.81$; $P < 0.0001$) and female ($F_{3,399} = 133.98$; $P < 0.0001$) pests toward the tested repellent were similar (Fig. 2).

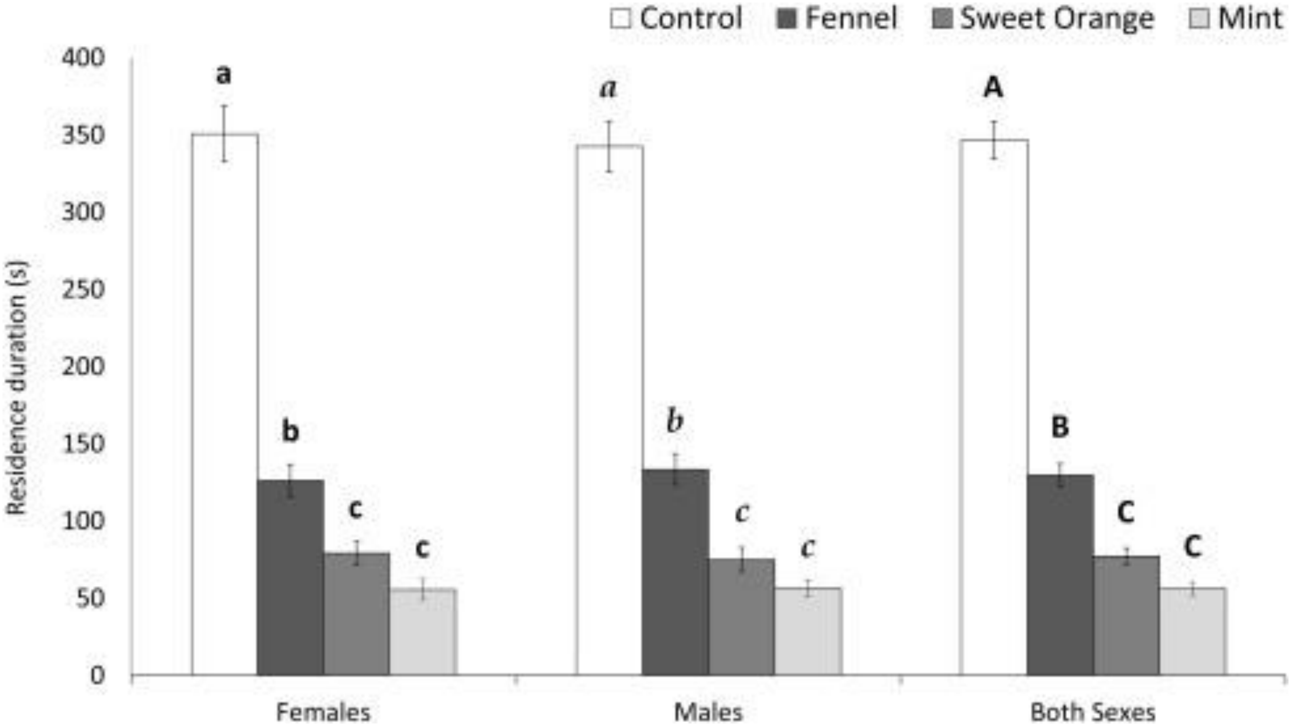

Fig. 2. Residence durations (mean ± SE, n = 200) of *R. dominica* adults in arena repellence trials with various EO-based formulations. Different letters (with the same style) indicate statistical differences among treatments within the same group (GLMM, $P < 0.05$).

3.4. Habituation characteristics for repellence of selected nano-emulsions

Both for SO and M nano-emulsion habituation processes took place. Considering both sexes, repeated exposure to repellent stimuli caused a decrease in the insect responses to the given stimulus during the training sessions (M: $F_{4,1176} = 69.65$, $P < 0.0001$; SO: $F_{4,1176} = 63.83$, $P < 0.0001$), and the decline was significantly accentuated at the lowest frequency (15 min) (M: $F_{2,294} = 7.74$, $P = 0.0005$; SO: $F_{2,294} = 24.46$, $P < 0.0001$). No statistical differences linked to the sex of the tested insect were highlighted (M: $F_{1,294} = 2.64$, $P = 0.11$; SO: $F_{1,294} = 0.59$, $P = 0.44$). Nevertheless, when considering each sex separately, it can be noted that usually females presented a strong decline of the responses (i.e., the stimuli were less repellent) at the last training session (Fig. 3). In comparison to the last training session, tested insects showed to significantly recover the innate negative responses to the repellent stimuli during both the testing phases (M: $F_{2,389} = 69.53$, $P < 0.0001$; SO: $F_{2,347} = 10.03$, $P < 0.0001$). Overall, the repellence was recovered just after 24 h from the last training and no statistical differences were noted in the residence durations between the testing phases (M: $F_{1,288} = 2.42$, $P = 0.12$; SO: $F_{1,288} = 1.40$, $P = 0.24$), neither according to frequency (M: $F_{2,288} = 1.02$, $P = 0.36$; SO: $F_{2,288} = 0.11$, $P = 0.89$) nor sex (M: $F_{1,288} = 0.45$, $P = 0.64$; SO: $F_{1,288} = 0.30$, $P = 0.58$). The recovery of the responses was complete; indeed, no statistical difference was highlighted respect to naïve insects either for M (24 h: $F_{3,242} = 1.28$, $P = 0.28$; 48 h: $F_{3,242} = 1.66$, $P = 0.18$) or SO nano-emulsion (24 h: $F_{3,242} = 0.41$, $P = 0.75$; 48 h: $F_{3,242} = 0.01$, $P = 0.99$). Nevertheless, just the females showed after 24 and 48 h a different recover in the responses caused by the repellent M nano-emulsion ($F_{1,153} = 4.05$, $P=0.04$), while no significant difference was attributable to frequency ($F_{2,153} = 0.50$, $P = 0.61$) (Fig. 3A).

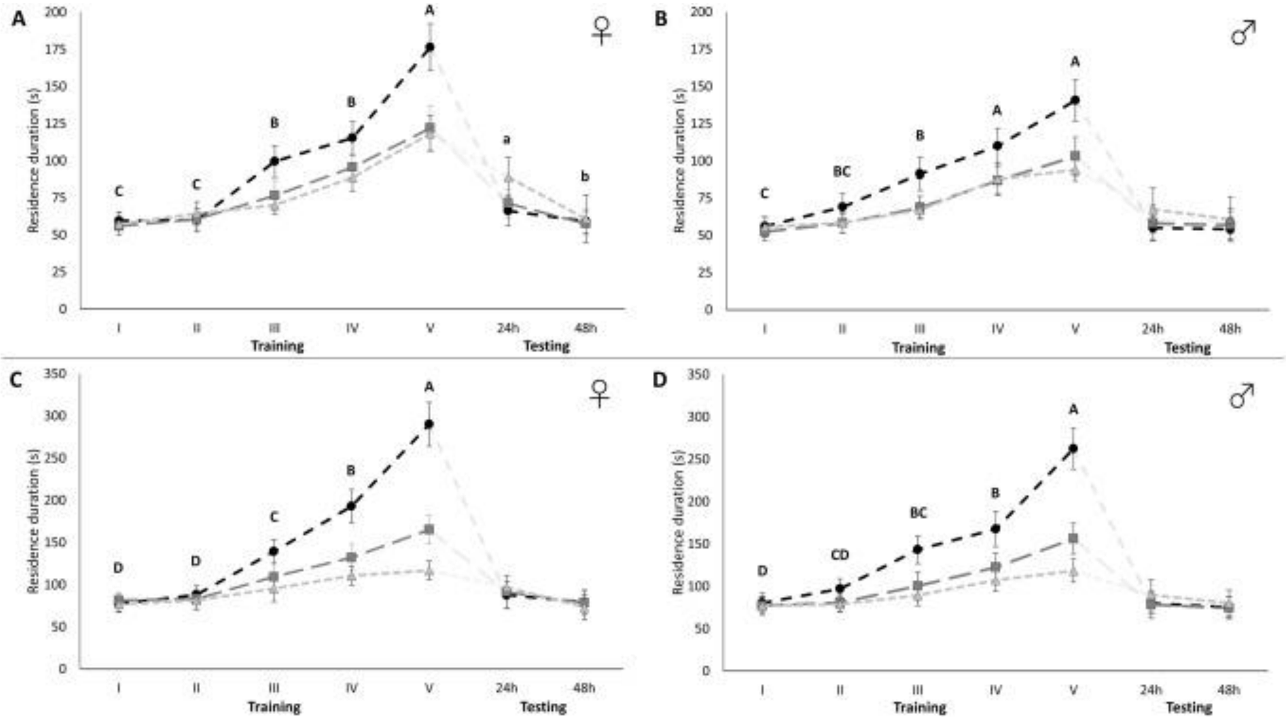

Fig. 3. Residence durations (mean ± SE, n = 100) of *R. dominica* adults in habituation trials. Habituation process (I-V training sessions and testing phases) displayed by female and males for M (A and B) and SO (C and D) nano-emulsions respectively.
Capital letters indicate statistical differences within the training sessions and lowercase within testing phases (GLMM, P < 0.05). Bars indicate standard errors. Training frequencies: ● - black = 15 min; ■ - dark grey = 30 min; ▲ - light grey = 60 min.

Stimulus specificity was noted for all the novel stimuli presented. Naïve and SO-trained insects responded equally to M ($F_{3,492}$ = 0.32, *P* = 0.81) and F nano-emulsions ($F_{3,492}$ = 0.06, *P* = 0.98), as well as M-trained *R. dominica* adults had comparable resident durations to untrained ones on novel stimuli (SO: $F_{3,492}$ = 0.04, *P* = 0.99; F: $F_{3,492}$ = 0.07, *P* = 0.98). Furthermore, the residence durations recorded for the novel stimulus were different compared to the those expected in the following training session (M nano-emulsion: $F_{2,822}$ = 38.01, *P* < 0.0001; SO nano-emulsion: $F_{2,822}$ = 127.44, *P* < 0.0001) and the decline of the repellence activity did not appear (**Supplementary Figure S5**).
Dishabituation tests demonstrated that the insects may recover their responses toward the original stimulus (i.e. shorter residence duration on the treated patches) when a novel strong stimulus was presented (Fig. 4) and the occurrence of this characteristic behavior proved the occurrence of real non-associative learning. M-habituated *R. dominica* after the exposure to SO nano-emulsion showed residence durations shorter than habituated insects ($F_{1,588}$ = 92.68, *P* < 0.0001), as well as SO-habituated adults recover their repellence toward the trained cue after the administration of M nano-emulsion ($F_{1,588}$ = 157.70, *P* < 0.0001). In contrast, when the F nano-emulsion (i.e. a week dishabituating cue), was proposed as dishabituating stimulus, insects did not recover their responses (M: $F_{1,588}$ = 2.13, *P* = 0.14; SO: $F_{1,588}$ = 1.20, *P* = 0.27) and the residence durations of *R. dominica* in the last training session were frequency-dependent (M: $F_{2,294}$ = 8.31, *P* = 0.0003; SO: $F_{2,294}$ = 35.87, *P* < 0.0001), similar to insects which did not experienced dishabituation.

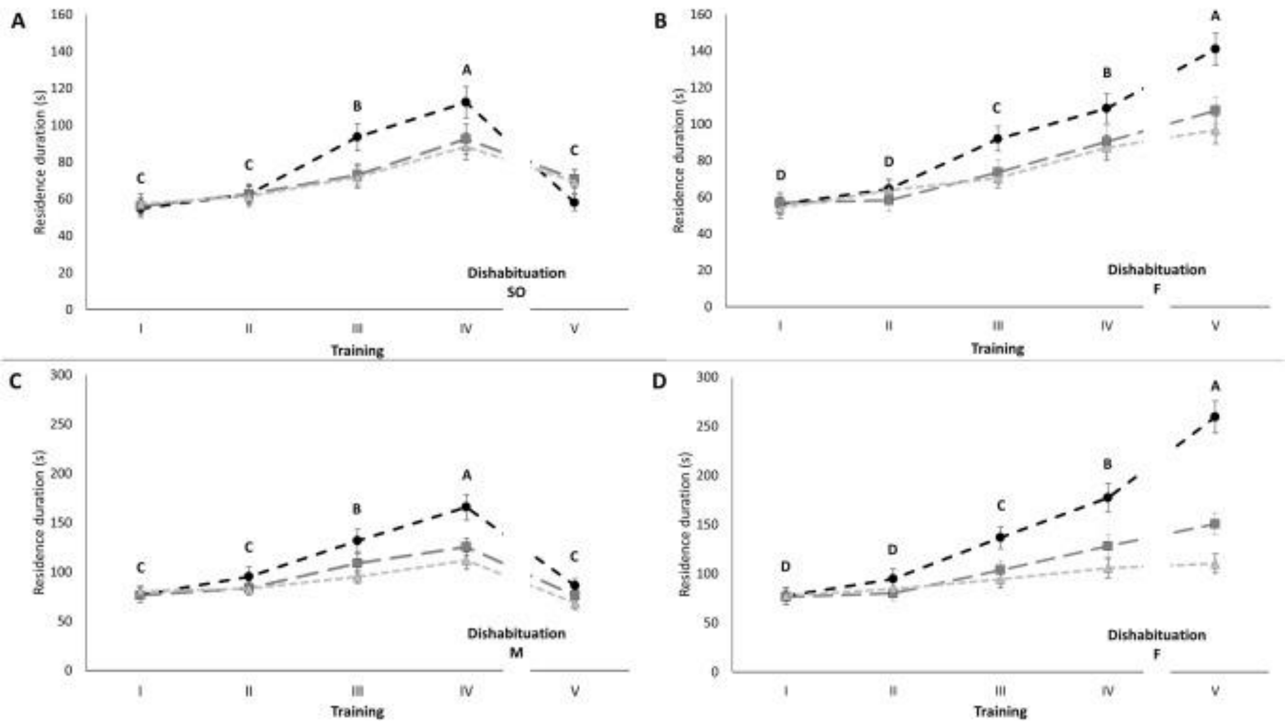

Fig. 4. Residence durations (mean ± SE, n = 100) of *R. dominica* adults in dishabituation trials. Dishabituation process (training V) for M-habituated (A and B) and SO-habituated (C and D) insects using a strong and a weak dishabituating stimulus respectively.
Capital letters indicate statistical differences within the training sessions (GLMM, $P < 0.05$). Bars indicate standard errors. Training frequencies: ● - black = 15 min; ■ - dark grey = 30 min; ▲ - light grey = 60 min.

4. Discussion

Plant-based nano-pesticides identify a variety of formulations presenting elements within the nano-metric scale, as nanoparticles and nano-emulsions (de Oliveira et al., 2014; Campolo et al., 2020b). In the present study, the developed nano-emulsions had droplet sizes lower than 200 nm, which are comparable to those of EO-base nano-emulsions obtained with higher surfactant/EO ratios (Hashem et al., 2018; Moghimi et al., 2016; Werdin González et al., 2014). Furthermore, the nano-emulsions presented low PDI, suggesting that the heterogeneous compositions of the EOs did not affect the homogeneity of the developed formulations. Using a non-ionic surfactant, the conductivity of the nano-emulsion is almost absent, and the stability is obtained by steric effect, while the surface charge is mainly attributable to elute phase characteristics (Li et al., 2016).

The selected EOs present distinctive compositions; indeed, the main components are monoterpenes hydrocarbons, oxygenated monoterpenes and phenylpropanoids for SO, M and F EO, respectively. The EO composition can explain the different physical characteristics of the developed nano-emulsions, as well as their bioactivity. However, all the tested nano-biopesticides were repellent toward *R. dominica* adults both in area choice trials as well as for residence durations in arena bioassays. Both the experiments highlighted that the repellence of the nano-emulsions is EO dependent, since M and SO-based formulations had a stronger repellent activity against this stored product pest respect to F-based one. These EOs are known to be repellent toward several stored product pests (Campolo et al., 2018; Kłyś et al., 2017) and they also exhibit toxic activity against *R. dominica,* when used as fumigant or contact insecticides (Bounoua-Fraoucene et al., 2019; Campolo et al., 2014; Shaaya et al., 1991). The formulation of the selected EOs in nano-emulsions increased the durability of the repellent effect toward the target pest. Indeed, also after 24 h from the application *R.*

dominica strongly avoided the treated filter paper halves, showing RDs similar to those calculated after 4 h of exposure. The durability of SO nano-emulsion and its repellence have been already successfully tested toward other stored product beetles [*Tribolium confusum* du Val (Coleoptera: Tenebrionidae) and *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae)], underlining the suitability of this technology to improve the applicability of botanical extracts under operative field conditions (Giunti et al., 2019).

In this context, insect ability to establish undesirable habituation behavior as response to the repellent presence can affect the effectiveness of this control strategy. Indeed, whilst a continuous exposure may have no impact on insect behavior, repeated distinct exposures to an adverse stimulus may reduce their repellent activity toward the target species. This kind of non-associative learning occurred in *R. dominica* adults exposed to M and SO nano-emulsions. This study demonstrates that the repellent responses of the lesser grain borer to EO-based nano-formulations show some typical characteristics of habituation, like the declining responsiveness dependent on training frequency (Rankin et al., 2009). There was little or no difference between the decline observed for the two longer training intervals compared to the shortest training frequency, consistent with previous literature on insect, mammalian and gastropod species (Abram et al., 2017; Byrne, 1982; Davis, 1970). Furthermore, the habituation seemed to be stronger for SO nano-emulsion than for M one. According to Rankin et al. (2009), the habituation is more rapid for low-intensity stimuli, considering that intense stimuli may lead to non-significant habituation. Thus, M nano-emulsion may be considered a stronger repellent compared to SO nano-formulation.

In contrast to results from response decline, the spontaneous recovery of the repellence was generally not frequency-dependent; however, this trait may be explained by the fast recovery expressed by *R. dominica* adults, which completely recovered their responsiveness also at the shorter testing interval (24 h). This is consistent with previous investigation on habituation in parasitic wasps, highlighting that the responses toward host kairomones can be recovered by parasitoid independently from training frequency (Peri et al., 2006). Furthermore, as suggested by Rankin et al. (2009), frequency-dependent recovery can be accounted when the asymptotical level was achieved in the responses; here, possibly at the longer training intervals this level of declined responsiveness was not achieved, allowing a quick recover.

To demonstrate the occurrence of learning over sensory adaptation or motor fatigue, two critical characteristics of habituation were tested: stimulus specificity and dishabituation (Rankin et al., 2009). Although these characteristics are fundamental to validate habituated responses, very few studies verified these assumptions when testing for habituation. Stimulus specificity relies on the hypothesis that habituation can reduce the responsiveness toward innocuous stimuli but allow the individuals to normally react to novel stimuli. Here, both M and SO-trained *R. dominica* adults displayed stimulus specificity. Furthermore, the specificity occurred either toward a strong or a weak repellent. Stimulus specificity generally occurred in arthropod species (Daly and Figueredo, 2000; Hemmi and Merkle, 2009), though habituation to deterrent compounds presented complex and asymmetric effects on the gustatory responsiveness of grasshoppers (Glendinning and Gonzalez, 1995).

Dishabituation process involves that after the presentation of different stimuli the responsiveness to the original stimulus increases. Traditionally, exclusively strong cues have been considered to produce response recovery (Thompson and Spencer, 1966), but further studies revealed that weak adverse stimuli may produce better dishabituated responses than strong ones (Marcus et al., 1988). In this research, *R. dominica* adults displayed dishabituation to the original repellent stimulus just when another intense repellent cue was proposed, while this behavioral response did not occur in presence of less intense dishabituating stimulus. Similarly, habituated responses of *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) to antifeedant compounds could be dishabituated after the exposure to a novel stimulus within the same stimulus modality (a noxious antifeedant) (Shikano et al., 2010), while other adverse cues (i.e., cold shock and CO_2 exposure) did not provoke the dishabituated responsiveness (Akhtar et al., 2003).

The present study highlighted that habituation can be a crucial behavioral process influencing the effectiveness of repellent bioinsecticides in real conditions; however, to date few information is available about the occurrence of this non-associative learning in target pest species. Several studies on the repellent and antifeedant activity of plant extracts exclusively focused on the repellence activity of compounds toward naïve pests, failing to investigate this physiological phenomenon that can impair the potential efficacy of crop and stored product protectants (Isman and Miresmailli, 2011). Bomford and Isman (1996) demonstrated that the tobacco cutworm, *Spodoptera litura* F. (Lepidoptera: Noctuidae), can quickly habituate also to azadirachtin, a potent antifeedant, almost nullifying the adverse response after just three exposures at 24 h intervals. Furthermore, the habituated responses toward certain feeding deterrents can be retained through metamorphosis and maintained after pupation by the emerging adults (Akhtar and Isman, 2003). Among stored product pests, habituation for pea fiber was observed after long continuous exposure (4 weeks) in *C. ferrugineus* (Fields et al., 2001). Although some studies focused on the decreasing responses of pests to protectants and repellents, generally the habituation characteristics (i.e. frequency-dependency, recovery, stimulus specificity and dishabituation) were not considered and investigated. Specifically, the crucial characteristic of dishabituation is fundamental to understand and predict insect behavior in field condition and to design appropriate integrated management programs. As example, if also stimulus specificity can be accounted, the synergic application of different repellents may prevent pests to quickly acquire undesirable responses, which can alter the potential insecticidal effect of the treatments.

5. Conclusions

The developed EO-based nano-emulsions presented good repellence against *R. dominica* adults, but also a decrease of the repellent activity after subsequent exposures. However, the habituation process taking place for these plant-borne formulations was moderate, as a complete recover of the responses was obtained just after 24 h for all the tested training frequencies. Furthermore, the occurrence of dishabituation in presence of intense adverse stimuli within the same stimulus modality and the stimulus specificity of the declined responsiveness are key characteristics of these promising bioinsecticides. In this scenario, it would be important to further investigate the behavioral responses of pests in a more realistic context and over a longer timescale, to validate the efficacy of this approach in IPM programs. Hence, the results presented in this paper highlighted that EO-based repellents, which have always been considered promising and effective control tools, need to be carefully used in real conditions to guarantee their actual efficacy. The occurrence of habituation toward repellent EOs in *R. dominica* should be considered when planning IPM programs, first, to avoid relying just on a single repellent; second, to alternate the repellent active ingredients during short periods; third, to place the dispensers to provide a homogeneous diffusion of the repellent; and last to use only strong repellents, which triggered low habituation outcomes in the target species. The limited behavioral resistance displayed by tested insects toward EO-based repellents suggests that this approach can be used also in combination with other biorational control tools to improve the overall effectiveness of the IPM programs against the target pests and to reduce pesticide residue and contamination of food.

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CRediT authorship contribution statement

Giulia Giunti: Conceptualization, Investigation, Formal analysis, Writing - original draft. **Orlando Campolo:** Conceptualization, Validation, Writing - original draft. **Francesca Laudani:** Formal analysis, Validation, Writing - review & editing. **Lucia Zappalà:** Funding acquisition, Project administration, Writing - original draft. **Vincenzo Palmeri:** Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Table S1 Chemical constituent of Sweet Orange Essential Oil identified by GC/MS

COMPOUND	**LRI Calculated**	**LRI**	**%Area**
α-thujene	928	931	0.01%
α-pinene	935	939	1.14%
camphene	950	953	0.01%
sabinene	975	976	0.50%
β-pinene	979	980	0.03%
β-myrcene	993	991	3.37%
octanal	1007	1001	0.33%
δ-3-Carene	1013	1011	0.18%
D-limonene	1033	1031	93.25%
terpinolene	1090	1088	0.02%
linalool	1102	1098	0.54%
trans-p-mentha-2,8-dienol	1123	1118	0.02%
cis-limonene oxide	1133	1134	0.04%
trans-limonene oxide	1138	1139	0.04%
citronellal	1155	1153	0.03%
α-terpineol	1192	1189	0.07%
decanal	1212	1208	0.10%
α-cubebene	1356	1351	tr
α-copaene	1367	1376	0.04%
β-cubebene	1381	1390	0.04%
Z-β-caryophyllene	1410	1406	0.02%
E-β-caryophyllene	1420	1418	0.04%
α-caryophyllene	1444	1454	0.01%
γ-muurolene	1468	1477	tr
germacrene D	1473	1481	0.02%
valencene	1484	1491	0.02%
α-muurulene	1492	1499	0.01%
δ-cadinene	1515	1524	0.04%
caryophyllene oxide	1578	1581	tr
TOTAL IDENTIFIED			**99.89%**

LRI= Linear retention index

Supplementary Table S2 Chemical constituent of Mint Essential Oil identified by GC/MS

COMPOUND	LRI Calculated	LRI	%Area
2,5-Diethyltetrahydrofuran	898	898	0.03%
Anisole	919	917	0.01%
α-Thujene	925	931	0.03%
α-Pinene	932	939	0.99%
Camphene	948	953	0.02%
Dehydrosabinene	951	957	0.02%
Sabinene	972	976	0.40%
β-Pinene	977	980	1.25%
2-Menthene	983	985	0.01%
β-Myrcene	990	991	0.30%
3-Octanol	995	993	0.37%
p-Mentha-1(7),8-diene (Pseudolimonene)	1005	1004	0.02%
δ-3-Carene	1011	1011	0.01%
α-Terpinene	1017	1018	0.04%
o-Cymene	1024	1022	0.12%
Limonene	1028	1031	3.64%
Eucalyptol (1,8-Cineole)	1031	1033	4.73%
(Z)-β-Ocimene	1036	1040	0.04%
(E)-β-Ocimene	1047	1050	0.03%
γ-Terpinene	1057	1062	0.08%
cis-Sabinene hydrate	1066	1068	0.19%
1-Octanol	1071	1070	0.09%
Terpinolene	1088	1088	0.06%
3-Nonanol	1098	1099	0.01%
Linalool	1100	1098	0.20%
Isopentyl isovalerate	1107	1103	0.02%
β-Thujone	1117	1114	0.01%
trans-p-Mentha-2,8-dien-1-ol	1121	1125	0.06%
cis-p-Mentha-2,8-dien-1-ol	1136	1138	0.03%
cis-Sabinol	1141	1143	0.10%
Isopulegol	1145	1145	1.52%
Menthone	1156	1154	18.23%
Isomenthone	1166	1164	13.56%
Lavandulol	1168	1166	0.16%
Menthol	1179	1173	36.72%
Isomenthol	1184	1178	1.05%
Neoisomenthol	1189	1183	0.24%
α-Terpineol	1191	1192	0.48%
Estragole	1197	1196	0.05%
trans-Dihydrocarvone	1202	1201	0.04%
trans-Carveol	1221	1217	0.02%
Citronellol	1230	1228	0.01%
cis-3-Hexenyl isovalerate	1234	1238	0.01%
Pulegone	1238	1237	1.20%

Carvone	1244	1242	0.06%
Piperitone	1253	1252	1.02%
Neomenthol acetate	1272	1275	0.19%
(E)-Anethole	1284	1283	2.64%
Menthyl acetate	1292	1294	5.79%
Isomenthol acetate	1306	1305	0.06%
cis-Pinocarvyl acetate	1310	1309	0.01%
δ-Elemene	1333	1339	0.07%
α-Cubebene	1346	1351	tr
Euguenol	1348	1356	0.01%
Piperitone oxide	1350	1363	0.01%
α-Ylangene	1367	1372	0.01%
α-Copaene	1371	1376	0.04%
β-Bourbonene	1381	1384	0.34%
β-Cubebene	1386	1390	0.01%
β-Elemene	1388	1392	0.10%
Longifolene	1395	1402	0.01%
Isocaryophillene	1402	1404	0.01%
α-Gurjunene	1405	1409	tr
cis-α-Bergamotene	1410	1415	tr
Caryophyllene	1415	1418	1.55%
β-Copaene	1424	1432	0.09%
trans-α-Bergamotene	1431	1439	0.02%
Aromadendrene	1434	1439	0.01%
(E)-geranylacetone	1440	1453	0.05%
α-Humulene	1445	1454	tr
α-Patchulene	1449	1456	0.21%
(E)-β-Famesene	1453	1458	0.04%
Alloaromadendrene	1456	1461	0.02%
α-Acoradiene	1459	1463	0.01%
γ-Gurjunene	1462	1473	0.03%
γ-Muurolene	1472	1477	0.04%
Germacrene D	1477	1480	0.52%
β-Selinene	1482	1485	tr
Valencene	1492	1491	0.26%
α-Muurolene	1496	1499	0.03%
cis-γ-Cadinene	1510	1514	0.03%
Cubebol	1513	1515	0.01%
δ-Cadinene	1519	1524	0.11%
Cadina-1,4-diene	1529	1532	tr
α-Cadinene	1534	1538	0.01%
α-Calacorene	1541	1542	tr
Sesquisabinene hydrate	1549	1547	0.01%
Aromadendrene epoxide	1563	1590	tr
Spathuneol	1575	1576	0.04%
β-Caryophyllene oxide	1579	1581	0.12%
Viridiflorol	1589	1590	0.05%

α-Humulene oxide	1606	1606	tr
epi-Cubenol	1626	1627	tr
epi-α-Cadinol (T-Cadinol)	1641	1640	0.02%
α-Cadinol	1655	1653	0.01%
TOTAL IDENTIFIED			**99.88%**

LRI= Linear retention index

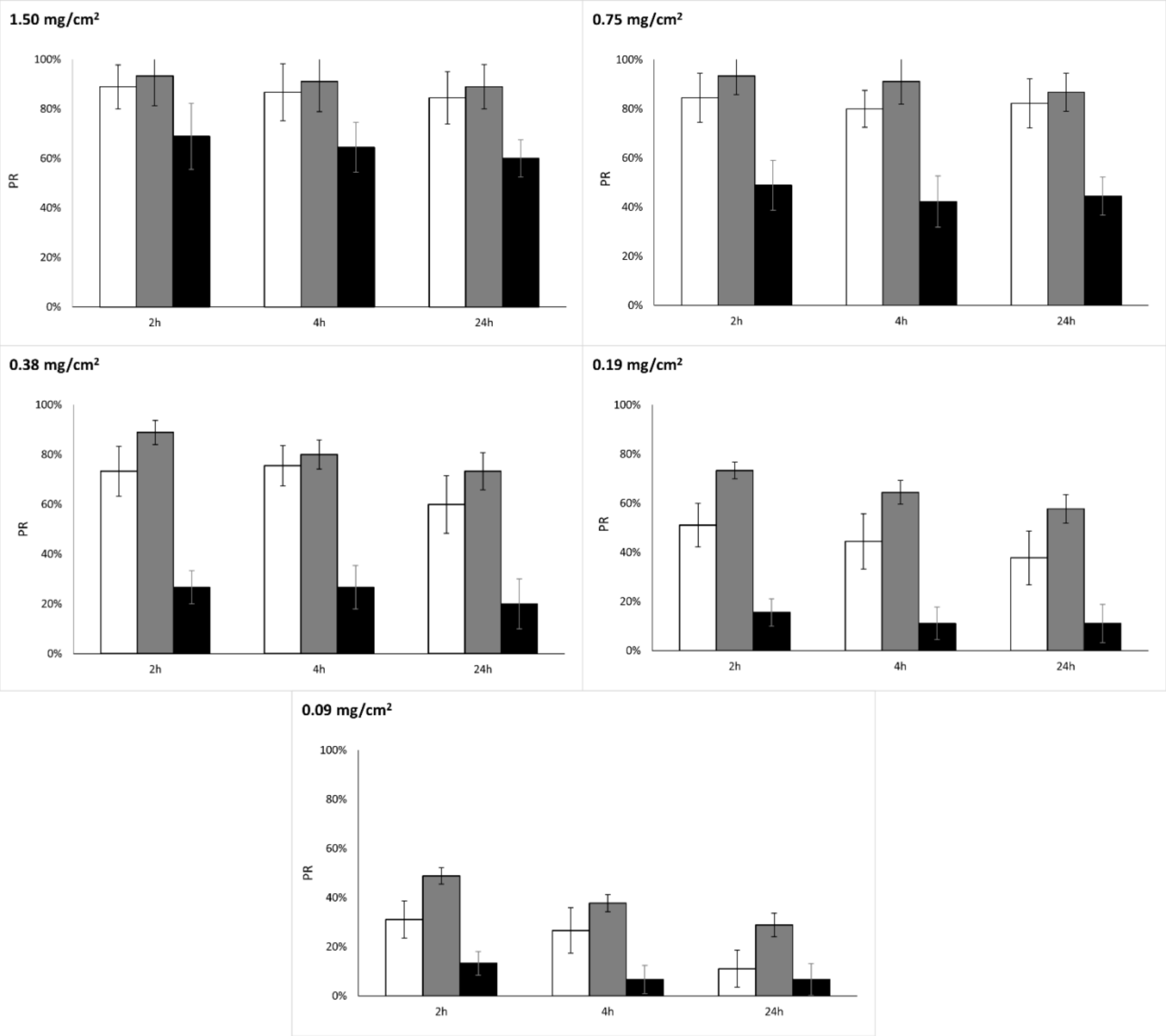
Supplementary Table S3 Chemical constituent of Fennel Essential Oil identified by GC/MS

COMPOUND	LRI Calculated	LRI	%Area
Tricyclene	923	926	0.01%
α-Thujene	926	931	0.05%
α-Pinene	933	939	2.79%
Camphene	948	953	0.39%
Thuja-2,4(10)-diene	954	957	0.00%
Sabinene	973	976	0.13%
β-Pinene	977	980	1.06%
β-Myrcene	990	991	1.21%
α-Phellandrene	1006	1005	3.33%
δ-3-Carene	1011	1011	0.36%
α-Terpinene	1017	1018	0.46%
o-Cymene	1025	1022	2.43%
Limonene	1031	1031	23.98%
(Z)-β-Ocimene	1036	1040	0.68%
(E)-β-Ocimene	1047	1050	0.06%
γ-Terpinene	1058	1062	0.19%
cis-Sabinene hydrate	1067	1068	0.00%
cis-Linalool oxide	1072	1070	0.01%
Fenchone	1090	1090	10.14%
Linalool	1099	1098	0.82%
α-Pinene oxide	1108	1105	0.00%
α-Fenchol	1113	1117	0.04%
β-Fenchol	1118	1122	0.01%
trans-p-Mentha-2,8-dien-1-ol	1121	1125	0.08%
Allo-Ocimene	1130	1132	0.01%
cis-limonene oxide	1133	1134	0.03%
cis-p-Mentha-2,8-dien-1-ol	1135	1138	0.05%
trans-limonene oxide	1138	1139	0.03%
trans-Pinocarveol	1139	1139	0.03%
Camphor	1144	1143	0.19%
Menthone	1155	1154	0.00%
Borneol	1165	1165	0.01%
3-Thujanol	1169	1166	0.01%
Dihydro-γ-Terpineol	1172	1178	0.04%
Terpinen-4-ol	1176	1177	0.16%
p-Cymen-8-ol	1186	1183	0.04%
α-Terpineol	1190	1192	0.17%
Estragole	1198	1196	3.15%
trans-Dihydrocarvone	1202	1201	0.27%
trans-Piperitol	1208	1205	0.01%
α-Fenchyl acetate	1218	1220	0.01%
trans-Carveol	1220	1217	0.02%
2-Hydroxy-1,8-cineole	1224	1229	0.02%
β-Fenchyl acetate	1232	1232	0.05%
Nerol	1235	1228	0.01%

cis-Carveol	1239	1233	0.01%
Carvone	1245	1242	0.02%
(Z)-Anethole	1253	1258	0.21%
p-Anisaldehyde	1260	1263	0.24%
(E)-Anethole	1290	1283	43.81%
2,3-Pinanediol	1315	1319	0.27%
δ-Elemene	1346	1339	0.00%
Euguenol	1356	1356	0.99%
α-Copaene	1372	1376	0.08%
Geranyl acetate	1381	1383	0.01%
β-Cubebene	1386	1390	0.01%
β-Elemene	1388	1391	0.03%
cis-α-Bergamotene	1410	1415	0.03%
Caryophyllene	1415	1418	0.25%
β-Copaene	1424	1429	0.01%
trans-α-Bergamotene	1431	1439	0.23%
Aromadendrene	1434	1439	0.02%
α-Humulene	1449	1454	0.03%
(E)-β-Famesene	1454	1458	0.04%
Alloaromadendrene	1455	1461	0.01%
γ-Muurolene	1472	1477	0.00%
Germacrene D	1477	1480	0.03%
γ-Curcumene	1480	1480	0.01%
Valencene	1489	1496	0.00%
Viridiflorene	1491	1493	0.03%
α-Muurolene	1496	1499	0.01%
(Z)-α-Bisabolene	1504	1504	0.04%
cis-γ-Cadinene	1510	1514	0.01%
δ-Cadinene	1519	1524	0.05%
Eugenol acetate	1530	1524	0.03%
Elemol	1546	1549	0.01%
(E)-Nerolidol	1561	1564	0.04%
Spathuneol	1574	1576	0.03%
β-Caryophyllene oxide	1579	1581	0.06%
Viridiflorol	1588	1590	0.02%
Guaiol	1590	1595	0.01%
Rosifoliol	1598	1599	0.01%
epi-γ-Eudesmol	1619	1622	0.00%
epi-Cubenol	1624	1627	0.00%
γ-Eudesmol	1629	1630	0.01%
epi-α-Muurolol (T-MuuroloI)	1639	1641	0.02%
α-Muurolol	1644	1645	0.00%
β-Eudesmol	1648	1649	0.00%
α-Cadinol	1652	1653	0.04%
Foeniculin (1-(3-Methyl-2-butenoxy)-4-(1-propenyl)benzene)	1680	1684	0.54%
TOTAL IDENTIFIED			99.91%

LRI= Linear retention index

Supplementary Figure S4 Percentage repellences (PR) calculated in area choice trials with thirty *R. dominica* adults (Mean ±SE, n=9) for three EO-based nano-emulsions at five different concentrations and three different time of exposure.
White bars = Sweet Orange-EO; Grey bars = Mint-EO; Black bars = Fennel-EO.



Supplementary Figure S5 Residence duration (Mean ±SE, n=100) induced by dishabituated cues to test stimulus specificity in *R. dominica* adults habituated with Sweet Orange-EO nano-emulsion (A= weak cue and B= strong cue) or Mint-EO nano-emulsions (C= weak cue and D= strong cue). Frequency of training during habituation trial: Grey bars = naïve insects; White bars = 60 min; Black bars = 30 min; Dotted bars = 15 min.