Repellence and acute toxicity of a nano-emulsion of Sweet Orange essential oil toward two major stored grain insect pests

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Abstract

Control strategies in stored-product facilities mainly rely on synthetic pesticides. The development of new environmentally friendly alternatives, such as essential oils (EOs), is a key issue. The aim of this research was to develop a stable nano-emulsion containing a high amount of sweet orange (*Citrus sinensis*) EO and to evaluate its insecticidal activity against *Tribolium confusum* du Val (Coloptera: Tenebrionidae) and *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae). The experimental nano-emulsion showed a low surface charge (ζ slightly higher than −30 mV) and a droplet size within the sub-micrometre range (131.37 nm ± 0.29), maintained also after 1 year of storage. The EO nano-emulsion presented good repellence toward both adult insects, which lasted until 24h of exposure at the highest dosages. Furthermore, the developed nano-formulation showed acute toxicity against both insects when tested as fumigant and cold aerosol. In fumigation trials, adults of *C. ferrugineus* were more susceptible than *T. confusum* adults. Moreover, the insecticidal activity of cold aerosol was 5 and 7-fold higher than fumigation against both *C. ferrugineus* and *T. confusum,* respectively. The EO-nano-emulsion was effective in controlling and repelling the target pests. Cold aerosol treatments with EO nano-formulations is a promising alternative method for the sanitation of production areas, warehouses, and machineries.

Keywords: *Tribolium confusum; Cryptolestes ferrugineus*; foodstuff pests; mortality; deterrence; fog treatment.

1. Introduction

Chemical control approaches are commonly employed to control pest infestation in stored product facilities. Synthetic pesticides and fumigants, such as methyl bromide and phosphine, have been used for decades to control insect pests on stored food, feedstuff, and other agricultural commodities (Boyer et al., 2012). However, the environmental consequences (e.g., bioaccumulation and ozone layer depletion) (Ristaino and Thomas, 1997; Serça et al., 1998), the rise of pest resistance phenomena (Bell and Wilson, 1995; Schlipalius et al., 2002) and the lack of specificity (Blümel et al., 1999; Desneux et al., 2007) increased the interest of researchers to develop new environmentally friendly alternatives to synthetic compounds (Pavela and Benelli, 2016). In this scenario, the bioactivity of botanical extracts, with particular reference to essential oils, have been widely investigated, but few commercial green pesticides for stored product pests' management are available on the market (Campolo et al., 2018). Essential oils (EOs) are promising alternative active ingredients, as they are nonpersistent in water and soils (Isman, 2000), present reduced onset of pest resistance (Regnault-Roger et al., 2012), may act as atractant for natural enemies (Hatt et al., 2018) and are relatively cost-effective (Isman, 2000). EOs are constituted by volatile and semi-volatile compounds, generally characterized by a strong odour, and synthesized by many plant species as secondary metabolites. EOs could act against stored product pests as adulticidal, larvicidal, antifeedant, fertility reducer, deterrent of oviposition, repellent, as well as they could impair larval development (Campolo et al., 2018). Among bio-pesticides, EOs are promising tools also for the control of bacterial and fungal pathogens (Romeo et al., 2008). In contrast, many EOs present really low mammalian toxicity, as reported for chamomile, citronella and eucalyptus (rat oral LD_{50} = 2-5 $g*kg^{-1}$) (Regnault-Roger et al., 2012).

Citrus EOs are considered by-products of citrus industries and are largely available in countries in which citrus represents an important agricultural production (e.g., subtropical regions, such as the Mediterranean basin). Thus, the concurrent large availability of *Citrus* EOs and their usual low cost (i.e. in particular for sweet orange, *Citrus sinensis* L.) make these compounds a potential alternative to synthetic chemical insecticides. The insecticidal activity of *Citrus* EOs has been widely investigated against mosquitoes (Campolo et al., 2016; Melliou et al., 2009; Michaelakis et al., 2009; Pavela, 2015), ticks (Benelli and Pavela, 2018), crop (Benelli et al., 2019; Campolo et al., 2017; Papachristos et al., 2009) and stored product pests (Campolo et al., 2018; Cosimi et al., 2009). For example, the fumigant bioactivity of five *Citrus* EOs was tested against the confused flour beetle *Tribolium confusum* du Val (Coloptera: Tenebrionidae). Campolo et al. (2014a) observed that EOs can effectively control *T. confusum* also at low concentrations, although the efficacy decreased in presence of debris, like flour residues. Furthermore, the efficacy of *Citrus* EOs were apparently associated to the content of monoterpenes and particularly of limonene (Ibrahim et al., 2003; Malacrinò et al., 2016).

Sweet orange EO (SO-EO) presented the highest amount of limonene among *Citrus* EOs, as well as the highest toxicity against insect pests (Campolo et al., 2014a; 2014b; Chaieb et al., 2018).

Despite their promising properties, EO-based pesticides may present some criticisms related to the standardization of the active ingredients. Indeed, considerable variation in chemical composition of EO can be related to several factors, as plant variety, season and climatic conditions (Fabroni et al., 2012; Thompson et al., 2003). Moreover, the chemical characteristics of EOs may cause some drawbacks, mainly related to the high volatility, the rapid degradation and the scarce water solubility of these botanical extracts (Moretti et al., 2002). Indeed, EOs are hydrophobic and generally lipophilic (i.e. their main compounds are non-polar or low-polar terpenes) and their density is often lower than that of water. The reduction to nano-size dimensions may improve EOs' solubility, as well as increase their stability and efficacy against the target pests (Kah et al., 2013). Indeed, loading/encapsulation of EOs by/in polyethylene glycol (PEG) nanoparticles (NPs) greatly improve the EO's water solubility and regulate the pesticide release (Campolo et al., 2017; Werdin González et al., 2014). Similarly, the incorporation of EOs inside nano-emulsion can provide several advantages, as a decreasing in volatility, increasing stability and obviously water dispensability. EO-based nano-emulsions are kinetically stable oil-in-water dispersions, having droplet covering the size range of 100–300 nm (Tadros et al., 2004). Nano-emulsions can be prepared using lower surfactant concentration than micro-emulsions and generally present long-term physical stability with no apparent flocculation or coalescence (Bouchemal et al., 2004; Golden et al., 2018).

The aim of this research was to develop a stable SO-EO nano-emulsion containing a high amount of EO. Furthermore, we wanted to evaluate the insecticidal activity of this emulsion against two economically important pests, which frequently damage processed cereals and flours, the confused flour beetle, *T. confusum*, and the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Cucujidae). Both insects are cosmopolitan external feeders, since these pests preferentially forage on damaged or previous infested kernels (Parkin, 1956) and both larvae and adults are responsible for the damages on various crops, including maize, wheat, sorghum, barley, and other cereals (Boyer et al., 2012; Oerke, 2006). These pests often occur together in stored grain in humid-continental regions (Madrid et al., 1990). First, we assessed the bioactivity of the experimental nano-emulsion as repellent and fumigant. Lately we tested an innovative administration method using a SO-EO cold aerosol as fogging agent.

2. Materials and methods

2.1 Insect colonies and rearing conditions

Insect species, *C. ferrugineus* and *T. confusum*, were reared for several generations at the laboratories of the Department of Agriculture, University *Mediterranea* of Reggio Calabria, Reggio Calabria, Italy. The original colonies

were collected from infested wheat provided by a local mill. Insects were reared on wheat flour mixed with yeast (15:1, w:w). The rearing conditions were: 25 ± 1°C, 50 ± 5% R.H., with a photoperiod of 12h:12h (L:D). To obtain coetaneous insect cohorts, about 300 unsexed adult insects were placed inside 5 L glass jars containing 500 g of rearing medium. After 5 days adult insects were removed and their progeny was used for the tests once developed in 5-10-day adults . Insects were collected from cultures using a 450-µm sieve (Technotest; Modena, Italy) and a mouth aspirator.

2.2 GC-MS chemical characterization of the SO-EO

Commercial sweet orange 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. 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 volts; scan time: 1.00 second; prescan ionization time: 100 microseconds; scan mass range: 30-300 m/z; ionization mode: EI). The following analytical conditions were employed: 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 1mL min^{-1}; injection, 0.2 µL (10% hexane solution); split ratio, 1:30. Identification of chemicals was based on the comparison of retention times (RT) and their linear retention indices (LRI), relative to the series of n-hydrocarbons, with those of pure chemicals, and on computer matching against the commercial libraries (NIST 05, Wiley FFNSC and ADAMS) and compared to 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). 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 analysed by GC-MS set at the identical conditions of the essential oil.

2.3 Nano-emulsion formulation and characterization

The SO-EO nano-emulsion was prepared following Bouchemal et al. (2004), with modifications. The nano-emulsion was produced using the spontaneous emulsification process which occurs when an organic phase and an aqueous phase are mixed. In brief, a mixture (3:1 w:w) of SO-EO and Tween 80® [Polyoxyethylene (20) sorbitan monooleate, Sigma Aldrich, Munich, Germany] was stirred for 30 mins. Then, double-distilled water was added to this mixture (4:1 respectively) and stirred for 60 min to attain a homogeneous emulsified phase. This coarse emulsion was sonicated for 5 min using an UP200ST ultrasonic immersion homogenizer (Hielsher©, Teltow, Germany) at 100W power to optimise its physical characteristics and reduce micellar dimension. The obtained oil in water nano-emulsion

was composed by 5% Tween 80®, 15% SO-EO and 80% water. Lastly, the SO-EO nano-emulsion was stored at 25 ± 0.5°C in an airtight glass bottle and used for the bioassays within the following 5 days.

A Dynamic Light Scattering (DLS) instrument (Zetasizer Nano, Malvern®) was used to assess qualitative analyses, as 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). To attain correct measurements from the instrument, 0.5 mL of SO-EO nano-emulsion were diluted in 100 mL of double-distilled water and aliquots (1 mL for droplet dimension and 0.75 mL for droplet surface charge) of the diluted emulsion was analysed. The physical characteristics of SO-EO nano-emulsion were tested after 24h from the preparation and after 1 year of storage in the dark at controlled conditions (25 ± 0.5°C in an airtight glass bottle). For each sample, three replicates of fourteen cycles were provided. Three samples were analysed as replicates.

2.4 Repellent activity of SO-EO nano-emulsion

To determine the repellent activity of SO-EO nano-emulsion an area preference bioassay was set up. The bioassays were conducted following the method described by Malacrinò et al. (2016). Insects were placed inside a glass Petri dish (9 cm Ø) covered with a filter paper (9 cm Ø, Whatman n°1) split in two identical halves, one treated with the putative repellent and the other one used as control. Here, half filter paper disks were treated with 150 µL of pure or water-diluted SO-EO nano-emulsion. Diluted nano-emulsions were obtained adding pure SO-EO nano-emulsion to double-distilled water and then gently mixed for 1 min. The other half paper disks were treated with 150 µL of Tween 80® water-solutions as control. The treated filter paper disks were dried under a fan. After drying, the disks were placed inside the Petri dishes and the insects placed in the central area of the disks. Petri dishes were covered with nylon meshes to prevent the odour saturation of the arena. The arenas were maintained at 25 ± 1°C, 50 ± 5% R.H., under constant light condition.

SO-EO nano-emulsion was diluted and the following EO-concentrations were obtained: 15% (i.e. pure SO-EO nano-emulsion), 7.5%, 3.75%, 1.88%, 0.94%, 0.47% and 0.23%, corresponding to EO-content of 0.71, 0.35, 0.18, 0.09, 0.04, 0.02 and 0.01 mg/cm^2. For every dose, 15 replicates (i.e. 10 unsexed *C. ferrugineus* or *T. confusum* adults for replicate) were carried out on different days to account daily variability. Insects were used only once. The number of insects on the two halves of the Petri dish was recorded after 2, 4, and 24h from the beginning of the test. The percent repellence (PR) of SO-EO nano-emulsion 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.

158 *2.5 Fumigant toxicity*

159 Trials were carried out under laboratory conditions at 25 ± 1°C, 50 ± 5% R.H. with a photoperiod of 16h:8h
160 (L:D), following the methodology described by Malacrinò et al. (2016) with modifications. Insects were exposed to the
161 SO-EO nano-emulsion in 300-mL glass tubes locked with airtight stainless-steel cap with a stainless-steel hook fixed on
162 the bottom. The parts of the fumigation chambers were completely washable with polar/nonpolar solvents and
163 autoclavable. A 3 cm^2 filter paper (Whatman® No. 1), soaked with a known quantity of SO-EO nano-emulsion, was
164 anchored to the hook fixed on the lid. Test insects were gently placed inside the tubes and maintained inside the
165 chamber for an exposure time of 24 or 48 h. Thus, the mortality was recorded for both insect species after 24 and 48h.

166 SO-EO nano-emulsion was diluted and the following EO-concentrations were tested: 15% (i.e. pure SO-EO
167 nano-emulsion), 11.25%, 7.5%, 5.63 and 3.75%. Different amount of SO-EO nano-emulsions were employed for the
168 pest species (i.e. 37.5 µL for *C. ferrugineus* and 150 µL for *T. confusum*). Preliminary trails with *C. ferrugineus* showed
169 that insect mortality was influenced by the humidity in the chamber. When applying 150 or 75 µL of double-distilled
170 water to the filter paper, *C. ferrugineus* adults presented a mortality rate higher than 15%, which resulted almost
171 nullified when a dose of 37.5 µL of water was provided. Thus, to obtain valuable data for statistical analyses a smaller
172 amount was applied to *C. ferrugineus*.

173 The following concentrations of SO-EO were fumigated inside the cage: 18.75, 14.06, 9.38, 7.03 and 4.69
174 µg*mL^{-1} of air for *C. ferrugineus* and 75.00, 56.25, 37.50, 28.13 and 18.75 µg*mL^{-1} for *T. confusum.* To evaluate the
175 impact of surfactant on insect mortality, control trials were carried out using a mixture of Tween 80® and water at the
176 highest concentration tested in the SO-EO nano-emulsions (i.e. 6.27 and 25 µg*mL^{-1} of Tween 80® against *C.*
177 *ferrugineus* and *T. confusum,* respectively). Additional control trials using just double-distilled water were performed.
178 For every treatment, 5 replicates (i.e. 20 insects each) were performed.

179

180 *2.6 Acute toxicity of SO-EO nano-emulsion as cold aerosol*

181 Trials were carried out under laboratory conditions at 25 ± 1°C, 50 ± 5% R.H. with a photoperiod of 16h:8h
182 (L:D). Test specimens were placed inside a Perspex cage (30 x 30 x 30 cm), presenting on one side a hole (height from
183 base 15 cm; 14 mm Ø) where an aerosol borosilicate-glass ampule (GammaDis Farmaceutici s.a.s., Civitanova Marche,
184 Italy) was plugged. A known quantity of SO-EO nano-emulsion was poured inside the aerosol ampule, which was
185 connected to an air delivery system, blowing purified air at 2 L min^{-1} constant flow. The air flow was turned off when
186 the ampule was almost empty (i.e. residues inside the glass ampula < 0.1 mL). The uniformity of distribution by the
187 aerosol equipment was proved for the tested air flow using water-sensitive papers. Tested insects were maintained
188 inside the cage for an exposure time of 24 h. After the exposure time, insects were removed from the cage, then gently

placed in a clean glass Petri dish and rearing medium (wheat flour mixed with yeast (10:1, w:w) was provided for the whole period of observation. The mortality was recorded for both insect species after 24, 48, 72, 96 and 120h from the beginning of the cold aerosol treatment.

Similar to fumigation trials, the SO-EO nano-emulsion was diluted with distilled water to obtain the following EO-concentrations: 15% (i.e. pure SO-EO nano-emulsion), 7.5%, 3.75%, 1.88%, 0.94% and 0.47%. For trials with *C. ferrugineus* adults, 2 mL of SO-EO nano-emulsion were spayed as cold aerosol, and six concentration of SO-EO were tested: 11.11; 5.56; 2.78; 1.39, 0.69 and 0.35 $mg*L^{-1}$ of air. In contrast, 4 mL of SO-EO nano-emulsion were applied against *T. confusum,* testing the adult mortality at 22.22, 11.11, 5.56, 2.78 and 1.39 $mg*L^{-1}$ of SO-EO in air. Indeed, preliminary trials carried out on *T. confusum* showed that its mortality was influenced not just by the absolute dose of EO sprayed, but also by the amount of nano-emulsion used to produce the aerosol. Although the treatment with 4mL lasted just 3-4 mins more than those with 2mL, *T. confusum* adults presented higher mortality (ca. 82%) when treated with 4mL of half-diluted SO-EO nano-emulsion than when they were sprayed with 2mL of pure SO-EO nano-emulsion (ca. 56%). Thus, to obtain valuable data for statistical analyses the highest quantity was applied against *T. confusum*.

To evaluate the potential impact of surfactant on insect mortality, control trials were carried out using formulations of Tween 80® in water at the same concentrations tested in the SO-EO nano-emulsions (i.e. 7.41, 3.70, 1.85, 0.93, 0.46, 0.23 and 0.12 $mg*L^{-1}$ of air). Additional control trials using just double-distilled water were performed. Nine replicates (i.e. 10 insects each) were provided both for control and EO trials.

2.7 Statistical analysis

Statistics were carried out using SPSS® 20 and JMP® 11 software. Data that did not met the ANOVA assumptions (normality and homoscedasticity) tested by Shapiro-Wilk and Levene's test respectively, were analysed by non-parametric tests. In repellence assays, for each insect species, binary choice data from different replicates of the same tested dose were analysed by χ^2 likelihood test ($\alpha = 0.05$). PR data calculated after 24h 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 for both insects. Values were considered significantly different if their 95% fiducial limits did not overlap. To assess differences in PR values, data were processed using a non-parametric model, the Kruskal-Wallis test, followed by Dunn's post-hoc test. PR values of each insect were separately analysed using "SO-EO dose" or "exposure time" as fixed factor. Furthermore, data of each exposure time were individually analysed using "SO-EO dose" as fixed factor. In fumigation and cold aerosol treatments, the efficacy of the tested formulation was not corrected for control mortality using Abbott's formula (Abbott, 1925), as no mortality was recorded in negative controls (i.e. pure double-distilled water). Probit analysis was performed in order to estimate the median lethal dose (LD_{50}) and 90% lethal dose (LD_{90}). For fumigation

trials, probit analysis was performed on mortality data recorded at 24 and 48h of exposure. For cold aerosol trials, mortality data of SO-EO and Tween 80® recorded at 24h and 120h from exposure were subjected to probit analysis. LD values between species were considered significantly different if their 95% fiducial limits did not overlap. To assess differences in mortality values, data were processed using a non-parametric model, the Kruskal-Wallis test, followed by Dunn's post-hoc test. Mortality values of each insect were analysed separately using as fixed factor "treatment" or "exposure time" for fumigation trials, and "treatment" or "time from exposure" for aerosol trials. Furthermore, data of each sampling time were individually analysed using "SO-EO dose" as fixed factor.

3. Results

3.1 GC-MS chemical characterization of essential oil

Thirty-five compounds were identified from GC-MS analyses (**Supplementary Figure S1**), representing 99.97% of the oil (**Table 1**). The sweet orange essential oil was almost entirely composed by monoterpene hydrocarbons (98.59%), followed by monoterpenes oxygenated (0.73%). The main constituents were *R*-limonene (93.35%), *β*-myrcene (3.38%), *α*-pinene (1.14%), linalool (0.54%) and sabinene (0.50%). Among non-terpene compounds, the most representative was the aldehydes octanal (0.33%) and decanal (0.10%). The analysed EO showed also several sesquiterpenes, although with a low relative percentage (0.22%).

Nano-emulsion formulation and characterization

SO-EO nano-emulsion showed an average size of the droplets within the nanometre range, either after 24 hours from its preparation, as well as after 1 year of storage (**Table 2**). Furthermore, the low values of the polydispersity index (0.12-0.19) indicated the size homogeneity of the formulation, since few or no aggregates were detected (**Supplementary Figure S2**). Finally, all the SO-EO nano-emulsion exhibited a negative surface charge (ζ) slightly higher than −30 mV, which decrease after 1 year (**Table 2**).

3.2 Repellent activity of SO-EO nano-emulsion

Both *C. ferrugineus* and *T. confusum* adults avoided the half filter paper disc treated with SO-EO nano-emulsion, preferring the control one at all the sampling times. However, *C. ferrugineus* showed no preferences after 24 h of exposure at the SO-EO dose of 0.04 mg/cm^2, and at the lowest tested dosage, i.e. and 0.02 mg/cm^2, repellence disappear just after 4h of exposure (**Supplementary Table S1**). Similarly, *T. confusum* presented no repellence for SO-EO after 24h at 0.02 mg/cm^2 with a PR value of 34.67% ± 5.33. However, analogous PR values were recorded for the

lowest tested dosage, i.e. 0.01 mg/cm^2, at all the considered exposure times, nullifying SO-EO repellence also after 2h (**Supplementary Table S1**).

According to Pearson test, PR values from repellence trials fitted the probit curve (*C. ferrugineus:* χ^2_4=0.09; *P*= 0.998*; T. confusum:* χ^2_5=0.93; *P*= 0.968), and RD_{50} and RD_{90} values from the insect species were not significant different (**Table 3**). For *C. ferrugineus* both the SO-EO doses (H= 72.83; df= 5; *P*<0.0001) and the exposure time (H= 6.11; df= 2; *P*= 0.047) significantly affected the repellence efficacy (**Supplementary Figure S3**), while for *T. confusum* the repellence was influenced by dose (H= 86.51; df= 6; *P*<0.0001), but not by exposure time (H= 3.04; df= 2; *P*= 0.219) (**Supplementary Figure S4**). Furthermore, a dose dependent repellence could be highlighted at all the tested exposure times both against *C. ferruginesus* (2h: H= 23.48; df= 5; *P*=0.0003; 4h: H= 26.92; df= 5; *P*<0.0001; 24h: H= 26.90; df= 5; *P*<0.0001) and *T. confusum* (2h: H= 29.37; df= 6; *P*<0.0001; 4h: H= 86.51; df= 6; *P*<0.0001; 24h: H=31.04; df= 6; *P*<0.0001).

3.3 Concentration-mortality response following fumigation and cold-aerosol exposures

Mortality from fumigation trials fitted the probit curve both at 24h (*C. ferrugineus:* χ^2_3=0.19; *P*= 0.979*; T. confusum:* χ^2_3=0.07; *P*= 0.995) and 48h (*C. ferrugineus:* χ^2_3=0.08; *P*= 0.993*; T. confusum:* χ^2_3=0.08; *P*= 0.994). Furthermore, the LD_{50} and LD_{90} values from the insect species were significant different at 48h, as their 95% fiducial limits did not overlap (**Table 4**). For both species, exposure time did not significantly alter the mortality (*C. ferrugineus:* H=2.32; df=1; *P*= 0.128*; T. confusum:* H=0.17; df=1; *P*= 0.680). Statistical differences were highlighted according to the treatment applied (*C. ferrugineus:* H=33.11; df=4; *P*<0.001*; T. confusum:* H=44.84; df=4; *P*<0.001) (**Supplementary Figure S5**). Tween 80® controls had lower toxicity toward both insect species (maximum mortality: 2.00% ± 1.50 after 48h) respect to SO-EO nano-emulsion. Fumigation with double distilled water caused no mortality. Mortality values from cold-aerosol trials were also subjected to Pearson test to prove the goodness of fit with probit model for the data recorded at 24h (*C. ferrugineus:* χ^2_4=2.51; *P*= 0.644*; T. confusum:* χ^2_3=0.39; *P*= 0.943) and 120h (*C. ferrugineus:* χ^2_4=6.02; *P*= 0.452*; T. confusum:* χ^2_3=0.70; *P*= 0.874). Both the LD_{50} and LD_{90} values estimated for the two species (**Table 5**) decreased in the survey carried out after 120h of exposure highlighting a delayed mortality induced by the nano-emulsion. Tween 80® mortality values also fitted with probit either for *C. ferrugineus* (24h: χ^2_4=0.04; *P*= 0.999; 120h: χ^2_4=0.04; *P*= 0.998), as well as for *T. confusum* (24h: χ^2_3=0.06; *P*= 0.996; 120h: χ^2_3=0.07; *P*= 0.995). However, the LD values calculated for the Tween 80® solution were heavily higher than those of SO-EO. After 120h from the exposure, LD_{90} values of Tween 80 were toward *C. ferrugineus* 14.58 mg*L^{-1} (LB-UB: 8.48-20.68) and toward *T. confusum* 25.11 mg*L^{-1} (LB-UP: 19.25-30.97). No differences were reported between the two insect species, since their 95% fiducial limits overlapped (**Table 5**). Mortality rates caused by SO-EO nano-emulsion were

281 significantly higher than those recorded for Tween 80®-control (*C. ferrugineus:* H=172.74; df=11; *P*<0.001*; T.*
282 *confusum:* H=144.01; df=9; *P*<0.001) (**Supplementary Figures S6-S7**). For both species, exposure time did not
283 significantly altered mortality (*C. ferrugineus:* H=2.71; df=4; *P*= 0.608*; T. confusum:* H=0.32; df=4; *P*= 0.989).
284
285 **4. Discussion**
286 The term nano-pesticide is used to identify a wide variety of products, defined as formulations that
287 intentionally includes elements in the nano-meter size range, such as nano-dispersions, nano-polymers, nanoparticles,
288 nano-capsules, nano-gels and nano-emulsions. Nanotechnologies may be applied to both synthetic (Nguyen et al., 2012;
289 Satehi et al.,2018) and botanical insecticides (de Oliveira et al., 2014). The aims of the nano-formulations are to
290 increase the poor solubility of some active ingredients, to guarantee a slow-constant release of the active ingredient
291 and/or to avoid premature degradation (Kah et al., 2013). Nano-emulsions are proposed for numerous applications
292 especially because of their capacity to dissolve non-polar active compounds (Gutiérrez et al., 2008). The nano-emulsion
293 produced from SO-EO presented a low droplet size (131.37nm ± 0.29), which slightly increased after 1 year of storage
294 (167.2nm ± 0.35). The size obtained for this nano-emulsion are comparable to those obtained for other EOs using
295 higher amount of surfactant (Hashem et al., 2018; Moghimi et al., 2016; Werdin González et al., 2014). Furthermore,
296 the dimension obtained using Tween 80® were smaller respect to nanoparticles obtained using polyethylene glycol
297 (PEG) with several citrus-peel EOs (Campolo et al., 2017). The homogeneity of the formulation (PI= 0.12-0.19) could
298 be attributable also the high homogeneity of the SO-EO, which contained a 93% of limonene. Stability of the nano-
299 emulsion was obtained mainly by steric effect, as Tween 80® is a nonionic surfactant,. However, nonionoic surfactants
300 do not alter the original zeta potential of the elute (Li et al., 2016). Thus, the surface charge recorded for SO-EO nano-
301 emulsion is attributable to the EO chemical characteristic. Since a minimum of ±30 mV of zeta potential is required for
302 a physically stable nano-suspension solely stabilized by electrostatic repulsion (Müller et al., 2001), here the droplets'
303 surface charges boosted the nano-emulsion stability. Nano-dimensions generally enhance both the stability and
304 effectiveness of botanical insecticides, boosting the gradual release of the active compounds (de Oliveira et al., 2014)
305 and also minimizing the negative effects on non-target organisms (Gogos et al., 2012).
306 Our results highlighted the good repellent and insecticidal activity of the SO-EO nano-emulsion against *T.*
307 *confusum* and *C. ferrugineus*. Repellence activity of SO-EO nano-emulsion was demonstrated for both the tested
308 insects. Except for the lower tested dosages, adult beetles avoided the treated side of the arena also after 24h. Our
309 results are consistent with previous studies investigating the repellence of SO-EO against pest species at shorter
310 exposure times (da Camara et al., 2015; Murugan et al., 2012). Repellent activity of SO-EO has been also noted against
311 *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), although no clear information about the actual dosages

applied were provided (Bilal et al., 2015). In contrast, this EO was not able to induce avoidance in *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae), highlighting a species-specific response to this plant-borne extract (Papachristos and Stamopoulos, 2002). Unfortunately, when testing the repellence activity, authors commonly did not calculate RD_{50} values, causing criticisms when comparing the outcomes between different EOs (Campolo et al., 2018). Nevertheless, regarding *T. confusum* the most interesting results were obtained with *Pistacia lentiscus* (Anacardiaceae) EO with RD_{50} = 0.025 µL/cm^2 after 24h (Bougherra et al., 2015). Our results accounted RD_{50} values at 24h of 0.10 mg/cm^2 of EO for *C. ferrugineus* and 0.07 mg/cm^2 of EO for *T. confusum,* showing to possess a good repellent activity toward both insect species.

SO-EO nano-emulsion show interesting insecticidal activity against both insect species when applied as fumigant, as well as cold aerosol. Adults of *C. ferrugineus* were more susceptible to fumigation than *T. confusum* ones, although contrast results were reported for the surfactant. Nevertheless, mortality data were mainly attributable to SO-EO activity, as Tween 80® presented low toxicity toward either insects. SO-EO nano-emulsion applied as fumigation showed good insecticidal activity immediately after 24h, as the LD_{50} values were non-significant different between the considered exposure times. Furthermore, applied as cold aerosol, the nano-emulsion induced higher mortality rate at lower dosages. Indeed, after 24h from the treatment, cold aerosol produced the mortality of 50% of *C. ferrugineus* at 2.19 mg*L^{-1} of air, while fumigation needed 11.03 mg*L^{-1} of air to exert the same mortality. Similarly, in *T. confusum* 50% mortality was achieved with 5.43 mg*L^{-1} of air for aerosol and 37.62 mg*L^{-1} of air for fumigation at 24h. The high efficacy of cold aerosol could be attributable to the different kind of application of the nano-emulsion, because when applied as cold aerosol SO-EO could act both as fumigant and contact insecticide. Comparing our results with the ones reported for commercial fogging agents, the SO-EO nano-emulsion presented promising insecticidal activity against *T. confusum.* As example, pyrethrin aerosol (23.4 g formulation/28 m^3 of headspace area) against *T. confusum* determined a mortality rate of 38.9% ± 4.3 after 7 days from exposure, which increased at 84.8% ± 3.2 after 14 days (Arthur, 2008).

The toxic activity of EOs against several stored product pests have been demonstrated form many insect species, including the beetles *T. confusum* and *C. ferrugineus* (for a dedicated review see Campolo et al., 2018). Among the plant secondary metabolites constituting the EOs, terpenes, and specifically monoterpenes, are considered the principal molecules responsible for the bioactivity against insect pests, as well as against microbes. The monoterpenes may penetrate inside the insect body via the cuticle (contact), the respiratory system (fumigation) and the digestive system (ingestion) (Prates et al., 1998). Although the exact mode of action of EOs has not been completely clarified yet, monoterpenes could cause the total breakdown of the nervous system, acting on the octopaminergic system of the insects (Isman, 2000; Price and Berry, 2006). Furthermore, *Carum carvi* L. EO showed prominent results for fumigant activity against *T. confusum,* causing also oxidative stress by altering the antioxidative defence system, catalase (CAT),

superoxide dismutase (SOD), and glutathione-S-transferase (GST) activities, as well as the level of lipid peroxidation (MDA) and the content of reduced glutathione (GSH) (Petrović et al., 2019). Monoterpenes are the main compounds constituting SO-EO (over 98%). Furthermore, this EO is characterized by the prevalent presence of a single molecule, *R*-limonene, whose toxicity against insects has been already accounted (Campolo et al., 2014b; Ibrahim et al., 2003; Malacrinò et al., 2016; Tripathi et al., 2003). Limonene naturally occurs in many plant species as two enantiomers: The *S*-limonene could be found in mint oils and has a turpentine-like odour, while the *R*-limonene, with a strong orange smell, is a floral compound of a number of plant species, as well as the main constituent of *Citrus* EOs (Adams, 1995; El-Sayed, 2014). Limonene is biodegradable in aerobic conditions (Schwartz et al., 1990) and, similar to phosphine (Glindemann et al., 2005), undergoes to phytochemical reactions, producing hydroxyl radicals, ozone, and nitrate radicals (Altshuller, 1983). Furthermore, this monoterpene presents a really short lifetime in the atmosphere, ranging from 12 to 48 minutes (Altshuller, 1983).

EOs extracted from citrus fruit have been used in many industrial applications, but their employment as biopesticide in agriculture and food-industry context is quite complex. The lipophilic nature, the ability to disrupt insect metabolism along with their biochemical, physiological and behavioral functions (Regnault-Roger et al., 2012) and the environment-friendly nature make EOs optimal competitors to synthetic chemical pesticides. The majority of researches related to stored product pests evaluated the insecticidal activity of EOs as fumigant or with their direct administration on foodstuff (Campolo et al., 2018). However, these techniques are unfeasible in field operative conditions. For fumigation of EO as such, the need of sealed spaces and the homogeneous distribution in large spaces are quite limiting, while the direct use on foodstuff may alter the qualities and sensory profile of food.

5. Conclusion

The SO-EO nano-emulsion was effective in controlling and repelling the target pests. This formulation was stable over 1 year of storage, maintaining good qualitative characteristics. Further work is needed to test its efficacy under more realistic operative conditions. In addition, considering the controversial opinions of biopesticides, it would be interesting to evaluate the potential side effects of these compounds on natural enemies used as biocontrol agents. Aerosol and fogging systems are rising interest among the scientific community as alternative methods to fumigation in commercial food storage facilities (Arthur, 2008; Scheff et al., 2018; Toews et al., 2006). The application of SO-EO nano-formulations as cold aerosol can be a promising method for the sanitation of production areas, warehouses, handling equipment and production machineries from stored product pests. Moreover, the scalability of the EO nano-emulsion process is realizable by pesticide industry because this process has been already used to produce some “new generation”insecticides.

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Competing Interests

The authors declare that they have no competing interests.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Table 1 Chemical characterization of sweet orange essential oil (SO-EO).

LRI	Name	%
928	*α*-thujene	0.01
935	*α*-pinene	1.14
950	camphene	0.01
975	sabinene	0.50
979	*β*-pinene	0.03
993	*β*-myrcene	3.38
1013	*δ*-3-Carene	0.18
1033	*R*-limonene	93.35
1090	terpinolene	0.02
	∑ Monoterpenes hydrocarbons	**98.59**

1102	linalool	0.54
1123	*trans*-p-mentha-2,8-dienol	0.02
1133	(*Z*)-limonene oxide	0.04
1138	(*E*)-limonene oxide	0.04
1155	citronellal	0.03
1192	*α*-terpineol	0.07
1258	carvone	tr
	∑ Monoterpenes oxigenated	**0.73**
1007	octanal	0.33
1212	decanal	0.10
	∑ Non-terpene aldehydes	**0.43**
1356	*α*-cubebene	tr
1367	*α*-copaene	0.04
1381	*β*-cubebene	0.04
1383	*β*-elemene	tr
1386	sativene	tr
1410	(*Z*)-β-caryophyllene	0.02
1420	(*E*)-β-caryophyllene	0.04
1444	*α*-caryophyllene	tr
1468	*γ*-muurolene	tr
1473	germacrene D	0.02
1484	valencene	0.02
1488	bicyclogermacrene	tr
1492	*α*-muurulene	tr

1497	germacrene A	tr
1515	*δ*-cadinene	0.04
	∑ Sesquiterpenes hydrocarbons	**0.22**
1549	elemol	tr
1578	caryophyllene oxide	tr
	∑ Sesquiterpenes oxigenated	**tr**
	∑ Identified compounds	**99.97**

Table 2 Dynamic Light Scattering (DLS) analyses of SO nano-emulsion after 24 hours from its preparation and after 1 year of storage at controlled conditions. Three replicates were provided for each test time. PDI = Polydispersity index; SE = standard error.

Test time	***Z*-average size ± SE (nm)**	**PDI ± SE**	**Zeta potential ± SE (mV)**
24 hours	131.37 ± 0.29	0.12 ± 0.003	-27.8 ± 0.58
1 year	167.2 ± 0.35	0.19 ± 0.004	-21.03 ± 0.37

Table 3 Repellence of SO-EO nano-emulsion toward two pest species after 24h. Repellent-dose values between species were considered significantly different if their 95% fiducial limits did not overlap. SO-EO = sweet orange essential oil; RD= repellent dose.

Insect species	**Probability**	**95% Confidence Limits SO-EO Dose (mg/cm^2)**		
		Estimate RD	**Lower Bound**	**Upper Bound**
Criptolestes ferrugineus	0.50	0.10	0.03	0.39
	0.90	0.41	0.11	0.71
Tribolium confusum	0.50	0.07	-0.24	0.38

0.90	0.43	0.14	0.72

574 No statistical differences (95% fiducial limits overlapped) were highlighted between the same RD (50 or 90) of the two
575 insect species.

576 **Table 4** Lethal doses of SO-EO nano-emulsion applied as fumigation toward two pest species. Lethal dose values
577 between species were considered significantly different if their 95% fiducial limits did not overlap. SO-EO = sweet
578 orange essential oil; LD= lethal dose.

Exposure Time	**Insect species**	**Probability**	**95% Confidence Limits SO-EO Dose (mg*L^{-1} of air)**		
			Estimate LD	**Lower Bound**	**Upper Bound**
24h	*Criptolestes ferrugineus*	0.50	11.03	4.18	29.10
		0.90	30.23	11.31	49.15
	Tribolium confusum	0.50	37.62	16.82	58.42
		0.90	56.17	35.50	76.84
48h	*Criptolestes ferrugineus*	0.50	9.28	0.29	18.28
		0.90	18.86	9.92	27.80
	Tribolium confusum	0.50	33.10	19.08	57.42
		0.90	54.39	30.77	78.01

579 Statistical differences (95% fiducial limits not overlapped) were highlighted between the same LD (50 or 90) of the two
580 insect species only 120h after exposure

581

582 **Table 5** Lethal doses of SO-EO nano-emulsion applied as cold aerosol toward two pest species. Absolute lethal dose
583 values between species were considered significantly different if their 95% fiducial limits did not overlap. SO-EO =
584 sweet orange essential oil; LD= lethal dose.

Time after Exposure	**Insect species**	**Probability**	**95% Confidence Limits SO-EO Dose (mg* L^{-1} of air)**		
			Estimate LD	**Lower Bound**	**Upper Bound**
24h	*Criptolestes ferrugineus*	0.5	2.19	-0.67	5.04
		0.9	5.09	2.23	6.77

	Tribolium confusum	0.5	5.43	-2.51	13.37
		0.9	13.18	5.25	21.11
120h	*Criptolestes ferrugineus*	0.5	0.93	-1.75	3.61
		0.9	3.46	-1.23	8.14
	Tribolium confusum	0.5	4.54	-2.34	11.43
		0.9	11.06	4.05	18.07

585 No statistical differences (95% fiducial limits overlapped) were highlighted between the same LD (50 or 90) of the two
586 insect species at the same time after exposure (24 or 120h).