Side effects of two citrus essential oil formulations on a generalist insect predator, plant and soil enzymatic activities

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Highlights

- Non-target effects of two formulations of citrus essential oil-based insecticides were studied.
- The generalist insect predator survival and reproduction were affected by some insecticides.
- Soil and tomato plant main enzymatic activities were not influenced by the treatments.
- The tested formulations were safer to *N. tenuis* than the reference commercial insecticides.

Abstract

The widespread use of chemical pesticides for crop protection, despite having contributed to ensure food security, have shown to exert negative impacts on the environment and on human health. In addition, the frequent emergence of resistance to pesticides and their adverse effects toward non-target organisms have generated the need to develop novel ecofriendly tools for pest control. Among these, plant essential oils (EOs) may play a central role in arthropod pest control. Recently, two formulations (Emulsion and PEG-nanoparticles) of three citrus EOs (lemon, mandarin and sweet orange) showed a promising potential against *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), a key tomato pest. Here, we evaluated the side effects of these experimental insecticides active substances toward (i) the generalist predator of several tomato pests, *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae); (ii) the soil enzymatic activities (dehydrogenase activity, alkaline phosphomonoesterase, acid phosphomonoesterase and urease) and (iii) the tomato plant antioxidant enzymes (ascorbate peroxidase, catalase, superoxide dismutase and polyphenol oxidase). Among the tested formulations, mandarin EO-based insecticide presented a significant impact on the predator survival and reproduction. Conversely, all the tested compounds proved to be harmless for the soil enzymatic and the plant antioxidant activities. Overall, these results provide solid bases for the development of novel biopesticides for sustainable tomato crop protection.

Keywords: Selectivity, *Nesidiocoris tenuis,* Nanoinsecticides, Botanicals, Enzymatic activity, Integrated pest management

1. Introduction

The use of synthetic pesticides is one of the causes of global environmental degradation and their use grows with the expansion of agriculture (Stehle and Schulz, 2015; Tilman et al., 2001). In this scenario, insecticides may exhibit a high toxicity to non-target organisms such as aquatic, pollinator, predator and parasitoid arthropods (Biondi et al., 2012; Desneux et al., 2007; Stehle and Schulz, 2015). The impact of invasive pests, having a worldwide increasing trend mainly due to climate change and global trade (Seebens et al., 2017), often leads to an intensive use of insecticides, that disrupt the existing integrated pest management (IPM) programs (Asplen et al., 2015; Ragsdale et al., 2011). This has been the case of the South American tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) which was first reported in Europe (Spain) in 2006 and since then it has been causing considerable damages to tomato crops (Biondi et al., 2018; Campos et al., 2017). The moth is currently reported worldwide in most tomato growing countries including China where it was very recently reported (Zhang et al., 2020) and with the exception of the North American and Australian continents (Han et al., 2018; Mansour et al., 2018; Verheggen and Fontus, 2019). Since its initial spread, the use of chemical insecticides was the control method most widely used by growers despite more than 70 arthropod species of which 20% predators and 80% parasitoids, were recorded attacking *T. absoluta* (Zappalà et al., 2013), some of which also applied in biocontrol programs (Biondi et al., 2018; Ferracini et al., 2019). Insecticide use in agriculture, apart from the cited unwanted effects on beneficial arthropods, may also affect the quality, fertility and health of the soil. Furthermore, toxic metabolites may be produced during the degradation processes of these insecticide molecules (Tilman et al., 2002). Therefore, their effect on the soil is one of the serious threats of their applications. Recently, Sanchez-Hernandez et al. (2018) showed that a single application of chlorpyrifos, a wide-spectrum organophosphate insecticide, negatively influenced soil enzyme activities (dehydrogenase, acid phosphatase and urease) within 14 days of its application. On the other hand, spinosad, a microbial biopesticide authorized for the control of several pests including *T. absoluta* also in organic farming, did not affect soil health and alkaline-phosphatase was not significantly influenced by the treatment within 28 days of its application (Telesiński et al., 2015). Moreover, pesticide applications can affect plant germination, growth, development, alteration in biochemical pathways, hence enzymatic activities (Parween et al., 2016). Some of these enzymes have an important protective role during the oxidative stress, such as polyphenol oxidase (PPO, EC 1.14.18.1), ascorbate peroxidase (APX, EC 1.11 0.1.11), catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, EC 1.15.1.1) which contribute jointly to strengthen the tomato plant natural antioxidant system (Barbagallo et al., 2009; Espín et al., 1998; Ricceri and Barbagallo, 2016; Vranova et al., 2002).

In addition, the selection of resistant populations to insecticides by the pest, as well as the side effects of chemical pesticides on non-target organisms, triggered the need for new alternative control strategies also in compliance with the EU Directive on sustainable use of pesticides (Directive 2009/128/EC). Among the alternative control methods, essential oil (EO)-based insecticides can play an important role in the development of new control strategies since their efficacy against different pests has been verified in a large number of studies (Campolo et al., 2018; Giunti et al., 2019, Isman, 2006; Regnault-Roger et al., 2012). EOs are secondary metabolites produced by plants for different purposes such as defense both from biotic and abiotic stresses and plant signaling including pollinator, parasitoid and predator attraction (Pavela, 2015; Smith et al., 2006; Walters, 2010; Zuzarte and Salgueiro, 2015). On the other hand, the application of EOs for plant pest control may also cause undesirable effects into the soil, caused by drip effect, interfering with the soil microbial populations therefore affecting their crucial role in the regulation of many enzymatic, physical and chemical processes occurring in this ecosystem. In fact, soil microorganisms excrete in the soil the enzymes which are involved in the principal biogeochemical cycles affecting soil fertility and health. Therefore, the study of soil enzymology is a very fast and useful method to study soil health (Nannipieri et al., 2012). To the best of our knowledge, little information is available on the effects of essential oils on the microbial communities regulating the principal biogeochemical cycles of soil

nutrients. Nevertheless, some authors reported an increase in the respiratory activity of the soil in the presence of essential oils obtained from officinal plants (Vokou et al., 1984; Vokou and Liotiri, 1999). Citrus peel essential oils were investigated as source of active ingredients aimed at developing new eco-friendly insecticides, due to their widespread availability, the relatively low cost and their efficacy against a series of crop, veterinary, medical and stored product pests (Campolo et al., 2014, 2016; Malacrinò et al., 2016). In a previous laboratory study, the efficacy of citrus EO-based emulsion and PEG nano-formulations against different *T. absoluta* instars was evaluated with promising results, through contact and ingestion exposure experiments (Campolo et al., 2017). In this study, we aim at assessing the side effects of the previously mentioned citrus EO insecticides, as well as of some commercial insecticides, on (i) the survival and reproduction of the predator *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae), a key biocontrol agent on many economically important vegetable crops (Biondi et al., 2013a,b, 2016; Calvo et al., 2009); (ii) on the main soil enzymatic activities and (iii) on the tomato plant antioxidant enzymes.

2. Materials and methods

2.1. Insecticides

The citrus EO-based insecticide formulations tested came from the same batch produced and characterized by Campolo et al. (2017); insecticides were formulated using pesticide-free certified commercial citrus peel EOs (Capua SRL, Campo Calabro Italy) extracted with the cold pressing technique from fruit grown in southern Italy. The EO-nanoparticles (NPs) were prepared by melting at 65 °C 100g of PEG (Polyethylene glycol, molecular weight 6,000). Then, 10 g of each essential oil were added to the melted PEG, while stirring the mixture using a T25 digital ULTRA-TURRAX® (IKA, Germany) for 30 min at 15,000 rpm. The mixture was then cooled at −4 °C for 2 h and completely ground in a refrigerated mortar. Finally, the product was sieved using a stainless-steel sieve (230 mesh), stored at 25 ± 0.5 °C in an airtight container, and used for the bioassay within the following 48 h. The EO-emulsion (EO-EM) was prepared using the self-emulsifying process, by adding 15% (w) of EO to 5% (w) of Tween 80. The mixture was stirred for 30 min at 10,000 RPM and then distilled water (80% w) was added. This mixture was stirred for 3 h at 10,000 RPM. Both types of formulation were produced using lemon (LE), mandarin (MA) and sweet orange (SO) essential oils. In the EOs used for the formulation preparations a total of 88 compounds were detected; the most abundant compound was limonene with 88.75, 59.19 and 52.80% detected in SO, MA and LE EOs, respectively. Monoterpene hydrocarbons ranged from 96.08% (SO) to 91% (LE). Oxygenated compounds (aldehydes, esters and alcohols) were more abundant in LE (8.91%) followed by MA (4.36%) and SO (3.28%) (Campolo et al., 2016). EO-NPs had dimensions ranging in the nanometric scale (212.5–240 nm) and high Zeta potential values (−27.80; −31.13 mV). For complete procedures and chemical characterization see Campolo et al. (2017). Indoxacarb (Steward®, DuPont™) and spinosad (Laser®, Dow Agrosciences™) were used as positive controls in trials aimed at evaluating the lethal and sublethal effects toward *N. tenuis* for their known toxic effects on the predator (Arnó and Gabarra, 2011; Dáder et al., 2019), as well as the impact of pesticides on plant enzymatic activities. While, chlorpyrifos (Dursban® – DOW Agrosciences™) and spinosad were used as reference treatments in the trials aimed at evaluating the soil enzymatic activities.

2.2. Insect and plant rearing

All the bioassays involving insects were carried out at the Department of Agriculture, Food and Environment of the University of Catania (Italy) inside climatic chambers set at 25 ± 1 °C, 50 ± 10% RH, 14:10 L:D. Tomato plants (cv. Shiren), used in both the bioassays and insect rearing, were grown in insect-proof cages placed in an external greenhouse. Tomato seedlings were transplanted into 1-litre pots using organic soil fertilized with a mineral complex fertilizer (N = 20; P = 5; K = 10). No pesticides were sprayed on plants used in the trials.

The *N. tenuis* colony originated from wild specimens collected in different tomato crops located in South-eastern Sicily (Italy). To avoid inbreeding, twice a year, wild specimens collected on tomato plants were introduced in the rearing cages. Tomato plants bearing 5–6 expanded leaves were used as feeding and oviposition substrate for mirid rearing (Naselli et al., 2017) and frozen *Ephestia*

kuehniella Zeller (Lepidoptera: Pyralidae) eggs were provided *ad libitum* on tomato leaves as factitious prey (Mollá et al., 2014). To obtain coetaneous insects, several unsexed newly-emerged *N. tenuis* adults were released inside polyester net cages (50 × 60 × 80 cm) containing four well developed tomato plants (height: 25 cm). The predators were left to lay eggs for three days and then removed from the cage. Newly emerged adults (2-5-days-old) were collected by a mechanical aspirator, then sexed and stored at low temperature (~7 °C) for max 12h. The adult mirid bugs (males and females) were placed together for at least 2h at ambient temperature before they were used in the experiments.

2.3. Side effects on *Nesidiocoris tenuis*

2.3.1. Lethal effects

The side effects of both the EO-EMs and EO-NPs were evaluated by assessing both the adult mortality and progeny produced by *N. tenuis* survived females. In detail, shoots were collected from plants sprayed 1h, 3 and 7 days before with the two formulations of the three EOs at the most effective concentrations (40 mg/mL) against *T. absoluta* (Campolo et al., 2017) and from both the negative (Water alone and Water + Tween 80) and positive (spinosad and indoxacarb) controls. Indoxacarb was sprayed at the highest recommended rate for tomato crop, whereas spinosad was tested at two different recommended rates: i) for the management of *T. absoluta* and thrips (25 mL/hL) and ii) for the management of agromyzid leafminers (75 mL/hL).

Plants were sprayed until run off using a 2 L power-pack aerosol hand sprayer (Dea®, Volpi, Italy). For each treatment and residue age, five couples of *N. tenuis* were released on tomato shoots placed inside an isolator made of a 600 mL plastic glass, provided with a hole (0.5 cm diameter) at the base, placed on top of another plastic glass. The stem of the shoot was inserted into the hole and then immersed in water contained in the bottom plastic glass (Biondi et al., 2012). After *N. tenuis* adult release, the upper opening of the isolator was covered with a fine mesh net to allow ventilation. The mirid mortality was assessed daily for three days by recording the number of live and dead adult males and females. Specimens were considered dead when they remained immobile after being stimulated with a fine paintbrush. Each treatment was replicated 10 times. Each replicate consisted in one tomato shoot and 5 *N. tenuis* couples.

2.3.2. Sublethal effects

To evaluate the side effects of insecticide treatment on the mirid progeny, after having checked the mortality for toxicity assessment (3d), survived adults were removed from the arenas and the number of emerged juveniles (nymphs) was recorded daily for additional ten days. To avoid cannibalism, emerged progeny was removed daily from the arena.

2.4. Non-target effects on soil enzymatic activities

In order to assess the effects of the developed nano-formulations on the soil enzymatic activities, soil coming from an organic farm was used. The agricultural top soil was collected (0–15 cm) in a plot on the slopes of Etna (Catania, Italy). This soil was not cultivated and was not subjected to anthropic manipulation, i.e. pesticide applications, fertilization, tillage, in the last two years. The soil samples were previously partially dried (15% humidity), sieved at 2 mm and stored at 4 °C for 3 days in PVC semi-closed boxes. pH (6.7), texture (sand, loam and clay were 66%, 17% and 16%, respectively), organic carbon (1.4%), total nitrogen (0.06%) and phosphorus content (56 ppm) in the soil were measured according to Violante (2000).

Soil samples (50 g dry weight) were aerated and hydrated 50% water holding capacity for 3 days before treatments (Barone et al., 2019; Dick, 2011) with the essential oils (both as emulsion and nanoparticles) and with the formulation ingredients (water, water + Tween 80 and water + PEG), by adding 1 mL (5 µg) of each to obtain a final concentration of 0.1 µg per g of dried soil. In order to evaluate the effect on soil enzymatic activities, two insecticides, spinosad and chlorpyrifos (at label rates at a final concentration of 0.8 and 20 µg/g dried soil, respectively) were used as reference treatments. In particular, spinosad was used because on the one hand it was proved effective in the control of *T. absoluta* and on the other it does not negatively affect the soil enzymatic activities (Telesiński et al., 2015). Whereas, chlorpyrifos was used as positive control treatment because its

negative effect on soil enzymatic activities is well proven in the literature (Sanchez-Hernandez et al., 2018). After treatments, soil samples were manually mixed, kept partially closed with a screw cap, incubated at 27 ± 0.5 °C, in dark for 4 days. For each treatment, 3 replicates were performed in 3 independent boxes and the experimental trial was executed twice. At the end of the incubation period, the samples were analyzed to evaluate the soil enzymatic activities as described in Barone et al. (2019). DHA (EC 1.1) was assayed according to von Mersi and Schinner (1991) in 1 M Tris(hydroxymethyl) aminomethane (TRIS buffer), pH 7, by mixing 1g of soil with 9.88 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazoliumchloride (INT) solution. Samples were incubated for 2 h at 40 °C and the reduced iodonitrotetrazolium formazan (INTF) was extracted by using a mixture of ethanol and dimethylformamide (1:1). Concentration of INTF in the samples was calculated photometrically (Jasco V-530 UV–vis spectrophotometer) at 464 nm from an INTF standard calibration curve and activity was expressed as μg of produced INTF per g of dried soil in 1 h. Acid (EC 3.1.3.2) and alkaline (EC 3.1.3.1) phosphomonoesterase activities (ACP and ALP, respectively) were performed by using a modified version of the two original methods (Eivazi and Tabatabai, 1977; Tabatabai and Bremner, 1969). Soil samples (1 g) were incubated at 37 °C for 1 h using 115 mM p-nitrophenyl phosphate as substrate in a buffer made of 28 mM TRIS, 28 mM maleic acid, 19 mM citric acid and 28 mM boric acid pH 6.5 and pH 11 for ACP and ALP, respectively. Reaction was then stopped by using 0.5 M calcium chloride and 0.5 mM sodium hydroxide. After sample filtration, the photometrically (Jasco V-530 UV–vis spectrophotometer) quantification at 400 nm was determined. The concentration of p-nitrophenol (PNP) released in the samples was calculated from a p-nitrophenol standard calibration curve and activity was expressed as μg of produced PNP per g of dried soil in 1 h. Finally, urease activity (URE, EC 3.5.1.5) was performed using a modified Berthelot method (Kandeler and Gerber, 1988). The soil samples (5 g) were mixed in a 720 mM buffered urea solution, incubated for 2 h at 37 °C, then treated with 2 M potassium chloride and filtered. Under alkaline pH conditions (pH 10), a green-colored complex was formed as a result of the reactions between NH_3 and sodium salicylate in the presence of sodium dichloroisocyanurate. The N released in the reaction was determined photometrically (Jasco V-530 UV–vis spectrophotometer) at 690 nm, calculated from a NH_4Cl standard calibration curve and activity was expressed as μg of N per g of dried soil in 1 h. Sodium nitroprusside was used as a catalyst and increased the sensitivity of the method about tenfold. Soil enzymatic activities were performed on three replicates of soil samples for each box and experiment.

2.5. Non-target effects on plant antioxidant and oxidative enzymes

The effects of the developed formulations on different endogenous antioxidant enzymes were evaluated 48 h after insecticide treatments (i.e. EO-Emulsions, EO-Nanoparticles, indoxacarb and spinosad) conducted as described above. The ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) enzymes were extracted from different plant organs (leaves, stem and roots) putting 1 g of homogenate plant tissues in contact with 10 mL of absolute ethanol at 4 °C for 30 min. This suspension was centrifuged at 12,000 x *g* at 4 °C, while the supernatant eliminated. The ethanol extraction was repeated twice. This pellet was subsequently suspended in 3 mL of 50 mM sodium-phosphate buffer (pH 7.0) containing 2 mM EDTA and 3% (w/v) PVP-40 (w/v), centrifuged and the supernatant thus collected was used for the enzymatic assays (Donahue et al., 1997).

Ascorbate peroxidase (APX, EC 1.11.1.11) was measured according to Ushimaru et al. (1997) by assessing the decrease in absorbance at 290 nm defining one unit (U) of APX equal to 1 mmol mL^{-1} ascorbate oxidized min^{-1} at 20 °C. Catalase (CAT, EC 1.11.1.6) was determined by measuring spectrophotometrically (240 nm) the decomposition of H_2O_2, as described by Aebi (1984). To avoid a rapid decrease of the initial velocity of the reaction, the assay was conducted using low concentrations of H_2O_2 (<0.05 M). The amount of enzyme able to decompose 1 μmol of H_2O_2 per minute represents an enzyme unit (U). Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) according to Masia (1998). One unit of activity (U) was defined as the amount of enzyme that would inhibit 50% of NBT photoreduction at 560 nm. Polyphenol oxidase (PPO) catalyzes the

hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity) (Robb, 1984). The catecholase activity of PPO was determined spectrophotometrically using a modified version of the method proposed by Espín et al., 1997, Espín et al., 1998.

Ten grams of homogenate plant organ (leaves, stems and roots) were added to 20 mL cold acetone (−20 °C) and continuously stirred for 10 min. The homogenate was filtered through Whatman No. 42 paper (Milan, Italy) under vacuum on Buchner funnel. The obtained acetonic powder was collected, suspended in 15 mL 0.1 M citrate buffer (pH 5.5) and kept overnight at 4 °C. It was then filtered again through Whatman No. 42 paper under vacuum on Buchner funnel. Finally, the clear solution was ultrafiltered in a cell equipped with a 50 kDa membrane (Millipore 8050, Milan, Italy). The enzymatic activity was assayed spectrophotometrically at 505 nm using catechol as phenolic substrate. The standard reaction mixture contained 0.9 mL of 0.04 M phenolic substrate, 0.1 mL of 0.093 M MBTH (3-methyl-2-benzothiazolinone hydrazone) chromophore coupling agent in methanol, 0.05 mL of DMF (N,N-dimethylformamide, 99,8%), 1.5 mL of 0.05 M sodium acetate buffer at pH 7.0 and 0.5 mL of enzymatic extract. The reaction was stopped at different times with 0.5 mL of 0.9 M H_2SO_4. Blank was prepared by inverting the order between the enzymatic extract and H_2SO_4. One unit of PPO activity was defined as the amount of enzyme which produces an increase in absorbance of 0.001 per min at 25 ± 0.5 °C under the conditions described above.

The activity of each enzyme was expressed on a protein basis (U mg^{-1}). Protein content was determined according to Bradford (1976) using bovine serum albumin as standard.

2.6. Data analysis

Dependent variables were tested for homogeneity and normality of variance (Levene and Shapiro-Wilk test respectively) and transformed (arcsin √x) whenever needed. Mortality data were corrected for control mortality using Abbott' formula (Abbott, 1925). Mortality and progeny production were subjected to univariate analysis of variance following the General Linear Model (GLM) procedure. Data related to enzyme concentrations were subjected to GLM with treatment, plant organ (leaves, stems and roots) and the interaction of these two factors on the content of the four investigated enzymes (APX, CAT, SOD and PPO) as predictor variables. In order to assess any significant effect of the formulation, a series of Wilcoxon non-parametric tests were carried out on each dataset specific for essential oil (EO and NP), plant organ and enzyme. Multiple comparisons were carried out using Tukey's HSD post-hoc test. All statistical analyses were performed using the software IBM SPSS v. 22 (IBM, Armonk, NY, USA).

Finally, to evaluate the potential harmfulness of the tested insecticides on the predator, taking into account both the toxic effect and the side effects on the progeny production, the reduction coefficient (Ex) was calculated according to Biondi et al. (2012):

$$Ex = 100\left\{1 - \left[\left(1 - \frac{E_{mx}}{100}\right)\left(1 - \frac{E_{fx}}{100}\right)\right]\right\}$$

where E_{mx} is the corrected mortality (Abbott, 1925) and E_{fx} is the corrected Predator reproductive capacity estimated using the formula:

$$E_{fx} = 100 - \frac{F_x 100}{F_c}$$

where F_x is the mean Predator reproductive capacity for pesticide x and F_c is the Predator reproductive capacity recorded in the control group (untreated group). The values (E_x) were then classified and interpreted according to the standards of the International Organization for Biological Control (IOBC) which include four categories: (1) harmless: $E_x < 30\%$, (2) slightly harmful: $30\% < E_x < 80\%$, (3)moderately harmful: $80\% < E_x < 99\%$, and (4) harmful: $E_x > 99\%$ (Sterk et al., 1999).

3. Results

3.1. Side effects on *Nesidiocoris tenuis*

3.1.1. Lethal effects

In the water control treatments, the mortality was 5.81% ± 2.38, 10% ± 5.48 and 8.5% ± 3.84 in the plants treated 1h, 3d and 7d before, respectively. Overall, the treatments (formulation, EO and age of residues) had a significant impact on the predator corrected mortality ($F = 80.89$; $df = 11$; $p < 0.001$). In the plants treated 1h before the predator exposure, *N. tenuis* mortality was significantly higher in the plants treated with the two commercial insecticides ($F = 22.03$; $df = 9$; $p < 0.001$), with indoxacarb able to kill 97.87% ± 2.12 of the exposed insects followed by spinosad at the highest dose (75 mL/hl) that killed 53.05% ± 10.37 of the adult predators (Fig. 1a). The developed EO formulations as well as the surfactant (Tween 80) used as additive during the formulation preparation process, had a low impact on the mirid mortality ranging from 24.56% ± 10.84 (mandarin NP) to 6.81% ± 4.22 (lemon EO). Instead, spinosad, applied at 25 mL/hl, was the least harmful toward adult predators (mortality 3.56% ± 0.89) (Fig. 1a).

Fig. 1. Lethal effect. Means (±SEM) of corrected mortality percentages of *Nesidiocoris tenuis* after 3 d of exposure to (A) 1-h-old, (B) 3-d-old and (C) 7-d-old insecticide residues. Columns bearing different letters are significantly different (ANOVA followed by Tukey HSD test at $p < 0.05$).

When exposing insects to 3-day-old residues, both the EO-emulsions as well as the EO-nanoparticles had a significantly lower impact ($F = 54.53$; $df = 9$; $p < 0.001$) on mirid mortality than indoxacarb (corrected mortality 86.96% ± 4.07). Among the EO-based formulations, lemon EO-NP was the most noxious toward *N. tenuis* adults (mortality: 9.01% ± 3.48) (Fig. 1B). The positive control spinosad, applied at 75 mL/hL, caused a mortality of the mirid about three times lower than that recorded on plants with 1h-old residues (18.03% ± 6.88 versus 53.05% ± 10.37) (Fig. 1A and B).
Seven days after the treatments, most of the insecticide residues had a negligible impact on the mirid survival. Except for indoxacarb ($F = 170.82$; $df = 9$; $p < 0.001$), which was able to kill almost all the insects exposed to the sprayed plants (94.86% ± 3.23); all the other treatments caused a corrected mortality lower than 7% (Fig. 1C). Seven days after the treatment, Lemon EO, both as emulsion and as nano-formulation, had no significant impact on the predator mortality.

3.1.2. Sublethal effects

The treatment ($F = 10.97$; $df = 11$; $p < 0.001$) as well as the age of residues ($F = 9.25$; $df = 2$; $p < 0.01$), significantly affected the progeny produced by the mirid females. The surfactant Tween 80 used during the formulation development process, had a negative impact on the progeny production compared with the negative control (water) but, on average, it was less influential in comparison with most of the other treatments (Fig. 2).

Fig. 2. Sublethal effects of insecticides on offspring production by *Nesidiocoris tenuis*. Means (±SEM) of total emerged nymphs of *N. tenuis* per female during 3 d of exposure to (A) 1-h-old, (B) 3-d-old and (C) 7-d-old insecticides residues. Columns bearing different letters are significantly different (ANOVA followed by Tukey HSD test at $p < 0.05$).

In plants treated 1h before *N. tenuis* release, the progeny produced by each female significantly varied among the treatments ($F = 9.69$; $df = 10$; $p < 0.001$) and both the EO ($F = 24.83$; $df = 2$; $p < 0.001$) and the formulation (EM or NP) ($F = 0.58$; $df = 1$; $p < 0.001$) influenced the offspring production. Among the tested EOs, mandarin, in both tested formulations, had a strong impact on the progeny production comparable to that recorded in plants treated with spinosad at the highest dose (75 mL/hL) and indoxacarb. On the contrary, sweet orange EO, in both formulations, as well as the surfactant Tween 80 had the lowest impact on the mirid progeny production (Fig. 2A).
In plants treated 3d before the mirid release, the progeny production significantly varied among the different treatments ($F = 16.30$; $df = 10$; $p < 0.001$), the EO ($F = 9.88$; $df = 2$; $p < 0.01$) and the formulation ($F = 4.54$; $df = 1$; $p < 0.05$). Overall, the NP formulations reduced the offspring production more than the corresponding emulsions ($F = 14.63$; $df = 1$; $p < 0.05$) whereas indoxacarb and spinosad applied at 75 mL/hL were less influential than the mandarin NP; conversely the other

EO based formulations had a lower impact on the progeny production than the synthetic insecticides (Fig. 2B).
When releasing the adult predators on plants treated 7 days before, the progeny produced by females was significantly influenced by the type of essential oil ($F = 7.78$; $df = 2$; $p < 0.01$) but not by the formulation (EM or NP) ($F = 0.01$; $df = 1$; $p = 0.59$). Among the synthetic insecticides used as positive controls, indoxacarb was the most harmful (Fig. 2C).

3.1.3. Reduction coefficient

Indoxacarb was the only insecticide classified as harmful (class 4) according to IOBC toxicity categories since its reduction coefficient (Ex) was >99% (Table 1). Its harmfulness was stable for all age residues tested (i.e. 1h, 3 and 7 days). Spinosad applied at 75 mL/hL, was classified as moderately harmful (IOBC class 3) when pesticide residues were 1-h- and 3-day-old (Ex = 94 and 91% respectively), whereas when pesticide residues were 7-day-old it was classified as slightly harmful (Ex = 58%; IOBC class 2). Among EOs, mandarin was the most toxic since both the developed formulations (i.e. EM and NP) were always classified as moderately harmful (IOBC class 3) with a reduction coefficient ranging from 94 to 82%.
Sweet orange EO-based formulations were the least toxic among all the developed formulations with Ex values ranging from 19% (IOBC class 1) to 89% (IOBC class 3) at 1-h- and 3-day-old residues, respectively (Table 1). In details, this EO encapsulated in nanoparticles highlighted a higher non-target toxicity than the respective emulsion.

Treatment	1-h-old residue		3-d-old residue		7-d-old residue	
	E_x (%)	IOBC Class	E_x (%)	IOBC Class	E_x (%)	IOBC Class
Indoxacarb (12.5 g/hl)	99	4	99	4	99	4
Mandarin EM	95	3	82	3	87	3
Mandarin NP	94	3	96	3	96	3
Spinosad (75 ml/hl)	94	3	91	3	58	2
Lemon EM	84	3	87	3	64	2
Lemon NP	68	2	81	3	59	2
Spinosad (25 ml/hl)	48	2	42	2	34	2
Control Tween	45	2	54	2	58	2
Sweet orange EM	19	1	62	2	78	2
Sweet orange NP	32	2	89	3	83	3

Table 1. Reduction coefficient (E_x) compared to the untreated control and IOBC toxicity classes estimated for *Nesidiocoris tenuis* after 3 d of exposure to 1-h-old, 3-d-old and 7-d-old insecticide residues.

3.2. Non-target effects on the soil enzymatic activities

The addition of all the essential oil formulations to the soil did not significantly affect the enzymatic activities of dehydrogenase, urease, alkaline and acid phosphomonoesterases, if compared to the controls (water, Tween 80 and PEG) (Table 2). Interestingly, the use of PEG and Tween 80 in the

formulation of the essential oils did not significantly influenced all the tested soil enzymatic activities (Table 2). As regard Spinosad, this insecticide did not impact soil enzymatic activities showing values always similar to the untreated controls (Table 2). On the contrary, chlorpyrifos greatly influence the soil enzymatic activities tested, reporting values always significantly lower than those measured in the control soils ($F = 46.6$; $df = 10$; $p < 0.01$).

	DHA (μg INTF $g^{-1}h^{-1}$)	ALP (μg PNP $g^{-1}h^{-1}$)	ACP (μg PNP $g^{-1}h^{-1}$)	URE (μg N $g^{-1}h^{-1}$)
Sweet orange EM	219.7±4.7a	141.2±1.3a	182.6±2.7a	173.9±3.0a
Lemon EM	226.3±6.6a	141.4±1.8a	183.8±1.5a	171.6±1.3a
Mandarin EM	223.6±9.6a	142.3±2.6a	184.7±3.1a	175.4±3.4a
Sweet orange NP	220.7±3.6a	140.1±1.1a	182.0±1.1a	176.3±3.8a
Lemon NP	218.6±5.0a	141.8±2.0a	183.7±3.1a	170.4±2.8a
Mandarin NP	220.7±3.8a	142.5±3.4a	184.3±3.3a	175.2±4.8a
Chlorpyrifos	120.7±3.2b	108.3±0.7b	125.3±0.9b	134.7±1.3b
Spinosad	219.6±5.9a	139.6±0.9a	182.0±2.1a	176.2±6.4a
Untreated control	221.0±5.1a	140.9±2.2a	181.9±2.4a	176.1±5.5a
Control Tween	218.2±1.8a	140.8±1.7a	183.8±1.4a	173.5±6.9a
Control PEG	218.8±3.5a	141.5±2.0a	183.2±1.4a	175.2±5.0a

Table 2. Soil enzymatic activities (DHA: Dehydrogenase activity; ALP: Alkaline phosphomonoesterase; ACP: Acid phosphomonoesterase; URE: Urease) in treated soils. Means (±SE) in each column followed by different letters are significantly different (ANOVA followed by Tukey HSD test at $p < 0.05$).

3.3. Non-target effects on plant antioxidant and oxidative enzymes

The activities related to the antioxidant enzymes (APX, CAT and SOD) as well as the oxidative PPOs in different tomato plant organs (roots, stem and leaves) are shown in Table 3. Insecticide treatments, plant organs and their interaction, significantly influenced the four enzyme activities. Conversely, the effects of the formulation (EM vs NP) for each essential oil on enzyme activity, were not significant ($p > 0.05$). As shown in Table 3, the two pesticides spinosad (75 ml/hL) and indoxacarb differently affected the enzyme activities in the leaves. In particular, spinosad did not significantly affect APX, CAT and SOD activities in all the plant organs, except that in the leaves where it significantly increased ($p < 0.01$) SOD activity (1.1 times higher than in the untreated control). On the contrary, in all plant organs, spinosad significantly reduced the PPO activities compared to the untreated control ($p < 0.01$). Indoxacarb influenced the antioxidant system of the plant by increasing only APX activity in the leaves as well as CAT activities in the stems and in the leaves. Finally, indoxacarb significantly reduced the PPO activities in the leaves and in the stems. The treatment carried out exclusively with Tween 80 showed values of all the antioxidant activities similar to the untreated control, whereas PPO activity was reduced both in the leaves and in the stems (1 and 1.1 times, respectively). Among EOs,

lemon and orange in both tested formulations (EM and NP) did not significantly affect the antioxidant activities (APX, CAT and SOD) in all plant organs. Interestingly, mandarin NP significantly increased APX activity compared to the untreated control (1.4 times) in the leaves as well as CAT and SOD activities in the stems (both 1.2 times compared to the untreated control). Moreover, mandarin EM significantly increased ($p < 0.01$) SOD activity in the stems (1.1 times compared to the control). Noteworthy, all antioxidant activities remained rather constant in the roots also in the plants treated with mandarin EO, both as EM and NP (Table 3). On the contrary, in the stems all EO formulations significantly decreased the values of PPO activities (1–1.1 times compared to the control).

Treatment	Leaves			
	APX	CAT	SOD	PPO
Untreated	14.17±0.36ab	8.91±0.27ab	73.99±0.87 abcd	160.68±2.69 e
Tween 80	12.63±0.13 a	7.72±0.18 a	73.32±0.80 abcd	158.02±1.06 cbd
Spinosad	15.24±0.41ab	9.26±0.29ab	80.54±1.08 e	147.95±6.67 abcd
Indoxacarb	20.88±0.41d	11.44±0.54 c	75.88±0.21 cde	150.05±4.25 abcd
Lemon EM	15.55±1.38ab	8.77±0.60ab	72.08±1.86 abc	154.19±6.62 abcd
Lemon NP	14.97±0.98ab	8.88±0.53ab	70.62±1.72ab	147.66±2.35 abcd
Sweet Orange EM	16.64±1.99 abcd	8.92±0.62ab	71.33±1.14 abc	144.44±1.27 abcd
Sweet Orange NP	15.96±1.85 abc	9.85±0.80 bc	69.92±0.67 a	141.81±0.83 a
Mandarin EM	18.52±1.50 bcd	10.74±0.32 bc	75.25±0.45 bcd	158.38±0.44 bcd
Mandarin NP	20.37±0.34cd	10.82±0.28 bc	77.47±0.39 de	160.55±0.77 e
One-Way ANOVA results	$F_{9,62}$=7.954P<0.001	$F_{9,62}$=6.992P<0.001	$F_{9,62}$=8.680P<0.001	$F_{9,62}$=4.821P<0.001	$F_{9,6}$

Table 3. Results of the biochemical analyses on the content (means ± SE of mg^{-1} protein) of Ascorbate Peroxidase (APX), Catalase (CAT), Superoxide dismutase (SOD) and Polyphenol oxidase (PPO) in leaves, stems and roots of tomato plants sprayed with the three citrus essential oils in two

formulations (EM and NP), two insecticides (Spinosad and Indoxacarb), the surfactant only (Tween) and with water only (untreated control).The results of the statistical analysis are reported at the bottom of each column. Values within the same column followed by different letters are significantly different (Tukey test; $p < 0.05$).

4. Discussion

The data collected demonstrated that the lethal and sublethal effects of the developed formulations on the mirid predator *N. tenuis* varied with the type of formulation, the citrus EO used and the different age of the residues. Despite none of the essential oils reached the highest IOBC toxicity class (i.e., 4: harmful) which was instead reached by one of the 2 positive control treatments (indoxacarb), mandarin EO, in both formulations, was the most toxic EO tested toward *N. tenuis* even when the specimens were exposed to 7-day-old residues. The differences in the toxicity of mandarin EO, compared to the other citrus EOs tested, could be due to its specific chemical composition. Indeed, the role of the single compounds or the synergistic effect of the different compounds present in the EOs is well known (Don-Pedro, 1996; Jiang et al., 2009). In the mandarin EO tested, some substances (i.e., γ-terpinen, α-sinensal, piperitone, α-terpinen, etc.) were either more abundant or exclusively present in this EO compared to the others (Campolo et al., 2016).

Side effects of biopesticides toward a variety of arthropods used as biocontrol agents have been extensively studied (Biondi et al., 2013a,b; Choi et al., 2004; Othira et al., 2009). The results of these studies highlighted that the impact of these active substances, often considered harmless, can largely vary depending on different factors taken into account (e.g. non-target organism, instar tested, pesticide, age of residue). However, few studies tested unwanted effects of EO-based insecticide formulations (Pavela, 2015; Pavela and Benelli, 2016; Regnault-Roger et al., 2012). Choi at al. (2004) assessing the potential of 53 plant EOs against the two spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) and their toxicity toward its predator *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), highlighted the toxicity of seven EOs toward the predatory mite suggesting the choice of an appropriate release timing to minimize the side effects of the EO residues. In the same paper, the authors reported that two Citrus EOs (i.e. bergamot and sweet orange) caused a mortality of 87 and 61% respectively of the two spotted spider mite whereas they were moderately toxic to *P. persimilis* adults. In our study sweet orange EO-based formulations were, among the other EOs tested, the least toxic toward the mirid predator whereas the same formulations were proved to be the most toxic against *T. absoluta* eggs and larvae (inside mines) when compared with mandarin and lemon EO-based formulations (Campolo et al., 2017).

Soares et al. (2019) observed that an orange essential oil and salt borax-based biopesticide (Prev-Am®) did not affect the longevity of *N. tenuis* while it significantly modified the predator behavior. Indeed, the predator resting, preying, plant feeding, walking and cleaning time were altered with possible consequences in terms of reduction in the capacity of prey location and capture changed after exposure to the insecticide. *Chenopodium ambrosioides* L var near *ambrosioides* EO-based commercial insecticide (UDA-245 – Codena Inc) had negligible contact toxicity toward the beneficial arthropods *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) and *Aphidius colemani* Viereck (Hymenoptera: Aphidiinae) when applied at the recommended rate for greenhouse pests (Bostanian et al., 2005). While, this formulation is known to have a high efficacy against different key crop pests such as *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), *Trialeurodes vaporariorum* (West.) (Hemiptera: Aleyrodidae) and *T. urticae* (Chiasson et al., 2004a, 2004b). On the other hand, Suthisut et al. (2011) highlighted that the two hymenopteran parasitoids *Anisopteromalus calandrae* (Howard) and *Trichogramma deion* Pinto & Oatmam were more susceptible than *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) when exposed to vapors of EOs extracted from three gingeraceae Thai plants. Similar results were obtained by Ketoh et al. (2002) when the bruchid *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) and its parasitoid, *Dinarmus basalis* (Rond.) (Hymenoptera: Pteromalidae), were exposed to *Cymbopogon*

nardus (L.), *Cymbopogon schoenanthus* (L.) and *Ocimum basilicum* EOs. Benelli et al. (2013) showed higher toxicity of *Melaleuca artenifolia* EO against *Ceratitis capitata* (Wiedemann) than toward its parasitoid *Psyttalia concolor* (Szépligeti) (Hymenoptera: Braconidae) both in contact and fumigation toxicity assessments.

When comparing the two formulations (i.e. EM and NP) per each EO, the results, both in terms of mortality and progeny production, were not significantly different despite the developed NPs contained a tenth part of essential oil compared to the emulsion. These results can be attributed to the effect of the formulation that is known to play a crucial role in the improvement of the stability and the gradual release of the active substance therefore influencing the product toxicity (de Oliveira et al., 2014). In the case of sweet orange EO, the increase in the E_x values is to be related to an increase of the sublethal effects of the EO while the acute toxicity decreases at increasing age of residues. In particular, the reduced offspring produced could be due to the high content of limonene (>88%) in this EO, known as oviposition deterrent and insect repellent (Campolo et al., 2016; Panzavolta et al., 2015; Shi et al., 2016).

As regards to soil enzymatic activities, our results are in partial accordance with Papatheodorou et al. (2014), who found that S-(+) carvone did not affect dehydrogenase activity as well as the alkaline phospho-monoesterase activity, whereas urease activity was significantly affected. Nevertheless, the differences among the substances used as well as the methodological procedures adopted, must be taken into account. Indeed, Papatheodorou et al. (2014) performed two treatments two weeks apart. Moreover, although carvone is a naturally occurring ketone found in citrus essential oils, its amount may be variable depending on the area of cultivation, on the variety, and extraction methods (Shaw, 1979). Interestingly, all the results we obtained suggest that EO-based formulations added to the soil did not affect the soil enzymatic activities and consequently the microbial population. These findings are also in accordance with Vokou and Liotiri (1999) who found that essential oils extracted from Origanum vulgare subsp. *hirtum*, *Rosmarinus officinalis*, *Mentha spicata*, and *Coridothymus* sp. are used as a carbon and energy source by soil microorganisms, stimulating their growth. The accuracy of the results was also proved by using spinosad and chlorpyrifos as reference treatments, confirming the data obtained by Telesinki et al. (2015) and Sanchez-Hernandez et al. (2018). Moreover, the incubation time (4 days) of soil with the essential oils was also proved to be long enough to observe a potential modification on the soil enzymatic activities, as previously showed by Barone et al. (2019).

The tests on plant enzyme activities showed that citrus EOs did not negatively affect the antioxidant activities (APX, CAT and SOD) of all the plant organs (Table 3). Biochemically, the increased SOD activity is involved in inactivation mechanism of O_2, while the enhanced CAT and APX activities would contribute to the greater elimination of H_2O_2 (Mittler, 2002). In fact, the protective action system includes SOD, catalyzing the dismutation reaction of this anionic radical to O_2 and H_2O_2; CAT that eliminates H_2O_2 generated by oxidase, and AXP, one of the enzymes of the ascorbate-glutathione cycle, which is also involved in the detoxification of H_2O_2, but rather labile and inactivated by high peroxide concentrations (Mittler, 2002).The involvement and the role of these enzymes in the protection against oxidative stress have been demonstrated in transgenic plants that express higher levels of some of them (Allen et al., 1997). The antioxidant activities are therefore crucial in enhancing the endogenous defense in a number of wild and cultivated plants, but relatively few studies in different organs of tomato are available. It has been reported that some stresses maintained significantly high levels of SOD, CAT and APX activities and the increase of superoxide radical production and oxidases in tomato has been inhibited (Barbagallo et al., 2009; Fan et al., 2008). Pesticides, including organophosphates, organochlorines and bipyridyl herbicides, have shown to trigger an oxidative stress with a consequent increase in ROS production and defense mechanisms due to the activity of antioxidant enzymes (Mishra et al., 2008). In our study, the natural insecticide spinosad resulted to increase only SOD activity in stems, whereas the benzimidazole indoxacarb raised the values of CAT activities in leaves and stems, partially according to Parween et al. (2016), as well as it increased also the APX value in leaves. Successfully, treatments with lemon and sweet

orange EOs (in both their formulations) are not putatively involved in ROS accumulation in tomato plants, since the antioxidant activities (APX, CAT and SOD) were kept at values similar to the untreated plants.
Among the tested compounds, mandarin EO formulations caused an increase in antioxidative activities in different plant organs; this inhibits, at least partially, the oxidative phenomena through the specific action against the ROS eventually produced.
As regard PPO enzymes, they play a negative action against the polyphenols stability, which consists in the hydroxylation of monophenols to *o*-diphenols (cresolasic activity) and subsequently oxidation of *o*-diphenols to *o*-quinones (catecolasic activity), main reaction products. The *o*-quinones are highly reactive, so they can undergo radical polymerization that results in the formation of melanoidins and consequently in a variation of chemical composition of plants for the involvement in the latter reaction of proteins and/or amino acids (Barbagallo et al., 2009; Espín et al., 1998). The different tested citrus EOs induced a reduction in oxidizing activity of the PPOs in different tomato plant organs (Table 3), in a scenario of total protection of the tomato against negative oxidative phenomena. Our results are in accordance with Fattouch et al. (2010) who found that in quince (*Cydonia oblonga*) benomyl (a benzimidazole fungicide), carbaryl (a carbamate insecticide), deltamethrine (a pyrethroid insecticide) and parathion methyl (an organophosphate pesticide) inhibited the in vitro PPO activity producing about 44%, 51%, 33% and 58% inhibition, respectively, suggesting a competitive inhibitory behavior. In other words, a reduction of oxidative stress in tomato plants might be one of the reasons to carry out citrus essential oil treatments, being potentially effective in the homeostasis of antioxidant enzymes and inhibiting PPO activities.

5. Conclusions

The developed formulations, despite containing natural active substances believed to be harmless, in some cases, showed a significant impact toward the predator *N. tenuis* also when exposed to longer aged residues. These results stress the importance of studying the toxic impact at differently aged residues (in order to intercept degradation patterns), as well as the sublethal effects (e.g. progeny production) for a complete risk assessment of new chemicals. However, before drawing final conclusions on these chemicals for their inclusion into IPM packages, further studies on sublethal concentrations (Ricupero et al., 2020), at varying environmental conditions (Abbes et al., 2015) and in real field scenario (Stark et al., 1995) are needed. These, together with the evaluation of side effects on the plant and on the soil, in a multilevel approach, draws a thorough picture of the potential unwanted effects of a pesticide. Indeed, in the case of biorational insecticides the great majority of the numerous papers published on the topic almost exclusively focus on target effects while overlooking their potential detrimental effects on human health as well as on beneficial arthropods (i.e. pollinators, parasitoids and predators) (Haddi et al., 2020). In particular, in the case of plant essential oils, the complexity of their composition makes the clear identification of selectivity mechanisms difficult. Besides, plant essential oils are known to have multiple modes of action, which may cause unintended effects on non-target organisms. These aspects should be taken into account for their implications both for the registration process of novel biopesticides and for the effective and sustainable inclusion of these tools in IPM strategies (Haddi et al., 2020).

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CRediT authorship contribution statement

Orlando Campolo: Methodology, Writing - original draft, Formal analysis, Conceptualization, Writing - review & editing. **Ivana Puglisi:** Methodology, Validation, Investigation, Formal analysis,

Writing - review & editing. **Riccardo Nunzio Barbagallo:** Methodology, Validation, Investigation, Writing - review & editing. **Asma Cherif:** Validation, Investigation. **Michele Ricupero:** Validation, Investigation. **Antonio Biondi:** Conceptualization, Writing - review & editing. **Vincenzo Palmeri:** Project administration, Conceptualization, Resources. **Andrea Baglieri:** Methodology, Validation, Investigation, Writing - review & editing, Resources. **Lucia Zappalà:** Project administration, Writing - review & editing, Conceptualization, Resources.

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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