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Bioactivity and physico-chemistry of garlic essential oil nanoemulsion in tomato

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13 **Bioactivity and physico-chemistry of garlic essential oil**  
14 **nanoemulsion in tomato**

15 **Short title:** Bioactivity of garlic essential oil nanoemulsion

16

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19

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29 **Abstract**

30 Tomato has an economic relevance worldwide but its production is threatened by several biotic  
31 factors, including the invasive South American tomato pinworm *Tuta absoluta*. The control of this  
32 pest mainly relies on the repeated applications of synthetic insecticides that can have considerable  
33 non-target effects; therefore, new sustainable approaches are required. The biocidal activity of  
34 garlic has been recognized and no risks for consumers and the environment are expected in its use.  
35 However, the practical implementation of garlic extracts is hampered by several draw backs that  
36 could be overcome by nanotechnologies. We developed and characterized a new garlic essential oil-  
37 based nanoemulsion (GEO-NE) and laboratory trials were carried out to investigate its insecticidal  
38 activity against *T. absoluta* involving different instars and exposure routes. GEO-NE side effects on  
39 the mirid predator *Nesidiocoris tenuis* and tomato plants were also assessed in the laboratory. The  
40 nanoformulation had dimensions belonging to the nanometric scale and good stability over time.  
41 GEO-NE showed significant toxicity toward *T. absoluta* eggs and larvae and repellence for  
42 ovipositing females. No lethal effect on *N. tenuis* adults was recorded but its progeny was  
43 significantly reduced on GEO-NE treated plants. By contrast, GEO-NE had no phytotoxic effects  
44 on sprayed tomato plants. Our findings suggested that GEO-NE can successfully control *T. absoluta*  
45 and its application deserves to be considered as a potential tool for tomato Integrated Pest  
46 Management.

47

48 **Keywords:** biopesticide, phytotoxicity, oviposition deterrence, botanicals, nanoinsecticide,  
49 selectivity

## 50 **Introduction**

51 Intensive agriculture is heavily reliant on pesticides for food protection but these chemical  
52 substances pose adverse impacts on human health, water quality and biodiversity on a global scale  
53 (Tang et al. 2021). For these reasons, public opinion and policymakers strongly encourage  
54 sustainable practices for pest control aiming at food safety and food security (Carvalho et al. 2006).  
55 Among botanical insecticides, plant essential oils (EOs) are considered environmentally friendly  
56 control tools mainly due to their rapid biodegradability, low risks of resistance phenomena and  
57 negligible toxicity towards non-target organisms (Regnault-Roger et al. 2012). Because of this  
58 promising evidence, botanicals have been regarded as a panacea for pest concerns over the last two  
59 decades (Campolo et al. 2014; Pavela & Benelli 2016; Galland et al. 2020; Pavela et al. 2020).  
60 Despite the massive body of literature produced, poor studies corroborate EO practical  
61 implementation which is limited by their constitutive drawbacks, e.g., stability and degradation  
62 patterns, changing toxicity towards the target and non-target organisms (Isman 2020). Nevertheless,  
63 nanotechnology could help overcome the intrinsic constraints often associated with the use of EOs  
64 (Athanassiou et al. 2018; Campolo et al. 2020a; Pavela et al. 2021; Sciortino et al. 2021).

65         Garlic, *Allium sativum* Linnaeus (Amaryllidaceae), is a commercial crop widely cultivated  
66 around the globe and China is its largest exporter worldwide (Rabinowitch & Currah, 2002). The  
67 long-standing use of garlic as food spice and medicine throughout human history has been  
68 associated with anticancer, cardiovascular and biocidal activities (Thomson & Ali 2003). The latter  
69 has been demonstrated in the laboratory against different pests including insects, mites and  
70 nematodes (Park et al. 2006; Vergel et al. 2011; Palermo et al. 2021). Although the non-target  
71 impact of garlic on beneficial arthropods is mostly unknown (Asadi et al. 2019), a recent pesticide  
72 peer-review published by The European Food Safety Authority (EFSA) recognizes no risk to  
73 consumers in the use of garlic as a plant protection product (Anastassiadou et al. 2020).

74         Tomato crop has a very high social and economic relevance worldwide and the South  
75 American tomato pinworm, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), negatively

76 affected the entire cropping system in Palaearctic, Afrotropical and Indomalayan realms during the  
77 last decade (Biondi et al. 2018). Synthetic insecticides are the most used control tool against this  
78 pest, but a plethora of adverse consequences have been continuously reported in their use (Desneux  
79 et al. 2007; Guedes et al. 2019; Soares et al. 2019a). Sustainable control tactics against *T. absoluta*  
80 have been developed across different world regions with promising results, but control failures by  
81 chemical pesticides and the high cost of biological and biotechnical solutions remain the biggest  
82 challenges for tomato growers worldwide (Desneux et al. 2022).

83 In previous researches, the use of EO-based insecticides against *T. absoluta* was assessed  
84 with promising results in both laboratory and field-applications (Campolo et al. 2017; Mansour &  
85 Biondi 2021; Desneux et al. 2022). Similarly, the non-target impact of EOs on the pest predator  
86 *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) was recently studied (Soares et al. 2019b;  
87 Campolo et al. 2020b). However, the toxicity of garlic EO on this biological system has not been  
88 investigated. Here, we tested garlic EO as insecticide against *T. absoluta* involving different instars  
89 and exposure routes. The egg was our first target stage since it is considered the least susceptible  
90 instar to both chemical and naturally-derived substances (Goudarzi Chegini & Abbasipour 2017;  
91 Campolo et al., 2017; Tomè et al., 2012). The LC<sub>50</sub> estimated for *T. absoluta* eggs and the  
92 maximum tested concentration were evaluated as larvicidal and oviposition deterrent. Further  
93 experiments were also addressed to evaluate the side effects of garlic-EO-based nanoformulation  
94 towards the aforementioned biological model. Our results can contribute for implementing  
95 sustainable control strategies of *T. absoluta* in the tomato cropping system.

96

## 97 **Materials and methods**

### 98 **GC-MS analysis and Nanoemulsion preparation**

99 Pharmaceutical grade *Allium sativum* (thereafter Garlic) EO was purchased by Esperis s.p.a. (Milan,  
100 Italy). The sample was diluted 1:100 with n-hexane and analysed with a Shimadzu GC 2010 Plus  
101 gas chromatograph coupled with a TQMS 8040 triple quadrupole mass spectrometer equipped with

102 a DB-5ms, 30 m, 0.25 mm i.d., 0.25  $\mu\text{m}$  film thickness non polar column (Supelco Sigma-Aldrich,  
103 Bellafonte PA, USA). The following conditions were used: injector temperature, 250  $^{\circ}\text{C}$ ; injection  
104 mode, split; split ratio, 1:100; oven temperature, 40  $^{\circ}\text{C}$  held for 2 min, then increased to 110  $^{\circ}\text{C}$  at a  
105 rate of 4  $^{\circ}\text{C}/\text{min}$  and to 240  $^{\circ}\text{C}$  at a rate of 3  $^{\circ}\text{C}/\text{min}$  and held for 3 min; carrier gas, helium at a  
106 constant flow of 1 ml/min; transfer line temperature, 240  $^{\circ}\text{C}$ ; ionization technique; electron impact  
107 (EI) at 70 eV; acquisition range, 40 to 400 m/z; scan rate, 3 scan/sec.

108 The identification of volatile compounds was conducted according to Cincotta et al. (2021).

109 Quantitative results were expressed as average peak areas of 3 replicates.

110 The Garlic EO-nanoemulsion (GEO-NE) was prepared using the self-emulsifying process  
111 followed by sonication according to the methodology described by Campolo et al. (2020a). The  
112 average droplet size and size distribution (Poly dispersion index), were measured by using a  
113 dynamic light scattering particle size analyser (Z-sizer Nano, Malvern Instruments) at 25  $^{\circ}\text{C}$ . In  
114 addition, the particle surface charge was quantified as zeta potential ( $\zeta$ ) using a Z-Sizer Nano,  
115 (Malvern Instruments) at 25 $^{\circ}\text{C}$ . Changes in droplet size and  $\zeta$  were measured over time up to 16  
116 weeks after the nanoemulsion preparation.

117

## 118 **Biological materials**

119 Tomato plants (*Lycopersicon esculentum* Mill., Solanaceae) used for both insect rearing and  
120 experiments were grown in greenhouse conditions in 1L pots, inside screened cages without  
121 pesticide application. *Tuta absoluta* laboratory rearing was established and maintained as described  
122 by Campolo et al. (2017). *Tuta absoluta* eggs and larvae of the same age were obtained by releasing  
123 about two hundred newly-emerged adults inside each cage containing four tomato plants when they  
124 reached the phenological stage of 3<sup>rd</sup> leaf on the main shoot unfolded. The moths were left 24 h to  
125 lay eggs and then removed. Eggs (72  $\pm$  12 h old) and newly-molted 2<sup>nd</sup> instar larvae were used for  
126 the bioassays. The *N. tenuis* colony was established and kept in the laboratory as described by  
127 Passos et al. (2022). Newly emerged (1-4-day-old) adults of *N. tenuis* were collected from the

128 rearing cages by a mechanical aspirator, coupled in plastic tubes and kept refrigerated ( $\sim 7^{\circ}\text{C}$ ) until  
129 their use.

130

### 131 **Bioassays**

132 The following bioassays were carried out at the Department of Agriculture, Food and Environment  
133 of the University of Catania (Italy) in climatic chamber under controlled environmental conditions  
134 ( $25 \pm 2^{\circ}\text{C}$ ,  $60 \pm 10\%$  RH, 14:10 L:D). The tested GEO-NE solutions were prepared by mixing the  
135 necessary amount of concentrated nanoemulsion (15% of EO) with distilled water in order to obtain  
136 the required concentration for the different bioassays. Because the developed nanoemulsion was  
137 able to disperse easily in water, a slight stirring (10 sec at 2,000 RPM) by means of a magnetic  
138 stirrer was needed for preparing the solutions.

139 A spinosad-based commercial insecticide (Laser<sup>TM</sup> Dow Agrosiences, applied at double  
140 highest label rate recommended in Italy for tomato crops, 150 mL/hL) was used as treated control in  
141 the bioassays involving *T. absoluta* because its use is widely recognized in Mediterranean basin  
142 organic tomato cultivation (Biondi et al. 2018). For the assessment of non-target impact towards *N.*  
143 *tenuis*, an indoxacarb-based insecticide (Steward<sup>®</sup>, DuPont<sup>TM</sup>, applied at the highest label rate  
144 recommended in Italy for tomato crops, 12.5 g/hL) was used since this active ingredient has been  
145 recognized as harmful towards the predator in laboratory conditions (Arnò & Gabarra 2011).  
146 Distilled water and TWEEN<sup>®</sup> 80 + distilled water were used as untreated controls.

147

### 148 **Toxicity toward *T. absoluta* juveniles**

149 Two bioassays were carried out for evaluating the efficacy of the developed formulation to control  
150 *T. absoluta* egg and larval stages, respectively. In the first bioassay, tomato plants bearing  
151 *T. absoluta* eggs, obtained as described above, were sprayed 72 hours later with seven different  
152 concentrations (from 0.015 to 3% W/W) of GEO-NE formulation until runoff by using a 2 L power-  
153 pack aerosol hand sprayer (Dea<sup>®</sup>, Volpi, Italy) and left to dry for one hour. For each replicate, ten

154 sprayed *T. absoluta* eggs were carefully transferred on untreated tomato leaves through a fine  
155 paintbrush inside a ventilated arena (Biondi et al. 2012). The egg mortality was daily checked up to  
156 48 hours after egg hatching.

157 In the second bioassay, tomato plants were sprayed with both the resulting LC<sub>50</sub> eggs and the  
158 maximum concentration tested against the egg stage (i.e., 3% of EO). These two concentrations  
159 were chosen for assessing the potential larvicidal activity that can be simultaneously determined by  
160 the ovicidal treatment. For each replicate, ten coetaneous 2<sup>nd</sup> instar *T. absoluta* larvae were  
161 transferred to sprayed tomato leave inside a ventilated arena according to the methodology  
162 described by Campolo et al. (2017). Larval mortality was assessed 24 and 72h after the spray. Non-  
163 reacting larvae when stimulated with a fine paintbrush were considered dead. Chronic toxicity was  
164 assessed by calculating the proportion of juveniles, alive 72 h after the spray, that reached the adult  
165 stage. Consequently, 14 and 12 days after exposing larvae to the chemicals, the isolators were  
166 checked daily to record adult emergence. Cumulative mortality (acute and chronic) was used to  
167 evaluate the efficacy of the developed formulation. Both bioassays were replicated five times for  
168 each tested concentration and the controls.

169

## 170 **Oviposition deterrence**

171 Choice and no-choice tests were carried out to evaluate the oviposition deterrence on *T. absoluta*  
172 adult females caused by the ovicidal treatments. To obtain coetaneous and mated females,  
173 *T. absoluta* pupae were sexed and, once adults had emerged, 5 females and 5 males were coupled  
174 and allowed to mate for 4 days. No oviposition substrate was provided during this period.

175 In both experiments, tomato plants were sprayed with the resulting LC<sub>50</sub> for eggs and left to dry.  
176 Only distilled water + Tween 80 was used as a control since no statistical difference ( $p > 0.05$ ) was  
177 recorded between this treatment and water alone (data not shown) in preliminary trials.

178 In the no-choice test, two sprayed tomato shoots with the base immersed in water, were  
179 placed inside a polyester net cage (50 × 60 × 80 cm), whereas in the choice-test, both treated and

180 control shoots were placed inside the cages. Ten *T. absoluta* adults (5 females and 5 males) were  
181 released in the cages and maintained in the same climatic conditions described above. After 72 h,  
182 the number of eggs laid both in the treated and control shoots were counted by using a  
183 stereomicroscope. Each experiment was replicated ten times.

184

### 185 **Side effects on *Nesidiocoris tenuis* and tomato plants**

186 To evaluate the side effects of *T. absoluta* ovicidal treatments towards *N. tenuis*, two different  
187 experiments were carried out, which aimed at evaluating the residual toxicity of GEO-NE on the  
188 survival and the progeny production of the predator. Shoots were collected 1h and 72 h later from  
189 tomato plants sprayed with LC<sub>50</sub> eggs, the highest application rate (3% of EO) used in the ovicidal  
190 bioassay, treated and untreated controls as described above. Also in this case, only distilled water +  
191 Tween 80 was used as a control since water alone had no effect on *N. tenuis* mortality and its  
192 progeny production ( $p>0.05$ ). Five couples of *N. tenuis* were released inside the above-described  
193 isolator provided with a sprayed shoot (1h or 72-h-old residues) and devitalized *Ephestia kuehniella*  
194 Zeller (Lepidoptera: Pyralidae) eggs as a food source. Mortality was assessed daily for three days  
195 by recording the number of alive and dead adult males and females. After three days, adults were  
196 removed and ten days later the number of nymphs was recorded daily for additional ten days. Each  
197 treatment was replicated ten times.

198 The toxic effect of the developed formulation on tomato plants was evaluated for two weeks  
199 by spraying five additional tomato plants with the seven application rates described above,  
200 following the methodology described in Campolo et al. (2017). Control treatments (i.e. indoxacarb,  
201 water + Tween 80 and water alone) were also included.

202

### 203 **Data analysis**

204 Mortality data were corrected for control mortality using the Abbott's formula (Abbott, 1925).

205 Dependent variables were tested for homogeneity and normality of variance (Levene and Shapiro-

206 Wilk test respectively) and transformed ( $\arcsin \sqrt{x}$ ) whenever needed. Probit analysis was  
207 performed in order to estimate the median lethal concentrations (LC<sub>50</sub> and LC<sub>90</sub>) with associated  
208 95% confidence intervals. Values were considered significantly different when their 95% fiducial  
209 limits did not overlap. Mortality and oviposition data in choice test, and progeny production, were  
210 subjected to univariate analysis of variance following the GLM procedure. Choice test data was  
211 subjected to the  $\chi^2$  goodness of fit analysis to test the null hypothesis that oviposition was not  
212 influenced by the treatment (response equal to 50:50). Multiple comparisons were carried out using  
213 Duncan's multiple range post-hoc test. To evaluate the effect on plants of the developed  
214 formulation, the Phytotoxicity index (Pi) was calculated according to the formula proposed by  
215 Campolo et al. (2017).

216 
$$P_i = \sum_{j=0}^n \left( \frac{DL_j}{TL} \times \frac{DC}{n-1} \right)$$

217 where DL is the number of damaged leaves for each damage severity class j, TL is the total number  
218 of leaves sprayed, DC is the damage severity class, and n is the number of damage severity classes.  
219 The Pi ranges from 0 (no damage) to 1 (dead leaves).

220

## 221 **Results**

### 222 **GC-MS analysis and Nanoemulsion preparation**

223 More than 70 volatile compounds were detected in the Garlic EO with more than 90% referred to  
224 sulphur compounds. Diallyl sulphides, from mono- to hexasulfide, quantitatively prevailed (Table  
225 S1). In particular, in our samples diallyl disulfide (29.66%) and diallyl trisulfide (21.50%)  
226 prevailed, diallyl tetrasulfide (13.19%) and diallyl sulfide (10.69%) followed. Other thiosulfates,  
227 including allyl methyl-, allyl 1-propenyl and methyl 1-propenyl di-, tri-, and tetrasulfides have been  
228 identified in small amount in the samples analysed.

229 The GEO-NE particles had dimensions belonging to the nanometric scale ( $176.23 \pm 0.9$  nm)  
230 and a surface charge ( $\zeta$  potential) of  $-23.16 \pm 0.29$  mV. The size distribution of the formulation (0.18)  
231 highlighted a close distribution of particle size in the analysed samples. Over time (Fig. 1), both the  
232 size and the  $\zeta$  potential increased still reaching values below 183 nm and -18 mV, respectively.  
233 During the first three weeks, the particle size remained almost constant ( $176.23 \pm 0.88$  nm) and only  
234 eight weeks after preparation a small increase in size was measured.

235

### 236 **Toxicity towards *Tuta absoluta* juveniles**

237 The mortality of eggs sprayed with the developed formulation had a concentration-dependent  
238 response with a value of  $LC_{50} = 0.124\%$  of EO (CI = 0.098-1.151) and a  $LC_{90} = 0.772\%$  of EO (CI=  
239 0.601-1.052) as estimated by the probit. analysis (Slope  $\pm$  SE =  $1.61 \pm 0.11$ ;  $\chi^2 84.612$ ;  $p = 0.084$ ).

240 Conversely, in the water control only  $4 \pm 1.63\%$  of eggs did not hatch. Statistical differences were  
241 highlighted among the egg corrected mortality registered in the GEO-NE treatments compared to  
242 the control treated with spinosad ( $F = 81.933$ ;  $df = 7$ ;  $p < 0.001$ ). Our nanoemulsion at 1.5 and 3%  
243 of EO concentrations killed 96.88 and 97.92% of the sprayed eggs, respectively. These results were  
244 similar to the spinosad-based control (Fig. 2). The formulation showed also promising larvicidal  
245 activity. Both the GEO-NE tested concentrations (i.e., 3% and  $LC_{50}$  eggs) caused 100% and

246 77.78±13.61% mortality of *T. absoluta* larvae, respectively. The GEO-NE efficacy was thus  
247 comparable to the spinosad-based control (F=2.667; df = 2; p=0.11).

248

### 249 **Oviposition deterrence**

250 In the choice test (Fig. 3), *T. absoluta* females oviposited significantly more eggs on control plants  
251 in comparison to plants sprayed with GEO-based formulation ( $\chi^2= 8.601$ ;  $p<0.01$ ). The mean  
252 number of eggs laid per female on control tomato shoots (5.75±1.39) doubled the amount of eggs  
253 laid on the GEO-NE sprayed shoots (2.08±0.6). In the no-choice test, untreated shoots resulted  
254 more attractive than GEO-NE sprayed shoots (F=90.556; df=1; p=0.01). Namely, the oviposition by  
255 *T. absoluta* females was significantly reduced on plants sprayed with the formulation in comparison  
256 to control plants (Fig. 3).

257

### 258 **Side effects of GEO-NE**

259 The overall mortality of *N. tenuis* caused by GEO-NE was affected by the age of the residues on  
260 tomato shoots (F=6.038; df=1; p=0.01). As expected, 1-h-old indoxacarb sprayed tomato plants  
261 negatively affected the survival of *N. tenuis* more than GEO-NE at both tested concentrations  
262 (F=44.431; df=2; p<0.001) (Table 1). Similarly, on the 3-day-old treated shoots, the mirid mortality  
263 was significantly higher for indoxacarb compared to both GEO-NE concentrations (F=148.816;  
264 df=2; p<0.001). In 1-h-old residue sprayed tomato shoots, indoxacarb and GEO-NE at 3% killed  
265 more than 80% and 60% of the exposed individuals, respectively. Conversely, only ~ 3% of dead  
266 *N. tenuis* individuals were recorded after the exposure to 1h GEO-NE residues at LC<sub>50</sub> estimated for  
267 *T. absoluta* eggs (F=27.356; df=2; p<0.001).

268 The progeny produced by *N. tenuis* females was significantly affected by GEO-NE  
269 (F=72.150; df=3; p<0.001). The offspring recorded for *N. tenuis* females exposed to 1-h-old 3%  
270 GEO-NE sprayed shoots was decreased (0.10±0.07) in comparison to the progeny recorded in water  
271 sprayed shoots (13.06±1.23) (Fig. 4). In the shoots treated with the LC<sub>50</sub> eggs, the number of progeny

272 was  $7.34 \pm 0.89$ . Within the same treatment, difference in the mean of progeny produced was  
273 observed only in the GEO-NE 3% treated shoots, in which the age of the residues affected the  
274 predator reproduction capacity.

275 Overall, no sign of toxicity was observed on tomato plants sprayed with the tested GEO-NE  
276 concentrations during two-weeks (data not shown). Consequently, the  $P_i$  was always equal to zero  
277 and classified as no-damage.

278 **Discussion**

279 The GEO nanoformulation we developed showed interesting potential for the control of *T. absoluta*  
280 and its effectiveness can be attributed both to the essential oil used and the formulation itself.

281 The Garlic EO we used mainly consisted of sulphur compounds which are responsible for the  
282 characteristic smell and taste of garlic (Amagase 2006; Satyal et al. 2017; Condurso et al. 2019).  
283 Diallyl disulfide and trisulfide, the most abundant compounds found in our samples, represent the  
284 main component of commercial garlic oils, in which diallyl trisulfide prevails in fresh garlic oil  
285 (Miething 1988; Jirovetz et al. 1992). These two compounds are known to be effective against  
286 stored product pests, mosquitoes, diptera sciaridae, termites, psillidae and psocoptera (Huang et al.  
287 2000; Park et al. 2006; Zhao et al. 2013; Liu et al. 2014).

288 Despite their promising insecticidal properties, EOs used as such present a series of  
289 problems mainly related to their chemical characteristics (e.g. poor water solubility, environmental  
290 degradation, phytotoxicity, volatility and flammability), therefore the development of  
291 nanoformulations is necessary for their use as insecticides under real operating conditions.

292 However, one of the main problems related to the widespread use of these control tools is related to  
293 the limited availability of registered nanoformulations depending on the variety of regulatory  
294 approval processes about natural derivatives adopted by different Countries. The nanoemulsion we  
295 developed helped solve many of EO constraints by enhancing its dispersion in water, reducing its  
296 phytotoxicity and, increasing its stability overtime. The low persistence of essential oils and other  
297 eco-friendly products, such as *Bacillus thuringiensis*, can represent a limitation, but at the same  
298 time it guarantees the consumer about the absence of insecticide residues in foods.

299 The nanoscale droplet diameters we obtained (i.e., less than 180 nm) likely contributed to  
300 the high effectiveness of the insecticide formulation against *T. absoluta*. Decreasing LC<sub>50s</sub> of  
301 permethrin and neem oil have been observed in nanoemulsions at decreasing droplet size (Anjali et  
302 al. 2012). Similar results have been reported by Mossa et al. (2018) in comparing the efficacy of  
303 both Garlic EO normal-emulsion and nanoemulsion against two eriophyid mites.

304 The amount of EO loaded, the surfactant-to-oil ratio (SOR) and the preparation method are critical  
305 factors in obtaining a stable and effective nanoemulsion (Donsì & Ferrari 2016). In our insecticidal  
306 formulation we were able to load 15% of EO with a SOR of 0.33 while ensuring good stability and  
307 a small particle size. In comparison to our nanoemulsion, most of the developed EO-based  
308 nanoemulsions contain less than 10% of EO while, formulations loading higher EO percentages  
309 (10-16.7) often require higher percentages of surfactant (SOR 1-2) or very high energy processes,  
310 such as high-pressure homogenization (Donsì & Ferrari 2016).

311 The obtained results highlighted a good insecticidal activity of the developed formulation in  
312 controlling *T. absoluta* preimaginal instars since both the tested concentrations (NE at 3% of GEO  
313 and the LC<sub>50</sub> eggs) caused high mortality rates in treated larvae. The evaluation of EOs as control tool  
314 of *T. absoluta* is still at an early stage probably because larvae feed on mesophyll tissues and EOs  
315 are not able to penetrate up to these tissues if not applied as nanoinsecticides (Campolo et al. 2017).  
316 Some researches tried to evaluate the fumigation efficacy of EOs against larvae despite, in our  
317 opinion, this approach, when applied towards crop pests, could be useful only to understand the  
318 potential of the tested EOs since this method is unlikely to be applied in the open field.  
319 Conversely, in stored product industry the use of EOs as fumigants could be a viable alternative to  
320 synthetic fumigants (Campolo et al. 2014). Garlic EO had strong fumigant activities against  
321 *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) adults and on F1 progeny (Yang et al.  
322 2010a) and against *T. castaneum* and *Sitophilus zeamais* (L.) (Coleoptera: Curculionidae) when  
323 combined with diatomaceous earth (Yang et al. 2010b). *Elettaria cardamomum* Maton  
324 (Zingiberaceae) EO extracted from seed revealed a good fumigation activity against *T. absoluta* 2<sup>nd</sup>  
325 instar larvae inside and outside the leaves (Goudarzv Chegini & Abbasipour 2017) as while  
326 *Artemisia absinthium* L (Asteraceae), *Eupatorium buniifolium* Hooker et Arnott (Asteraceae) EOs  
327 applied as fumigants (e.g. vapors) and by contact toxicity route (Umpiérrez et al. 2017).

328 *Tuta. Absoluta* eggs are considered less susceptible both to EOs and chemical insecticides  
329 than larvae (Tomé et al. 2012; Goudarzv Chegini & Abbasipour 2017). Our results confirm this

330 aspect since the lethal concentration that killed the 50% of the eggs was able to kill almost 80% of  
331 the treated larvae which feed mainly protected inside the mines. Our results suggest that Garlic EO  
332 seem to be more effective against the moth eggs than other EOs. Campolo et al. (2017) evaluating  
333 the efficacy of Lemon, Mandarin and Sweet Orange Citrus peel EOs emulsion against *T. absoluta*  
334 eggs highlighted that the all the tested concentrations (from 2.5 to 40 mg of EO x mL<sup>-1</sup>), much  
335 higher than that used in this study, were not able to kill the 50% of the treated eggs. LC<sub>50</sub> value of  
336 *E. cardamomum* EO applied as fumigants against *T. absoluta* eggs was significantly higher than  
337 that of the 2<sup>nd</sup> instar larvae inside mines (351.19 vs 7.88 µl L<sup>-1</sup> air respectively) (Goudarzv Chegini  
338 & Abbasipour 2017).

339 GEO-NE revealed also a good oviposition repellence both in the choice and no choice tests  
340 by reducing the eggs laid by female more than 50% on the treated shoots. Oviposition repellence  
341 due to EOs in *T. absoluta* was also highlighted by Yarou et al. (2018), by treating tomato plants  
342 with *Ocimum gratissimum* L. (Lamiaceae) and *Ocimum basilicum* L. (Lamiaceae) EOs (0.5 and 1  
343 mg) formulated with paraffin oil. In addition, the same Authors highlighted a reduction in eggs laid  
344 on tomato plants when associated with basil plants which might have masking tomato VOCs and  
345 preventing *T. absoluta* females from recognizing tomato plants. In *Tetranychus urticae* Koch  
346 (Acari: Tetranychidae) sublethal concentrations of *Piper marginatum* Jacq (Piperaceae) EO and its  
347 major compounds affected the fecundity of females (Ribeiro et al. 2016).

348 Despite the EOs were largely tested as pesticides, only few studies targeted the adverse  
349 impact on plants and non-target organisms (Pavela & Benelli, 2016). In our study, both the tested  
350 application rates of the developed formulation (LC<sub>50</sub> eggs and GEO-NE 3%) had an impact on  
351 *N. tenuis* adult survival lower than the indoxacarb-based treated control. When the mirid was  
352 released 72h after the treatments, the residues had low effects on the predator mortality. Conversely,  
353 the developed formulations had an important influence on the mirid progeny production and only at  
354 the lowest tested concentration (LC<sub>50</sub> eggs) the progeny produced by females was significantly higher  
355 in comparison to the treated control. Moreover, the age of residue had negligible effects on the

356 offspring production. Our results suggest that GEO-NE acts, as recorded for *T. absoluta*, as  
357 oviposition deterrent since the treatments in which most of females survived (i.e. shoots treated with  
358 GEO-NE 3% 72h before the mirid release) the progeny produced by females was similar to that  
359 registered in the positive control in which most females died.

360 Biopesticides are generally considered ecologically-sound since they are thought selective,  
361 less threatening to the environment and human health. Despite these beliefs, several studies prove  
362 that this issue cannot be generalized. For instance, although the survival analysis of *N. tenuis*  
363 predators exposed to citrus oil-based insecticide residues at different concentrations indicated no  
364 significant differences from the untreated control (Soares et al. 2019), borax + citrus oil based  
365 formulation had the same adverse impacts (mortality and progeny production) of indoxacarb on the  
366 predator *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) (Biondi et al. 2012).

367 Umpierrez et al. (2017) reported that *A. absinthium* and *E. buniifolium* EOs were toxic to  
368 honeybees when applied at the concentrations effective against *T. absoluta* larvae. Essential oils had  
369 negative effects also on the predatory mite *Amblyseius swirskii* Athias-Henriot (Acari, Phytoseiidae)  
370 by affecting both the female survival as well as egg laying (Amer & Momen 2002). Conversely,  
371 *Piper marginatum* EO applied as fumigant against the two-spotted spider mite *T. urticae* and the  
372 generalist mite predator *N. californicus* was the less toxic to the natural enemy than the pest  
373 (Ribeiro et al. 2016). Oregano EO and its different compounds affected the survival of green  
374 lacewing *Chrysoperla externa* (Hagen) (Neuroptera: Chrysopidae), having often sublethal effects  
375 on its fecundity and fertility (Castilhos et al. 2018).

376 Kimbaris et al. (2010) showed that the coccinellid predators *Adalia bipunctata* L  
377 (Coleoptera: Coccinellidae) and *Coccinella septempunctata* L (Coleoptera: Coccinellidae) were 2 to  
378 five times more susceptible to *Mentha* spp. EOs applied as fumigant compared to their prey  
379 *Acyrtosiphon pisum* Harris (Hemiptera: Aphididae) and *Myzus persicae* Sulzer (Hemiptera:  
380 Aphididae); whereas, orange EO had LC values higher in the coccinellids than in the aphids. Also,  
381 *Origanum vulgare* and *Thymus vulgaris* L (Lamiaceae) EOs applied as fumigants were selective

382 toward *Trissolcus basalis* (Woll.) (Hymenoptera: Scelionidae) (González et al. 2013).  
383 *Piper aduncum* (Piperaceae) EO when applied via contact and immersion routes against  
384 *Euschistus heros* (F.) (Hemiptera: Pentatomidae) caused deleterious effects to different stages of the  
385 stink bug without effects toward its natural enemies, *Telenomus podisi* (Ashmead) (Hymenoptera:  
386 Platygasteridae) and *Trissolcus urichi*(Crawford) (Hymenoptera: Platygasteridae) (Turchen et al.  
387 2016).

388 In our study, no phytotoxic effects on the treated plants were highlighted at all the tested  
389 concentrations. EOs due their extremely heterogeneous pool of secondary metabolites may have  
390 different impact on plants depending also on the concentration and the kind of formulation. The  
391 phytotoxic effect on tomato plants caused by citrus peel EOs was concentration-dependent, and the  
392 EO emulsions caused more damage than the PEG EO-nanoparticles formulation (Campolo et al.  
393 2017). The EO adverse effects on plants are considered negative for plant protection from insects  
394 but they represent a resource for the development of bio-herbicides. Rolli et al. (2014) screened the  
395 phytotoxicity of 25 EOs at pre and post-emergence growth using *S. lycopersicum* and highlighted  
396 *Pelargonium capitatum*(L) (Geraniaceae ) and *Aniba rosaeodora* Ducke (Lauraceae) EOs eligible  
397 as herbicides since these EOs strongly affected both the seed germination as well as plant survival.

398 In conclusion, the newly developed GEO-based formulation in this research showed  
399 promising results in controlling different *T. absoluta* stages with low mortality towards the predator  
400 *N. tenuis* and no phytotoxicity on tomato plants. Specifically, the ovicidal and larvicidal activities  
401 together with the oviposition deterrence and the lack of phytotoxicity are noteworthy because these  
402 different effects can act in parallel synergism in controlling one of the most important tomato pests.

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611 **Figure legends**

612 **Figure 1.** Mean value ( $\pm$  SE) of average size and surface charge trend of the GEO-NE measured  
613 during the 16 weeks of survey.

614 **Figure 2.** Mean percentages ( $\pm$ SE) of mortality of *Tuta absoluta* eggs sprayed with different GEO-  
615 NE application rates. Spinosad-based treated control was sprayed at label rate. Different letters  
616 indicate statistical differences among the treatments for  $P < 0.05$  (Univariate analysis of Variance  
617 followed by Duncan post-hoc test).

618 **Figure 3.** Mean number ( $\pm$ SE) of eggs laid by *Tuta absoluta* females in choice and no-choice tests.  
619 Different letters indicate statistical differences between the treatments for  $P < 0.05$  (choice test:  $\chi^2$   
620 goodness of fit; no-choice test: Univariate analysis of Variance followed by Duncan post-hoc test).

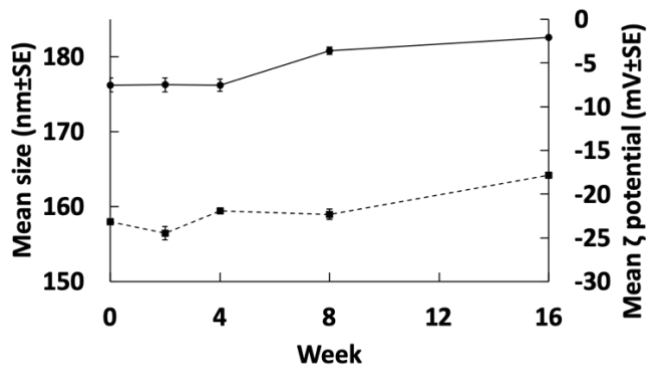
621 **Figure 4.** Bars show the mean number ( $\pm$ SE) of progeny produced by *Nesidiocoris tenuis* females  
622 during 3 d of exposure to 1h and 72h old different concentrations of GEO-NE residues on tomato  
623 shoots. Different letters indicate statistical differences between each treatment for  $P < 0.05$   
624 (Univariate analysis of Variance followed by Duncan post-hoc test). Dashed line indicates the mean  
625 number ( $\pm$ SE) of progeny produced in the control.

626 **Table 1.** Female, male and total mean percentages ( $\pm$ SE) of mortality of *Nesidiocoris tenuis* adults  
 627 exposed to 1h and 72h old different concentrations of GEO-NE residues on tomato shoots. Different  
 628 letters indicate statistical differences between the same treatment for  $P < 0.05$  (Univariate analysis of  
 629 Variance followed by Duncan post-hoc test).

<b>Treatment</b>	<b>Residual age</b>	<b>Male mortality</b>	<b>Female mortality</b>	<b>Total mortality</b>
GEO-NE LC <sub>50</sub> eggs	1	4.74 $\pm$ 2.41a	3.33 $\pm$ 2.22a	3.16 $\pm$ 1.61a
	72	2.22 $\pm$ 1.48a	0 $\pm$ 0a	0.43 $\pm$ 0.29a
GEO-NE 3%	1	66.84 $\pm$ 13.08a	54.17 $\pm$ 11.11a	60 $\pm$ 11.07a
	72	4.44 $\pm$ 3.39b	1.49 $\pm$ 1.49b	2.83 $\pm$ 1.72b
Indoxacarb	1	91.58 $\pm$ 6.43a	77.5 $\pm$ 10.15a	84.21 $\pm$ 8.04a
	72	73.33 $\pm$ 8.64a	68.09 $\pm$ 7.27a	70.65 $\pm$ 5.39a

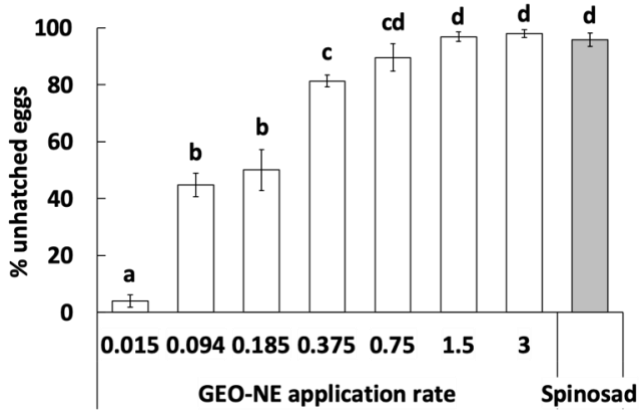
630

631 **Figure 1**



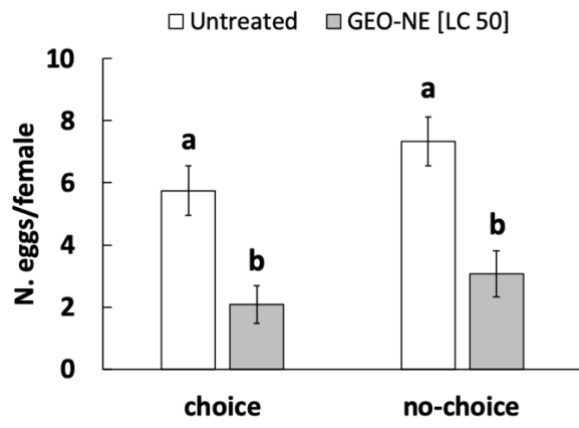
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633 **Figure 2**



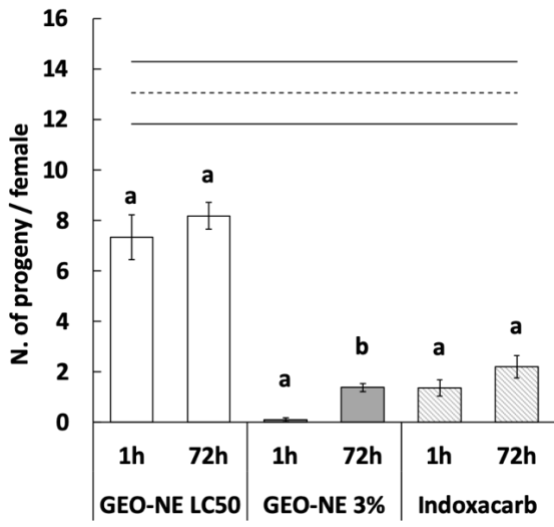
634

635 **Figure 3**



636

637 **Figure 4**



638

640 **Table S1.** Essential oil composition (Average area %) of garlic (*Allium sativum*)

Compound	LRI <sup>a</sup>	%
Allylmethylsulfide	699	0.62
Dimethyldisulfide	739	0.05
Hexan-3-one	788	* b
Hexan-2-one	797	0.02
Hexanal	807	*
4-Methylthiazole	822	0.01
Allylisopropylsulfide	826	0.01
Furfural	832	0.01
1,2-Dithiolane	842	0.03
Diallylsulfide	857	10.69
2,4-Dimethyl thiophene	864	*
Allylpropylsulfide	872	0.03
Allyl (E)-1-propenyl sulfide	890	*
Allylmethylsulfide	914	2.60
Methylpropylsulfide	930	0.01
2-Ethoxythiazole	944	0.01
Methyl (E)-1-propenyl disulfide	947	0.11
(E)-2-Hexenal	952	0.02
3H-1,2-Dithiolene	957	0.16
Dimethyltrisulfide	967	0.06
Benzaldehyde	969	0.04
3-(Methylthio)-1-propanol	979	0.01
2-Carboxaldehyde thiophene	1016	*
Allylisopropylsulfide	1050	0.16
2,5-Dimethyl-4-ethylthiazole	1054	*
1-(Methylthio)-3-pentanone	1067	0.01
Diallyldisulfide	1080	29.66
Allyl (Z)-1-propenyl disulfide	1093	0.22
Allyl (E)-1-propenyl disulfide	1099	0.01
Allylmethyltrisulfide	1138	3.22
4-Methyl-1,2,3-trithiolane	1156	0.95
Methyl (E)-1-propenyl trisulfide	1166	0.01
4,5-Dimethyl-2-propylthiazole	1174	0.01
3-Vinyl-4H-1,2-dithiine	1189	0.03
4H-1,2,3-Trithiine	1200	0.03
Allicin (diallylthiosulfinate)	1208	0.02
2-Vinyl-4H-1,3-dithiine	1215	0.05
4,5-Dimethyl-2-butylthiazole	1226	0.07
Allylisopropyltrisulfide	1266	0.06
4-(Hydroxymethyl)-1,2-dithiepane	1278	0.01

4-Methyl-1,2,5-trithiepane	1285	0.05
Diallyltrisulfide	1303	21.50
Allylpropyltrisulfide	1314	0.12
Allyl (E)-1-propenyl trisulfide	1323	*
(E)-3,5-Diethyl-1,2,4- trithiolane	1342	0.05
Allylmethyltetrasulfide	1357	0.01
5-Methyl-1,2,3,4-tetrathiane	1367	0.06
(Z)-3,5-Diethyl-1,2,4-trithiolane	1374	0.29
2-Heptyl thiophene	1381	1.32
3,6-Dimethyl-1,2,5-trithiepane	1428	0.02
4-Ethyl-2,3,5-trithia-6-octene	1444	0.33
4,6-Dimethyl-1,2,5-trithiepane	1460	0.06
Diallyltetrasulfide	1544	13.19
Allylmethylpentasulfide	1573	0.01
7-Methyl-4,5,8-trithia-1,10-undecadiene	1583	0.33
4-Ethyl-6-methyl-1,2,3,5-tetrathiolane	1588	0.05
6-Methyl-4,5,8-trithia-1,10-undecadiene	1592	1.21
N-propyl-2-Thiopheneacetamide	1622	0.06
4-Methyl-1,2,3,5,6-pentathiepane	1649	0.18
6-Ethyl-4,5,7,8-tetrathianonane	1658	0.39
N-isobutyl-2-Thiopheneacetamide	1663	0.24
Hexathiepane	1680	0.29
Diallylpentasulfide	1755	1.03
Allylmethylhexasulfide	1781	0.47
8-Methyl-4,5,6,9-tetrathia-1,11-dodecadiene	1815	5.54
Diallylhexasulfide	1897	0.22
Allylmethylheptasulfide	1922	0.16
2-Methyl-1,3-benzothiazole	1957	0.36
5-Ethyl-7-pentyl-1,2,3,4,6-pentathiepane	2005	0.32
Cyclooctasulfur	2044	0.08
9-Methyl-4,5,6,7,10-pentathia-1,12-tridecadiene	2051	0.68
8-Methyl-4,5,6,7,10-pentathia-1,12-tridecadiene	2056	0.68

641 <sup>a</sup> Linear Retention Index calculated on a DB-5ms column; <sup>b</sup> < 0.01%.