



Green pest control strategies: essential oil-based nano-emulsions for *Delottococcus aberiae* management

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Abstract

Increasing restrictions on synthetic pesticides due to environmental and health concerns have driven the search for alternative environmentally friendly pest management strategies. Essential oils (EOs) from plants like garlic (*Allium sativum*), clove (*Syzygium aromaticum*), and eucalyptus (*Eucalyptus camaldulensis*) have shown promise as bioinsecticides. However, their volatility, low water solubility, and short persistence limit their practical application in Integrated Pest Management programs. To address these challenges, we developed nano-emulsions of these EOs using a high-pressure microfluidization technique, achieving stable formulations with nano-sized droplets (< 200 nm) and optimal polydispersity index and zeta potential values. The insecticidal efficacy of these EO-based nano-emulsions was tested against the invasive citrus pest *Delottococcus aberiae*, with garlic nano-emulsion (GNE) exhibiting the highest mortality (100% within 24 h), significantly outperforming clove and eucalyptus formulations. GNE exhibited a dose–response mortality against *D. aberiae* while demonstrating no toxicity (100% of survival) toward *Cryptolaemus montrouzieri* and no phytotoxicity on citrus plants. Moreover, gene expression analysis revealed that GNE application triggered the overexpression of key genes involved in plant defense pathways, including *ICS2*, *NPR1*, *PAL*, and *MYC2*, suggesting the activation of both salicylic acid and jasmonic acid signaling pathways. This dual action—direct pest control and enhancement of plant defenses—positions GNE as a powerful tool in sustainable citrus pest management, with potential applications in real-world pest control. The study underscores the potential of EO-based nano-emulsions as a safe, effective, and environmentally sound alternative to chemical insecticides.

Keywords Citrus pest · Botanicals · EOs nano-emulsions · Nontarget organisms · Plant defenses · Sustainable agriculture

Introduction

The mealybug *Delottococcus aberiae* (De Lotto) (Hemiptera: Pseudococcidae) is an invasive pest native from South Africa that has recently spread to European citrus orchards (Beltrà et al. 2012; Pérez-Rodríguez et al. 2018). It has been reported to feed on several tropical and subtropical plants including citrus, coffee, guava, pear and olive (De Lotto 1961; Miller and Giliomee 2011). This mealybug causes severe damage to citrus fruits, leading to deformities and reduced fruit size. Furthermore, the excreted honeydew promotes the growth of sooty mold fungi, which hinder photosynthesis. These negative effects, combined with the absence of *D. aberiae* effective natural enemies, resulted in significant economic and environmental consequences (Martínez-Blay et al. 2018; Tena et al. 2017). Among the natural enemies, *Cryptolaemus montrouzieri* (Mulsant) (Coleoptera: Coccinellidae),

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a well-known predator of mealybugs in citrus orchards, contributes to the control of *D. aberiae* (Pérez-Rodríguez et al. 2019). However, on its own, it is not sufficient to fully manage the pest. Consequently, since the *D. aberiae* introduction in Europe, control strategies have predominantly relied on synthetic pesticides (Franco et al. 2009; Pérez-Rodríguez et al. 2017; Tena et al. 2017; Vacas et al. 2019). The extensive use of conventional pesticides has led to adverse effects, including environmental pollution and potential health risks to humans (Mata et al. 2024; Saroop and Tamchos 2024). In response to these negative consequences, the efficacy of natural substances, in particular essential oils (EOs), as insecticides has been extensively studied over the last decade (Assadpour et al. 2024; Pavela and Benelli 2016; Said et al. 2017). EOs are complex phytocomplexes containing a wide range of substances with properties that make them suitable as bioinsecticides, such as proven toxicity against several pests, wide availability and distribution, and low toxicity toward nontarget organisms such as natural enemies, pollinators, or mammals (Giunti et al. 2022).

The biological activity of EOs from different botanic families (e.g., Lamiaceae, Myrtaceae, Amaryllidaceae, Rutaceae, Apiaceae) has been investigated against other mealybugs (Avila et al. 2022). Mostafa, et al. (2018) demonstrated the efficacy of different EOs against *Phenacoccus solenopsis* (Tinsley) (Hemiptera: Pseudococcidae), noting that *Thymus vulgaris* EO exhibited good insecticidal activity with LC₅₀ values of 29.03 and 15.04 mg/kg 24 and 72 h post-exposure, respectively. Similarly, other studies have highlighted the insecticidal effectiveness of EOs from *Salvia rosmarinus*, *Cymbopogon citratus*, *Eucalyptus melliodora*, and *Mentha* spp. against the same pest (Roozdar et al. 2020; Saad et al. 2021). Furthermore, other studies have reported the efficacy of these EOs against *Planococcus ficus* (Signoret), *Planococcus citri* (Risso), *Ferrisia virgata* (Cockerell), and *Nipaecoccus nipae* (Maskell) (Hemiptera: Pseudococcidae) (Elhefny et al. 2023; Erdemir and Erler 2017; Hassan et al. 2023; Karamaouna et al. 2013; Peschiutta et al. 2017).

Despite the biological efficacy of the EOs as insecticides, certain limitations, such as volatility, photo-lability, poor water solubility, or flammability, pose challenges for their practical application. In recent years, nanotechnology has shown promise in overcoming these limitations, providing new environmentally friendly tools based on EOs (Giunti et al. 2023). In Integrated Pest Management (IPM), nanotechnology typically involves the encapsulation of active ingredients within nano-delivery systems (e.g., nano-emulsions and nanoparticles) that exhibit nanoscale physical properties (Bakkali et al. 2008; Burt 2004; Campolo 2021). Research has demonstrated that nano-insecticides based on EO formulations not only improve the biological efficacy of EOs but also enhance their stability over time,

outperforming raw or unformulated EOs (Campolo et al. 2020a; Modafferi et al. 2024a).

Recently, it has been demonstrated that the application of EOs, due to their high content of secondary metabolites, can activate the defenses of the plants to which they are applied. In this context, EO treatments increased the expression of genes related to jasmonic acid (JA) and salicylic acid (SA) signaling pathways, enhancing the plants' resistance to pests and pathogens in tomatoes and peppers (Ben Abdallah et al. 2023; Ricupero et al. 2023). However, whether this defense activation could be achieved with EO-based nano-emulsions in a perennial crop like citrus still needs to be discovered.

This study aimed to develop stable and effective EO-based nano-emulsions with a high EO:surfactant ratio (3:1) through the high-pressure microfluidization technique. Firstly, the EOs (i.e., garlic, clove, and eucalyptus) and their nano-emulsions were chemically and physically characterized through gas chromatography-mass spectrometry (GC-MS) and dynamic light scattering (DLS) analyses, respectively. Then the EO-based nano-emulsions were used to investigate the biological activity against the second instars of *D. aberiae*, and the most efficacy nano-emulsion (i.e., garlic nano-emulsion) was subjected to further investigations in order to assess its toxicity on nontarget organisms such as *C. montrouzieri* and citrus plants. At the same time, the effects of garlic nano-emulsion on transcriptional levels of different genes involved in plant defense mechanisms were also investigated.

Materials and methods

Plant material

Carrizo citrange (*Citrus sinensis* × *Poncirus trifoliata*) plants used in the experiments were obtained from seeds and cultivated in a growth chamber of the Instituto Valenciano de Investigaciones Agrarias (IVIA) (Valencia, Spain) at 25 ± 1 °C, relative humidity of 60%, and a photoperiod of 16:8 h light:dark (L:D). Plants were used when they reached 3 months old and had 8 to 9 fully expanded leaves.

Insect rearing

Delottococcus aberiae was reared for several generations at the facilities of the IVIA on lemon fruits [*Citrus × limon* (L.) Osbeck (Sapindales: Rutaceae)] in a dark climate chamber under constant climatic conditions (25 ± 1 °C and 60 ± 5% relative humidity RH). *Cryptolaemus montrouzieri* adults were purchased from Bioline AgroSciences Iberia (Almería, Spain).

Chemical compositions of EOs

Commercial *Allium sativum* EO (GEO) and *Syzygium aromaticum* EO (CEO) were purchased from Esperis S.p.A. (Milan, Italy). *Eucalyptus camaldulensis* EO (EEO) was steam extracted from leaves collected from plants growing in southern Italy (Reggio Calabria, Italy).

The chemical characterization of the EOs was carried out following the methods described by Giunti et al. (2019). Briefly, a Thermo Fisher TRACE 1300 GC with a MEGA-5 capillary column (30 m × 0.25 mm; coating thickness = 0.25 µm) and a Thermo Fisher ISQ LT mass detector (ionization mode: EI; scan time: 1.00 s; scan mass range: 30–300 m/z) were used setting injector and transfer line at 250 and 240 °C, respectively, and a temperature ramp from 60 to 240 °C at 3 °C min⁻¹ (carrier gas: He 1 mL min⁻¹). The pure EO was diluted (1:10 v:v) in hexane (95%, Sigma-Aldrich, Munich, Germany), and 0.2 µL was injected at a split ratio of 1:30. The identification of peaks was made using computer matching against the commercial libraries (NIST 05, Wiley FdavisFNCS, and ADAMS) comparing linear retention indices (LRI). The LRIs were calculated using the formula of Van den Dool and Kratz (1963) by comparing the retention times of the compounds to be annotated with those of a standard mixture of alkanes (C8–C20 saturated alkanes standard mixture, Supelco®, Bellefonte, PA, USA) which was analyzed in GC–MS under the identical operating conditions as the sample (van Den Dool and Kratz 1963; Masada 1976; McLafferty and Stauffer 1989; Davies 1990; Jennings 2012; Adams et al. 2017).

Formulation and physical characterization of nano-emulsions

Allium sativum nano-emulsion (GNE), *E. camaldulensis* nano-emulsion (ENE), and *S. aromaticum* nano-emulsion (CNE) were obtained using a high-pressure microfluidization (HPM) technique, following the methods described by Modafferi et al. (2024c). Firstly, EO and Tween 80® (Polyoxyethylene (20) sorbitan monooleate, Sigma-Aldrich, Munich, Germany) (ratio 3:1 w:w) were mixed using a magnetic stirrer (5 min at 6000 rpm) to obtain a homogeneous organic phase. Then, the raw emulsion was prepared by slowly adding (1 mL/min) double distilled water to the organic phase (ratio 4:1 w:w). The resulting raw emulsion (EO 15%, Tween 80 5%, and water 80% w:w:w) was mixed for 5 min at 7000 rpm. The final mixture was homogenized five times at 30,000 psi through a high-pressure microfluidization (HPM) apparatus (LM20 Microfluidizer™ Processor, USA) to obtain a homogeneous nano-emulsion. The interaction chamber was immersed in an ice bath to maintain a low temperature (< 10 °C) during the HPM process. The

resulting nano-emulsion was then stored in aluminum bottles and kept at 4 °C. This process was repeated for each EO.

The developed EO-based nano-emulsions were then physically characterized to assess the droplet dimension (Z-average size), polydispersity index (PDI), droplet surface charge (ζ-potential), and stability over time (1 day, 1 month, and 1 year). The analyses used a dynamic light scattering (DLS) apparatus (Zetasizer Nano, Malvern®). The measurements were conducted by diluting the developed nano-emulsions in double distilled water (ratio 1:200 v:v). To assess the droplet size and droplet surface charges, 1 mL and 0.75 mL of diluted formulations were used, respectively.

Insecticidal activity against target insect

The insecticidal activity of the developed nano-emulsions was evaluated through residual contact toxicity trials using the leaf-dip method (Modafferi et al. 2024c). To assess the efficacy of each nano-formulation and to discard that they did not have the potential to be used in real field conditions, preliminary trials were carried out at a high amount of EO (2.5%) by diluting the required amount of developed nano-emulsion (15% of EO) in double distilled water (w:w). Bioassays were carried out by individually immersing rectangular bean pod sections (2 × 4 cm) for 10 s in the emulsion (2.5% of EO). After that, the treated sections were dried at room temperature and then placed in ventilated plastic Petri dishes (Ø 5.5 cm). Subsequently, 15 *D. abieriae* second instar individuals were gently placed on the treated bean pod surface. The trials were conducted under laboratory conditions, and the experimental arenas were maintained under the same rearing conditions as the abovementioned ones. Insects were considered dead if immobile after stimulation with a fine brush. Mortality data were recorded 24, 48, and 72 h after the exposure. The preliminary results showed that only GNE provided a good mortality efficacy, so only this oil was used for further bioassays (see result Sect. "Toxicity of EO-based nano-emulsions against *Delottococcus abieriae*"). The efficacy of serial dilutions of GNE (0.46, 0.625, 0.93, 1.25, and 1.87% w:w of EO), was evaluated against *D. abieriae* as described above. Each dilution was replicated eight times, and water and deltamethrin (Decis, Bayer Crop Science, San Joan Despí, Barcelona, SP) were used as negative and positive control treatments, respectively. Each dilution was prepared as described for preliminary trials.

Side effects on *Cryptolaemus montrouzieri*

Toxicity on *C. montrouzieri* adults was assessed through a residual contact toxicity trial using the LD₅₀ (1.14% of EO) and LD₉₀ (2.20% of EO) estimated 72 h after the exposure against *D. abieriae* (see Table 2). The experiment was replicated ten times for each LD value, negative control

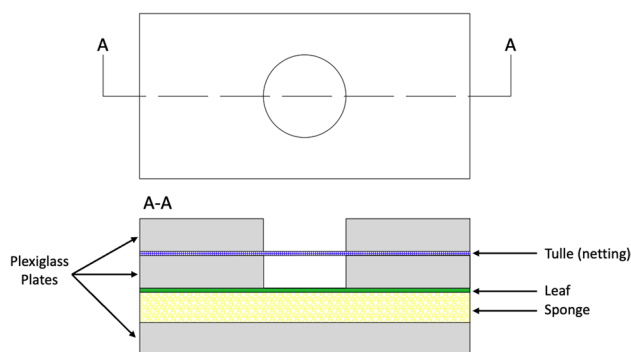


Fig. 1 Schematic representation of the modified Huffaker cell used in the residual contact toxicity with *Cryptolaemus montrouzieri*

(water), and positive control (deltamethrin, Decis, Bayer Crop Science, San Joan Despí, Barcelona, SP). The positive control was tested at the maximum label concentration (15 ml/hl). The bioassays were conducted on non-infested, fully expanded detached leaves from *C. citrange* seedlings. The experimental unit was a modified Huffaker cell (Fig. 1) (Abad-Moyano et al. 2009). This unit consisted of a bottom PVC plate (80 × 40 × 5 mm) and two top PVC plates (80 × 40 × 10 mm). The top plates had a central circular hole (Ø 2 cm). On each bottom plate, a *C. citrange* leaf treated with the corresponding treatment was placed on a wet cleaning cloth (80 × 40 mm) (Vileda®, Freudenberg Home and Cleaning Solutions GmbH, Weinheim, Germany), ensuring that the leaf substrate formed the bottom of the arena. A muslin cloth was placed between the two bottom plates to allow ventilation in the arena. Then, 5 adults were placed on the citrus leaf surface, and the arena was assembled by covering the bottom plate with both top plates. The three plates were secured together with two rubber bands (Supplementary Fig. 1 SF.1). The arenas were kept in a climate chamber at 25 ± 2 °C, 65 ± 10% R.H., and a photoperiod of 14:10 h (L:D) throughout the experiment. Mortality was recorded 24, 48, and 72 h after the exposure. Insects were considered dead if they were not able to walk. Eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) were offered daily as food to the predators until the end of the experiment.

Phytotoxicity on citrus seedlings

The phytotoxic effects of GNE were observed on *C. citrange* plants. Three treatments were carried out using the estimated LD₅₀, LD₉₀ (1.14 and 2.20% of EO, respectively) (see Table 2), and negative control (water). Each treatment was replicated six times. Citrus seedlings with an average height of 25 cm and approximately the same number of leaves were used. The plants were individually treated with a hand sprayer until runoff, placed in different climate chambers (one per treatment), and maintained under the same

conditions (25 ± 2 °C, 65 ± 5% R.H. and a photoperiod of 14:10 h L:D). The phytotoxic effects of GNE were checked 24 h, 3, 7, and 14 days after treatment, recording the number of damaged leaves and the severity of the damage. The severity of damage was classified following the method described by Campolo et al. (2017):

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

where *DL* is the number of damaged leaves for each damage severity class *j*; *TL* is the total number of leaves sprayed; *DC* is the damage severity class (0 = no damage; 1 = leaf surface with only chlorosis; 2 = leaves with evident necroses and 3 = dead leaves; *n* is the number of damage severity classes. The *Pi* ranges from 0 (no damage) to 1 (dead leaves).

Plant defenses on citrus seedlings

The ability of GNE to induce the expression of genes involved in plant defenses was evaluated at the same concentrations used in the phytotoxicity assessment (see Sect. 2.5.2). Five genes were analyzed via real-time PCR (rt-PCR). Specifically, the expression levels of *ICS2* (Ischorismate Synthase 2, Chloroplastic), *NPR1* (Non-Pathogenesis Related Protein 1), and *PAL* (phenylalanine ammonia-lyase), associated with the salicylic acid (SA) pathway, as well *JAR1* (jasmonate resistant 1) and *MYC2* (Transcription factor MYC2, DNA binding protein), which are marker for Jasmonic acid (JA) pathway, were investigated (Alfarez et al. 2018; Pérez-Hedo et al. 2024). *GADPH* (Glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control for normalization. The nucleotide sequences of the gene-specific primers are provided in Supplementary Table 1 (ST1).

The experiment included seven replicates for each LC value and control (water) treatment. Citrus seedlings were treated under the same conditions as those used in phytotoxicity trials (see Sect. 2.5.2). Twenty-four hours post-treatment, the apical region of each plant was excised, wrapped in foil paper, and immediately ground in liquid nitrogen. Samples were stored at − 80 °C until the RNA extraction. Following the manufacturer's recommendations, total RNA was extracted using NZYol (NZYTech, Lisboa, Portugal). Then, 10 µg of total RNA was treated with a TURBOTM DNA-free kit (Ambion, Life Technologies, CA, USA) to remove the DNA contamination. Reverse transcription was performed using PrimeScript RT reagent kit (TAKARA BIO INC., Japan) starting from 1 µg of RNA–DNA-free template. Real-time PCR amplification was performed in LightCycler 480 System (Roche Molecular Systems, Inc., Switzerland), using 5 µl of NZYSupreme qPCR Green Master Mix (2×) (NZYTech,

Lisboa, Portugal), 0.5 µl of forward and reverse primers (10 mM), and 2 µl of cDNA, resulting in a total reaction volume of 10 µl. The reaction was performed using the following PCR conditions: 1 cycle at 95 °C for 2 min, 40 cycles at 95 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s, and a final melting cycle at 95 °C for 5 s and 65 °C for 1 min. The relative fold gene expression of the samples was calculated using the 2^{-ΔΔCT} method described by Ricupero et al. (2023).

Statistical analysis

Changes in the physical characteristics of the EO-based nano-emulsions (GNE, ENE, and CNE) over time were analyzed using analysis of variance (ANOVA) with particle size, polydispersity index (PDI), and zeta potential as dependent variables, and the nano-emulsions used as fixed factors. All data met the assumptions required for parametric testing, including normality and homoscedasticity of variance ($P > 0.05$). Mortality data for *D. abieriae* were corrected for control mortality using Abbott's formula (Abbott 1925). Differences in exposure times in the preliminary test were also subjected to ANOVA analysis. The concentration–mortality response was evaluated by probit analysis, in which the LD₅₀ and LD₉₀ values and their fiducial limits were estimated using the results obtained 24, 48, and 72 h after the treatment with GNE. LD values were considered significantly different if their 95% fiducial limits did not overlap. The effects on *C. montrouzieri* adult mortality following treatment with LD₅₀ and LD₉₀ were assessed

with the Kruskal–Wallis test. The ability of GNE dilutions to enhance plant defenses on *C. citrange* plants was analyzed using ANOVA, and multiple comparisons were conducted using Tukey's HSD post hoc test. All statistical analyses were performed using IBM® SPSS® StatistICS2 v. 23 (IBM Corp. Released 2015. Armonk, NY, USA).

Results

Characterization of EOs and EO-based nano-emulsions

The GC–MS analysis identified twenty-three, twenty-six, and sixty-three components, accounting for 97.87%, 99.45%, and 95.33% of the total area for GEO, CEO, and EEO, respectively (SF.2). GEO consisted primarily of sulfur compounds (97.87%) (ST.2). At the same time, CEO was composed of aldehydes (0.03%), monoterpenes (0.05%), oxygenated monoterpenes (0.06%), phenylpropenes (88.65%), esters (0.16%), sesquiterpenes (8.69%), and oxygenated sesquiterpenes (1.83%) (ST.3). In contrast, EEO included aldehydes (0.01%), monoterpenes (61.03%), oxygenated monoterpenes (17.56%), phenylpropenes (6.76%), esters (0.01%), sesquiterpenes (1.40%), and oxygenated sesquiterpenes (8.56%) (ST.4).

The physical characteristics of the EO-based nano-emulsions developed are presented in Table 1. All formulations demonstrated good stability over time, with particle size in the nanometric range, as confirmed by their negative zeta potential and low PDI values. The physical properties of the nano-emulsions varied depending on the type of EO used in each formulation. Specifically, the ENE displayed the smallest droplet size (62.5 ± 1.1 nm) 1 day post-development ($F = 4399.1$; $df = 2$; $P < 0.01$), whereas GNE showed the smallest droplet size at both one month (96.4 ± 1.8 nm; $F = 3681.9$; $df = 2$; $P < 0.01$) and one year (215.7 ± 2.4 nm; $F = 513.1$; $df = 2$; $P < 0.01$) after the formulation. In contrast, the CNE exhibited the largest droplet size throughout the experiment, with particle size values ranging from 161.3 ± 0.7 nm on day one to 268 ± 2.5 nm after one year. A high-energy system allowed to develop nano-emulsions with low PDI values (< 0.25). In detail, one day after development, no statistical differences were observed between GNE and ENE ($F = 12$; $df = 2$; $P < 0.01$) with very low PDI values close to zero (0.07 ± 0.01 and 0.09 ± 0.03 for GNE and ENE, respectively) after 1-month statistical differences were observed among all the developed nano-emulsions ($F = 105.6$; $df = 2$; $P < 0.01$), while 1 year after development, only GNE highlighted statistical differences ($F = 24.4$; $df = 2$; $P < 0.01$) with PDI values less than 0.2. Furthermore, considering the surface charge ($P < 0.05$), the EO plant source resulted in significant differences. Particularly, CNE registered the

Table 1 Physical characteristics (particle size, PDI, and zeta potential) of different developed EO-based nano-emulsions during storage

TIME	NE ¹	Size (nm)	PDI ²	Zeta Potential (mV)
1 day	GNE ³	69.3 ± 2.1a	0.07 ± 0.01a	− 15.3 ± 0.4a
	ENE ⁴	62.5 ± 1.1b	0.09 ± 0.03a	− 11 ± 0.5b
	CNE ⁵	161.3 ± 0.7c	0.15 ± 0.01b	− 24.7 ± 2.3c
1 month	GNE	96.4 ± 1.8a	0.09 ± 0.01a	− 22.8 ± 1.6a
	ENE	101.3 ± 1.7b	0.18 ± 0.01b	− 15.7 ± 0.4b
	CNE	193.2 ± 0.9c	0.14 ± 0.01c	− 27.1 ± 0.2c
1 year	GNE	215.7 ± 2.4a	0.16 ± 0.02a	− 30.6 ± 0.6a
	ENE	230.5 ± 0.7b	0.21 ± 0.01b	− 21.8 ± 0.4b
	CNE	268 ± 2.5c	0.21 ± 0.01b	− 35.9 ± 0.4c

¹Nano-emulsion

²Polydispersity index

³*Allium sativum* EO-based nano-emulsion

⁴*Eucalyptus camaldulensis* EO-based nano-emulsion

⁵*Syzygium aromaticum* EO-based nano-emulsion

Values represent the mean (± SD standard deviation) of three replicates. Different letters indicate statistical differences among nano-emulsions at the same storage time ($P < 0.05$)

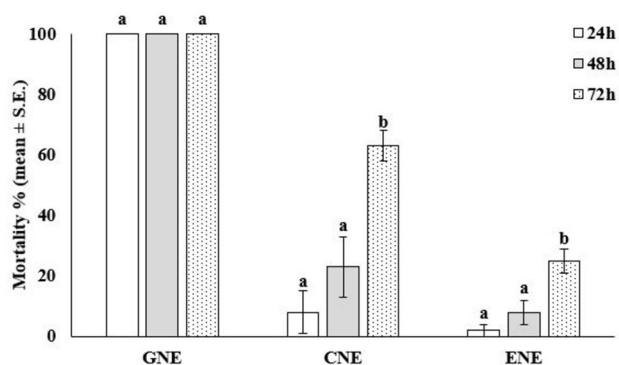


Fig. 2 Mortality % (mean ± S.E.) at 24, 48, and 72 h after exposure to different developed EO-based nano-emulsions against *D. abieriae* in the preliminary trials (2.5% of EO). Different letters indicate statistical differences among exposure times within the same EO-based nano-emulsion ($P < 0.05$)

lowest negative surface charge at all observed times with values ranging from -24.7 ± 2.3 to -35.9 ± 0.4 mV 1 day ($F = 79.7$; $df = 2$; $P < 0.01$) and 1 year ($F = 598.5$; $df = 2$; $P < 0.01$), respectively.

Toxicity of EO-based nano-emulsions against *Delottococcus abieriae*

At the maximum application rates (2.5% EO), the different formulations displayed variable toxicity against second instars of *D. abieriae*, depending solely on the EO used (Fig. 2). In the water control treatments, no mortality was observed, whereas all specimens exposed to deltamethrin at the labeled dose (positive control) experienced 100% mortality. GNE exhibited very high toxicity against the mealybugs, achieving 100% mortality within 24 h post-exposure. In contrast, the maximum mortality caused by CNE and ENE increased gradually over time. Specifically, CNE showed low mortality 24 and 48 h after exposure ($8 \pm 7\%$ and $23 \pm 9\%$, respectively), which rose to $63 \pm 5\%$ at 72 h post-exposure. Conversely, ENE showed very low efficacy, with mortality rates reaching approximately $25 \pm 4\%$ at 72 h post-exposure.

Table 2 Estimated LD_{50} and LD_{90} of GNE against second instar of *D. abieriae* at 24, 48, and 72 h after exposure

LD^1	Time (h^2)	Estimated	Lower Bound	Upper bound	X (df^3)	P level
LD_{50}	24	1.49	0.78	6.11	5.973(3)	0.113
	48	1.29	1.06	1.56	4.507(3)	0.212
	72	1.14	0.93	1.36	1.899(3)	0.549
LD_{90}	24	2.80	1.79	6.03	5.973(3)	0.113
	48	2.57	2.00	4.59	4.507(3)	0.212
	72	2.20	1.75	3.50	1.899(3)	0.549

Table 2 (Continued)

²hours

³Degrees of freedom

Values were considered statistically different if their 95% fiducial limits did not overlap

¹Lethal dose

Given the high efficacy of GNE, this nano-formulation was the only one further tested against the pest. Mortality data recorded at all observed times for the selected EO-based nano-emulsion (i.e., GNE) showed a dose–response relationship and fit well with the probit model ($P > 0.05$) (Table 2). However, no significant differences were observed across times within the same LD, as indicated by the overlapping fiducial limits.

Side effects on *Cryptolaemus montrouzieri*

The biological activity of the selected EO-based nano-emulsion (GNE) on the nontarget predator *C. montrouzieri* is presented in Fig. 3. Adults were unaffected by GNE, with no mortality recorded at any observed times (24, 48, and 72 h). Furthermore, no significant differences ($P > 0.05$) were observed between the LD_{50} , LD_{90} (1.14 and 2.20% of EO, respectively), and the negative control (water). In contrast, the positive control (deltamethrin) exhibited high toxicity toward nontarget insect adults, with no survivors observed just 24 h after treatment.

Phytotoxicity and plant defenses on citrus seedlings

No phytotoxicity ($P_i = 0$) was observed in *C. citrange* plants at any of the tested times (24 h, 3 d, 7 d, and 14 d) across the tested LD_{50} and LD_{90} (1.14 and 2.20% of EO, respectively). The capacity of GNE to enhance plant defenses in

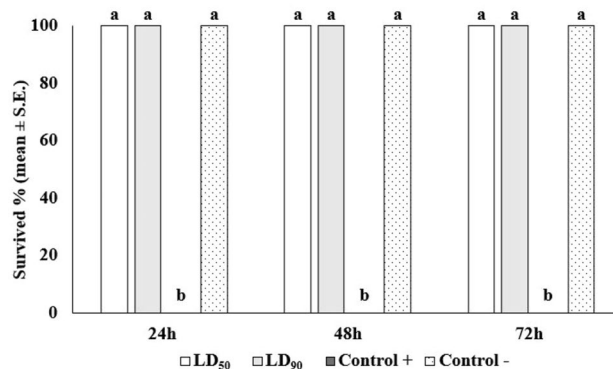


Fig. 3 Survival % (mean ± S.E.) of *C. montrouzieri* adults 24, 48, and 72 h after exposure to lethal doses (LD_{50} and LD_{90}) of GNE, the negative control (water), and the positive control (deltamethrin). Different letters indicate statistical differences among the treatments within the same exposure time ($P < 0.05$)

citrus seedlings is shown in Fig. 4. Transcriptional analysis revealed that the GNE application at the estimated LD₅₀ (1.14% of EO) and LD₉₀ (2.20% of EO) doses significantly increases the relative expression (RE) levels of *ICS2*, *NPR1*, *PAL* associated with the SA pathway, as well as *MYC2*, associated with the JA pathway. Specifically, significant differences were observed for *ICS2* ($F = 8.94$; $df = 2$; $P < 0.01$) and *NPR1* ($F = 8.65$; $df = 2$; $P < 0.01$) among the control treatment (0.69 ± 0.07 and 0.74 ± 0.06 RE for *ICS2* and *NPR1*, respectively), LD₅₀ treatment (1.31 ± 0.16 and 1.11 ± 0.09 RE for *ICS2* and *NPR1*, respectively), and LD₉₀ treatment (1.51 ± 0.17 and 1.17 ± 0.08 RE for *ICS2* and *NPR1*, respectively). No significant differences, however, were found between the LD₅₀ and LD₉₀ doses. In the *PAL* gene experiment, only LD₉₀ (1.95 ± 0.18 RE) significantly increased the relative expression level compared to the control ($F = 9.05$; $df = 2$; $P < 0.01$). The *MYC2* gene, involved in the JA pathway, also shows significant differences between the lethal doses (LD₅₀ = 1.22 ± 0.1 and LD₉₀ = 1.67 ± 0.17 RE) and the control (0.71 ± 0.1 RE) ($F = 13.12$; $df = 2$; $P < 0.001$). Conversely, *JAR1* gene expression showed no statistical differences across the treatments ($P > 0.05$).

Discussion

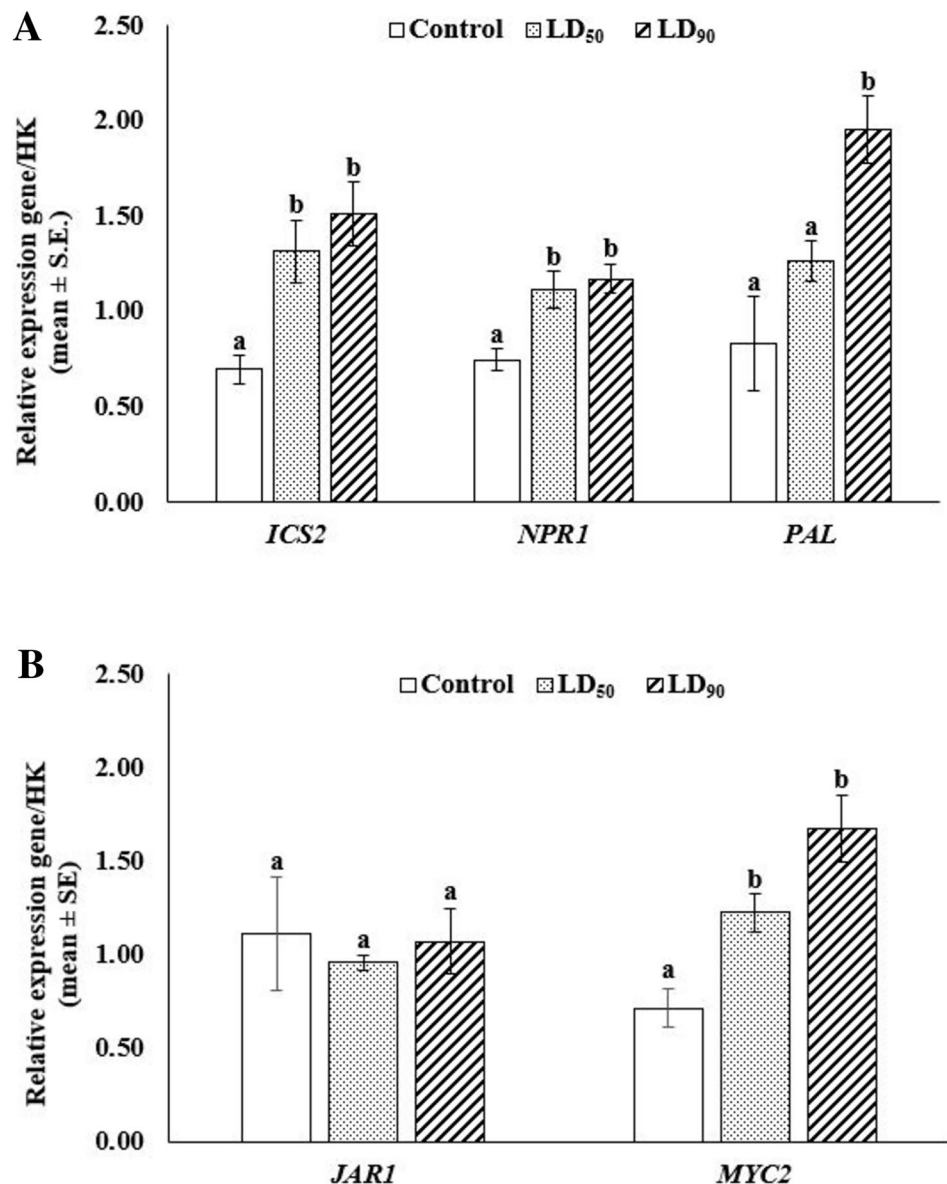
This study aimed to develop highly stable EO-based nano-emulsions (i.e., GNE, CNE, and ENE) and to investigate their toxicity against *D. aberiae*. Although the stability of nano-emulsions is influenced by the chemical profile of the essential oil (EO), this chemical composition of EOs can also be affected by various factors, including environmental conditions, plant parts used, extraction and drying methods, and analytical techniques (Campolo et al. 2020a; Li et al. 2015), our results are consistent in terms of the chemical composition and relative abundance of the different compounds, in agreement with those reported by other authors (Figueiredo et al. 2008; Barra et al. 2010; Dziri et al. 2014; Mossa et al. 2018; Li et al. 2020; Selles et al. 2020; Pant et al. 2021; Modafferi et al. 2024b). This analytical consistency suggests that nano-emulsion production could be feasible across various EO sources. Such reproducibility strengthens the potential for scalable nano-emulsion formulations using diverse plant-derived EOs, broadening the range of sources that could be employed effectively.

The use of nanotechnology to enhance the biological efficacy of botanical-based insecticides is rapidly expanding, with various approaches (e.g., low or high-energy ones) playing a crucial role in achieving high-stable EO-based nano-emulsion (Donsì and Ferrari 2016; Singh and Pulikkal 2022). In this study, the EO-based nano-emulsions were developed using the high-energy approach, specifically high-pressure microfluidization, which is generally favored

over low-energy techniques due to its ease of production, scalability for industrial applications, and superior physical properties (Barradas and de Holanda e Silva 2021; Kumar et al. 2025; Tanuku et al. 2024). Supporting these findings, Modafferi et al. (2024b) recently demonstrated that high-energy approaches like sonication and microfluidization produced garlic EO-based nano-emulsions with particle sizes under 200 nm, maintaining stability throughout a 100-day testing period. In contrast, the self-emulsification technique yielded unstable nano-emulsions, with particle sizes exceeding 500 nm. In our study, the physical characterization of EO-based nano-emulsions indicated outstanding stability up to one year post-development, with particle sizes consistently within the nanoscale range (215.7 ± 2.4 , 268 ± 2.5 , and 230.5 ± 0.7 nm for GNE, CNE, and ENE, respectively) and optimal PDI values (always ≤ 0.22 PDI). Additionally, the negative surface charge values observed (-30.6 , -21.8 , and -35.9 mV for GNE, CNE, and ENE, respectively) contributed to enhanced surface adherence and robust repulsive properties (steric and electrostatic), which further supported long-term stability (Akbari and Nour 2018; Campolo et al. 2020a). These findings align with previous studies that underscore the efficacy of high-energy methods in producing EO-based nano-emulsions (Kaur et al. 2019; Liu et al. 2022; Ricupero et al. 2022; Shahavi et al. 2016). For instance, Giuliano et al. (2024) successfully obtained a GNE with optimal particle size and PDI values (141.0 ± 1.37 nm and 0.146 ± 0.009 PDI, respectively) through the microfluidization technique. Similarly, Das et al. (2024) employed sonication to produce CNE with a smaller particle size (approximately 125.3 nm) than our nano-emulsion (161.3 ± 0.7 nm). However, this method resulted in a notably higher PDI (0.46), indicating less stability over time. Additionally, the sonication method applied by Sugumar et al. (2014) yielded extremely small particles for ENE (ranging from 20 to 40 nm) with exceptionally low PDI values (0.06). However, this formulation required twice as much surfactant relative to the EO (ratio 2:1). In contrast, our study prioritized minimizing surfactant usage to mitigate potential phytotoxicity effects on treated plants (Appah et al. 2020; Falk et al. 1994; Temple and Hilton 1963). In this regard, we opted for a higher EO-to-surfactant ratio (3:1) in our formulations, balancing stability with reduced surfactant concentration. This approach addressed toxicity concerns and maintained desirable physical characteristics in our nano-emulsions. Our results suggest that this formulation strategy, emphasizing high EO content and reduced surfactant usage, may be advantageous for applications where plant health is a priority.

To our knowledge, this study demonstrated, for the first time, the efficacy of EO-based nano-emulsion (GNE) against *D. aberiae*. Although nano-formulations have

Fig. 4 Relative expression of HK/gene (mean $\pm$ SE) for *ICS2*, *NPR1*, *PAL* (4 A), *JAR1*, and *MYC2* (4B) in the apical portion of *C. citrange* seedlings 24 h after treatment with control (water), LD₅₀ (1.14% of EO), and LD₉₀ (2.20% of EO) of GNE. Transcript levels were normalized to the expression of the housekeeping gene (*GADPH*). Different letters indicate statistical differences among treatments within the same gene ($P < 0.05$)



shown effectiveness against various pests, including crop pests, stored product pests, and blood-feeding insects, their biological activity against scale insects has been underexplored (Campolo 2021; Esmaili et al. 2021; Menossi et al. 2021; Modafferi et al. 2024a). A limited number of studies have reported on the efficacy of GNE against *P. citri*. For instance, Modafferi et al. (2024c) demonstrated high insecticidal activity of GNE in both topical and residual toxicity assays against the second instar of *P. citri* with LD₅₀ and LD₉₀ values estimated at 0.78% and 1.08% EO for residual application and 0.24% and 0.96% EO for topical application, respectively, 48 h after treatment. Similarly, Modafferi et al. (2024b) showed that three GNEs developed using high-energy methods (sonication and/or microfluidization) were effective against

P. citri, with an LD₉₀ consistently below 1.5% EO. While our results (LD₅₀ = 1.14% EO; LD₉₀ = 2.20% EO after 72 h) are slightly less effective than those reported by Modafferi et al. (2024b), they still underscore the potential of GNE against mealybugs.

One of the most promising attributes of botanical insecticides, whether formulated into nano-delivery systems like GNE or used as conventional EOs, is their potential selectivity toward nontarget organisms (e.g., predators, parasitoids, pollinators, plants, and invertebrates), which is crucial for their practical application in real-world settings. In this context, our study confirms the safety of GNE for the nontarget organism *C. montrouzieri*. However, the impact of EOS—whether used in their natural form or nano-formulated—on nontarget organisms still needs to be

investigated. Some authors have reported minimal impact of *Mentha pulegium* EO, citrus EO, and a D-limonene-based formulation on the survival of *C. montrouzieri* larvae and adults (Attia et al. 2022; Bibi et al. 2022; El Aalaoui et al. 2019). Conversely, *Thymus capitatus* EO has shown substantial toxicity, with *C. montrouzieri* adult mortality reaching about 90% (Alloui-Griza et al. 2022). The potential negative effects of natural substances on nontarget insects are influenced by various factors, including the type of plant and insect species involved, exposure route, or application rate (Gahukar and Das 2020; Giunti et al. 2022). Data on the effects of garlic (whether as aqueous extract or EO) on nontarget organisms are limited. Among the few studies available, Fand and Kamra (2012) evaluated garlic aqueous extract at concentrations of 0.5, 1, and 1.5% against *C. montrouzieri* larvae, observing a mortality rate of less than 20% at 72 h post-exposure. Similarly, Modafferi et al. (2024a, b, c) found no toxicity to *C. montrouzieri* adults in topical toxicity trials using a garlic EO-based nano-emulsion.

On the other hand, the adverse effects of EOs on plants, particularly phytotoxicity, are well documented. Several studies have reported that EOs can inhibit photosynthesis and mitochondrial respiration, induce genotoxic effects, interfere with enzymes and phytohormones, alter membrane properties, and promote the formation of reactive oxygen species (ROS) (De Almeida et al. 2010; Pinheiro et al. 2015; Verdeguer et al. 2020; Werrie et al. 2020). However, encapsulating EOs in nano-delivery systems appears to mitigate these negative effects. For instance, various studies have shown that botanical-based nano-formulations (such as EO-based nano-emulsions and/or nanoparticles) did not produce adverse effects on plants like garlic, tomato, and maize (Campolo et al. 2020b; Qian et al. 2023). Similarly, Pasquoto-Stigliani et al. (2017) noted that the adverse effects of botanical nano-insecticides on garlic and maize plants were dose-dependent. In the case of GNE, previous studies reported its safe application on tomato plants, with *Pi* values comparable to those observed in *C. citrange* seedlings in our research (*Pi* = 0) (Ricupero et al. 2022). Likewise, Giuliano et al. (2024) observed that GNE application on pepper plants (*Capsicum annuum* L., cv Makko F1) did not affect the growth, leaf number, weight, and chlorophyll content. However, mild phytotoxic effects were noted on the leaves, with low *Pi* levels (0.13 ± 0.01), and a significant reduction in the number of fruits was observed compared to the control.

One of the most promising aspects of using EOs or their nano-formulations for crop protection is their potential to enhance natural plant defenses. Studies have shown that applying EOs can boost defense enzyme activities (e.g., chitinase, 1,3- β -glucanase, phenylalanine ammonia-lyase, and peroxidase) (Kaneko et al. 2024; Kesraoui et al. 2022).

Additionally, EO treatments can induce systemic resistance by overexpressing genes involved in plant defense (Andres et al. 2024; Sellamuthu et al. 2013; Werrie et al. 2022). In our study, GNE increased the expression levels of several genes involved in the SA and JA pathways. Previous research showed that the foliar application of *Achillea millefolium* and *Allium sativum* EOs provided a dual benefit on tomato plants (Ben Abdallah et al. 2023), leading to a decrease in *Phthorimaea absoluta* (Meyrick) (Lepidoptera: Gelechiidae) populations and an increase in the expression levels of proteinase inhibitor II (*PIN2*) and abscisic acid stress ripening 1 (*ASR1*), associated with the JA and ABA signaling pathways, respectively. Similarly, Ricupero et al. (2023) showed that garlic and peppermint EOs increased the expression levels of the basic PR1 protein precursor (*PR1*) and *PIN2* compared to the control treatment.

Conclusion

In conclusion, this study demonstrates, for the first time, the efficacy of botanical-based nano-insecticides, specifically GNE, against *D. abieriae*. The high-pressure microfluidization technique enabled the development of a highly stable formulation with optimal physical properties featuring a high EO-to-surfactant ratio (3:1) that prevented phytotoxic effects on citrus seedlings. Additionally, the solid insecticidal activity against the target pest and the absence of side effects on the nontarget coccinellid predator *C. montrouzieri* supports its potential integration into IPM programs. The overexpression of defense-related genes in the SA and JA pathways further suggests an additional plant-protective effect, enhancing resilience against pest damage. However, to ensure the safe application of GNE and similar formulations, further research is necessary to investigate their toxicological impacts on a broader range of nontarget organisms, including pollinators, beneficial invertebrates, and aquatic organisms. Future studies should also explore the efficacy of these nano-formulated botanical insecticides against other economically significant plant pests, both in laboratory settings and under field conditions.

Author contributions

AM, AU, OC, and MPH were involved in conceptualization; AM, GG, AU, and MPH were responsible for data curation; VP, AU, and MPH were responsible for funding acquisition; AM, FL, and CMA participated in investigation; AM, GG, OC, and MPH analyzed data. AM was involved in writing the original draft. All authors read and approved the manuscript.

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Data availability The data are available at the following link: Campolo, Orlando (2024), "Modafferi et al., 2024 data," Mendeley Data, V1, <https://doi.org/10.17632/vjz5x5yg5v.1>

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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