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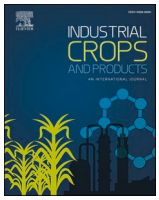

Aniseed essential oil botanical insecticides for the management of the currant-lettuce aphid

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ABSTRACT

Nasonovia ribisnigri Mosley (Hemiptera: Aphididae) is the most damaging aphid species of lettuce grown in open fields. Populations of *N. ribisnigri* are developing resistance to insecticides, making their control difficult. Botanicals are an alternative for pest control. Aniseed (*Pimpinella anisum* L.) is a relevant crop in the production of essential oils. The effect of aqueous nano-formulations of this oil and its main compound *(E)*-anethole were tested against *N. ribisnigri* in a growth chamber, a greenhouse (in spring for two years, 2019 and 2020) and in the open field in a plot in the Southeast of Spain (Torrepacheco, Murcia) in May 2019. Aniseed essential oil nano-emulsions were prepared using a laboratory dispersing machine at a high-speed regime (10 min, 7940 revs/min, 15 °C) using Tween80 as a surfactant at a 1:2 ratio. Foliar applications of aniseed essential oil at concentrations of 0.2% and 0.4% (0.1 and 0.2 mL respectively) to lettuce plants infested with homogeneous populations of *N. ribisnigri* reduced the number of insects compared with the control in the laboratory (efficacies > 50%) and greenhouse (efficacies > 25%, 48 h after treatment) experiments. During the field trial, a reduction in the aphid populations was also produced after the application of the products, without any phytotoxic effects observed on the crop. Likewise, *(E)*-anethole gave similar results as aniseed essential oil (with efficiencies of up to 47% with respect to the control) without damaging the plant.

1. Introduction

Lettuce (*Lactuca sativa* L.) (Asteraceae) is grown in large areas of monoculture in the Mediterranean basin. In 2019, more than 4 M tons were produced in the European Union. The principal producers are Spain, Italy, Belgium, and France (FAO, 2019). In Spain, most of the production takes place in the southeast, in Murcia.

The currant-lettuce aphid, *Nasonovia ribisnigri* Mosley (Hemiptera: Aphididae) is considered the most damaging species in open field lettuce crops. Crop damage is mainly due to insect feeding and the presence of aphids on lettuce during commercialization (ten Broeke et al., 2013). *N. ribisnigri* is the Lettuce necrotic leaf curl virus vector (LNLCV) as well (Verbeek et al., 2017).

The control of aphids in lettuce is mainly based on chemical control, using contact insecticides such as carbamates or pyrethroids (Barrière et al., 2014). The use of insecticides authorized is overused and very toxic to natural enemies and pollinators (Gentz et al., 2010). Lettuce cultivars resistant to *N. ribisnigri* are also used. However, resistant populations of *N. ribisnigri* to carbamates, organochlorines, and organophosphates have appeared (Rufingier et al., 1997). Since 2007 new biotypes of *N. ribisnigri* are overcome resistances of cultivars in Europe (Cid et al., 2012; Walley et al., 2017).

Some plants of the Apiaceae family contain pure compounds that repel or have toxic effects against insects (Pavela et al., 2018). Aniseed (*Pimpinella anisum* L.) (Apiaceae) is one of the most important crops for the production of essential oils (Rocha and Fernandes, 2016). It has traditionally been used in medicine and it currently has a wide range of applications in the food and beverage industry. Its oil is mainly composed of the phenylpropanoid *(E)*-anethole (Hashem et al., 2018). Formulations of essential oils in water is difficult due to their insolubility (Isman, 2020). The formulations of essential oils in nanoparticle range increases their solubility and their biological activity (Pascual-Villalobos et al., 2019).

The aim of this work was to study the toxic effect of nano-formulations of aniseed essential oil (EO) and its main compound *(E)*-anethole, and the pure compound farnesol against the currant–lettuce aphid *Nasonovia ribisnigri*. These essential oils were selected based on previous works on different aphid species (*Rhopalosiphum padi* L.,

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Macrosiphum euphorbiae Thomas and *Myzus persicae* Sulzer) (Pascual--Villalobos et al., 2017, 2019; Cantó-Tejero et al., 2021). We performed laboratory, semi-field, and field experiments for two years.

2. Material and methods

2.1. Essential oils and pure compounds. Preparation of nanoemulsions

The essential oil (EO) of aniseed (*P. anisum*) and its main compound, the phenylpropanoid *(E)*-anethole were studied. Also, the pure compound farnesol was tested. Aniseed EO was obtained from Distilleries Muñoz Gálvez S.A. (Murcia, Spain), and the pure compounds from Sigma Aldrich (St. Louis, MO, USA). Aniseed essential oil composition were analyzed in a previous work where the main compounds is *(E)*-anethole (96.9%) (Pascual-Villalobos et al., 2017). Essential oils were formulated in water (O/W) as nanoemulsions with Tween80® (Panreac, Barcelona, Spain) as a surfactant (1:2). Nanoemulsions were prepared 2–4 days before the treatment with a high-speed rotor (IKA-Labor Pilot 2000/4, IKA-Werke GmbH and Co. Staufen, Germany) at 7940 rev/min for 10 min at 15 °C, and used for this study.

2.2. Aphids

Nasonovia ribisnigri were collected from lettuce plants in Orihuela (Alicante, Spain) in 2017. The aphids were maintained on lettuce plants (*L. sativa*, cv Bondena, Syngenta Seeds, USA) for several generations in a growth chamber in our laboratory under a 14:10 (L:D) photoperiod, at 22 ± 1 °C and 60 ± 10% relative humidity. All aphids used in the experiment were parthenogenetic females.

2.3. Growth chamber experiment

We studied the toxic effect against *N. ribisnigri* using the bioassay by Ribeiro et al. (2014). Ten plants per treatment were used (5–7 true leaves developed). Each group of plants was kept in different cages in the same growth chamber under a 14:10 (L:D) photoperiod, at 23 ± 1 °C and 60 ± 10% relative humidity. Lettuce plants (*Lactuca sativa*, cv Bondena, Syngenta Seeds, USA) were cultivated in pots (0.33 L) filled with a mixture of peat (Klasmann TS3, Klasmann-Deilmann GmbH, Germany) and perlite (Projar, S.A. company, Valencia, Spain) in a 3:1 ratio. Plants were watered twice a week (≈70 mL/plant), one with a NPK (15−15−15) fertilizer diluted in water (10 g/L).

Ten wingless female adults of *N. ribisnigri* were released per plant two weeks before the treatment. Aqueous nanoformulations of aniseed EO, *(E)*-anethole, and farnesol were sprayed at a concentration of 0.4% (v/v) (for 100 mL: 0.4 mL of EO, 0.8 mL of Tween80 and 98.8 mL of water) with a manual (atomizer) sprayer (Berry 1.5, Matabi, Goizper Group, Gipuzkoa, Spain) at a rate of ≈ 50 mL/plant. Only Tween80® diluted in water (as same concentration than treatments) was used as a control. Live aphids were counted before and 1, 2, 3, and 6 days after the treatments.

2.4. Greenhouse experiments

Semi-field experiments were conducted for two consecutive years (2018–2019) between March-April in a greenhouse located at the Instituto Murciano de Investigación y Desarrollo Agrario y Medioambiental (IMIDA) (La Alberca, Murcia, Spain) (37º56′18.1′’N 1º08′01.1′’W).

Lettuce plants (*Lactuca sativa*, cv Bondena, Syngenta) were cultivated in pots (0.33 L) in a mixture of peat (Klasmann TS3, Klasmann-Deilmann GmbH, Germany) and perlite (Projar, S.A. company, Valencia, Spain) (3:1). Plants were watered twice a week according to the to the needs of the crop, one with NPK (15−15−15) fertilizer diluted in water (10 g/L).

A randomized block was designed with four replicates of 12 plants each one. Each treatment tested in all the experiments had 48 plants and the concentrations ranged between 0.2% and 0.4% (v/v). Each experiment had four treatments, except in the second year, which had five treatments. Tween80® as a control at the same dose as the treatments (1:2 EO: Surfactant), and pyrethrins as a reference product (Pirecris®, Seipasa company, Valencia, Spain) at 0.4% (v/v), were used in each experiment. Aniseed EO and *(E)*-anethole at 0.4% (v/v) were tested in the first experiment in the first year, whereas in the second experiment a dose of 0.2% was tested. In the second year, aniseed EO, *(E)*-anethole and farnesol were tested at 0.4% (v/v). Essential oils were formulated at a dose of 2% (v/v) and then diluted to the test concentration. Treatments were sprayed using a hand sprayer (Polita 7, Matabi, Goizper Group, Gipuzkoa, Spain) at a rate of ≈ 50 mL/plant. A lettuce leaf with 5–10 wingless female adults of *N. ribisnigri* was released on each plant (=9 true leaves developed) two weeks before the first count to rear the aphid colonies. After this time, the number of aphids on all plants were counted (day −1) and treated the next day with the products described above (day 0). After the treatments, aphids per plant were counted at after 1, 2, 3, and 6 days.

2.5. Open field experiment

The field experiment was conducted in 2018 between May and June at the Torreblanca Experimental Station (Torrepacheco, Murcia, Spain) (37°46’24.8"N 0°53’56.6"W) with a Mediterranean climate. In May, the average temperature was 19.5 °C (minimum of 16.34 and maximum of 23.09). Average of wind intensity was 2.02 m/s and solar radiation of 297.99 w/m2. The total precipitation was of 1.1 mm (on 24 may) during the experiment.

Baby Lettuce plants susceptible to aphids (*L. sativa*, cv Bondena, Syngenta) were planted on 2 May and harvested after five weeks (5 June). Plants were cultivated on ridges (with separation of 1 m) at a density of 12 plants/m^2 (0.12 ×0.10 m). Plots of 10 m^2 (3 ×3.33 m) were replicated four times in a randomized block design (1 m distance between blocks). Plants were watered thrice a week under drip irrigation, one with NPK (15–15–15 or 0–21–5) fertilizer diluted in water according to the to the needs of the crop (Rincon, 2008). Treatments were aniseed EO and *(E)*-anethole, each at a concentration of 0.2% and 0.3% (v/v). Only Tween80® diluted in water (as same concentration than treatments) was used as a control and pyrethrins as a reference product (Pirecris®) at 0.4% (v/v) were used. Essential oils were formulated at a dose of 2% (v/v) and then diluted to the test concentration. Treatments were sprayed with a backpack sprayer (Super 16, Matabi, Goizper Group, Gipuzkoa, Spain) at a rate of ≈ 200 mL/plant 20 days after planting. The crop was sampled twice a week for monitoring aphid populations. To set up a homogeneous inoculation of the pest, lettuce leaves with aphids (10–20 aphids in each leaf) were distributed all over the field (450 leaves) before the first count, when 50% of lettuce plants sampled had aphids. Aphids per plant were counted in 30 lettuce plants from each experimental plot (120 per treatment) at − 1, + 1, 3, 7 days. Production (size and weight of lettuces) of each treatment was assessed (14 days after treatment). Thirty lettuce plants were sampled from each experimental unit to count the natural enemies of aphids present in the field. Exemplars of natural enemies of aphids were collected for their identification in the laboratory. Syrphid larvae were fed with aphids in laboratory conditions (24 °C and 60% RH.) to rear adults for their identification.

2.6. Statistical analysis

The data from the growth chamber experiment were analyzed using Statgraphics (Centurion 18.1.6.). The normality of the data and homogeneity of variance were assessed with the Shapiro–Wilk and Levene’s tests, respectively. The data were analyzed with by a one-way ANOVA and the means were separated by Fisher’s LSD test with the significance level set as $P \leq 0.05$.

The data from greenhouse and open field experiments were analyzed

with the "R" software, version 4.0.5 (R Core Team, 2021). The data were adjusted to a negative binomial model, using the glm.nb function from the MASS package (Venables and Ripley, 2002), where the number of aphids per plant was the variable response, and the treatment and the block, the factors. The significance of the treatment factor was verified by comparing this model with the restricted model (without the treatment factor), using the anova.negbin function found in the same package. Pairwise comparisons of estimated marginal means among treatments were made for each day using the emmeans package (Length, 2021), fitting the p-values with Tukey's test.

The data of natural enemies from the open field experiment were fitted to a Poisson model, using the glm function from the Stats package (R Core Team, 2021). Multiple comparisons were made between treatments for each experiment date using the emmeans package (Length, 2021), fitting the p-values with Fisher's LSD test.

Efficacy was calculated with respect the control (efficacy means from different blocks) using the Henderson-Tilton (1955) formula:

$$Efficacy \quad (\%) = (1 - \frac{T_a \times C_b}{T_b \times C_a}) \times 100$$

Where tb and ta are the insects in the treated group before (T_b) and after (T_a) the treatment. C_b and C_a are the number of insects in the group control before (C_b) and after (C_a) the treatment.

The Instantaneous population growth rate (ri) was calculated for growth chamber and greenhouse experiments using the formula described in Stark and Banks (2003):

$$ri = \frac{Ln\frac{N_f}{N_o}}{\Delta_T}$$

Where N_f is the number of aphids on each plant treated the day after treatment application, and No is the initial number of aphids in the same plant. Δ_T are the days between both counts. To avoid the error that occurs when Nf = 0 or No= 0 (Ln=+-∞), the 0 has been replaced by 0.5, in a similar way to Berkson's adjustment (Hubert, 1992). Values of ri were analyzed using Statgraphics (Centurion 18.1.6.) and "R" software, version 4.0.5. The normality of the data and homogeneity of variance were assessed with the Shapiro–Wilk and Levene's tests, respectively. The data were analyzed with by a one-way ANOVA and the means were separated by Fisher's LSD test with the significance level set as $P \leq 0.05$. When the data are not normal were analyzed with Kruskal–Wallis test ($P \leq 0.05$) followed by Dunn's pairwise comparisons. Kruskal-Wallis and Dunn tests were carried out using the 'Dunn test' package (Dinno, 2017).

3. Results

3.1. Growth chamber experiment

The initial populations of *N. ribisnigri* were between = 50–70 aphids per plant. The initial population of the control was similar to aniseed EO but greater than in *(E)*-anethole and farnesol ($P = 0.0108$) (Table 1). Farnesol at 0.4% (v/v) decreased the aphid populations in the first day (ri= −0.37) but phytotoxicity was observed in the lettuce plants. Aniseed EO and *(E)*-anethole treatments reduced *N. ribisnigri* populations to a greater degree than in the control ($P < 0.001$) during the experiment. High reductions in the aphid populations of plants treated with aniseed EO (ri= −0.49) or *(E)*-anethole (ri= −0.43) were produced one day after treatment, while growing in the control (ri= 0.38) (Table 1). Aniseed EO and *(E)*-anethole treatments at a dose of 0.4% (v/v) did not produce phytotoxicity on lettuce plants.

3.2. Greenhouse experiments

The products were evaluated in three semi-field experiments under greenhouse conditions. Two experiments were conducted in March and April, 2019 at doses of 0.4% and 0.2% (v/v). In March 2020, the essential oils were evaluated again at 0.4% (v/v) to compare with the results obtained the previous year, in 2019 (Table 2).

3.2.1. Experiment 1 (Year 2019)

The initial aphid populations were homogeneous (*LR. stat* = 3.3, $P = 0.3411$). After one day from the application of the insecticide treatments, a lower number of aphids was counted in the treatment plots as compared with the control, with statistical differences for aniseed EO (*LR. stat* = 28.8, $P < 0.001$). Aniseed EO, or *(E)*-anethole at 0.4% (v/v) decreased the aphid populations after three days (ri= −0.08 and −0.09, respectively). Treatment efficacies of 40% were obtained for aniseed EO and *(E)*-anethole at 0.4% (v/v) three days post-treatment, similar to pyrethrins at 0.2% (Efficacy of 52.9%).

3.2.2. Experiment 2 (Year 2019)

The initial populations of *N. ribisnigri* were homogeneous between treatments (= 44–47 aphids/plant) (*LR. stat* = 0.4, $P = 0.949$).

Table 1
Aphid populations of *Nasonovia ribisnigri* in lettuce plants before and after (in days) spraying with nanoemulsions in a growth chamber.

Product	Aphids per plant[a]					Efficacy (%)[b]				Instantaneous rate of population growth (ri)[c]			
	0	1	2	3	6	1	2	3	6	1	2	3	6
Aniseed EO 0.4%	68.8 ± 5.2a	45.7 ± 6.6b	45.7 ± 6.8b	48.0 ± 8.7b	56.9 ± 8.7b	53.8	64.8	70.2	68.2	-0.49 ± 0.13b	-0.24 ± 0.08c	-0.17 ± 0.08c	-0.05 ± 0.03b
(E)-anethole 0.4%	47.6 ± 3.8c	32.9 ± 4.6b	40.3 ± 2.4b	45.3 ± 4.1b	95.3 ± 12.9b	51.9	55.1	59.4	23.1	-0.43 ± 0.09b	-0.08 ± 0.03 BCE	-0.02 ± 0.04 BCE	0.11 ± 0.03a
Farnesol 0.4%	51.4 ± 4.5 BCE	37.5 ± 4.9b	54.2 ± 5.6b	65.3 ± 8.4b	139.7 ± 17.4a	49.2	44.1	45.8	-4.4	-0.37 ± 0.09b	0.02 ± 0.04b	0.07 ± 0.04b	0.16 ± 0.03a
Control[d]	63.9 ± 5.7ab	91.8 ± 6.1a	120.5 ± 12.3a	149.7 ± 11.9a	166.3 ± 13.5a					0.38 ± 0.05a	0.31 ± 0.08a	0.29 ± 0.04a	0.16 ± 0.03a
F value/P value	4.3/ 0.0108	23.2/ < 0.001	23.8/ < 0.001	32.0/ < 0.001	12.84/ < 0.001					18.7/ < 0.001	14.4/ < 0.001	13.0/ < 0.001	12.0/ < 0.001
P S. Wilk/P Levene[e]	0.159/ 0.248	0.296/ 0.432	0.267/ 0.003	0.062/ 0.105	0.523/ 0.448					0.493/ 0.070	0.455/ 0.130	0.522/ 0.139	0.478/ 0.821

[a] Means (± SE) of aphids/plant followed by different letters within the column indicate significant differences among the treatments (ANOVA followed by Fisher LSD test; $P > 0.05$; *F value d.f.* (3, 36)

[b] Efficacy calculated by means of Henderson and Tilton (1955) formula.

[c] Instantaneous rate of population growth (ri) = (ln Nf/No)/ΔT), where Nf is the number of aphids on each day after treatment, No is the number of aphids on the first day and ΔT are the days among them. Means (± SE) of ri followed by different letters within the column indicate significant differences between the treatments (ANOVA followed by Fisher LSD test; $P > 0.05$; *F value d.f.* (3, 36)

[d] Tween 80 0.8%

[e] Probability of normality (Shapiro-Wilk) and homogeneity of variance (Levene) tests.

Table 2
Aphid populations of Nasonovia ribisnigri in lettuce plants before and after (in days) spraying with nanoemulsions in greenhouse experiments.

		Product	Aphids per plant[a]					Efficacy (%)[b]				Instantaneous rate of population growth (ri)[c]			
			-1	1	2	3	6	1	2	3	6	1	2	3	6
1st year (2019)	Experiment 1	Aniseed EO 0.4%	19.0 ± 1.0a	15.5 ± 0.8c	14.2 ± 0.9b	13.8 ± 0.8 BCE	20.8 ± 1.1b	17.4	27.4	40.1	25.0	-0.11 ± 0.03 BCE	-0.11 ± 0.02b	-0.08 ± 0.01b	0.01 ± 0.01b
		(E)-anethole 0.4%	21.5 ± 1.2a	19.4 ± 1.3ab	14.7 ± 0.9b	16.5 ± 1.1b	25.9 ± 1.4a	14.7	37.6	40.7	21.6	-0.07 ± 0.03b	-0.15 ± 0.02b	-0.09 ± 0.02b	0.03 ± 0.01b
		Pyrethrins 0.2%	20.8 ± 1.2a	15.5 ± 0.9 BCE	13.2 ± 0.9b	12.8 ± 0.8c	18.7 ± 1.2b	36.9	41.6	52.9	38.9	-0.17 ± 0.03c	-0.17 ± 0.02b	-0.13 ± 0.02b	-0.02 ± 0.01c
		Control[d]	21.4 ± 1.3a	22.9 ± 1.3a	24.1 ± 1.3a	27.1 ± 1.6a	31.4 ± 1.6a	–	–	–	–	0.04 ± 0.02a	0.05 ± 0.02a	0.06 ± 0.01a	0.06 ± 0.01a
		Statistic/Pr.[e]	3.3/0.3411	28.8/ < 0.001	55.8/ < 0.001	87.8/ < 0.001	49.6/ < 0.001					30.7/< 0.001	59.3/< 0.001	67.4/ < 0.001	29.9/ < 0.001
	Experiment 2	Aniseed EO 0.2%	44.8 ± 2.6a	29.3 ± 1.8a	43.3 ± 2.5b	39.6 ± 2.5a	42.8 ± 2.6b	16.3	22.7	15.0	25.9	-0.22 ± 0.03a	-0.01 ± 0.02a	-0.04 ± 0.02a	-0.01 ± 0.01b
		(E)-anethole 0.2%	45.6 ± 2.5a	29.8 ± 2.1a	46.3 ± 2.9b	40.7 ± 2.8a	40.7 ± 3.1b	18.1	20.7	16.5	32.2	-0.24 ± 0.03a	0.00 ± 0.03a	-0.04 ± 0.02a	-0.03 ± 0.01b
		Pyrethrins 0.4%	47.5 ± 3.2a	8.3 ± 0.7b	14.6 ± 0.9c	14.6 ± 1.0b	18.3 ± 1.1c	76.4	73.7	68.3	69.5	-0.92 ± 0.05b	-0.39 ± 0.03b	-0.29 ± 0.02b	-0.13 ± 0.01c
		Control[f]	44.5 ± 2.4a	35.2 ± 2.0a	57.1 ± 3.5a	47.0 ± 2.7a	58.2 ± 2.9a	–	–	–	–	-0.11 ± 0.03a	0.08 ± 0.03a	0.02 ± 0.02a	0.04 ± 0.01a
		Statistic/Pr.[e]	0.4/0.949	154.8/ < 0.001	161.8/ < 0.001	120.9/ < 0.001	114.5/ < 0.001					93.2/< 0.001	86.0/< 0.001	81.2/ < 0.001	78.7/ < 0.001
2nd year (2020)	Experiment 3	Aniseed EO 0.4%	13.2 ± 0.9ab	11.4 ± 1.1b	9.5 ± 0.8b	9.1 ± 0.8 BCE	11.2 ± 0.8 BCE	31.8	47.1	47.5	44.1	-0.13 ± 0.05b	-0.13 ± 0.04c	-0.12 ± 0.02c	-0.02 ± 0.01b
		(E)-anethole 0.4%	16.1 ± 1.5a	13.7 ± 1.1b	12.2 ± 1.0b	12.2 ± 1.3b	14.3 ± 1.6b	30.0	42.2	41.8	41.9	-0.10 ± 0.04b	-0.11 ± 0.03 BCE	-0.09 ± 0.02c	-0.04 ± 0.02b
		Farnesol 0.4%	11.0 ± 1.0b	12.6 ± 1.2b	11.0 ± 1.0b	12.4 ± 1.2b	15.2 ± 1.6b	12.0	26.3	17.9	20.01	0.04 ± 0.04a	-0.04 ± 0.03b	-0.01 ± 0.03b	0.02 ± 0.02a
		Pyrethrins 0.4%	14.5 ± 0.9ab	5.0 ± 0.7c	5.2 ± 0.8c	6.5 ± 0.8c	8.8 ± 1.0c	72.4	73.0	67.8	60.5	-0.72 ± 0.08c	-0.49 ± 0.06d	-0.29 ± 0.04d	-0.11 ± 0.02c
		Control[d]	16.4 ± 1.1a	21.0 ± 1.4a	22.6 ± 1.5a	22.1 ± 1.7a	25.3 ± 1.7a	–	–	–	–	0.12 ± 0.03a	0.11 ± 0.02a	0.07 ± 0.02a	0.06 ± 0.01a
		Statistic/Pr.[e]	16.9/0.002	83.5/ < 0.001	90.6/ < 0.001	74.9/ < 0.001	60.0/ < 0.001					92.0/< 0.001	82.5/< 0.001	77.7/ < 0.001	47.4/ < 0.001

[a] Means (± SE) of aphids/plant followed by different letters within the column indicate significant differences among the treatments. Data were analyzed using R software with GLM negative binomial model, calculated with function glm.nb (package MASS), and differences between treatments were separated by Tukey's test of Estimated marginal means (emmeans-package).

[b] Efficacy calculated by means of the Henderson and Tilton (1955) formula.

[c] Instantaneous rate of population growth (ri) = (ln Nf/No)/ΔT), where Nf is the number of aphids on each day after treatment, No is the initial number of aphids and ΔT are the days between them. Means (± SE) of ri followed by different letters within the column indicate significant differences among the treatments (Kruskal Wallis followed by Dunn test; $P < 0.05$; d.f. =3 in 1st year and d.f. =4 in 2nd year).

[d] Tween 80 0.8%

[e] Likelihood ratio statistic (*LR Stat*) and *Chi-square* probability for aphids per plant columns; and Kruskal Wallis statistic (*K*) *Chi-square* probability and probability for ri columns.

[f] Tween 80 0.4%

Pyrethrins were the most effective treatment, reducing the initial population throughout the entire experiment (efficacies between 68.3% and 76.4%), with better results than essential oils treatments. However, aniseed EO or *(E)*-anethole at 0.2% (v/v) also reduced aphid populations with respect to the control (Tween80 0.4%) the day after treatment application (ri= −0.22 and −0.24, respectively) (*K. stat* = 93.2, $P < 0.001$) and stopped aphid development six days after (ri= −0.01 and −0.03, respectively).

3.2.3. Experiment 3 (Year 2020)

The initial aphid populations were homogeneous between pyrethrins, aniseed EO *(E)*-anethole, and the control (= 14–16 aphids/plant), whereas for the farnesol plots, the starting populations were lower in number (*LR. stat* = 16.9, $P = 0.002$). Again, the pyrethrins were the most effective product (efficacies between 60.5% and 73%), but aniseed EO and *(E)*-anethole also caused a significant reduction in the population of aphids one day after the treatment (ri= −0.13 and −0.10, respectively) *K. stat* = 92.0, $P < 0.001$). During the experiment, the aphid populations were lower in the treatments than the control (Tween80 0.8%), almost half (= 11–14 aphids/plant) with respect to the control (25 aphids/plant) on the last day of the experiment (*LR. stat* = 60.0, $P < 0.001$).

Aniseed EO and *(E)*-anethole did not produce phytotoxic effects on the lettuce plants during the experiments. However, when farnesol was sprayed at 0.4% some leaf burns in the plants were observed.

3.3. Open field experiments

The initial populations were homogeneous, except for the control, which had a smaller number of aphids than aniseed EO (*LR. stat* = 13.46, $P = 0.019$). At one day post-treatment, significant reductions were produced by pyrethrins and essential oils than the control (*LR.stat* = 56.54, $P < 0.001$) (Table 3). Whereas no statistical differences were obtained between pyrethrins and *(E)*-anethole at 0.3% after two days, some differences were found with the other treatments (highest number of aphids). After 48 h, all the essential oils treatments reduced the number of aphids in comparison with the control (Tween80 0.6%) (*LR. stat* = 26.70, $P < 0.001$). A similar efficacy was obtained between essential oils (efficacies of = 40–50%), but pyrethrins were the best in reducing the aphid population (efficacy of 58.1%) at two days (Table 3). Yields of 3.28 Kg/m^2 were harvested from the experimental plot, without differences between treatments ($P > 0.7134$). No phytotoxic effects were observed in the crop during the experiment with aniseed EO and *(E)*-anethole treatments at 0.2% and 0.3% (v/v) doses.

The natural enemies of aphids present during the experiment were identified. Three species of syrphids (Diptera: Syrphidae): *Sphaerophoria rueppellii* (Wiedemann), *Sphaerophoria scripta* L., and *Episyrphus balteatus* De Geer; ladybugs *Coccinella sectempuntata* L. (Coleoptera: Coccinellidae), and the mirid *Zelus renardii* Kolenati (Heteroptera: Reduviidae). Also, parasitoids of syrphids from the genera *Diplazon sp.* (Hymenoptera: Ichneumonidae) and *Pachyneuron sp.* (Hymenoptera: Pteromalidae) were identified.

Natural enemies were present on the crop during the experiment (Fig. 1). The number of natural enemies per lettuce was lower in the plants treated with pyrethrins (0 ladybugs and 0.017 syrphids per plant) two days after the treatment (Fig. 1). However, the populations of natural enemies of aphids were higher in the plants treated with essential oils two days post-treatment (0.04–0.07 ladybugs and 0.07–0.12 syrphids per plant) (Fig. 1). Statistical differences were found between the essential oils and the pyrethrins treatments on syrphids populations, two days post treatment (*LR. stat* = 13.73, $P = 0.017$). After seven days, the natural enemies populations were homogeneous between the pyrethrins and aniseed EO treatments for syrphids (*LR. stat* = 7.59, $P = 0.18$).

4. Discussion

Our results confirm that there is a toxic effect of aniseed EO and its main compound *(E)*-anethole against the currant-lettuce aphid, *N. ribisnigri*. The literature reports the repellent and toxic effects of aniseed against different groups of insects (Pascual-Villalobos et al., 2021; López et al., 2008; Park et al., 2006; Spinozzi et al., 2021; Cantó-Tejero et al., 2021). Other authors have also reported the repellent or toxic effect of plant essential oils (where *(E)*-anethole is the main compound) belonging to different botanical families against insects, such as Magnoliaceae (Li et al., 2017; Ho et al., 1995), Lamiaceae (de Paula et al., 2003) or Rutaceae (Guo et al., 2017) among others. According to Cantó-Tejero et al. (2021), the toxic effect caused by aniseed EO is due to *(E)*-anethole, and the results from our greenhouse and field experiments confirm this.

The key factors that determine the suitability of essential oils for biopesticide production are activity against the pest, availability, price, and regulatory approval (Isman, 2020). *(E)*-anethole is one of the main compounds present in the essential oils of aniseed and fennel (*Foeniculum vulgare* Miller) (Pavela, 2018). Anise is one of the most important crops in the production of essential oils due to its wide range of applications in medicine or the food industry (Hashem et al., 2018). In this sense, aniseed EO is a good option, because there is a large-scale market production with an acceptable price (7–9 €/Kg) (Lubbe and Veeporte, 2011). In addition, its use is regulated in Europe and is classified as GRAS (Generally Recognized As Safe) by the USFDA (Food and Drug Administration) and EPA (Environmental Protection Agency) in the United States (Rocha and Fernandes, 2016; Pavoni et al., 2020).

Spraying nanoemulsions of aniseed EO and *(E)*-anethole at 0.4% (v/v) on *N. ribisnigri* populations reduced and stopped colony development for one week. Other works have also studied the effect of *(E)*-anethole on other species of aphids. For instance, Benelli et al. (2018) obtained a

Table 3
Aphid populations of Nasonovia ribisnigri in lettuce plants before and after (in days) spraying with nanoemulsions in an open field experiment.

Product	Aphids per plant[a]					Efficacy (%)[b]		
	-1	1	2	7	14	1	2	7
Aniseed EO 0.2%	9.5 ± 0.7ab	8.9 ± 0.8 BCE	14.9 ± 1.2b	3.5 ± 0.9 BCE	0.02 ± 0.0a	62.6	51.8	17.1
Aniseed EO 0.3%	10.7 ± 0.7a	12.2 ± 1.0ab	15.0 ± 1.3ab	5.0 ± 1.0ab	0.01 ± 0.0a	47.6	52.0	-18.3
(E)-anethole 0.2%	9.0 ± 0.6ab	10.9 ± 1.0b	15.5 ± 1.3ab	2.2 ± 0.6 BCE	0.00 ± 0.0a	44.2	39.8	-8.8
(E)-anethole 0.3%	9.2 ± 0.7ab	9.5 ± 0.9 BCE	10.8 ± 1.2b	2.1 ± 0.6c	0.01 ± 0.0a	49.0	50.5	38.4
Pyrethrins 0.4%	9.6 ± 0.7ab	7.3 ± 0.8c	12.1 ± 1.2b	7.8 ± 1.3a	0.07 ± 0.0a	68.2	58.1	-124.9
Control[c]	7.4 ± 0.5b	18.3 ± 1.3a	23.5 ± 1.6a	3.3 ± 0.8abc	0.01 ± 0.0a			
LR Stat / Pr. (Chi)[d]	13.46/0.019	56.54/< 0.001	26.70/< 0.001	22.35/< 0.001	9.93 < 0.077			

[a] Means (± SE) of aphids/plant followed by different letters within the column indicate significant differences between the treatments. Data were analyzed using R software with GLM negative binomial model, calculated with function glm.nb (package MASS), and differences among treatments were separated by Tukey's test of Estimated marginal means (emmeans-package).

[b] Efficacy calculated by means of the Henderson and Tilton (1955) formula.

[c] Tween 80 0.6%

[d] Likelihood ratio statistic (*LR Stat*) and Chi-square probability.

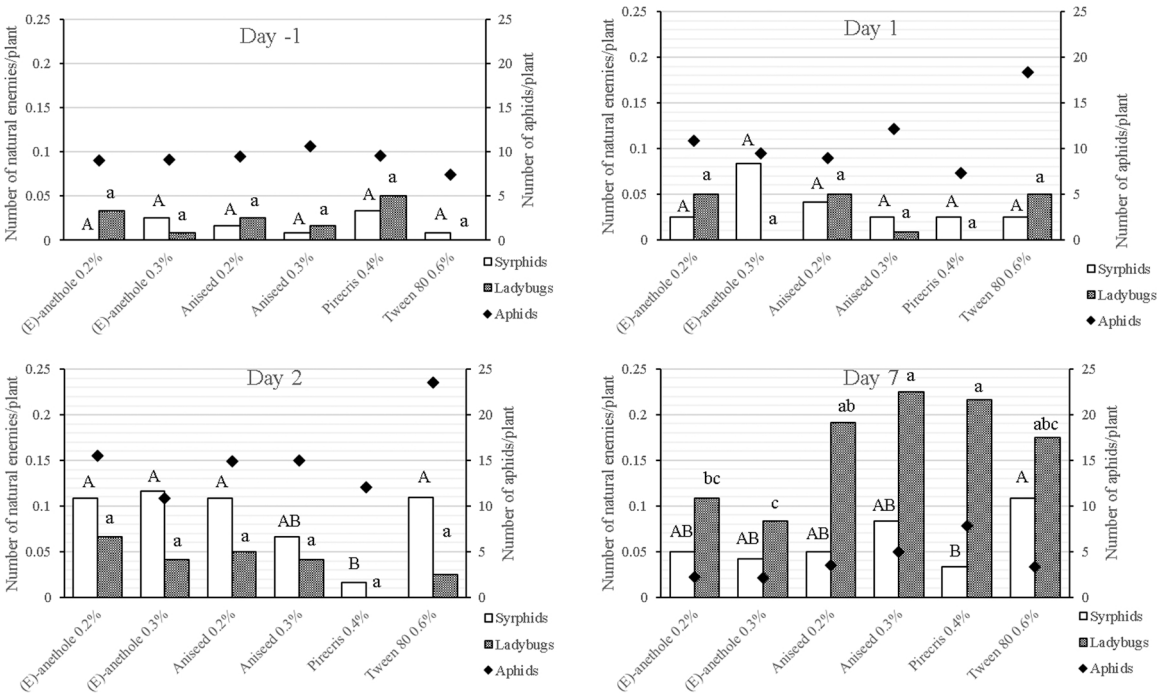

Fig. 1. Natural enemies (syrphids and ladybugs) and aphid populations (insects/plant) in the lettuce crop on different days (before and after treatment) during the field experiment. Letters within the column indicate significant differences among the treatments for syrphids and ladybugs (each one separately). Data were analyzed using R software with the GLM Poisson model, calculated with function glm.nb (package MASS), and differences between treatments were separated by Tukey's test of Estimated marginal means (emmeans-package).

lethal dose 50 and 90 (0.43% and 0.95% (v/v)) of aniseed EO against *M. persicae*. In a similar bioassay, they obtained a LC90 = 0.24% against *M. persicae* by spraying a formulation of fennel (67.9% of *(E)*-anethole) (Pavela, 2018). In our previous work on *M. persicae* and *M. euphorbiae,* we reported the mortality and reduction in the development of aphid populations sprayed with nanoemulsions of *(E)*-anethole at 0.2% (v/v) (Cantó-Tejero et al., 2021).

Chemical treatments are usually carried out preventively on lettuce crops, since aphid spots cannot be detected in large areas of monocultures (Barrière et al., 2014.). Morales et al. (2013) established economic damage thresholds for *N. ribisnigri* of 0.06–0.13 aphids or over per plant. Plants must be treated before forming the head of lettuce to avoid cosmetic damage in the crop. The control exerted by predators (syrphids and ladybugs) over aphids in the crop can be useful if it is combined with insecticide treatments when needed (if products are compatible). The presence of natural enemies in the early stages of lettuce crops when the pest appears can help reduce the treatments needed to manage the aphids. Fagan et al. (2010) reduced the number of treatments with the insecticide imidacloprid to control *N. ribisnigri*, combining its use with the action of natural enemies that appeared in the crop. However, neonicotinoid insecticides such as imidacloprid are known to be toxic against syrphids, ladybugs, and bees (Jansen, 1998; Youn et al., 2003; Cressey, 2017). Other authors reported that the presence of hoverflies in the crop was common, favouring their appearance using reservoir plants such as coriander (*Coriandrum sativum* L.), chrysanthemum (*Chrysanthemum coronarium* L.) or sweet alyssum (*Lobularia maritima* L.) (Pascual-Villalobos et al., 2004, 2006; Gillespie et al., 2011). However, Pascual-Villalobos et al. (2006) indicated that the installation of natural enemies in the crop was conditioned by a previous establishment of aphid populations. Only the larval stages of hoverflies act as aphids' predators (Amorós-Jiménez et al., 2015), and due to this, their settlement in the crop is necessary to develop their biological cycle and to therefore exert aphid control.

During our field experiment, the aniseed EO and *(E)*-anethole treatments proved to be compatible with hoverflies and ladybugs. After spraying the nanoformulations, the number of natural enemies was maintained and even increased. On the other hand, pyrethrins were toxic to syrphids. Cantó-Tejero et al. (2021) reported that larvae of the hoverfly *Sphaerophoria rueppellii* (Wiedemann) (Diptera: Syrphidae) exposed to nanoemulsions of (*E*)-anethole 0.3% (v/v) were not damaged (less than 10%). In other studies, it was reported that the topical application of fennel EO (67.9% of (*E*)-anethole) at 0.37% (v/v) was not toxic against the ladybug *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) (Pavela, 2018).

5. Conclusions

The results obtained from this work are promising. The nanoemulsion of aniseed essential oil at a concentration of 0.4% was toxic to *N. ribisnigri* and compatible with their natural enemies (hoverflies and ladybugs). In the future, its use could be combined with other tools to manage aphids (natural enemies or cultivars resistant to aphids).

However, more research is necessary to improve formulations so that they are more efficient and to enhance the insecticidal properties of aniseed essential oil.

CRediT authorship contribution statement

M. Cantó-Tejero: Conceptualization, Methodology, Experiments, Writing – original draft. **M. J. Pascual-Villalobos:** Conceptualization, Methodology, analyzed the data. **P. Guirao:** Conceptualization, Methodology, analyzed the data. All authors contributed critically to the drafts and gave final approval for publication.

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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