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Toxicity of *Ocimum basilicum* essential oil combined with thiamethoxam to cotton aphid

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ABSTRACT: *Aphis gossypii* Glöver is one of the main cotton insect pests, causing significant losses in the crop. In order to reduce these damages, frequent applications of insecticides are carried out, despite reported cases of resistance. One auxiliary tool of resistance management is the combination of natural and synthetic products because the combinations can deliver comparable or greater pest toxicity while reducing the necessary dose of the synthetic molecules, thus maintaining its effectiveness. Therefore, the present work evaluated the toxicity of *Ocimum basilicum* essential oil, the synthetic insecticide thiamethoxam and the combination of these in the control of cotton aphid. The acute toxicities of thiamethoxam and the essential oil were obtained through dose response assays; then, combinations of the two products were assessed. The essential oil had a high concentration of linalool and it exhibited toxicity to the cotton aphid. The combination of the two products caused high mortality, particularly when they were applied as the LD_{50} of the thiamethoxam + LD_{50} of the essential oil and the 50% LD_{50} of the thiamethoxam + 50% LD_{50} of the essential oil. This work concludes that *O. basilicum* L. essential oil combined with thiamethoxam may be an alternative in the population management of the cotton aphid and may contribute in the future to the management of resistance and reduce the environmental impact of pesticides.

Key words: *Aphis gossypii*; insecticide resistance management; linalool; neonicotinoids; synergism

Toxicidade do óleo essencial de *Ocimum basilicum* combinado ao inseticida tiametoxam para o pulgão-do-algodoeiro

RESUMO: *Aphis gossypii* Glöver é um dos principais insetos-pragas do algodoeiro, causando perdas significativas na cultura. Para reduzir tais perdas, são realizadas aplicações frequentes de inseticidas, apesar do relato de casos de resistência. Uma ferramenta auxiliar no manejo da resistência de pragas a inseticidas é a combinação de produtos naturais e sintéticos, já que tais combinações podem causar toxicidade semelhante ou maior às pragas, reduzindo a dose necessária dos inseticidas, e ainda mantendo a sua eficácia. Portanto, o presente trabalho avaliou a toxicidade do óleo essencial de *Ocimum basilicum*, do inseticida sintético tiametoxam e da combinação destes no controle do pulgão-do-algodoeiro. As toxicidades dos produtos foram obtidas por ensaios do tipo dose-reposta. O óleo essencial apresentou alta concentração de linalol e exibiu toxicidade para o pulgão. A combinação entre os produtos naturais e sintéticos causou alta mortalidade quando foram combinadas DL_{50} tiametoxam + DL_{50} óleo essencial e 50% da DL_{50} de tiametoxam + 50% DL_{50} do óleo essencial. Concluiu-se que o óleo essencial combinado com tiametoxam pode ser uma alternativa no manejo populacional do pulgão-do-algodoeiro, podendo contribuir futuramente para o manejo da resistência, e ainda, reduzir o impacto ambiental do uso exclusivo dos pesticidas.

Palavras-chave: *Aphis gossypii*; manejo de resistência a inseticidas; linalol; neonicotinóides; sinergismo

Introduction

Aphis gossypii Glöver (Hemiptera: Aphididae) is the main insect pest that causes economic losses to agricultural crops, such as cotton, cucurbitaceous, citrus, and solanaceous crops, under tropical conditions (De Little et al., 2017). Because it is a polyphagous species with a high reproductive potential and capacity for infestation (Kataria & Kumar, 2012), its control has faced several difficulties. One difficulty is the need for a constant application of synthetic insecticides (Herron & Wilson, 2017).

Damage can occur when aphids remove plant photoassimilates and subsequently inject toxins into the plant foliar tissue, which causes leaf wrinkling and the deformation of shoots and leads to the temporary inhibition of plant growth (Goggin et al., 2017). This pest also secretes honey dew (a sugar-rich substance) on the leaf surface, which supports the development of the fungi *Capnodium* spp., thus reducing the photosynthetically active area of the plant (Ambarish et al., 2017; Stevens et al., 2017). These direct damages can be further aggravated by indirect damages due to the transmission of viruses, leading to the reduction of the yield of the crops (Stevens & Lacomme, 2017).

A. gossypii is not affected by Bt transgenic plants, thus the application of neonicotinoids has been the first-line option for aphid management adopted by crop growers (Funichello et al., 2012). Consequently, one problem with the application of neonicotinoids is that cotton aphid populations, constantly exposed to these insecticides, have become resistant to these synthetic molecules (Matsuura & Nakamura, 2014).

This highlights an urgent need to reduce the selection pressure of synthetic products by using alternative insecticides, like plant-based products, in order to reduce the development of pest resistance. Natural products have been tested in different parts of the world in isolated use and in combination with synthetic molecules in the same formulation (Herron & Wilson, 2011; Silva et al., 2016), due the advantages that it can promotes at synergic combination.

It is known that combinations between different products can promote greater toxicity to insects, since a compound can potentiate the action of another active ingredient by weakening the insect detoxification system, acting on the inhibition of P450 enzymes and esterases (Khan et al., 2013; Fazolin et al., 2016). This method may allow the reduction of insecticide doses, while maintaining toxicity to pests. In the case of *A. gossypii*, it may increase the toxicity of the insecticide to the pest, because the natural compound presents a synergistic effect with the synthetic insecticide (Khan et al., 2013), potentiating the synthetic insecticide.

Naturally, essential oils (terpenoids) play an important role in the indirect plant defence against pest attacks, and these compounds have presented different levels of toxicity to various pests (Lima et al., 2013). Some studies have shown that these compounds inhibit cellular respiration enzymes, nervous system enzymes such as acetylcholinesterase (AChE), and enzymes of the detoxification system such as P450 and esterase (Fazolin et al., 2016), thus weakening insecticide metabolism in insects.

Linalool is an example of a terpenoid that jointly operates with other compounds in the cholinergic system of insects, promoting the rapid breakdown of the nervous system. As one of the major natural sources of this terpenoid, the essential oil of *Ocimum basilicum* L. (Lamiaceae) varieties is, on average, 80% linalool.

Another point that should be considered is that insecticide resistance develops more slowly when essential oil-based products are used, due to the diversity of active compounds that characterize the products (Bailen et al., 2013).

Moreover, one advantage using combined products is to preserve and maintain the effectiveness of insecticide molecules, which have been harshly developed by the chemical industry and are about to lose their agricultural applicability, associating them with natural products, which can act as effective synergists (Radhika & Sahayaraj, 2014).

Although essential oils and neonicotinoids present different modes of action, their combination has already demonstrated efficacy to control other aphids (Faraone et al., 2015). However, more studies are needed to demonstrate the effectiveness of these combinations against the cotton aphid. Therefore, this work aimed to evaluate the toxicity of *O. basilicum* essential oil alone and in combination with thiamethoxam to improve the management of *A. gossypii*.

Material and Methods

Production of *Ocimum basilicum* L. and essential oil

The variety of *O. basilicum*, chemotype linalool, was obtained from accession PI 197442, originating from the Germplasm Bank of the North Central Regional PI Station, Iowa State University, United States of America. Seeds were obtained from the aromatic plant breeding programme of the Federal University of Sergipe and cultivated during the spring of 2016 at the Gloria Experimental Farm of Federal University of Uberlândia, at Uberlândia City, Brazil. In full bloom, fresh leaves were harvested for essential oil extraction using a Clevenger-type hydrodistillation apparatus (Blank et al., 2007).

Chemical characterization of essential oil

Chemical analyses of the essential oil were performed using a gas chromatograph coupled with a mass spectrometer (Shimadzu GC-2010 + QP-5000) and equipped with a DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25 µm). The operation mode was as follows: the carrier gas was helium at 1.7 mL min^{-1}, the injector temperature was 240 °C, the detector temperature was 230 °C, and a programmed increase in temperature from 60 to 240 °C with at 3 °C min^{-1}. Half of the flow was split, and the flow rate was 1 mL min^{-1}. The identification of compounds was performed by comparing their mass spectra with system databases and literature and by determining the Kovats retention indices and comparing them with the literature (Adams, 2007).

The quantification of the compounds was performed using a gas chromatograph coupled with a flame ionization detector (Shimadzu GC-2010/FID) and a DB5 capillary column. The carrier gas was helium with a flow rate of 1.0 mL min^{-1} and a split ratio of 1/20, the injector temperature was set to 240 °C, the detector temperature was set to 230 °C, and the temperature was ramped from 60 to 165 °C at 4 °C min^{-1} and from 165 °C to °C at 10 °C min^{-1}.

Cotton aphid collection and rearing

Initially, *A. gossypii* was collected in four distinct places in the agricultural region of Uberlandia City, Minas Gerais State, Brazil. The cotton aphid population was kept *in vitro* at laboratory conditions inside Petri dishes that contained cotton leaves in a 1% water/agar layer. For this, the leaves were immersed in a 1% sodium hypochlorite solution for 10 min and then soaked in distilled water for 15 min. Subsequently, the leaves were placed in a water/agar layer.

Cotton leaves were obtained from nontransgenic cotton plants (variety Delta Pine 555, Monsanto Corporation) cultivated in greenhouse conditions. Seeds were provided by the cotton breeding programme of the Federal University of Uberlândia (UFU). In addition, the plants were cultivated under the protection of a cage with anti-aphid material.

Toxicological evaluations

Dose-response assays were performed with the essential oil from the *O. basilicum* chemotype linalool and the technical product thiamethoxam (99.6%, Pestanal, Sigma-Aldrich). The tests were performed on cotton leaf discs of 90 mm in diameter inserted in Petri dishes of 100 mm in diameter on a 1% agar/water layer. The plates were maintained in a climatic chamber (23 ± 1 °C temperature, 70 ± 10% relative humidity and 12 h photophase). Ten adult aphids were placed in each plate. Thiamethoxam was chosen for toxicity assessment because of its great agricultural use in order to control this pest.

Solutions for the determination of lethal doses were prepared in acetone. For this, a range of dilutions of products were prepared, ranging from 0 to 50 ng $insect^{-1}$ for thiamethoxam and 0 to 60 nL $insect^{-1}$ for the essential oil. The applications were done using a microsyringe coupled to an automatic dispenser that released 0.2 µL of solution on each individual aphid. This volume was chosen based on the mean body mass of an adult aphid (0.4807 mg), as obtained from the mass evaluation of 350 adult aphids that were weighed in 10 groups of 35 individuals on a precision scale with five decimal places.

For each concentration evaluated, 4 replicates of 10 aphids were used. The evaluations were performed every 24 h after the treatments, and the number of dead aphids was counted. A negative control treatment that used the application of only acetone was also applied.

Assessment of the combinations of thiamethoxam and essential oil

This assessment studied the effects of combinations of thiamethoxam (th) and essential oil (eo) on aphids, using previously measured lethal dose (LD_{50}) values as references. Pairwise comparisons were performed using seven random mixtures with four replications: (1) LD_{50} th + LD_{50} eo; (2) 50% LD_{50} th + 50% LD_{50} eo; (3) 25% LD_{50} th + 25% LD_{50} eo; (4) 25% LD_{50} th + LD_{50} eo; (5) LD_{50} th + 25% LD_{50} eo; (6) 25% LD_{50} th + 75% LD_{50} eo. All procedures during the bioassays were carried out as previously described. The number of dead aphids was counted at 24 h after the treatments, but it was counted death individuals until 48 h, when was observed no deaths.

Data analysis

The mortality data were subjected to the dose-response analysis using the Probit tool of the software SPSS 20. Based on the mathematical model adjusted for the observed data, the lethal dose (LD_{50}) values, and the confidence interval, the chi-square and degrees of freedom were determined. The data of effectiveness of control of the combinations between the products were first submitted to a Shapiro-Wilk test of normality of distribution of the residuals and a Levene test of homogeneity of the variances using 0.01 as the significance value. Then, the F-test was performed for analysis of variance, and comparison of the means was carried out with the Scott-Knott test at a 0.05 significance threshold with the statistical program SPSS 20.

Results and Discussion

Chemical composition of *Ocimum basilicum* L. essential oil

A total of 19 compounds were identified in the essential oil of *O. basilicum* (Table 1 and Figure 1). The major compounds were linalool, 1,8-cineole and geraniol, which composed 95% of the total oil composition. The essential oil content and yield were 2.34% and 13.57 g $plant^{-1}$, respectively. Similar results were also obtained by Govindarajan et al. (2013), with linalool as the major compound.

Acute toxicity to cotton aphids

The topical application of the thiamethoxam and essential oil caused acute toxicity to *A. gossypii* (Figures 2 and 3). The LD_{50} values, including the obtained parameters, are shown in Table 2. No mortality was observed in the control treatment.

After applying different doses of essential oil, a stationary behaviour of the aphids was observed, characterized by loss of motor coordination, reduced feeding, starvation and death. Similar behaviours were observed after the application of thiamethoxam, with mortality recorded up to 48 h. The LD_{50} value for the essential oil was on the order of 5 times higher than the thiamethoxam value. Even so, essential oil proved to be a very promising toxin, especially at higher concentrations.

These results agree with Faraone et al. (2015), who studied the toxicity of *Lavandula angustifolia* Mill. (Lamiaceae) essential oil, which is 54% linalool and 38.56% linaline acetate. They found a lethal concentration (LC_{50}) of 5.06 ppm for *Myzus persicae* Sulzer (Hemipetra: Aphididae). For the same pest, an LC_{50} of 5.27 ppm was measured using *Thymus vulgaris* L. (Lamiaceae) essential oil, which is 72% thymol. In that same study, imidacloprid presented an LC_{50} of 0.79 ppm.

Table 1. Chemical composition of *Ocimum basilicum* essential oil as determined by gas chromatography.

Peak	RT[1]	ICR[2]	IRL[3]	Compound	% Area	% GC-FID[4]
1	8.809	923	932	α-pinene	0.01	0.15
2	10.029	963	969	sabinene	0.11	0.12
3	10.156	967	974	β-pinene	0.53	0.49
4	11.895	1021	1026	1,8-cineole	6.02	5.00
5	14.214	1091	1095	linalool	77.34	79.29
6	17.095	1181	1186	α-terpineol	0.48	0.41
7	18.587	1229	1235	neral	0.12	0.11
8	18.950	1241	1249	geraniol	9.86	9.05
9	19.447	1258	1264	geranial	0.16	0.87
10	19.982	1275	1287	bornyl acetate	0.24	0.53
11	22.700	1369	1359	neryl acetate	0.04	0.10
12	23.091	1382	1389	β-elemene	0.30	0.30
13	24.000	1415	1417	caryophyllene	0.20	0.18
14	24.260	1424	1432	α-(E)-bergamotene	1.91	1.46
15	24.413	1430	1437	α-guaiene	0.12	0.10
16	25.660	1476	1484	germacrene D	0.43	0.36
17	26.272	1498	1509	α-bulnesene	0.12	0.10
18	26.495	1507	1513	γ-cadinene	0.58	0.33
19	29.729	1636	1638	epi-a-cadinol	1.43	1.06

[1]Retention Time; [2]Index of Calculated Retention; [3]Index of Literature Retention; [4]Gas Chromatography - flame ionization detector.

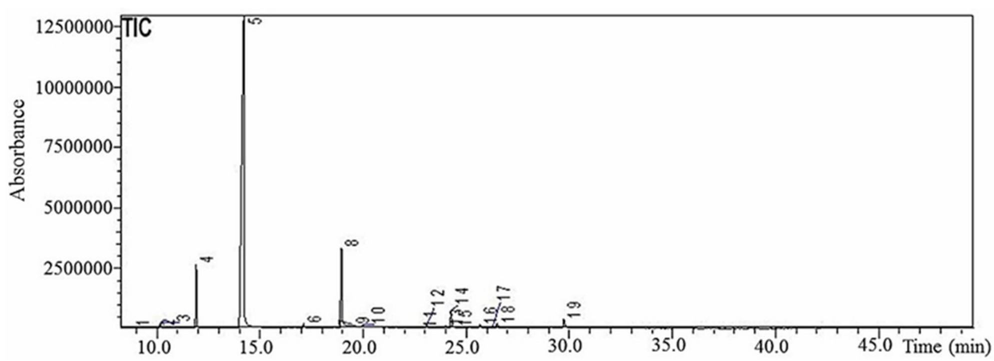

Figure 1. Major compounds in *Ocimum basilicum* essential oil as obtained by gas chromatography and mass spectrometry. The compounds 1,8-cineole, linalool and geraniol are represented by peaks 4, 5 and 8, respectively.

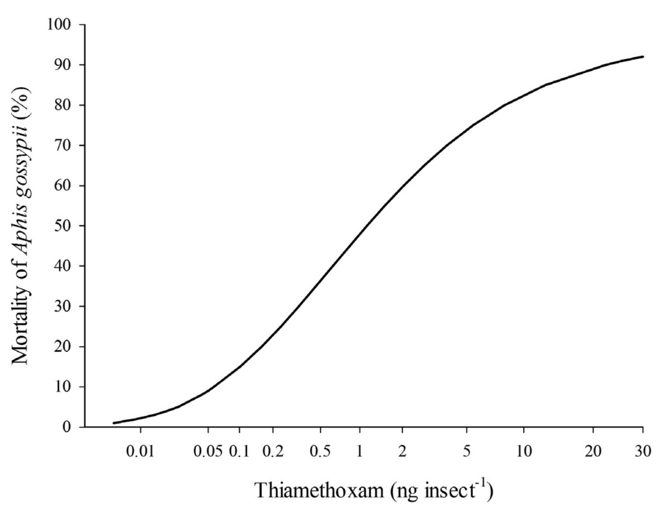

Figure 2. Mortality (%) of *Aphis gossypii* 24 h after intoxication with different doses of thiamethoxam (ng insect^{-1}).

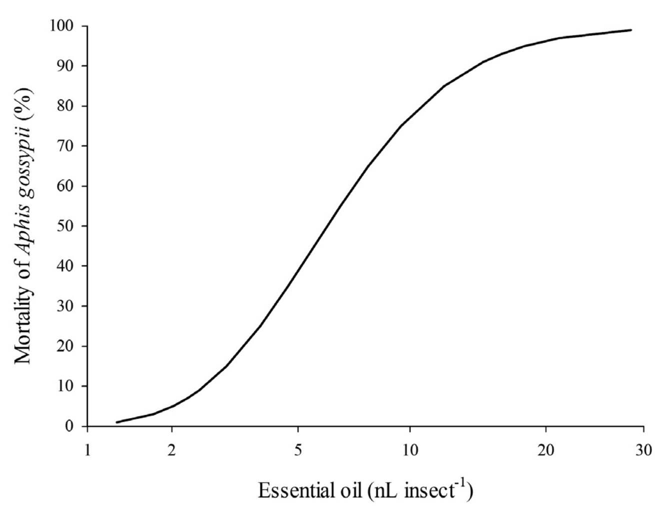

Figure 3. Mortality (%) of *Aphis gossypii* 24 h after intoxication with different doses of de *Ocimum basilicum* essential oil (nL insect^{-1}).

Ebrahimi et al. (2013) found LC_{50} values of 1.96, 2.28 and 31.6 µL L^{-1} to *Azadirachta indica* A. Juss. (Meliaceae), *Eucalyptus camaldulensis* Dehnh. (Myrtaceae) and *Laurus nobilis* L. (Lauraceae) essential oils to cotton aphid nymphs, respectively.

Bagavan et al. (2009) also observed high LC_{50} values for cotton aphids, 80.99 and 130.19 ppm, using plant extracts of *Ocimum canum* L. (Lamiaceae) and *Ocimum sanctum* L. (Lamiaceae), respectively. These results indicate that

Table 2. Summary of the parameters obtained during the acute toxicity assays of *Ocimum basilicum* essential oil and thiamethoxam against A*phis gossypii*.

Product	LD_{50}[a]	C.I. 95%[b]	*D.F.*[c]	χ^2 [d]
Essential oil	6.05 nL $insect^{-1}$	4.387 – 8.239	8	22.061
Thiamethoxan	1.22 ng $insect^{-1}$	0.248 – 2.290	5	27.079

a. Lethal dose; b.Confidence interval; c. Degrees of freedom; d. Chi-square.

LD_{50} values can vary according to the plant species, the concentration and amount of compounds in the oil, and the insecticide metabolism capacity of the pests in question.

Abdel-Aziz et al. (2015) investigated the effect of *Rosmarinus officinalis* L. (Lamiaceae), *Salvia officinalis* L. (Lamiaceae) and *Curcuma longa* L. (Zingiberaceae) essential oils against *Aphis craccivora* Koch (Hemipetra: Aphididae). They found above 93% and up to 100% of mortality with only 0.5% of the essential oils mentioned. The lowest LC_{50}, 0.0069 ppm, was from the *R. officinalis* essential oil. Significant reductions in the activities of the enzymes acetylcholinesterase (AChE) and glutathione-S-transferase (GST) were observed in the aphids that received the oil applications, a result that shows high inhibition of these enzymes relative to the control. Possibly, this result was caused by the presence of 1,8-cineole in high concentrations (42%) in the essential oil that was tested.

AChE is a key enzyme that terminates nerve impulses by catalysing acetylcholine hydrolysis in the nervous system. GST is a detoxification enzyme that acts on an important route for insect survival after exposure to endogenous or exogenous xenobiotics. The inhibition of these key enzymes demonstrates the potential of essential oils to facilitate the performance of other insecticides in pest management. These results also agree with other studies that have demonstrated a reduction of enzymatic activity after exposing different pests to several essential oils (Liu et al., 2014; Waliwitiya et al., 2012).

In the present study, linalool was the most abundant compound in the *O. basilicum* essential oil. For Praveena & Sanjayan (2011), linalool formed a stable intermolecular complex with AChE, inhibiting its interaction with acetylcholine. This may indicate that high concentrations of linalool in the applied oil prevent the full functioning of nerve impulse transmission in *A. gossypii*, causing slowed movement and subsequent death in this aphid.

Another hypothesis further suggests that the secondary constituents of essential oils, despite their lower concentrations, were important in helping with synergism, since plants defend against insect attacks using a set of compounds rather than isolated compounds. These secondary constituents can be synergists by increasing the efficiency of the major constituents and also by acting on similar metabolic routes.

Essential oil combined with thiamethoxam

The combination of essential oil and thiamethoxam presented high toxicity to cotton aphids; at the 24 h and 48 h post-treatment evaluations, the LD_{50} th + LD_{50} eo and 50% LD_{50} th + 50% LD_{50} eo treatments produced the highest aphid mortality rates (Table 3). In addition, it was possible to achieve insect control even when reducing the dose of the synthetic insecticide by 50%.

The other tested combinations did not differ, but all produced mortality levels above 50% up to 48 h after the applications. The obtained results agree with Isman et al. (2011), who evidence that mixtures of essential oils and conventional insecticides efficiently control pests such as thrips and aphids. Other studies have also confirmed that a mixture of neonicotinoids and essential oils are effective against aphids of agricultural importance (Işik & Görür, 2009).

Faraone et al. (2015) reduced the dose of imidacloprid required to manage the population of *M. persicae* (LC_{50} of 0.79 ppm) 15.8 times in the presence of *L. angustifolia* essential oil (LC_{50} of 0.05 ppm), which has a high concentration of linalool and linaline acetate, and 19.8 times in the presence of *T. vulgaris* essential oil (LC_{50} of 0.04 ppm), which has a high amount of thymol. They tested the toxicity of the same neonicotinoid mixture with the major essential oil compounds, but the results were lower than those obtained with the essential oils, having LC_{50} values of 0.28 ppm for linalool and 0.25 ppm for thymol. These results confirm a good synergistic relationship between essential oil compounds and neonicotinoids.

The combinations between natural products and neonicotinoids showed high and moderate toxicities and were effective and compatible mixtures against the aphids.

Table 3. Mortality (%) of A*phis gossypii* due to the combined use of different doses of thiamethoxam (th) and *Ocimum basilicum* L. essential oil (eo).

Combinations	Thiamethoxam (ng $insect^{-1}$)	Essential oil (nL $insect^{-1}$)	Mortality (%)	
			24 h	48 h
LD_{50} th + LD_{50} eo	1.2200	6.0500	90.0 a[(1)]	100.0 a
50% LD_{50} th + 50% LD_{50} eo	0.6100	3.0250	82.5 a	95.0 a
25% LD_{50} th + 25% LD_{50} eo	0.3050	1.5125	52.5 b	72.5 b
25% LD_{50} th + LD_{50} eo	0.3050	6.0500	47.5 b	67.5 b
LD_{50} th + 25% LD_{50} eo	1.2200	1.5125	45.0 b	65.0 b
25% LD_{50} th + 75% LD_{50} eo	0.3050	4.5375	45.0 b	62.5 b
Control (Acetone)	-	-	0	0

[(1)] Means followed by letters in the columns differ based on the Scott-Knott test at the 0.05 significance level; Fc_{24h} = 0.0001; $Fc_{48\,h}$ = 0.0001; $VC_{24\,h}$ = 25.10%; $VC_{48\,h}$ = 15.97%.

In this case, aphids were subjected to the combination of two products that attack different target sites; they were thiamethoxam, an agonist of nicotinic acetylcholine receptors in the postsynaptic cleft, and the essential oil, which inhibits acetylcholinesterase.

When examining the joint action of insecticides, true synergism in a mixture of two or more insecticidal compounds is rarely found. When synergism happens, it provides a new tool with great potential for *A. gossypii* resistance management programmes and is a safer product for the environment.

Finally, tests in field conditions that focus on such combinations are crucial to elucidate their biological efficacy and to complement and emphasize the first results that were obtained.

Conclusions

O. basilicum essential oil presents relevant toxicity to *A. gossypii* at 6.05 nL insect^{-1}. In laboratory was possible to control cotton aphid population while reducing the thiamethoxam dose more than 50% in the presence of natural compound.

The combination of natural and synthetic products is an important tool to be developed and used in integrated pest management, specifically to insecticide resistance management. Furthermore, it may reduce the environmental impact of pesticide use.

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