D-limonene combined with *Bacillus thuringiensis* against *Spodotera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae)

D-limonene combinando com *Bacillus thuringiensis* no manejo de *Spodotera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae)

D-limonene combined with *Bacillus thuringiensis* in the management of *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae)

DOI: 10.34188/bjaerv7n2-014

Submetido: 19/01/2024
Aprovado: 01/03/2024

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**ABSTRACT**

Focusing on alternatives for better control of *S. frugiperda*, research has proven the efficiency of using natural compounds to reduce the emergence of resistance. Consequently, the combination of different modes of action of natural insecticide, capable of enhancing the action of each molecule, promoting a barrier to the pest resistance. So, this work evaluated the toxicity of d-limonene associated with entomopathogenic microorganisms against *S. frugiperda*. A population of caterpillars was assembled in the laboratory to conduct in vitro toxicity assays. A range of insecticide dilutions was carried out and then applied to larvae, with the aim of obtaining the dose-
response curve. After determining the lethality of each product and aiming to determine the synergistic potential between them, several binary combinations were established. D-limonene essential oil promoted high mortality of caterpillar at very low concentrations, and the subspecies of bacilli used ensured high caterpillar mortality. However, the strain of *B. bassiana* used promoted caterpillar mortality only at a very high dose. *B. bassiana* + d-limonene presents synergistic properties with very high toxicity to *S. frugiperda*. *B. thuringiensis* + d-limonene, although resulting in toxicity for larvae, does not appear to be synergistic.

**Keywords:** fall armyworm, terpenoids, *Bacillus thuringiensis*, *Beauveria bassiana*, synergism, resistance management.

**RESUMO**
Em busca de alternativas para melhor controle de *S. frugiperda*, pesquisas comprovaram a eficiência do uso de compostos naturais para reduzir o surgimento de resistência de insetos praga. Consequentemente, a combinação de diferentes modos de ação de produtos naturais, capaz de potencializar a ação de cada molécula, promove uma barreira à resistência da praga. Assim, este trabalho avaliou a toxicidade do d-limonoeno associado a microrganismos entomopatogênicos contra *S. frugiperda*. Uma população de lagartas foi montada em laboratório para realização de ensaios de toxicidade in vitro. Foram realizadas diversas diluições do inseticida e, posteriormente, aplicadas nas larvas, com o objetivo de obter a curva dose-resposta. Após determinar a letalidade de cada produto e visando determinar o potencial sinérgico entre eles, foram estabelecidas diversas combinações binárias. O óleo essencial de d-limonoeno promoveu alta mortalidade de lagartas em concentrações muito baixas, e as subespécies de bacilos utilizadas garantiram alta mortalidade de lagartas. No entanto, a cepa de *B. bassiana* utilizada promoveu mortalidade de lagartas apenas em doses muito elevadas. *B. bassiana* associado ao d-limonoeno apresentou propriedades sinérgicas com altíssima toxicidade para *S. frugiperda*. E *B. thuringiensis* associado ao d-limonoeno, embora resulte em toxicidade para larvas, não parece ser sinérgico.

**Palavras-chave:** lagarta do cartucho, terpenoides, *Bacillus thuringiensis*, *Beauveria bassiana*, sinergismo, manejo da resistência

**RESUMEN**
Centrándose en las alternativas para un mejor control de *S. frugiperda*, la investigación ha demostrado la eficacia del uso de compuestos naturales para reducir la aparición de resistencias. En consecuencia, la combinación de diferentes modos de acción de insecticidas naturales, capaz de mejorar la acción de cada molécula, la promoción de una barrera a la resistencia de la plaga. Así, en este trabajo se evaluó la toxicidad del d-limonoeno asociado a microorganismos entomopatógenos frente a *S. frugiperda*. Se reunió una población de orugas en el laboratorio para realizar ensayos de toxicidad in vitro. Se realizó una serie de diluciones del insecticida y se aplicó a las larvas, con el fin de obtener la curva dosis-respuesta. Tras determinar la letalidad de cada producto y con el objetivo de determinar el potencial sinérgico entre ellos, se establecieron varias combinaciones binarias. El aceite esencial de D-limonoeno promovió una elevada mortalidad de la oruga a concentraciones muy bajas, y la subespecie de bacilos utilizada garantizó una elevada mortalidad de la oruga. Sin embargo, la cepa de *B. bassiana* utilizada sólo favorecía la mortalidad de las orugas a dosis muy elevadas. *B. bassiana* + d-limonoeno presenta propiedades sinérgicas con una toxicidad muy elevada para *S. frugiperda*. *B. thuringiensis* + d-limonoeno, aunque produce toxicidad para las larvas, no parece ser sinérgico.

**Palabras clave:** gusano cogollero, terpenoides, *Bacillus thuringiensis*, *Beauveria bassiana*, sinergismo, gestión de la resistencia.
1 INTRODUCTION

The corn crop is the main cereal produced in Brazil, but several factors can compromise its productivity, being the fall armyworm, *Spodoptera frugiperda* (J. E. Smith, 1797), one of the most limiting biological factors.

*S. frugiperda* presents a biological cycle during around 35 days, at an average temperature of 25°C (Santos, 2012). Females lay, approximately, 800 eggs, laid in gray layers, protected by scales left by the females. After hatching, the larval period ranges from 15 to 25 days, with 4 to 7 instars depending on aspects such as temperature, host plant, sex, and biotype. The adult moth can have a wingspan of 35 mm and has a longevity of up to 15 days (Omoto *et al.*, 2013).

For this pest control, the exclusive application of chemical control has encountered difficulties due to the rapid emergence of resistance to conventional insecticides. In Brazil, resistance of *S. frugiperda* has been reported to conventional insecticides such as pyrethroids (Diez-Rodriguez & Omoto 2001; Carvalho *et al.*, 2013), organophosphates (Carvalho *et al.*, 2013), benzoylureas (Nascimento *et al.*, 2016), spinosyns (Okuma *et al.*, 2018), diamides (Bolzan *et al.*, 2019) and Bt proteins (Omoto *et al.* 2013; Farias *et al.* 2014).

In contrast, the application of entomopathogenic microorganisms has grown in recent years, demonstrating good results and viability in controlling caterpillar populations, as *S. frugiperda* larvae are susceptible to these microorganisms, and are also ecologically safer for fauna (Shahzad *et al.*, 2021). Entomopathogenic fungi, such as *Beauveria bassiana*, can cause mortality in all stages of insects, and especially in the youngest stages, as they can adjust to external habitats, different from their origin, making them very efficient and suitable candidates for biological control (Bamisile *et al.*, 2019).

Added to this, the search for more alternatives to chemical control has increased throughout the world, one of which is the application of essential oils (terpenoids, such as limonene). Such oils can present acute neurotoxicity to insects (lepidoptera and others) and cause high mortality (Silva *et al.*, 2017; Marques *et al.*, 2019). These are products with a high potential for toxic interference in basic biochemical processes, with irreversible physiological and behavioral consequences in insect organism (Monteiro *et al.*, 2021).

More recently, synergism, that is, the combination of insecticides with different modes of action, has emerged as a great tool for Integrated Pest Management, guaranteeing greater success in control, by enhancing the action of each molecule, consequently promoting a greatest barrier to the development of pest resistance (Khann *et al.*, 2013; Silva *et al.*, 2020).

In view of this, the association of essential oils and entomopathogenic microorganisms is of considerable interest in the integrated management of *S. frugiperda*, because they act in different
modes of action on insects, and the synergistic effects can function as additives is capable of preventing the functioning of their enzymatic defense and detoxification system, and its adoption is more advantageous as it uses doses lower than those recommended for each product separately (Almeida et al., 2017).

Specifically for *S. frugiperda*, there are no studies that evaluated the combination of entomopathogenic microorganisms, such as *Bacillus thuringiensis* and *Beauveria bassiana*, and the essential oil derived from orange peel with d-limonene compound. Therefore, the objective of this work was to evaluate the efficacy of d-limonene associated with *B. thuringiensis* and *B. bassiana* to fall army worm control.

2 MATERIAL AND METHODS

*Dose-response bioassays*

The research was carried out at the Agricultural Entomology Laboratory, located at the Institute of Agricultural Sciences, belonging to the Federal University of Jequitinhonha and Mucuri Valley’s (UFVJM), Campus Unaí, Minas Gerais State, Brazil.

The caterpillars used were obtained through laboratory stock breeding, initiated through insect populations donated by the company Corteva Agriscience, collected in the field in the Bahia, Goiás, Paraná and Minas Gerais states. Adult populations of *S. frugiperda* were also collected in corn plantations at the Santa Paula Experimental Farm, belonging to our university, under geographic coordinates 16° 44' 28.46" S and 46° 90' 15.96" W.

The caterpillars were individualized in pots, provided with a diet produced in the laboratory, following the methodology of Parra & Carvalho (1984), and maintained at a temperature of 25 ± 2ºC, relative humidity of 60 ± 10% and a 12-hour photo-phase. Upon reaching the pupal stage, the caterpillars were placed in a Petri dish containing filter paper and kept inside screened cages. The adults were kept in cylindrical cages, fed with honey and brewer's yeast.

In order to determine the acute toxicity of d-limonene (obtained from Quinar®, purity 96.9%) to *S. frugiperda*, dose-response bioassays were carried out with third-instar larvae (±1cm), in accordance with adapted methodology proposed by OECD-OECD (1998). Solutions containing d-limonene were prepared in a mixture containing water plus emulsifier (Tween 80 adjuvant, concentration 0.05%), at the following concentrations: 0.5%, 1.0%, 2.0%, 5.0%, 10.0%, 15.0%, 25.0% and 50.0% of the essential oil. In each treatment, the caterpillars received 500 µL of the solutions, with each solution applied using an automatic pipette onto a Petri dish (60 mm), containing 20 third-instar caterpillars.
After 15 seconds of agitation and 15 minutes of rest, the caterpillars were individualized in cell trays with sixteen wells, containing 2 mL of modified artificial diet (excluding formaldehyde and sorbic acid), and kept in a room with following conditions: 25 ± 2°C of temperature; 50 ± 5% relative humidity, 12 hours of photo-phase. Assessments occurred every 24 hours for 7 days after application, counting the number of dead caterpillars.

D-limonene is a colorless, volatile, and oily liquid naturally found in the peels of citrus fruits, especially lemons and oranges, and some pine trees. It is even responsible for the strong characteristic odor of these fruits. It is a low toxicity solvent and is currently widely used in the chemical and food industries.

Toxicity tests with entomopathogenic fungus *B. bassiana* for *S. frugiperda* was performed using the commercial product Boveril®, WP PL63 (Koppert of Brazil Holding, 1x10⁸ viable conidia per gram). In solutions containing water plus emulsifier (Tween 80 adjuvant, concentration 0.05%), 12 treatments were prepared using 6.65 g L⁻¹ of the commercial product, as initial dosage (as recommended in the label), using the eucalyptus weevil (*Goniobasus scutelatus*), as a reference. Thus obtaining, through serial dilution, treatments with following concentrations: 10.0%, 20.0%, 30.0%, 40.0%, 50.0%, 60.0%, 70.0%, 80.0%, 90.0%, 100.0% and 200.0% of the entomopathogenic fungus, together with the control treatment containing only water and emulsifier. The treatments were carried out and maintained according to the method and conditions described previously.

The toxicity tests with *B. thuringiensis* were carried out with Xentari® (composition: spores of *B. thuringiensis* subsp. *anzawai* GC – 91 (Sumitomo Corporation of Brazil, 540 g L⁻¹). Nine treatments were prepared using the initial dose of 3.35 g L⁻¹, as recommended in the label for *S. frugiperda*, thus obtaining, through serial dilution with the following concentrations 1.0, 2.5%, 5.0%, 12.5%, 25.0%, 50.0%, 75.0% and 100.0%, and the control (water). Again, 20 third-instar caterpillars were used for each of the treatments carried out and 100 µL of each concentration was added to the diet, spreading with a glass rod. The mortality was assessed up to 96 h after applications.

**D-limonene combined to Beauveria bassiana**

Firstly, to determine the synergistic potential of these products, the following binary combinations were established: 50.0% x 0.5%, 50.0% x 1.0%, 50.0% x 2.0%, 100% x 0.5%, 100.0% x 1.0%, 100.0% x 2.0%, 200.0% x 0.5%, 200.0% x 1.0%, 200.0% x 2.0% of *B. bassiana* and d-limonene, respectively.

The procedures and environmental conditions of the bioassays with larvae of *S. frugiperda* followed those described previously. However, after 15 seconds of light agitation and 15 minutes of rest, they were individualized in six-well cell culture plates, using 3 replicates per treatment.
Bioassays with all binary combinations, as well as the preparation of isolated components, were carried out simultaneously. The mortality was assessed up to 96 h after applications.

**D-limonene combined to Bacillus thuringiensis**

To determine the synergistic potential between the products, the following binary combinations were established for study: 12.5% x 12.5%, 12.5% x 25%, 12.5% x 50%, 25% x 12.5%, 25% x 25%, 25% x 50%, 50% x 12.5%, 50% x 25%, 50% x 50% of the insecticide Xentari® and d-limonene, respectively. Again, the procedures and environmental conditions of the bioassays with young forms of *S. frugiperda* followed those described previously. Mortality assessments of the combinations occurred up to 96 h after applications.

**Data analysis**

Insecticide mortality data were subjected to dose-response analysis, using the “Probit” tool from SPSS Software (2022). With the mathematical model adjusted to the observed data, the lethal dose or lethal concentration 50 (LC$_{50}$) values of the products were determined, as well as the value of the confidence interval, chi-square and degrees of freedom.

The data about the efficacy of product mixtures were subjected to Shapiro Wilk's normality of residual distribution tests, Levene's homogeneity of variances and the means were compared using the Scott-Knott test, at 5% probability.

**3 RESULTS AND DISCUSSION**

**Dose-response bioassays**

D-limonene presented insecticidal activity against *S. frugiperda*, at all concentrations evaluated. I was obtained larvae lethality from 0.5% d-limonene (Figure 1 and Table 1).
After 48h, in different concentrations of essential oil, mortality differed significantly from the control with increasing concentration. From 1% of oil concentration, mortality was approximately 50% of the caterpillars, reaching 100% mortality, around 5% of d-limonene. Insects that received highest concentrations of d-limonene showed “knock-down” behavior (shock effect), reducing their feeding activity and consequently, death, a few hours after the intoxication.

These results corroborate with Cruz (2016) who tested different essential oil compounds, including d-limonene, from different aromatic plants, proving adverse biological and biochemical toxic effects for \textit{S. frugiperda} caterpillars, influencing parameters essential to their survival.

Niculau \textit{et al.} (2013) also found high toxicity of lemon balm (\textit{Lippia alba}) essential oil, which has limonene (36%) and carvone (54.8%) as its main compounds, revealing LD$_{50}$ of 1.20 µg mg$^{-1}$ and LD$_{90}$ of 3.08 µg mg$^{-1}$ of insect for \textit{S. frugiperda}, via topical application, proving the insecticidal action of these compounds. They obtained approximate mortality of 90%, although the high mortality was mainly associated with the presence of carvone.

Among the twelve concentrations of \textit{B. bassiana} tested, there was mortality above 50% only for the highest concentration, representing a proportion of 200% of the dosage indicated in the label (equivalent to 13.2 µg µL$^{-1}$), expressing moderately to low toxic. No mortality was observed in the control treatment (Figure 2 and Table 2).

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Table 1. Summary of parameters obtained to d-limonene after toxicity tests for \textit{S. frugiperda}.

<table>
<thead>
<tr>
<th>Time</th>
<th>LD$_{50}^{a}$</th>
<th>C.I. 95%$^{b}$</th>
<th>D.F.$^{c}$</th>
<th>$\chi^2$ $^{d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h</td>
<td>1.49%</td>
<td>1.39-1.61</td>
<td>12</td>
<td>2.37</td>
</tr>
<tr>
<td>48h</td>
<td>1.21%</td>
<td>1.01-1.31</td>
<td>11</td>
<td>3.62</td>
</tr>
</tbody>
</table>

$^{a}$ Lethal dose; $^{b}$ Confidence interval; $^{c}$ Degrees of freedom; $^{d}$ Qui-square

---

After 48h, in different concentrations of essential oil, mortality differed significantly from the control with increasing concentration. From 1% of oil concentration, mortality was approximately 50% of the caterpillars, reaching 100% mortality, around 5% of d-limonene. Insects that received highest concentrations of d-limonene showed “knock-down” behavior (shock effect), reducing their feeding activity and consequently, death, a few hours after the intoxication.

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Among the twelve concentrations of \textit{B. bassiana} tested, there was mortality above 50% only for the highest concentration, representing a proportion of 200% of the dosage indicated in the label (equivalent to 13.2 µg µL$^{-1}$), expressing moderately to low toxic. No mortality was observed in the control treatment (Figure 2 and Table 2).

Figure 2. Mortality of third-instar larvae of \textit{S. frugiperda}, 96 hours after exposure to different concentrations of Boveril® (\textit{Beauveria bassiana} (Bals.) Vuill., Koppert of Brazil, strain PL63, minimum of 1 x 10$^8$ viable conidia g$^{-1}$); 50 g kg$^{-1}$.
Table 2. Summary of parameters obtained for d-limonene after toxicity tests for *S. frugiperda*.

<table>
<thead>
<tr>
<th>Time</th>
<th>LC₅₀ a</th>
<th>C.I. 95% b</th>
<th>D.F. c</th>
<th>χ² d</th>
</tr>
</thead>
<tbody>
<tr>
<td>48h</td>
<td>155.5%</td>
<td>152.89-157.61</td>
<td>13</td>
<td>24.69</td>
</tr>
<tr>
<td>96h</td>
<td>166%</td>
<td>163.8-168.11</td>
<td>12</td>
<td>23.01</td>
</tr>
</tbody>
</table>

*a Lethal concentration; b Confidence interval; c Degrees of freedom; d Qui-square*

The fungal spores of these species infect insects through the integument and then multiply inside their bodies. Then, the entomopathogenic fungus releases certain toxins that destroy tissues, leading to the insect’s death (Cagán *et al.*, 2022).

In a study carried out by Ramanujam *et al.* (2020), larvae of the second instar of *S. frugiperda* were immersed in a suspension containing isolates of the fungus *B. bassiana* (ICAR-NBAIR Bb-5ª d strain) resulted in mortality of 28.6%, in agreement with our find, which caused 25% mortality of caterpillars using 100% of the recommended dose of Boveril®. Aktuse *et al.* (2019) investigated twenty isolates of entomopathogenic fungi, including six strains of *B. bassiana*, concluding that the isolate *B. bassiana* ICIPE 676 caused 30% mortality in second instar larvae of *S. frugiperda*, a fact also observed in our study.

Despite that, to Ramos *et al.* (2020), the effects of in vitro inoculation of *B. bassiana* in *S. frugiperda* resulted in 100% mortality of second instar larvae and up to 87% mortality of fourth instar larvae. Among the entomopathogenic fungi tested, *B. bassiana* was reported to be more toxic than *Metarhizium anisopliae* to *S. frugiperda*, causing larval mortality above 79% (Shahzad *et al.*, 2021).

For *B. thuringiensis*, among the concentrations tested, there was mortality above 50% of larvae, from a concentration of 12.5%, equivalent to 0.41 g L⁻¹ of Xentari® (in relation to the recommended dose), expressing high toxicity. No mortality was observed in the control treatment (Figure 3 and Table 3).

The application of the entomopathogenic microorganism caused mortality at a slower rate, as its effects are related to the ingestion of protein crystals produced by *B. thuringiensis*. Ingestion of such crystals leads to the destruction of the midgut membrane of insects, and consequently, their paralysis and death. In this case, the insects did not show behavioral changes such as the “knock-down” effect.
Figure 3. Mortality of third-instar larvae of *S. frugiperda*, 96 h after application of different concentrations of Xentari (*Bacillus thuringiensis* subsp. anzawai GC – 91, Sumitomo Corporation of Brazil, 540 g L⁻¹), in vitro.

Table 3. Summary of parameters obtained for d-limonene after toxicity tests for *S. frugiperda*.

<table>
<thead>
<tr>
<th>Time</th>
<th>L.C₅₀ᵃ</th>
<th>C.I. 95%ᵇ</th>
<th>D.F.ᶜ</th>
<th>χ²ᵈ</th>
</tr>
</thead>
<tbody>
<tr>
<td>48h</td>
<td>13.15%</td>
<td>15.61-11.52</td>
<td>08</td>
<td>3.17</td>
</tr>
<tr>
<td>96h</td>
<td>12.51%</td>
<td>14.21-11.11</td>
<td>09</td>
<td>2.89</td>
</tr>
</tbody>
</table>

ᵃLethal concentration;ᵇConfidence interval;ᶜDegrees of freedom;ᵈQui-square

**D-limonene combined to Beauveria bassiana**

Compared to d-limonene or *B. bassiana* used alone, the association between the two compounds demonstrated greater efficacy, resulting in very high toxicity to *S. frugiperda*, causing 100% mortality in all treatments investigated (Table 4).

Table 4. Mortality of third-instar larvae of *S. frugiperda* after application of different combined concentrations of d-limonene and *Beauveria bassiana*, in vitro.

<table>
<thead>
<tr>
<th>d-limonene (%)</th>
<th>B. bassiana (%)</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>50</td>
<td>3.32</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>3.32</td>
<td>6.65e</td>
</tr>
<tr>
<td>0</td>
<td>200</td>
<td>6.67e</td>
<td>23.22d</td>
</tr>
<tr>
<td>0.5</td>
<td>4.15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>8.30</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>16.60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>4.15</td>
<td>50</td>
<td>3.32</td>
</tr>
<tr>
<td>1</td>
<td>8.30</td>
<td>50</td>
<td>3.32</td>
</tr>
<tr>
<td>2</td>
<td>16.60</td>
<td>50</td>
<td>3.32</td>
</tr>
<tr>
<td>0.5</td>
<td>4.15</td>
<td>100</td>
<td>6.65</td>
</tr>
<tr>
<td>1</td>
<td>8.30</td>
<td>100</td>
<td>6.65</td>
</tr>
<tr>
<td>2</td>
<td>16.60</td>
<td>100</td>
<td>6.65</td>
</tr>
<tr>
<td>0.5</td>
<td>4.15</td>
<td>200</td>
<td>13.20</td>
</tr>
<tr>
<td>1</td>
<td>8.30</td>
<td>200</td>
<td>13.20</td>
</tr>
<tr>
<td>2</td>
<td>16.60</td>
<td>200</td>
<td>13.20</td>
</tr>
</tbody>
</table>

Control (water + Tween) 0 0

P 0.000* 0.000*

CV (%) 3.17 6.56

Means followed by different letters differ according to the Scott-Knott test at 5% probability; P: probability value; CV (%): coefficient of variation; *Commercial product: Boveril® (*Beauveria bassiana* (Bals.) Vuill., Koppert of Brazil, PL63 strain (minimum of 1 x 10⁸ viable conidia g⁻¹); 50 g kg⁻¹).
There was no lethality in the control treatment. These results contrast with those obtained previously, where isolated bioassays resulted in moderate mortality at lower concentrations. Thus, we observed a synergistic effect between the fungus and the essential oil.

Regarding the possibility of combining entomopathogenic fungi and essential oils (d-limonene), this practice can be successful, since concentrations of d-limonene associated with fungal concentrations improve the suspension of conidia, producing an emulsion and increasing spore density (Bateman, 1993). This process would facilitate the adhesion of conidia to the larval cuticle to cause greater infection and mortality.

Gonçalves (2017) studied the compatibility of essential oils with *B. bassiana*, verifying that for *B. bassiana* there is a significant beneficial correlation between mycelial growth and sporulation when subjected to essential oils of oregano (*Origanum vulgare*), citronella (*Cymbopogon winterianus*), clove (*Syzygium aromaticum*), melaleuca (*Melaleuca alternifolia*), and mugwort (*Artemisia vulgaris*), presenting a Pearson correlation coefficient of 0.99, 0.79, 0.88, 0.79 and 0.64 respectively, which represents good compatibility between the oils and the fungus.

According to Mattos et al. (2021) the use of essential oils to control corn pests, whether used individually or in conjunction with other active components, has positive evidence of insecticidal and repellent functions. D-limonene essential oil as well as other oils with biological effects on insects, acts in a neurotoxic way, leading to the inhibition of the enzyme acetylcholinesterase, during nervous synapses (Lopez & Pascual-Villalobos, 2010).

Even though it is a route already sensitized by many neurotoxic insecticides, it is believed that the rapid action of limonene and its great absorption in the insect organism made it difficult to reverse this cellular mechanism, and consequently, the survival of *S. frugiperda*. After its application, evident signs of hyperexcitation, extreme agitation, paralysis and death were observed in the affected individuals.

The fungus entomopathogenic, as evidenced by the control results, promoted caterpillar mortality and a large drop in the number of individuals at the site. Its mechanism of action is associated with the parasitism of the fungus in insects, starting with the germination and penetration of conidia into the insect integument, colonizing it internally and leading to its death. This mechanism of action becomes even more complex for the insect defense system, which indicates rare possibilities for the development of resistance in the field.

Finally, the association of d-limonene with *B. bassiana*, based on the results obtained, allows us to state that the two mechanisms of action of these products combined is an even greater obstacle to the rapid development of resistance in caterpillars, indicating that synergism between products is
a valuable tool and powerful, and could contribute greatly to reducing the damage caused by this pest.

**D-limonene combined to Bacillus thuringiensis**

Regarding the combination of insecticides, the greatest control efficacy was obtained at concentrations of 12.5% x 50%, followed by the combination of 12.5% x 25% of d-limonene and *B. thuringiensis*, respectively (Table 5).

Table 5. Mortality of third-instar larvae of *S. frugiperda* after application of different combined concentrations of d-limonene and *B. thuringiensis*, in vitro.

<table>
<thead>
<tr>
<th>d-limonene (%)</th>
<th><em>Bacillus thuringiensis</em> (%)</th>
<th>d-limonene µg µL⁻¹</th>
<th><em>Bacillus thuringiensis</em> µg µL⁻¹</th>
<th>72h Mortality</th>
<th>96h Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>42.00c</td>
<td>45.00c</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.82</td>
<td>54.00b</td>
<td>54.00b</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>50.0</td>
<td>1.64</td>
<td>74.00a</td>
<td>75.00a</td>
</tr>
<tr>
<td>12.5</td>
<td>103.75</td>
<td>12.5</td>
<td>0.41</td>
<td>48.00c</td>
<td>48.68c</td>
</tr>
<tr>
<td>12.5</td>
<td>103.75</td>
<td>25.0</td>
<td>0.82</td>
<td>55.00b</td>
<td>56.12b</td>
</tr>
<tr>
<td>12.5</td>
<td>103.75</td>
<td>50.0</td>
<td>1.64</td>
<td>73.00a</td>
<td>74.68a</td>
</tr>
<tr>
<td>25.0</td>
<td>207.5</td>
<td>12.5</td>
<td>0.82</td>
<td>6.00g</td>
<td>6.50g</td>
</tr>
<tr>
<td>25.0</td>
<td>207.5</td>
<td>25.0</td>
<td>1.64</td>
<td>42.85d</td>
<td>43.12d</td>
</tr>
<tr>
<td>50.0</td>
<td>415.0</td>
<td>12.5</td>
<td>0.41</td>
<td>37.56e</td>
<td>37.56e</td>
</tr>
<tr>
<td>50.0</td>
<td>415.0</td>
<td>25.0</td>
<td>0.82</td>
<td>6.00g</td>
<td>6.06g</td>
</tr>
<tr>
<td>50.0</td>
<td>415.0</td>
<td>50.0</td>
<td>1.64</td>
<td>5.23g</td>
<td>5.93g</td>
</tr>
</tbody>
</table>

Control (water + Tween) 0.00 0.00

P 0.000* 0.000*

CV (%) 2.18 2.32

* Means followed by different letters, in the column, differs according to the Scott-Knott test at 5% probability. P: probability value; CV (%): coefficient of variation.

As observed, high doses of both products were necessary to obtain higher fall armyworm mortalities, a fact very different from other previous tests, i.e., in these combinations, when d-limonene concentrations were greater than 12.5%, a reduction in caterpillar mortality was obtained, even at higher concentrations of *Bacillus thuringiensis*. This may indicate a possible antagonistic effect between the products and a reduction in the insecticidal potential of both, when d-limonene concentrations are high, a fact evidenced mainly when the lowest control of caterpillars is observed, in the combination of 50% concentration of both products. Even though more than 70% of mortality was obtained using 12.5 x 50% d-limonene and *B. thuringiensis*, the same synergistic relationship between these insecticides was not found in relation to the combination of d-limonene and *B. bassiana*.

Knaak et al. (2010), despite not having evaluated different doses, studied the association of *B. thuringiensis*, also using Xentari®, with different plant extracts from aromatic plants. These authors obtained mortality of *S. frugiperda* larvae, but with smaller (and slower) intestinal changes.
when extracts of *C. citratus*, an aromatic species composed of terpenoids similar to d-limonene, were used in relation to the other plant extracts tested.

All these results are promising and indicate that more studies should be conducted in order to clarify whether the use of combined insecticides like natural products and entomopathogenic organisms are effective to pest control. Consequently, field tests have also become crucial to provide further clarification of the timing and residual effect of these combinations on the environment as part of integrated pest management.

4 CONCLUSIONS

D-limonene essential oil promoted high mortality of caterpillar at very low concentrations, and the subspecies of bacilli used ensured high caterpillar mortality.

However, the strain of *B. bassiana* used promoted caterpillar mortality only at a very high dose.

*B. bassiana* + d-limonene presents synergistic properties with very high toxicity to *S. frugiperda*.

*B. thuringiensis* + d-limonene, although resulting in toxicity for larvae, does not appear to be synergistic.

ACKNOWLEDGEMENTS, FINANCIAL SUPPORT AND FULL DISCLOSURE

Thanks to “Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG” for all financial supporting of this work.
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