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Nanoformulation prototype of the essential oil of *Lippia sidoides* and thymol to population management of *Sitophilus zeamais* (Coleoptera: Curculionidae)

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ABSTRACT

Sitophilus zeamais is a pest of global significance and it is difficult to control due to the high indices of resistance to insecticides showed by the populations. As an alternative to the management of *S. zeamais* populations, in the present study, we evaluated the toxicity of essential oil (EO) of *Lippia sidoides*, its major compound (thymol − 68.5%) and prototypes of nanoformulations (NF) (18%) based on these compounds on *S. zeamais* populations (N = 5) from different regions of Brazil. Toxicity bioassays were performed to determine lethal and chronic toxicity doses and times to test the efficiency of prototypes in the treatment of stored grains. Additionally, we study the efficiency and stability of stored NFs. The lethal doses of EO of *L. sidoides* and thymol required to kill 50% of *S. zeamais* populations ranged from 7.1 to 19.9 μg/ mg^{-1} and 17.1 to 25.7 μg/ mg^{-1}, respectively. The populations of Jacarezinho-PR and Maracaju-MS were, respectively, the most tolerant and susceptible to the EO of *L. sidoides*. EO of *L. sidoides*, thymol and its NFs acted fast on the populations of *S. zeamais*. Increasing of NF concentrations led to reduced grain consumption and total population mortality. NFs stored for up to seven months maintained high mortalities on *S. zeamais*. This work indicates that the prototypes of NFs based on the EO of *L. sidoides* and its major compound are promising for the management of *S. zeamais* populations.

1. Introduction

Post-harvest losses resulting from insect activity are usually unrecoverable and consist of a growing food security constraint, especially, in tropical developing countries. Infestations, which often begin in the field, persist in the storage phase where cause considerable losses (20–60%) in the weight of grains and seeds (Castro-Álvarez et al., 2015).

The maize weevil *Sitophilus zeamais* Mots. 1855 (Coleoptera: Curculionidae) is the most important primary pest of stored grains (*e.g.* corn, rice, wheat) in the world. Conventional control of *S. zeamais* populations is based on continuous applications of synthetic insecticides, which can result in several problems, such as: insecticide residues above of acceptable limits and, especially, the selection of resistant populations to insecticides (Li et al., 2013; Nukenine et al., 2011; Wei et al., 2014).

The resistance of *S. zeamais* to synthetic insecticides and the concerns about the risks associated with these insecticides to human health have been driven the search for alternative methods to control this pest (Sousa et al., 2016). Among the alternative methods, the essential oils (EOs) of medicinal and aromatic plants − constituted of a mixture of organic compounds (*e.g.* mono- and sesquiterpenes) have been considering promising. Besides their biological activity against a range of organism (Albuquerque et al., 2013; Bacci et al., 2015; Campos et al., 2015; Peixoto et al., 2015); EOs are environmentally safer (Lima et al., 2013). Due to the low molecular weight of their compounds, EOs are highly volatile and therefore have low persistence in the environment, presenting lower toxicity to non-target organisms (Campos et al., 2015;

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Freires et al., 2015; Lima et al., 2013; Montefuscoli et al., 2014). However, some characteristics of these compounds (*e.g.* water insolubility, chemical instability, high volatility, short residual activity due to degradation by temperature and light) may be obstacles to their use in natural situations (Attia et al., 2013; González et al., 2014; Montefuscoli et al., 2014).

The development of NFs (droplets in nanometer between 10 and 100 nm) from EOs may allow the efficient use of these compounds in natural situations, since such technology protects bioactive substances from degradation and losses by evaporations, in addition to allowing a controlled release of these compounds (González et al., 2014). NFs can also contribute to increase the efficacy of the compounds once they have higher specific area, solubility and mobility when compared to conventional insecticides (González et al., 2015; Montefuscoli et al., 2014).

Studies have been shown that the EOs of the shrub *Lippia sidoides* Cham. (Verbenaceae) − a native plant from the semiarid of Brazilian northeast − has bioactivity against different organisms (Gomes et al., 2014; Veras et al., 2012). This bioactivity seems to be due, at least in part, to thymol (Faraone et al., 2015; Ma et al., 2014), a major compound in the EO of this plant. However, till the present moment, no study has developed a NF of this *L. sidoides* EO to control the *S. zeamais*.

Once insect populations are spatially isolated and can be subjected to different selective pressures, they are expected to present different levels of tolerance or susceptibility to conventional and natural insecticides (*e.g.* EOs from plants). Thus, in this study, we analysed the toxicity of EO of *L. sidoides*, its major compound and NFs based in these compounds on populations of *S. zeamais*. Some of the *S. zeamais* populations used in the present study have already had their resistance proven to conventional insecticides (Braga et al., 2011; Corrêa et al., 2011a, 2011b). The results from the present study will allow to evaluate not only the bioactivity of the *L. sidoides* EO and its major compound over different populations, but also the efficacy of their respective NFs.

2. Materials and methods

2.1. S. zeamais populations

We used populations of *S. zeamais* from all regions of Brazil (Fig. 1): north (municipality de Rio Branco, Acre), northeast (Aracaju, Sergipe), south (Jacarezinho, Paraná); southeast (Sete Lagoas, Minas Gerais) and midwest (Maracaju, Mato Grosso do Sul). Populations of *S. zeamais* were kept at the Clínica Fitossanitária of the Federal University of Sergipe (UFS), located in the municipality of São Cristóvão (10°54′ S, 37°04′ W and altitude 7m), Sergipe, Brazil, where the bioassays were conducted. For multiplications of *S. zeamais* populations, corn kernels were first washed and kept in freezer (−10 °C) for 30 days in order to eliminate possible residues of insecticides and organism present in them. Then, kernels were transferred to plastic container (1 L), where the insects were placed. Plastic containers were sealed with organza and kept under controlled conditions (26 ± 3 °C, 50% of humidity and 12-h photoperiod).

2.2. EO of L. sidoides

The EO from leaves of *L. sidoides* used in this study was obtained by steam drag and supplied by the company PRONAT (Horizonte, Ceará, Brasil).

The composition of EO was analysed using a GC–MS/FID (QP2010 Ultra, Shimadzu Corporation, Kyoto, Japan) equipped with an autosampler AOC-20i (Shimadzu). Separations were accomplished using an Rtx®-5MS Restek fused silica capillary column (5%-diphenyl-95%-dimethyl polysiloxane) of 30 m × 0.25 mm i.d, 0.25 mm film thickness, at a constant Helium flow rate of 1.0 mL min^{-1}. The injection temperature was 280 °C and 1.0 μL (10 mg/mL^{-1}) of sample was injected, with a slip ratio of 1:30. The oven temperature was programmed from 50 °C (isothermal during 1.5 min), with an increase of 4 °C/min^{-1}–200 °C, then 10 °C/min^{-1}–300 °C, ending with a 5 min isothermal at 300 °C.

For GC/MS, molecules were ionized by ionization of electrons with energy of 70 eV. The fragments were analyzed by a quadrupole system programmed to filter fragments/ions with *m/z* in the order of 40–500 Da and detected by an electron multiplier. The data processing was performed with CG/MS software Postrun Analysis (Labsolutions-Shimadzu). The ionization process for CG/FID was performed by the flame from hydrogen gases 5.0 (30 mL min-1) and synthetic air (300 mL min-1).

Identification of constituents was performed by comparison of retention indices in the literature (Adams, 2007). Retention indices were obtained with equation proposed by Van den dool and Kratz (1963) in relation to a series of *n*-alkanes (nC_9 − nC_{18}). Libraries from GC–MS were also used to compare acquired mass spectra using a similarity index of 80%.

The tymol, constituent in greater proportion in the EO of *L. sidoides,* was acquired from SIGMA-ALDRICH company.

2.3. Prototype of the NFs

NFs were prepared at the Pharmacotechnical Development Laboratory (Department of Pharmacy − UFS), by the dropwise addition of 72 mL of Procetyl® AWS (surfactant) in a glass container under magnetic stirring for 10 min. Then, 18 mL of the active ingredient (*L. sidoides* EO or thymol − oil phase) were added slowly. The mixture (surfactant and the active ingredient in oil phase) was homogenized by magnetic stirring at room temperature. After 20 min, 10 mL of ultra pure water miliq (aqueous phase) was added. The system was kept under agitation according to the ratios determined at room temperature until its complete homogenization (El Maghraby, 2008). NFs were stored in a 200 mL amber vials in the dark at room temperature (26 ± 3° C) and 50 ± 10% of humidity.

2.3.1. Chemical and physical characterization

The active ingredients (a.i) contained in the EO of NFs were isolated by hydrodistillation with Clevenger type apparatus adapted with the addition of 1 mL of the emulsified system in 2 L of water in a volumetric flask of 3.0 L for a boiling period of 140 min. The EO was conditioning in an amber bottle (5 mL). The chemical analysis was performed as previously described (CG-MS/FID). Chemical characterization was performed at 30, 90, 150 and 210 days of NFs storage.

Physical characterization of NFs was performed considering the Zeta potential, microparticle size, pH and polydispersity index. The Zeta potential is related to the repulsive forces existing between the microparticles in an emulsified system, where the increase of these forces gives the NFs greater stability, thus avoiding the agglomeration thereof. The polydispersity index, in turn, indicates how much the particle size has deviated from the mean. The microparticle size and polydispersity indexes were determined by photon correlation spectrometry after dilution of the sample with 1: 500 (v: v) ultrapurified water in Zetasizer Nanoseries Nono-Zs (Malvern Instruments, Worcestershire, RU). The electrical conductivity of isotropic systems (IONLAB CON-500) was measured at scale up to 2000 uΩ/cm with standard KCl solution of 1412 uΩ/cm at room temperature. The pH was measured through microprocessed bench pH meter, with Pt100 sensor in stainless steel electrode. All characterizations were performed at 25 °C and in triplicate.

2.3.2. Microscopy of polarized light

The identification of isotropy was performed by polarized light microscopy, where one drop of each sample was transferred to a glass slide, covered by a coverslip and then analyzed under polarized light. Analysis were performed with an Olympus BX-51 microscope equipped with a Color Evolution LC digital camera (PL-A662) and PixelLINK

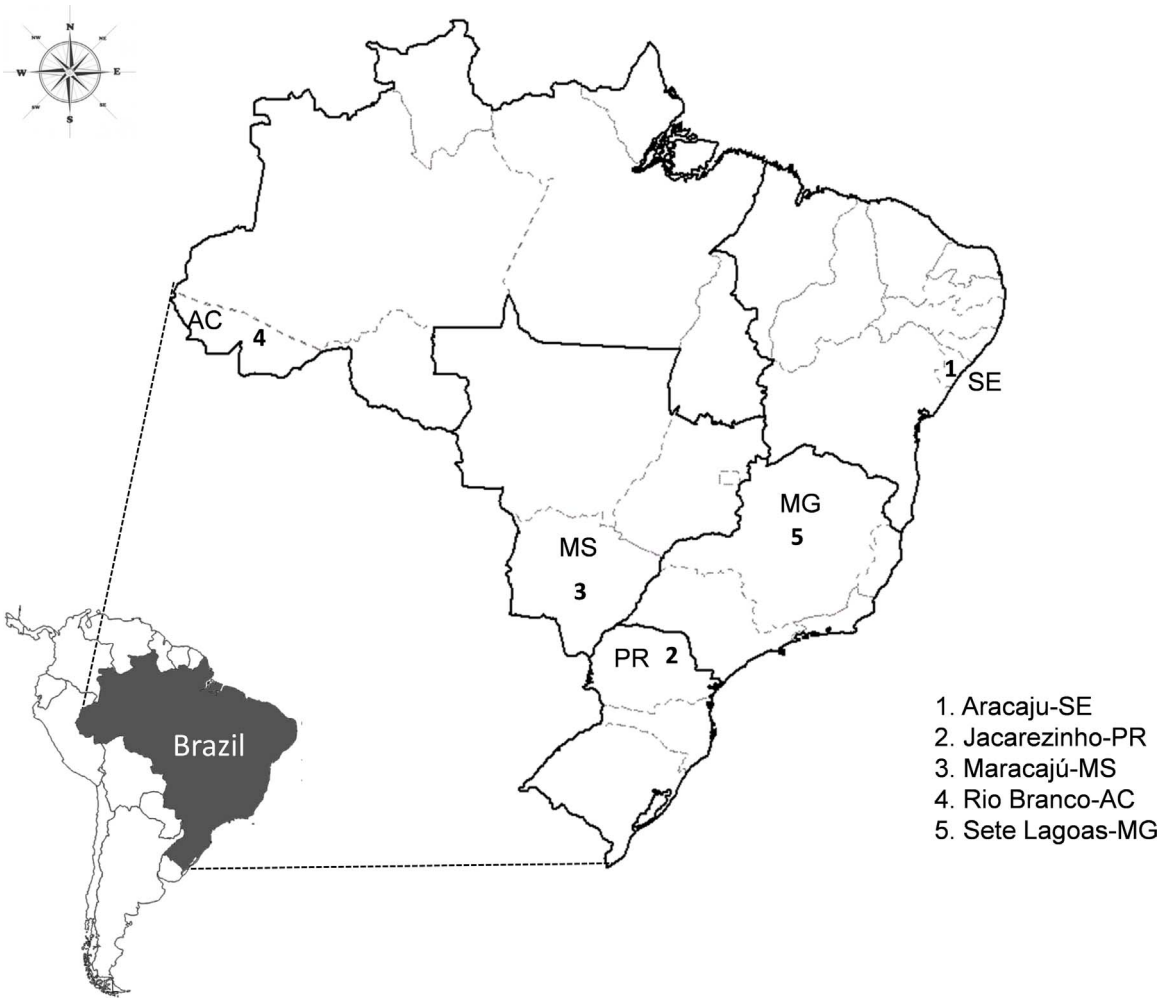


Fig. 1. Collection sites of *Sitophilus zeamais* populations from all Brazilian regions [Northeast (Sergipe: Aracaju), South (Paraná: Jacarezinho); North (Acre: Rio Branco), Midwest (Mato Grosso do Sul: Maracaju) and Southeast (Minas Gerais: Sete Lagoas)].

image analyzer software, with a minimum of 5 days after obtaining NFs.

2.4. Bioassays

Bioassays were conducted with populations of *S. zeamais* to evaluate: *(i)* acute toxicity (doses and lethal time) of EO from *L. sidoides*, thymol and its NFs, *(ii)* effectiveness of NFs prototypes in the treatments of grains and, *(iii)* effectiveness of NFs over time (*e.g.* storage period). All bioassays were conducted using non-sexed adult insects of *S. zeamais*. The treatments were diluted with acetone (Panreac, UV-IR-HPLC-GPC PAI-ACS, 99.9% purity), except for grain consumption bioassays. On the control group was used only the solvent acetone. Preliminary tests showed that solvent acetone and the surfactant Procetyl® AWS, presenting NFs, did not interfere in the survival of insects.

2.4.1. Acute toxicity

Each experimental unit consisted of a Petri dish (6 × 1.5 cm), covered with filter paper, containing 10 individuals of *S. zeamais.* Petri dishes were kept in the freezer for 45 s to reduce the insects' activity and to allow the application of treatments. Preliminary tests have shown no effect of this procedure in the survivorship of insects.

The EO of *L. sidoides*, thymol and its NFs were applied topically (0.5 μL) in the prothoracic region of each insect with a microsyringe (Hamilton®, 10 μL). After application of treatments, the Petri dishes were covered with plastic film and placed in a biochemical oxygen demand (BOD) incubator at 25 ± 1 °C, 70% of relative humidity and photoperiod of 12 h. Insects were considered dead in all treatments when they were immobile or with uncoordinated movements.

To determine the lethal doses, the experimental design was completely randomized with four replicates for each combination of treatments × doses × populations of *S. zeamais* ($N = 5$: for EO of *L. sidoides* and thymol; $N = 2$: for its NFs, the most susceptible and tolerant populations). Bioassays were initially performed with three doses (1, 5 and 10 μg of substances/mg of insects). Posteriorly, it was determined intermediates doses (6–12 doses) to obtain dose-mortality curves. The number of dead and living insects was registered up to 72 h after application.

To determine the lethal time, the experimental design was completely randomized with 10 replicates for each combination of treatments × populations of *S. zeamais* (N = 2: the most susceptible and tolerant population). The LD_{95} determined in the lethal doses bioassays were used to determine the lethal time (LT_{50}) as well as the survival curves of treatments on the two populations of *S. zeamais*. The mortality was evaluated in intervals of 10 min to complete 30 min; every 30 min until completing two hours, and every two hours until the mortality of control group reaches 80%.

2.4.2. Treatment of corn kernels

Treatments consisted of corn kernels treated with NFs from the EO of *L. sidoides* and thymol. In the control group, untreated grains were used. It was performed four replicates for each combination of concentration (N = 7) × *S. zeamais* population (N = 2: the most

susceptible and most tolerant population) × treatment.

NFs were applied with a micropipette (Labmate Pro) directly onto the corn kernels (250 g and 14% humidity) which were kept in a glass vial (0.8 L). After application, vials were agitated to homogenization during 40 s. Non-sexed adult insects (N = 30) were inserted in each vial, which were hermetically sealed and stored under controlled conditions (27 ± 2 °C, 70 ± 10% of relative humidity and 12-h of photoperiod). After 90 days, the mass of the grains in each vial was measured.

2.4.3. Effectiveness of NFs over time

Toxicity bioassays were conducted to determine the temporal effectiveness during the storage of NFs prototypes of *L. sidoides* OE and thymol on the populations of *S. zeamais* (N = 2). Bioassays were performed weekly from the stabilization of them to seven months of storage (210 days).

To perform these bioassays, the DL_{90} determined in the acute toxicity bioassays were used (item 3.4.1). The procedures of bioassays were the same and the mortality was evaluated 72 h after the begging of the bioassays.

2.5. Statistical analysis

The mortality rates of insects submitted to treatments were corrected in relation to the control group using Abbott's formula (1925). Probit analyses were performed to determine the doses-mortality curves, for each treatment × population after 72 h. Curves whose probability of acceptance of the null hypothesis (that the data have Probit distribution) by the $\chi 2$ test was greater than 0.05 were accepted. Curves and the lethal concentrations (LD_{50} and LD_{90}) with their respective confidence intervals at 95% were obtained using the software SAS (SAS, Institute).

For each treatment × population, survival curves were obtained using Kaplan-Meier estimators in the software SigmaPlot 11.0. Through these survival curves, it was possible to estimate the times necessary to cause the mortality of 50% of individuals of each population (LT_{50}).

The grain consumption (g) was determined by the subtraction of initial mass of grains by the grain mass at the end of the experiment. For each combination of treatment × population, the variation of grain consumption rate in relation to the treatments was analysed with analysis of variance (ANOVA; PROC GLM, SAS). Exponential regressions were adjusted to data of grain consumption. To evaluate the effectiveness and stability of NFs, regression analysis over time were performed in the software SigmaPlot 11.0.

3. Results

3.1. Chemical composition of L. sidoides EO and NFs

The major compound present in the EO of *L. sidoides* was thymol (68.45%), followed by ρ-cymene (10.66%) and (*E*)-caryophyllene (7.28%) (Table 1). The others compounds were present in concentrations less than 3% of the total of OE of *L. sidoides*.

Thymol, ρ-cymene and (*E*)-caryophyllene remained in larger proportions in the EOs extracted from NF prototype during the four periods of storage. However, the concentrations of these terpenes showed temporal variations, with an average increase of 17% in the thymol concentrations and a reduction of 40 and 10% of ρ-cymene and (*E*)-caryophyllene, respectively. Some components present in lower concentrations in the EO were not detected in the NFs after seven months of storage (*e.g.* γ-terpinene) (Table 1).

3.2. Physical characterization of NFs

The microscopy images from the NFs prototypes of *L. sidoides* EO and thymol showed that the particles formed were spherical and with similar diameter (Fig. 2).

NFs showed particle size between 58 to 78 nm, zeta pontencial of −27 and −31 mV; polyspersion index of 0.37 and 0.28; and pH of 6.97 and 8.85 for NFs contaning EO of *L. sidoides* and thymol, respectively (Fig. 2).

Table 1
Chemical composition of the essential oil of *L. sidoides* before preparation of NF (pré-formulation) and in the NF over the storage time [storage (days)].

Compound	RT[a]	Concentration (%)				
		Pre-formulation	Storage (days)			
			30	90	150	210
Tricyclene	8.38	0.57	0.10	0.10	0.10	0.10
α-Thujene	8.61	0.61	0.10	0.10	0.10	0.10
Myrcene	10.27	2.58	0.94	0.55	0.40	0.70
δ-3-Carene	11.01	0.17	0.10	0.10	–	–
α-Terpinene	11.21	1.06	0.52	–	–	–
ρ-Cymene	11.42	10.66	7.99	5.89	5.95	6.42
Limonene	11.55	0.79	0.65	0.35	0.42	0.39
1,8-Cineole	11.65	0.75	0.76	0.60	0.65	0.45
γ-Terpinene	12.58	2.12	0.57	0.25	–	–
Linalool	13.86	0.34	0.34	0.49	0.35	0.35
3-Thujen-2-one	16.42	0.54	1.16	–	0.36	1.34
Terpinen-4-ol	16.85	0.85	0.15	1.58	1.20	0.11
Methyl Thymol	18.08	0.99	1.29	1.05	1.26	1.10
Thymol	19.99	68.45	73.93	81.75	82.01	80.20
α-Copaene	22.43	0.31	0.36	0.16	0.16	0.19
(*E*)-Caryophyllene	23.75	7.28	8.03	4.50	4.61	6.54
α-E-Bergamotene	24.09	0.20	0.18	–	–	–
Aromadendrene	24.27	0.52	0.66	0.33	0.42	0.39
α-Humulene	24.66	0.32	0.37	0.15	0.29	0.25
Viridiflorene	25.76	0.36	0.45	0.20	0.25	0.30
δ-Cadinene	26.41	0.15	0.20	0.10	0.28	0.13
Caryophyllene oxide	28.13	0.38	1.14	1.22	1.17	0.89

[a] Retention index calculated using the Van den dool and Kratz (1963) equation for a homologous series of n-alkanes (nC9–nC18).

3.3. Lethal doses

Populations of *S. zeamais* showed different levels of suceptibility to treatments. Maracaju was the population more suceptible, both to the *L. sidoides* OE and thymol, with LD_{50} of 7.10 and 17.08 μg/mg, respectively. It was necessary higher concentrations of *L. sidoides* OE (2.8x) and thymol (1.5x) to kill the same 50% of population from Jacarezinho. The population from Aracaju, Rio Branco and Sete Lagoas showed intermediate suceptiilities to these compounds (Table 2).

For all populations, the EO of *L. sidoides* was more toxic than thymol, with exception of population from Sete Lagoas, in which there were no significant differences in the concentrations of these treatments to kill 50% of this population (Table 2).

When treated with NFs prototypes, populations showed the similar pattern of susceptibility. The population from Maracaju was around 36 and 34% more suceptible to NFs of EO and thymol than the population from Jacareinho, respectively (Table 3). However, the pattern of toxicity among treatments was altered: NFs of thymol was more toxic for all populations of *S. zeamais* compared with NFs of *L. sidoides* EO (Table 3).

3.4. Lethal time

The survival of *S. zeamais* populations from Maracaju (Log-Rank: $\chi^2 = 516{,}6$; $p < 0{,}001$) and Jacarezinho (Log-Rank: $\chi^2 = 339{,}9$; $p < 0{,}001$) were reduced over time (Fig. 3). It was necessary, in average, 50 and 91 h to cause the mortality higher than 90% in the populations from Maracaju and Jacarezinho, respectively (Fig. 3A–B).

The lethal time required to kill 50% of *S. zeamais* populations varied from 5.8 to 62 h. The *L. sidoides* EO showed faster action for both *S. zeamais* populations compared to its major compound thymol

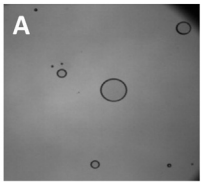
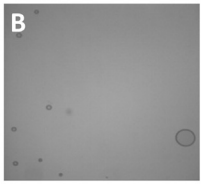
Image (Increase of 20,000 x)	Particle size (nm)	Zeta potential (mV)	Polydispersity	pH
	58,0	-27,0	0,37	6,97
	78,0	-31,0	0,28	8,85

Fig. 2. Image obtained by polarized light microscopy and physical properties of NFs containing EO of *L. sidoides* (A) and thymol (B).

Table 2
Toxicity by contact of essential oil of *L. sidoides* and thymol against adults of *Sitophilus zeamais* populations after 72 h of exposure.

Population	N. of insects	LD_{50} (CI95%) (μg/mg)	LD_{90} (CI95%) (μg/mg)	Slope	$\chi 2$	*p*
EO of *L. sidoides*						
Maracaju	360	7.10 (5.77–8.67)	43.63 (31.81–66.83)	1.62	0.56	0.75
Aracaju	360	14.28 (12.33–16.02)	44.72 (35.05–68.63)	2.58	1.06	0.59
Rio Branco	520	15.37 (13.01–18.36)	79.16 (54.70–142.65)	1.79	0.26	0.87
Sete Lagoas	520	17.77 (16.94–18.70)	26.88 (24.87–29.77)	7.12	0.09	0.95
Jacarezinho	520	19.93 (18.38–21.45)	41.15 (35.52–51.91)	4.06	1.93	0.61
Thymol						
Maracaju	360	17.08 (15.26–18.79)	43.07 (36.28–56.03)	3.18	2.05	0.35
Aracaju	360	25.55 (21.95–34.51)	108.64 (61.90–335.00)	2.03	0.03	0.98
Rio Branco	440	24.71 (20.32–30.92)	172.78 (104.94–413.36)	1.51	0.80	0.67
Sete Lagoas	360	17.76 (14.41–23.33)	100.68 (62.71–214.75)	1.69	0.91	0.63
Jacarezinho	360	25.71 (20.39–38.47)	41.12 (36.74–48.75)	6.27	0.73	0.70

Table 3
Toxicity by contact of NFs (18%) containing essential oil of *L. sidoides* and thymol against adults of *Sitophilus zeamais* populations after 72 h of exposure.

Population	N. of insects	LD_{50} (CI95%) (μg/mg)	LD_{90} (CI95%) (μg/mg)	Slope	$\chi 2$	*p*
EO *L. sidoides*-NF						
Maracaju	440	26.44 (23.27–31.18)	81.72 (61.48–124.80)	2.61	0.31	0.95
Jacarezinho	480	35.96 (31.81–40.54)	114.23 (83.53–215.23)	2.55	0.27	0.87
Thymol-NF						
Maracaju	480	20.75 (19.13–22.32)	40.55 (37.01–45.42)	4.40	0.28	0.96
Jacarezinho	520	27.71 (24.86–30.51)	61.04 (54.12–71.14)	3.73	0.05	0.97

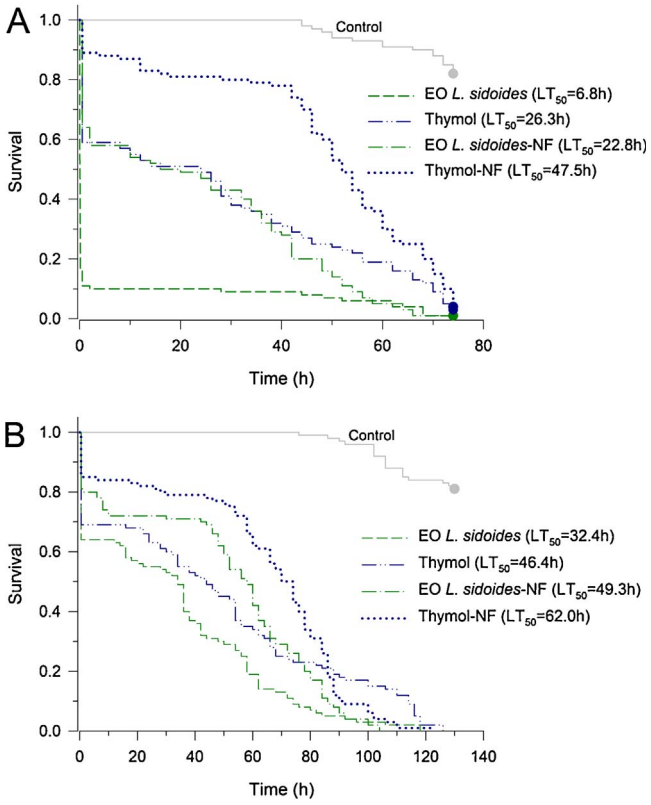


Fig. 3. Survival curves and lethal time (TL_{50}) of *Sitophilus zeamais* populations from Maracaju (A) and Jacarezinho (B) exposed by contact (DL_{95}) to essential oil of *L. sidoides*, thymol and its NFs.

(Fig. 3A–B). In all cases, NFs take longer to cause the same mortality (Fig. 3A–B).

3.5. Consumption of grain

Increasing concentrations of NFs containing OE of *L. sidoides* or thymol resulted in a reduction of grain consumption in both populations (Fig. 4A–B). The reduction of this parameter was more pronounced in the population from Maracaju (Fig. 4).

Low concentrations of NFs (*e.g.* 50 μL kg^{-1}) resulted in a small increase in the grain consumption when compared with control group (Fig. 4A–B). At the dose of 1600 μL/kg of maize, all insects were killed (Fig. 4A–B).

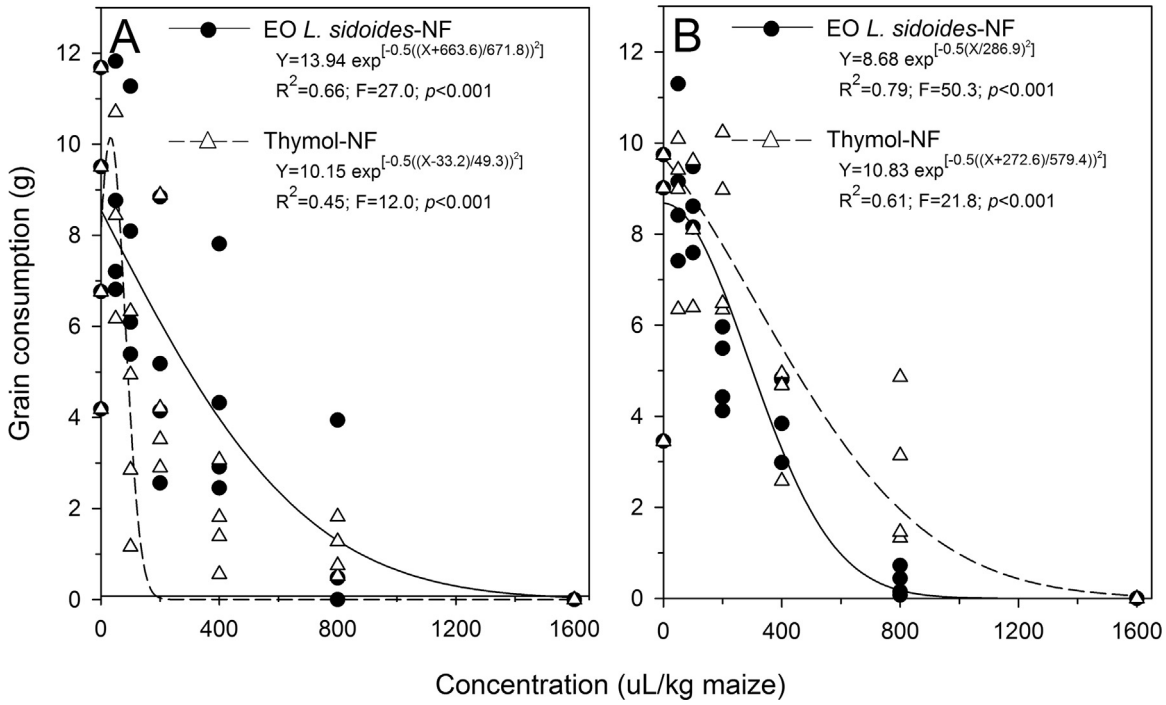


Fig. 4. Grain consumption (g) of *Sitophilus zeamais* populations from Maracaju (A) and Jacarezinho (B) after 90 days of exposure to NFs containing essential oil of *L. sidoides* and thymol in different concentrations.

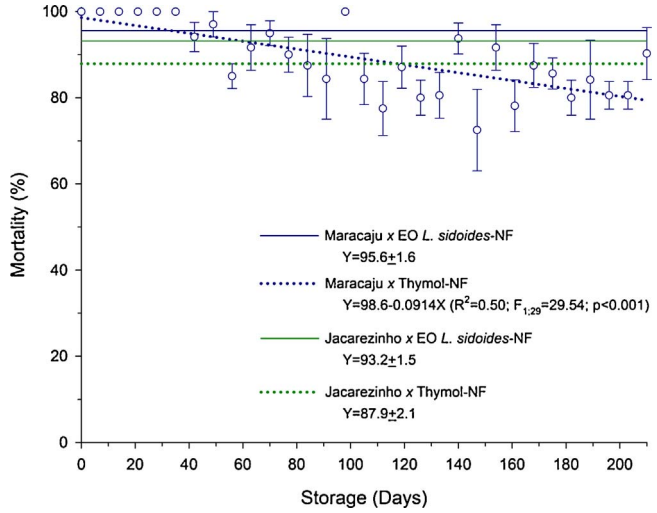


Fig. 5. Mortality of *Sitophilus zeamais* from Maracaju and Jacarezinho exposed by contact (DL_{90}) to NFs containing essential oils of *L. sidoides* and thymol stored for 120 days.

3.6. Effectiveness of NFs over time

NFs of *L. sidoides* and thymol stored for seven months caused high mortality of *S. zeamais* populations over time. The average mortality caused by NFs of *L. sidoides* in the populations from Maracaju and Jacarezinho and, by NFs of thymol in the population of Jacarezinho were 95,6%; 93,2% e 87,9%; respectively (Fig. 5). Although the mortality of population from Maracaju reduced over time of storage by NFs of thymol, this one still remained high (80%) after 210 days of storage (Fig. 5).

4. Discussion

The chemical analysis of EO of *L. sidoides* and the NF based on this oil showed that both are composed by mono- and sesquiterpenes and that thymol is the major compound present in the EO of *L. sidoides* as well as in the NF. The concentration of thymol was similar to found in previous studies (Chagas et al., 2016; Gomes et al., 2012; Santos et al., 2015). The variations found in the composition of EO may be related to biotic and abiotic factors, such as: genetic and climatic factors, geographic origin, harvest, fertilization and cropping systems (Luz et al., 2014; Santos et al., 2015, 2016).

The EO of *L. sidoides* in the NF prototype showed variations in its chemical composition during storage period. These variations are related to reactions of the compounds during the manufacturing process of NF and/or storage. Increases in the thymol concentrations in NF during storage are directly related to some enzymes that use some precursors, such as γ-terpinene and ρ-cymene which are structurally related and occur in this EO for thymol biosynthesis. In fact, these precursors had their concentrations decreased at the same time that the thymol concentration increased. The thymol is biosynthesized by the aromatization of γ-terpinene in ρ-cymene, followed by the hydroxylation process of this compound (Costa et al., 2014; Marchese et al., 2016). This indicates that the NF potentiates the concentration of thymol.

The results found in the present study are promising for the management of *S. zeamais* populations with different levels of resistance to conventional insecticides. Here, we find that lethal doses necessary to kill 50% of the *S. zeamais* are near or below to those reported by other studies with another EOs for this same insect (Suthisut et al., 2011; Yang et al., 2011; Li et al., 2013; Liu et al., 2012).

It was possible to observe different levels of susceptibility in the *S. zeamais* populations to EO of *L. sidoides* and thymol. However, these variations were small compared to the variations observed for these same populations exposed to organosynthetic insecticides (*e.g.* Maracaju and Jacarezinho were 25 and 56 times more tolerant to the pyrethroids and permethrin than Sete Lagoas, respectively) (Corrêa et al., 2011b). This may occur because EOs are a complex mixtures of compounds that can interact (*e.g.* synergism, additive and antagonism) and act on more than one insecticide target site (Tak and Isman, 2015; Tak et al., 2016). In addition, the major compound – thymol – has been shown to inhibit the acetylcholinesterase enzyme in insects (Jukic et al., 2007), a different mechanism of action of pyrethroids (sodium

channels) for which the *S. zeamais* populations studied here shown resistance.

The higher doses and lethal times observed for the population of Jacarezinho, resistant to pyrethroids (Braga et al., 2011; Corrêa et al., 2011a), indicate that compounds from OE or at least part of them can also act in target sites of pyrethroids and/or present lower sensitivity to the detoxifying enzyme glutathione S-transferase (Araújo et al., 2011; Lopes et al., 2010).

The EO of *L. sidoides* and thymol showed fast insecticidal activity indicating that these compounds act in target sites in the nervous system of insects. The lowest LT_{50} observed in the *S. zeamais* populations exposed to the EO of *L. sidoides* in relation to thymol are probably due to synergic interactions between compounds present in the EO (Tak et al., 2016). In our study, LT_{50} was bellow to those observed for pyrethroid deltamethrin, which is a neurotoxic insecticide normally used to control *S. zeamais* (Morales et al., 2013).

In the present study, NF prototypes developed were very efficient for the control of *S. zeamais* populations, reducing the survival and grain consumption with fast action and maintained high toxicities during long periods of storage.

Considering that the NF contained only 18% of the a.i. (active ingredients: EO of *L. sidoides* or thymol) it is observed that the prototypes potentiated the action of the EO of *L. sidoides* and thymol. Even with the a.i. concentration being 5.6 x lower, the lethal doses required to kill 50% of *S. zeamais* populations increased at rates ranging from 1.1 to 3.7. On the other hand, the high efficiency of NF in the reduction of grain consumption is due, in addition to the effect of contact, to exposures by ingestion and fumigation, once the insects walked on the treated grains and remained in an enclosed environment (glass containers).

The potentiation effect of EO of *L. sidoides* and thymol is probably due to some properties in the NFs, such as: *(i)* smaller particle size, increasing the contact surface, *(ii)* controlled release, avoiding excessive losses (*e.g.* volatilization) and *(iii)* higher solubility, facilitating insect penetration (Kah and Hofmann, 2014; Oliveira et al., 2014).

The NFs prototypes still showed good efficiency in the control of *S. zeamais* populations during storage. This efficiency is due, in addition to the toxicity of the a.i. (EO of *L. sidoides* and thymol), to the physical properties provided by the surfactant Procetyl® AWS. Most of the work involving NFs to control insect pests uses other surfactants such as Tween (Montefuscoli et al., 2014) and polyethylene glycol (González et al., 2014) or also encapsulating agents such as β-cyclodextrin (Galvão et al., 2015), poly-β-hydroxybutyrate and poly-ε-caprolactone (Carvalho et al., 2015). Thus, our work is a pioneer in the use of Procetyl® AWS as a surfactant for EO of *L. sidoides* on *S. zeamais* and shows that this was important for particle size formation, stability (Zeta potential) and uniformity (Polydispersity) of NFs.

Both NFs had average particle sizes within the range of 0–100 nm, which indicates nanometer scale (Kah and Hofmann, 2014). NF containing thymol was more stable and homogeneous compared to NF based on EO of *L. sidoides*. The zeta potential values of −27 and −31 mV found in our NFs containing the EO of *L. sidoides* and thymol indicate incipient and moderate stability, respectively (Honary and Zahir, 2013a). This potential refers to the repulsive forces between the approaching EO of *L. sidoides* or thymol particles, and expresses the potential difference between the dispersion medium and the stationary phase of fluid bound to the dispersed particle (Dias et al., 2014; Honary and Zahir, 2013b). Negative values may be associated with the free acids or other components present in the EO, as well as the surfactant composition (Dias et al., 2014).

The NFs containing the EO of *L. sidoides* and thymol presented polydispersity indexes of 0.37 and 0.28, showing smaller and greater homogeneity in the size of the particles present in the NF, respectively. The values of this index below 0.3 indicate that the particle population is homogeneous (Damasceno et al., 2011; Dias et al., 2014; Flores et al., 2011; González et al., 2014). The pH values are inversely proportional to the values of the polydispersity index (Chen et al., 2015; Ghiasvand et al., 2015).

In summary, our work demonstrates the efficiency of NFs prototypes based on the EO of *L. sidoides* and thymol for the management of *S. zeamais* populations with different levels of resistance to conventional insecticides.

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