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ORIGINAL PAPER

An alternative to reduce the use of the synthetic insecticide against the maize weevil *Sitophilus zeamais* through the synergistic action of *Pimenta racemosa* and *Citrus sinensis* essential oils with chlorpyrifos

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Abstract

Sitophilus zeamais attacks stored corn kernels and is traditionally controlled using synthetic pesticides. However, the frequent use of these toxic compounds leads to environmental and health damage and to the development of resistant insect populations. Essential oils (EOs) represent an alternative to conventional pesticides for pest control. The objective of the present study was to evaluate the effect of the combination of EOs with chlorpyrifos against *S. zeamais* in order to reduce the effective applied dose of the synthetic insecticide. The most active EOs against *S. zeamais* were *Rosmarinus officinalis, Pimenta racemosa* var. *ozua* and *Citrus sinensis*. Moreover, all the binary mixtures (1:1 volume of EO/volume of EO) of these EOs (*P. racemosa–R. officinalis, P. racemosa–C. sinensis* and *R. officinalis–C. sinensis*) showed a higher fumigant activity and repellency than the individual EOs, with the combinatory index (CI) values of these binary mixtures indicating synergism for fumigant activity. The *P. racemosa–C. sinensis*–chlorpyrifos (16:1 volume of binary mixtures of EOs/volume of chlorpyrifos) combination had a lower LC_{95} value compared to the respective binary mixtures of EOs, revealing a synergistic effect. In addition, this combination did not produce phytotoxicity on maize grains. The binary mixture *P. racemosa–C. sinensis* in combination with chlorpyrifos synergized the effect of the synthetic pesticide without affecting germination of the maize grains. In addition, the use of this mixture (*P. racemosa–C. sinensis*–chlorpyrifos) decreased the net quantity of the synthetic insecticide, thus making it an interesting alternative for the control of the maize weevil *S. zeamais*.

Keywords Maize weevil · Binary mixture · Chlorpyrifos · *Pimenta racemosa* · *Citrus sinensis*

Key message

- This work searches for an alternative to reduce synthetic insecticide use against the maize weevil *Sitophilus zeamais* by combining chlorpyrifos with essential oils.
- The mixture *Pimenta racemosa–Citrus sinensis* repels *S. zeamais* and, in combination with chlorpyrifos, synergizes the effect of the insecticide without affecting the germination of the maize grains.
- The use of the mixture *P. racemosa–C. sinensis*– chlorpyrifos decreases the amount of synthetic insecticide required and is therefore an interesting alternative for the control of the maize weevil *S. zeamais*.

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Introduction

Maize (*Zea mays*) is one of the most important crops in Argentina, with a world ranking of fifth in terms of production and third in exports (USDA 2018). The cultivated area dedicated to maize in Argentina exceeds 6 million hectares, producing more than 40 million tons of grain (Bolsa de Cereales 2019). However, during storage, numerous species of insects attack corn grains and this leads to great economic losses (De Groote et al. 2013). The maize weevil *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae) is considered to be one of the major primary pests of stored maize (Erenso and Berhe 2016), due to the damage caused to grains through its feeding and reproductive habits (Trematerra et al. 2013). The adult bores a hole in the grain for feeding and then lays eggs in the holes, with the emerging larvae starting the most voracious stage of the weevil life cycle, which feed on the maize grain until reaching maturity (Nwosu 2018). Moreover, the frequent movement of these insects favours the growth of fungi that are already present in the grains, increasing the dispersion of their spores and the production of mycotoxins. (Ferreira-Castro et al. 2012; Brito et al. 2019). The fungi present in maize produce various mycotoxins that can induce toxic responses in humans and animals after their ingestion (Grenier and Oswald 2011; Queiroz et al. 2012, Leggieri Camardo et al. 2019).

Synthetic pesticides have traditionally been used for grain protection from spoilage caused by pests (Kim et al. 2019). However, the frequent use of high doses of these compounds has been associated with problems in human and animal health (Nasr et al. 2016; Mosquera Ortega et al. 2018), as well as having negative effects on the environment (Liu et al. 2018; Knillmann and Liess 2019) and leading to the development of resistant insect populations (Sevastos et al. 2018; Kim et al. 2019; Hawkins et al. 2019). Organophosphorus pesticides are extensively used throughout the world to control agricultural pests. Among these, chlorpyrifos is one of the most commonly employed pesticides, which is classified as being a moderately toxic compound (Ministerio de Salud 2016; Brancato et al. 2017). As a result, it has been reported that chlorpyrifos has hazardous effects on various organisms from different environments (Rivadeneira et al. 2013, Uzun and Kalender 2013; Cacciatore et al. 2015; Deeba et al. 2017; Srivastav et al. 2017), including humans (Tripathi and Srivastav 2010). In this context, the development of new strategies for pest control is urgently required. Consequently, in recent years, there has been an increasing interest in the use of natural bioactive compounds, such as essential oils (EOs), as alternatives to conventional pesticides. The insecticidal (Herrera et al. 2014; Dhifi et al. 2016; Liao et al. 2016; Pavela 2016; Peschiutta et al. 2016; 2017; Amoabeng et al. 2019) and repellent activity of EOs (Nerio et al. 2010; Deletre et al. 2016; Arena et al. 2017; Alcala-Orozco et al. 2019) against a wide variety of insects has been extensively studied. Many EOs have been reported to have activity that protects stored grains as they are highly effective against different insect pests (Ngamo et al. 2007; Abdelgaleil et al. 2009; Caballero-Gallardo et al. 2011; Chaubey 2019). The EOs that are used in storage systems have been applied as repellents, antifeedants, growth inhibitors, oviposition inhibitors, ovicides and insecticides (Said-Al Ahl et al. 2017). Previous investigations have reported toxic effects of certain EOs against *Sitophilus oryzae* (Singh and Mall 1991; Yadav et al. 2008), *Rhyzopertha dominica* (Brooker and Kleinig 2006), *Tribolium castaneum* (Jaya et al. 2014) and *Sitophilus granarius* (Rahman et al. 2003) in stored wheat. In addition, Dey and Sarup (1993) showed that several EOs are highly effective in protecting stored maize against *S. oryzae*. This line of investigation has resulted in numerous patents and formulations having been developed using EOs for control of stored grain pests (Said-Al Ahl et al. 2017). Some EOs-based formulations are commercially produced by different companies for pest control, such as Rockwell Labs Ltd. (USA), EcoSMART (USA) and Bayer (Germany) (Mossa 2016; Pavela 2016).

Essential oils of aromatic plants are complex mixtures of volatile organic compounds, comprising terpenes (monoterpenes, sesquiterpenes, diterpenes) and aromatic (phenylpropanoids) and aliphatic compounds with a variety of functional groups (Bakkali et al. 2008). The activity of a particular EO is usually attributable to its major constituents. However, it has been reported that the activities of the individual components of an EO do not fully explain the activity of the EO, revealing synergistic or antagonistic interactions to be taking place among its components (Kim et al. 2016). These interactions could also occur between components of different EOs, thereby enhancing their repellent or insecticidal activity (Arena et al. 2017; Bustillos Hernández and Cabrera Narváez 2018). Furthermore, Arena et al. (2018) reported an increased insecticidal activity against *Alphitobius diaperinus* when a conventional insecticide was combined with certain EOs, which reduced the amount of the insecticide required and consequently its negative effects. However, such synergy does not always occur. Faraone et al. (2015) reported an antagonistic action between certain EOs and synthetic insecticides.

The aim of the present study was to evaluate the effect of mixtures of EOs with chlorpyrifos against *S. zeamais* in order to reduce the effective applied dose of the synthetic insecticide. Thus, the insecticidal and repellent activities of *Pimenta racemosa* var. *ozua* (Mill.) J.W. Moore (Myrtaceae), *Rosmarinus officinalis* L. (Lamiaceae), *Pimenta* *haitiensis* (Urb.) Landrum (Myrtaceae), *Citrus sinensis* (L.) Osbeck (Rutaceae) and *Illicium verum* Hook. f. (Illiciaceae) EOs and their combinations were analysed in order to identify the most bioactive mixture against the maize weevil.

Materials and methods

Insects

The maize weevils *Sitophilus zeamais* (Motschulsky) were obtained in Córdoba, Argentina and reared in sealed containers for one year with maize grains free from insecticide exposure (Brito et al. 2019), under controlled conditions of temperature and humidity (27 ± 1°C and 65 ± 2%, respectively) and in complete darkness (FAO 1974). Weevils without differentiation of sex and age were used for all the bioassays.

Essential oil compositions

The EOs of *Pimenta racemosa* var. *ozua* (Mill.) J.W. Moore (Myrtaceae), *Rosmarinus officinalis* L. (Lamiaceae), *Pimenta haitiensis* (Urb.) Landrum (Myrtaceae), *Citrus sinensis* (L.) Osbeck (Rutaceae) and *Illicium verum* Hook. f. (Illiciaceae) were purchased from Santo Domingo's local market (Dominican Republic).

The composition of the EOs was determined by gas chromatography–mass spectrometry (GC-MS) using a PerkinElmer SQ8 chromatograph–mass spectrometer, equipped with a mass selective detector in the electron impact mode (70 eV), and the compounds were separated using a DB-5 capillary column (30 m x 0.25 mm, film thickness 0.25 mm). The injector temperature was 200 °C, and the oven temperature was programmed linearly at 60°C for 5 min, ramped up to 170°C at 4°C/min, and then increased to 250°C at 20°C/min. The detector temperature was 250°C. The carrier gas used was He at 1 ml/second, and diluted samples of 1 µL (1/100 in *n*-heptane, v/v) were manually injected in the split-less mode. The Kovats retention index (KI) of each compound was obtained after an analysis of a homologous series of *n*-alkanes C8-C21 (Sigma-Aldrich Co. Buenos Aires, Argentina) under the same chromatographic conditions. Identifications were conducted by matching their mass spectra and KI values with those from the Adams Library, the NIST-14 Mass Spectral Library, and those of pure compounds by co-injection of standards (Sigma-Aldrich Co. Buenos Aires, Argentina). Compound concentrations were expressed as relative percentages by peak area normalization.

Chemicals

Clorfox (Gleba, Argentina) was provided by Alejo Fabian Bonifacio (Universidad Nacional de Córdoba, Córdoba, Argentina). Clorfox is a commercial liquid formulation that contains 48% w/v of chlorpyrifos (0,0-diethyl 0-[3,5,6-trichloro-2-pyridinyl] phosphorothioate) and 52% of solvent and emulsifiers. Although chlorpyrifos is used as an insecticide against *S. zeamais*, it has no repellent effect against this weevil (Pereira et al. 2009).

Effect of essential oils on *Sitophilus zeamais*: fumigation toxicity and repellent/attraction activity assays

To evaluate the insecticidal activity of the EOs against *S. zeamais*, a fumigation toxicity test described by Huang et al. (2000) was carried out, with some modifications. The EOs at concentrations of 300, 150, 75, 37.5 and 18.75 µl/l air were placed separately on Whatman filter paper disks of 2 cm diameter. Each filter paper disk was placed on the underside of the screw cap of a glass vial (30 ml) covered with nylon gauze to avoid direct contact of the weevils with the EOs. Then, ten weevils were placed in each vial. Chlorpyrifos was used as a positive control, and all experiments were performed in five repetitions carried out twice per concentration.

Lethal concentrations causing 50% and 95% mortality (LC_{50} and LC_{95}) were determined after 24 h of exposure, according to Finney (1971), using the SPSS Statistics program version 17.0 (SPSS Inc. 2008). Once the LC_{50} and LC_{95} had been calculated, the most active EOs were mixed in a ratio of 1:1 (volume of EO/volume of EO) in binary mixtures, which were tested at concentrations corresponding to 75, 37.5, 18.75 and 9.375 µl/l air. For all mixture treatments, the combinatorial index (CI) was determined using the CompuSyn software (Chow and Martin 2007), with values of $CI < 1$, $CI = 1$ and $CI > 1$ indicating synergistic, additive and antagonistic effects, respectively.

A two-choice olfactometer bioassay was carried out to evaluate the behavioural response of *S. zeamais* to individual EOs and to the binary mixtures that had shown the highest insecticidal effects (Herrera et al. 2015). Briefly, two glass Erlenmeyer flasks (250 ml) were connected by a glass tube (30 cm x 1 cm diameter) with a central hole (1 cm x 1 cm). The connections between the two flasks and the tube were sealed with rubber plugs, which were covered with parafilm to prevent gas leakage (Fig. 1). A 2-cm-diameter filter paper was placed in one flask, where EOs or binary combinations mixed in a ratio of 1:1 (volume of EO/volume of EO) were added at concentrations of 0.05, 0.40 and 4.00 µl/l air. In the other flask, a filter paper free of EOs was placed. Twenty insects, deprived of food for 24 h, were

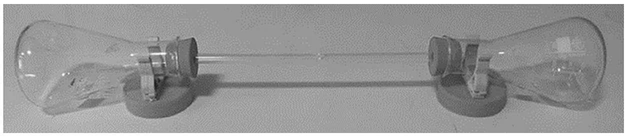


Fig. 1 Two-choice olfactometer system used to assess the repellent/attraction activity of individual and binary mixtures of EOs in *S. zeamais*

then placed in the hole of the glass tube. The experiments were conducted under dark conditions at 27 ± 1°C and 65 ± 2% relative humidity for 2 h in a climatic chamber. The position of the flasks was changed in each repetition. The insects that showed no behavioural response in the experiment were not considered in the calculation of the response index (RI). The experiments were performed five times for each EO concentration, and each insect was only used once. Independent controls were carried out (both flasks without any compound) to determine whether the movement of the insects towards the flasks was random. Propionic acid was used as the repellence positive control. In each test, the RI was calculated using the following equation: RI = [(*T* - *C*)/Tot] × 100, where *T* is the number of insects that respond to the treatment, *C* is the number of insects that respond to the control, and Tot is the total number of insects released. Positive values of RI indicate attraction to the treatment, while negative values indicate repellency (Herrera et al. 2015).

Combinations of binary mixtures of essential oils and chlorpyrifos: fumigant toxicity against *Sitophilus zeamais* and their effect on maize grain germination

The most bioactive binary mixtures of EOs were combined with chlorpyrifos. The ratio of each combination was 16:1 [volume of binary mixtures of EOs/volume of chlorpyrifos (48 % w/v)], with the effect of these combinations being studied on *S. zeamais* mortality and maize grain phytotoxicity.

To evaluate mortality on *S. zeamais*, we conducted the fumigant toxicity technique described above. The concentrations tested were from 2.20 to 58.36 µl/l air. The control treatments were carried out under the same conditions but without the presence of EOs or chlorpyrifos.

To study the effect of the mixtures of EOs with chlorpyrifos on maize grains, a seed germination assay was carried out (Herrera et al. 2015). LC_{50} and LC_{95} values of each mixture of EOs and chlorpyrifos were placed on a filter paper inside aluminium containers (2 cm diameter). These containers were then placed in the centre of a Petri dish (9 cm diameter) with two paper filters covering the bottom of the plate, after which, ten healthy maize grains were placed around the containers and 5 ml of sterile distilled water was added to each Petri dish. As control samples, Petri dishes without the addition of the mixture of EOs–chlorpyrifos and with chlorpyrifos were carried out. The plates were incubated at 27 ± 1°C for 8 days, and a germination count was performed daily. The rate of germination was calculated using the formula germination rate = $\Sigma\ (nd\text{-}1)$, where n = number of germinated seeds per day and d = number of days elapsed since the beginning of the test (Agrawal 1980). Then, the seed vigour was calculated, relative to the control treatment (100% germination). All experiments were repeated twice in quintuplicate.

Statistical analyses

All analyses were performed using InfoStat/Professional 2010 p (Di Rienzo et al. 2010).

Once the dose-mortality values were obtained, the lethal concentrations (LC_{50} and LC_{95}) and confidence limits (95%) of the EOs and the different combinations were calculated using the probit regression analysis of the SPSS software. Lethal concentration values were considered significantly different if their confidence limits did not overlap.

The mean RI of each treatment of the repellency bioassays was evaluated by a two-way analysis of variance (ANOVA) and subsequently classified using the Fisher's LSD test ($p \leq 0.05$).

To analyse the effect of the EOs on seed germination, the germination rates were evaluated by a one-way ANOVA and a DGC posteriori test (Di Rienzo et al. 2002). The results with values $p \leq 0.05$ were considered significantly different from the control.

Results

Essential oil compositions

The chemical compositions of the EOs are shown in Table 1. According to the chromatographic analyses, the main components of the EOs from *Pimenta racemosa* var. ozua were 1,8-cineole (45.25%) and p-cymene (33.54%). On the other hand, 1,8-cineole (53.48%), α-pinene (15.85%) and camphor (10.17%) were the major components of *Rosmarinus officinalis* EO, while estragole (32.53%), linalool (18.43%), 1,8-cineole (14.95%) and methyl eugenol (14.23%) characterized the EOs of *Pimenta haitiensis*. Finally, the essential oils from *Citrus sinensis* and *Illicium verum* were composed mainly of limonene (96.14%) and anethole (E) (77.35%), respectively.

Table 1 Essential oil compositions of *Pimenta racemosa* var. *ozua, Rosmarinus officinalis, Pimenta haitiensis, Citrus sinensis* and *Illicium verum*

KI (theoretical)	KI (calculated)	Compounds	*Pimenta racemosa var. racemosa*	*Rosmarinus officinalis*	*Pimenta haitiensis*	*Citrus sinensis*	*Illicium verum*	Methods of identification
921	928	Tricyclene	–	0.13	–	–	–	GC–MS, KI
924	928	α-Thujene	0.20	–	0.10	–	–	GC–MS, KI
932	935	α-Pinene	1.17	15.85	0.37	0.37	0.26	GC–MS, KI, CO
945	952	α-Fenchene	–	0.13	–	–	–	GC–MS, KI
946	955	Camphene	–	3.67	–	–	–	GC–MS, KI
974	982	β-Pinene	–	0.47	–	–	–	GC–MS, KI, CO
979	989	3-Octanone	–	–	0.28	–	–	GC–MS, KI
988	993	Myrcene	–	0.72	0.37	1.17	0.11	GC–MS, KI, CO
988	1001	3-Octanol	–	–	0.32	–	–	GC–MS, KI
1002	1007	α-Phellandrene	–	–	0.15	0.38	0.11	GC–MS, KI
1008	1013	δ-3-Carene	–	–	0.15	–	0.45	GC–MS, KI, CO
1014	1019	α-Terpinene	0.24	0.18	0.10	–	0.13	GC–MS, KI
1020	1027	p-Cymene	33.54	3.47	–	–	–	GC–MS, KI, CO
1022	1029	o-Cymene	–	–	–	–	0.63	GC–MS, KI
1024	1032	Limonene	4.98	–	1.33	96.14	4.78	GC–MS, KI, CO
1026	1035	1,8-Cineole	45.25	53.48	14.95	–	0.63	GC–MS, KI
1044	1051	(E)β-Ocimene	–	–	0.11	–	–	GC–MS, KI
1054	1062	γ-Terpinene	–	–	0.22	–	0.17	GC–MS, KI, CO
1067	1074	cis-Linalool oxide (furanoid)	–	–	0.20	–	–	GC–MS, KI
1084	1089	trans-Linalool oxide	–	–	0.27	–	–	GC–MS, KI
1086	1089	Terpinolene	–	–	–	–	0.21	GC–MS, KI, CO
1089	1093	p-Cymenene	–	–	–	–	0.10	GC–MS, KI
1095	1102	Linalool	0.20	0.55	18.43	0.90	–	GC–MS, KI, CO
1118	1123	exo-Fenchol	–	0.11	–	–	–	GC–MS, KI
1122	1130	α-Campholenal	–	0.10	–	–	–	GC–MS, KI
1141	1150	Camphor	–	10.17	–	–	–	GC–MS, KI, CO
1158	1163	Bicyclo[3.1.1]heptan-3-one, 2,6,6-trimethyl-	–	0.14	–	–	–	GC–MS, KI
1165	1175	Borneol	–	2.52	–	–	–	GC–MS, KI, CO
1174	1183	Terpinen-4-ol	9.75	0.47	0.87	–	0.20	GC–MS, KI
1186	1197	α-Terpineol	3.91	2.26	2.37	0.16	0.53	GC–MS, KI, CO
1195	1200	Estragole	–	–	32.53	–	5.79	GC–MS, KI, CO
1201	1208	Decanal	–	–	–	0.22	–	GC–MS, KI, CO
1204	1209	Verbenone	–	0.13	–	–	–	GC–MS, KI
1227	1253	Nerol	–	–	1.07	–	–	GC–MS, KI
1247	1257	p-Anisaldehyde	–	–	–	–	5.62	GC–MS, KI, CO
1247	1344	Chavicol	–	–	0.43	–	–	GC–MS, KI
1249	1255	Anethole (Z)	–	–	1.07	–	–	GC–MS, KI
1282	1289	Anethole (E)	–	–	7.31	–	77.35	GC–MS, KI, CO
1287	1287	Bornyl acetate	0.15	0.42	–	–	–	GC–MS, KI
1356	1354	Eugenol	–	–	0.89	–	–	GC–MS, KI, CO
1371	1375	Methyl p-anisate	–	–	–	–	0.10	GC–MS, KI
1372	1373	Ylangene	–	0.17	–	–	–	GC–MS, KI
1374	1380	α-Copaene	–	0.67	–	–	–	GC–MS, KI

Table 1 (continued)

KI (theoretical)	KI (calculated)	Compounds	*Pimenta racemosa var. racemosa*	*Rosmarinus officinalis*	*Pimenta haitiensis*	*Citrus sinensis*	*Illicium verum*	Methods of identification
1403	1401	Methyl eugenol	–	–	14.23	–	–	GC–MS, KI
1412	1382	p-Anisyl acetate	–	–	–	–	1.22	GC–MS, KI
1411	1417	α-cis-Bergamotene	–	–	–	–	0.10	GC–MS, KI
1417	1424	β-Caryophyllene	–	0.95	0.50	–	0.10	GC–MS, KI, CO
1432	1464	α–trans-Bergamotene	–	–	–	–	0.20	GC–MS, KI
1452	1460	α-Humulene	–	0.16	–	–	–	GC–MS, KI
1458	1443	Alloaromadendrene	–	0.38	–	–	–	GC–MS, KI
1478	1479	γ-Muurolene	-	0.39	–	–	–	GC–MS, KI
1491	1495	Methylisoeugenol (E)	–	–	0.43	–	–	GC–MS, KI
1500	1502	α-Murolene	–	0.20	–	–	–	GC–MS, KI
1505	1522	β-Bisabolene	–	–	–	–	0.47	GC–MS, KI
1506	1510	α-Bisabolene	–	0.11	–	–	–	GC–MS, KI
1522	1522	δ-Cadinene	–	0.38	–	–	–	GC–MS, KI
1521	1525	Calamenene	–	0.18	–	–	–	GC–MS, KI
		Trace compounds	0.56	1.71	0.63	0.66	0.51	GC–MS, KI

Compound concentrations are expressed as relative percentages. The compounds are listed according to elution order in a DB-5 column; *KI* Kovats indices, *Co* co-injection with standard. Trace compounds: compounds lower than 0.1%

Table 2 Fumigant toxicity against *Sitophilus zeama*is adults after 24 h of exposure to essential oils and their binary combinations. Combinatorial index (CI) of mixtures[a]

Essential oils	LC$_{50}$ (μl/l)	95% Confidence interval (μl/l)	LC$_{95}$ (μl/l)	95% Confidence interval (μl/l)	Z	CHI^2	CI
Pimenta racemosa	40.58	36.09–45.64	69.92	62.25–81.50	8.44	26.61	–
Rosmarinus officinalis	37.14	34.52–40.57	54.30	48.55–66.14	5.36	13.18	–
Pimenta haitiensis	107.44	82.42–138.81	266.64	216.05–361.00	9.28	60.53	–
Citrus sinensis	75.80	69.97–84.27	105.85	94.20–131.39	4.93	7.30	–
Illicium verum	273.58	226.44–353.74	609.75	489.49–837.38	6.21	30.53	–
Pimenta racemosa–Rosmarinus officinalis	26.88	23.46–30.46	43.59	38.64–51.72	9.57	44.83	0.69
Pimenta racemosa–Pimenta haitiensis	37.46	28.89–38.76	74.30	41.83–89.97	9.40	143.74	0.45
Pimenta racemosa–Citrus sinensis	30.48	26.01–35.52	52.27	44.94–65.88	10.24	101.9	0.61
Pimenta racemosa–Illicium verum	42.10	33.62–53.51	77.40	63.09–108.43	9.96	99.86	0.57
Rosmarinus officinalis–Pimenta haitiensis	44.35	33.29–58.90	97.30	77.05–142.72	9.28	86.33	0.54
Rosmarinus officinalis– Citrus sinensis	33.21	27.30–41.08	62.27	51.40–84.99	10.56	146.19	0.49
Rosmarinus officinalis–Illicium verum	39.92	33.24–48.57	72.31	60.79–93.06	9.58	49.72	0.47
Pimenta haitiensis–Citrus sinensis	47.32	39.71–56.46	93.74	79.77–117.47	9.96	47.67	0.52
Pimenta haitiensis–Illicium verum	60.41	50.29–75.76	121.79	99.39–166.61	8.03	46.75	0.38
Citrus sinensis–Illicium verum	47.29	38.85–59.07	98.20	80.68–131.11	8.56	41.71	0.65
Chlorpyrifos	1.07	0.68–1.43	3.80	2.75–4.57	7.79	72.98	–

Probit regression analysis using SPSS software. Lethal concentration values were considered significantly different if their confidence limits did not overlap. The experiment was performed in five repetitions twice per concentration.[a] The values CI < 1, CI = 1 y CI > 1 indicate synergistic, additive and antagonistic effects, respectively

Effect of essential oils on *Sitophilus zeamais*: fumigation toxicity and repellent/attraction activity assays

The fumigant insecticidal activity of individual EOs was evaluated against adults of *S. zeamais* (Table 2). After 24 hours of exposure, the most active EOs were *R. officinalis* and *P. racemosa*, with LC_{95} values of 54.30 and 69.92 µl/l, respectively. A moderate toxicity was observed for the EOs of *C. sinensis* and *P. haitiensis*, with LC_{95} values of 105.85 and 266.64 µl/l, respectively. Finally, the EO of *I. verum* exhibited a high LC_{95} value of 609.750 µl/l.

Considering the results from the fumigant assay, the most active EOs (*R. officinalis*, *P. racemosa* and *C. sinensis*) were selected to evaluate the toxicity of binary combinations of these EOs against the maize weevil. These results revealed that these combinations presented a higher fumigant activity than the insecticidal effect of the individual EOs (Table 2), indicating synergistic activity with CI <1. The most active binary mixtures were *P. racemosa–R. officinalis, P. racemosa–C. sinensis* and *R. officinalis–C. sinensis*, with LC_{95} values of 43.59, 52.27 and 62.27 µl/l, respectively.

The repellent/attraction activity of the most toxic individual and binary mixtures of EOs was evaluated on *S. zeamais* (Fig. 2). The individual EOs of *P. racemosa*, *R. officinalis* and *C. sinensis* showed repellency at the tested concentrations, with *R. officinalis* being the most repellent EO with RI values of −52.06 ± 8.04, −50.30 ± 13.86 and −53.42 ± 12.54 at concentrations of 4 µl/l, 0.4 µl/l and 0.2 µl/l, respectively. However, the binary mixtures of the EOs showed higher RI values compared to the individual EOs. The mixtures that had the highest repellent effects were *P. racemosa–R. officinalis* with RI values of −62.92 ± 7.31, −67.46 ± 8.41, −70.62 ± 6.30, followed by *R. officinalis–C. sinensis* with RI values of -55.78 ± 17.13, −57.93 ± 13.12, −57.50 ± 7.80 at 4 µl/l, 0.4 µl/l and 0.2 µl/l, respectively. In addition, *P. racemosa–C. sinensis* also showed repellency with RI values of −52.46 ± 13.71, −35.90 ± 18.91 and −20.66 ± 13.18 at 4 µl/l, 0.4 µl/l and 0.2 µl/l, respectively.

Combination of binary mixtures of essential oils and chlorpyrifos: fumigant toxicity against *Sitophilus zeamais* and their effect on maize grain germination

The fumigant insecticidal activity of the most active binary mixtures of EOs combined with chlorpyrifos was evaluated against adults of *S. zeamais* (Table 3). When the EOs were used in combination with chlorpyrifos, only the *P. racemosa–C. sinensis*–chlorpyrifos mixture revealed synergism

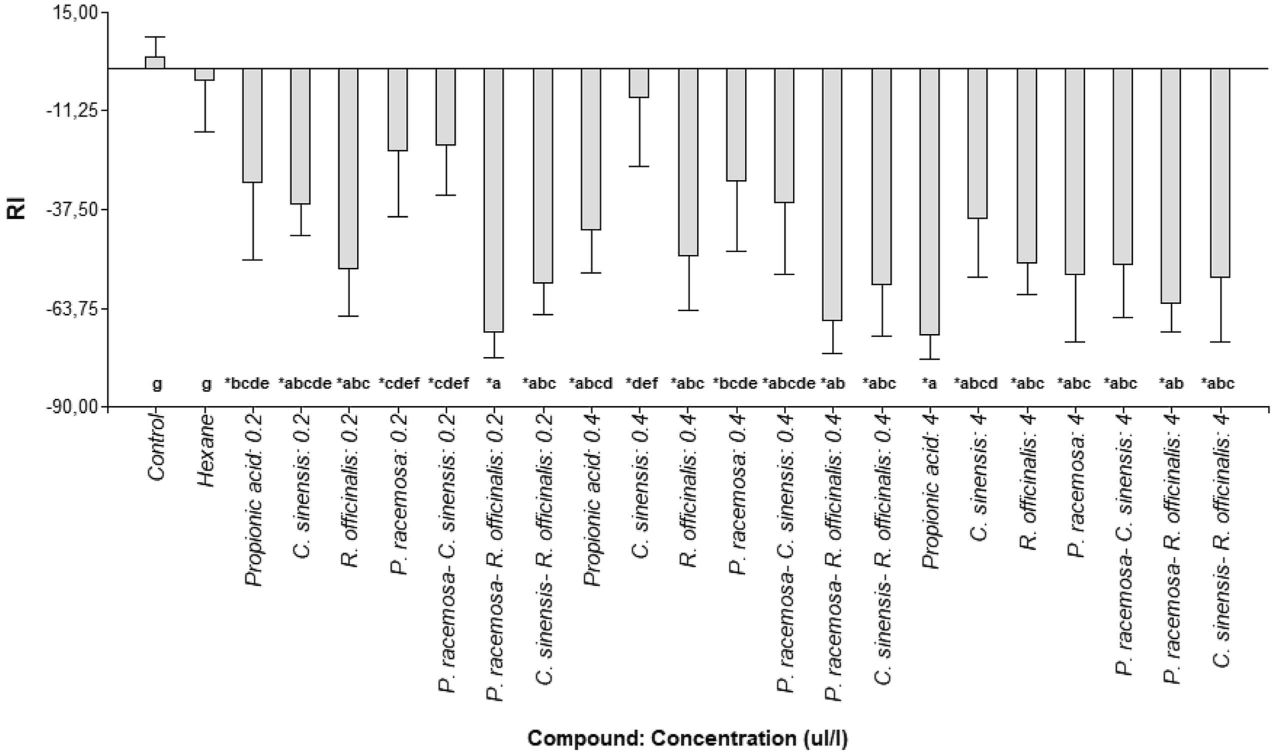


Fig. 2 Response of *Sitophilus zeamais* adults to individual and mixtures of EOs, evaluated at 4.0, 0.4 and 0.2 µl/l air in a two-choice olfactometer system. * Indicates significant difference with the control. Values with different letters are significantly different according to Duncan's multiple range test at $p \leq 0.05$ ($n = 5$); (−) values of RI indicate repellency; (+) values of RI indicate attraction

Table 3 Fumigant toxicity against *Sitophilus zeamai*s adults after 24 h of exposure to the combination of binary mixtures of essential oils and chlorpyrifos. Combinatorial index (CI) of combinations[a]

Essential oils	LC_{50} (µl/l)	95% Confidence interval (µl/l)	LC_{95} (µl/l)	95% Confidence interval (µl/l)	*Z*	CHI^2	CI
Pimenta racemosa–Rosmarinus officinalis–chlorpyrifos	35.97	27.00–55.87	85.31	62.20–163.50	5.44	37.41	1.59
Pimenta racemosa– Citrus sinensis–chlorpyrifos	8.05	0.72–14.38	47.84	35.74–77.82	7.14	96.23	0.05
Rosmarinus officinalis–Citrus sinensis–chlorpyrifos	108.80	43.63–303.53	220.24	155.51–413.37	4.71	58.08	3.35

Probit regression analysis using the SPSS software. Lethal concentration values were considered significantly different if their confidence limits did not overlap. The experiment was performed in five repetitions twice per concentration. a The values CI < 1, CI = 1 y CI > 1 indicate synergistic, additive and antagonistic effects, respectively

(CI = 0.005) against *S. zeamais*, with values for LC_{50} of 8.05 µl/l and LC_{95} of 47.84 µl/l.

The phytotoxic effects of the binary mixtures of EOs combined with chlorpyrifos on maize grains are shown in Table 4. The mixture of *P. racemosa–C. sinensis*–chlorpyrifos, evaluated at the concentrations corresponding to LC_{50} and LC_{95}, did not show significant differences with the control (DG= 2; *p*= 0.29), with a seed vigour of 115.64% ± 2.79 % for LC_{50} and 100.55 % ± 9.21 % for LC_{95}. Conversely, the mixtures of *R. officinalis–C. sinensis*–chlorpyrifos and *P. racemosa–R. officinalis*–chlorpyrifos inhibited grain germination. Maize grains exposed to LC_{95} of *R. officinalis–C. sinensis*–chlorpyrifos and LC_{50} and LC_{95} from *P. racemosa–R. officinalis*–chlorpyrifos showed vigours of 77.65% ± 12.01%, 81.84% ± 9.21% and 76.53% ± 6.14%, respectively.

Discussion

In the present study, the effect of five EOs on *S. zeamais* was investigated. The EO that showed the highest insecticidal activity was *R. officinalis,* followed by *P. racemosa* var. *ozua* and *C. sinensis*. The effect of *R. officinalis* (Duarte et al. 2015; Kiran and Prakash 2015; Khoobdel et al. 2017) and *C. sinensis* (Akono et al. 2016; Araujo et al. 2016; Oboh et al. 2017) EOs on many species of insects has been previously reported. However, to our knowledge, this is the first study reporting the insecticidal activity of *P. racemosa.* The efficiency of EOs as insecticides may be due to their high monoterpene content (Lee et al. 2004; Rozman et al. 2007). Monoterpenes can penetrate through the cuticle, respiratory and digestive systems of the insects (Gnankiné and Bassolé 2017), thereby affecting several neuronal pathways such as GABA and the cholinergic and octopaminergic systems (Rattan 2010). The insecticidal effect of 1,8-cineole, a natural monoterpene and the main component of *P. racemosa* and *R. officinalis* EOs, has been widely documented on a broad range of insects (Rossi and Palacios 2015; Cicera et al. 2017; Chaaban et al. 2017; Adjou et al. 2019). In addition, limonene, the major component of *C. sinensis* EO, has been reported as a contact insecticide against *S. zeamais* adults (Wang et al. 2015; Fouad and Da Camara 2017; Kamanula et al. 2017) and other insects (Jalaei et al. 2015; Botas et al. 2017; Prado-Rebolledo et al. 2017; El Aalaouia et al. 2019; Showler et al. 2019).

The results obtained in the present study revealed a repellent activity for all the evaluated EOs against *S. zeamais*, with *R. officinalis* being the most active EO. Previous studies have reported a repellent effect of this EO against *Sitophilus oryzae* (Kiran and Prakash 2015; Jayakumar et al. 2017) and *Sitophilus granarius* (Karakas 2017). Insects have sensory hairs in the antennae that perceive chemical substances, leading to different behavioural responses (Abd El-Ghany and Abd El-Aziz 2017; Romani et al. 2019), such as the repellent effect reported in the present study.

The binary mixtures of the EOs showed higher insecticidal and repellent effects compared to the individual EOs, possibly due to the synergic action of their main components. The synergistic effect of EOs on insects could be related to the ability of certain components of the EO to facilitate the uptake of bioactive compounds (Armstrong et al. 1951; Wang et al. 2005; Ahmad et al. 2006). Thus, this could explain why 1,8-cineole promoted the entry of minority compounds of certain EOs (Tak and Isman 2015, 2016, 2017), while inhibiting the activity of cytochrome P450 and carboxylesterases (Ruttanaphan et al. 2019), and may also be the reason for the synergism observed between the EOs evaluated in the present study. In agreement, some previous investigations found that the insecticidal and repellent activities of mixtures of different EOs against *S. zeamais* (Arena et al. 2017), *S. oryzae L.* and *Bruchus rugimanus Bohem* (Liu et al. 2006) were significantly higher compared to the individual EOs.

In order to try to reduce the use of chlorpyrifos, we evaluated the insecticidal effect of mixtures of the most active EOs with this insecticide. The *Pimenta racemosa–C. sinensis*–chlorpyrifos mixture improved the insecticidal effect against *S. zeamais*, which may have been due to synergism between the EO components and the synthetic insecticide. Moreover, the *P. racemosa–C. sinensis*–chlorpyrifos mixture did not affect the germination of maize grains. Related

Table 4 Seed vigour (%) of maize grains treated with LC$_{50}$ and LC$_{95}$ of mixtures of essential oils–chlorpyrifos[a]

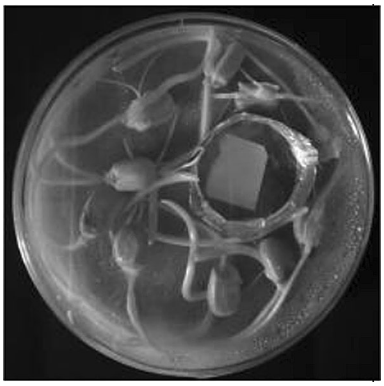
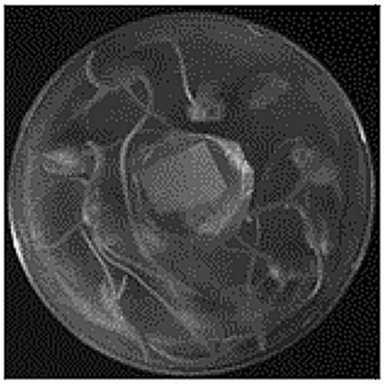
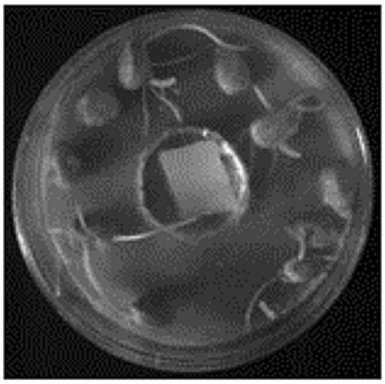
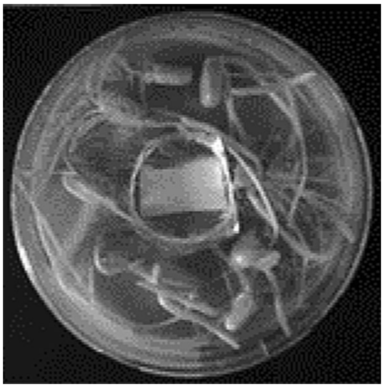
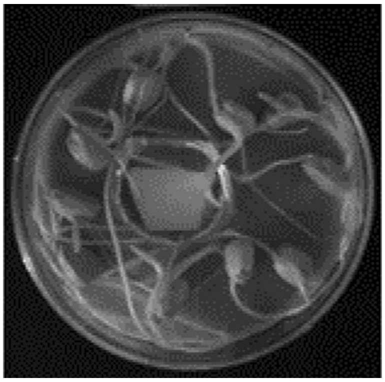
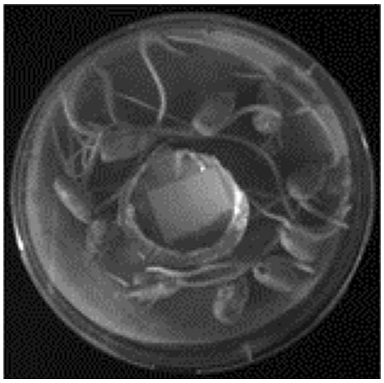
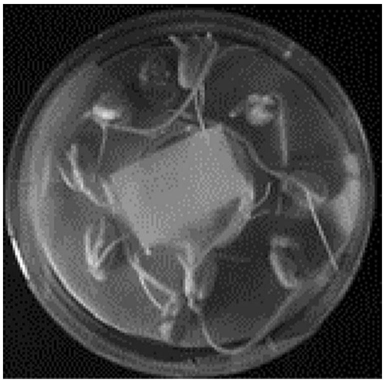
Treatments	Concentration	
	LC$_{50}$ (µl/l)	LC$_{95}$ (µl/l)
Chlorpyrifos	108.10 ± 7.82	111.45 ± 7.54
		
Pimenta racemosa–Rosmarinus officinalis–chlorpyrifos	81.84 ± 9.21*	76.53 ± 6.14*
		
Pimenta racemosa–C. sinensis–chlorpyrifos	115.64 ± 2.79	100.55 ± 9.21
		

Table 4 continued

Treatments	Concentration	
	LC_{50} (µl/l)	LC_{95} (µl/l)
Rosmarinus officinalis–*C. sinensis*–chlorpyrifost	98.04 ± 2.51	77.65 ± 12.01*
		

[a] Values were expressed as means ± SE. *Significant differences with the control according to DGC test ($p \leq 0.05$). To study the effect of the mixtures of EOs–chlorpyrifos on maize grains, the experiments were repeated twice in quintuplicate

to this, Saad and Abdelgaleil (2014) revealed a correlation between the chemical composition of EOs and their effects on germination, with the main compounds of *P. racemosa* and *C. sinensis*, 1,8–cineole and limonene, having been reported to be inhibitors of seed germination (Boukaew et al. 2017). However, the effect of mixtures of EOs on the germination of grains depends not only on the individual effects of the main constituents of each EO, but also on the interactions occurring between them (El-Bakry et al 2016).

In Argentina, chlorpyrifos is one of the most frequently applied insecticides in agricultural production (Lepori et al. 2013), and consequently, its presence as a residue in food has been reported worldwide (Australian Veterinary Pesticides and Medicines Authority 2009; Brancato et al. 2017). Chlorpyrifos residues above the maximum residual limit (MRL) have been detected in fruits, vegetables (Hjorth et al. 2011) and meat (Stefanelli et al. 2009). In addition, chlorpyrifos has been reported to contaminate soil as well as surface and underground water (Etchegoyen et al. 2017). Therefore, it is necessary to reduce the amount of chlorpyrifos to mitigate these negative effects related to its excessive use, but without losing its effectiveness. The LC_{95} value of the *P. racemosa*–*C. sinensis*–chlorpyrifos mixture was found to be higher than the LC_{95} value of chlorpyrifos, but considering that the mixing ratio between EOs and chlorpyrifos was 16:1, the net quantity of chlorpyrifos employed in the mixture is significantly reduced, evidencing a synergistic effect between the EOs and the insecticide. Future studies should now focus on the adaptation of this laboratory-based study for its practical application in storage technology.

In conclusion, the binary mixture *P. racemosa*–*C. sinensis* repels *S. zeamais* and, in combination with chlorpyrifos, has an insecticidal effect without affecting the germination of maize grains. Thus, the use of the *P. racemosa*–*C. sinensis*–chlorpyrifos combination decreases the amount of the synthetic insecticide required, providing an interesting alternative for the control of the maize weevil, *S. zeamais.*

Author contributions Statement

VDB wrote the manuscript and conducted experiments; FA wrote the manuscript and conducted experiments; RPP was involved in the research design; ARS contributed essential oils and provided ideas; EAGT donated essential oils and contributed ideas; JAZ performed essential oil analyses; MPZ performed statistical data analysis. All authors have read and approved the manuscript.

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