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An insecticide formulation of terpene ketones against *Sitophilus zeamais* and its incorporation into low density polyethylene films

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ABSTRACT

The use of active natural compounds derived from plants appears as an interesting alternative for pest control in stored products. In this study, the fumigant toxicity of several terpene ketones against maize weevil *(Sitophilus zeamais)* adults was assessed, and the effect of their combination with piperonyl butoxide (PBO), a cytochrome P450 inhibitor commonly used as synergistic agent, was investigated. A mixture of the most active ketones (*R*-(+)-pulegone and thymoquinone) was then tested on *S. zeamais*, and finally this mixture was incorporated into low density polyethylene (LDPE) films by supercritical CO_2 impregnation, in order to study the fumigant toxicity of this formulation against *S. zeamais* under laboratory conditions, as well as the release profile of the active ketones. PBO showed antagonistic effects in combination with terpene ketones, decreasing the toxicity of the individual compounds and therefore increasing their lethal concentrations. The mixture of ketones showed a lower lethal concentration (LC_{50} value of 7.12 μL/L air) than the individual compounds, thus indicating a synergistic effect between them. Finally, the impregnated films revealed high toxicity values (up to 95% mortality), and maintained a moderate level of activity even after 8 days of exposure. In conclusion, the mixture of these active terpene ketones incorporated into a polyethylene film, material commonly used for hermetic storage of maize grains, could be applied as a potential technology against insect pests.

1. Introduction

The large number of products obtained from maize for human and animal nutrition, as well as for industrial use, makes it one of the most important crops worldwide from an economic point of view. World production in the 2016/2017 season is estimated to be 1028.4 million tons, Argentina being the main exporting country, and contributing 36.5 million tons (USDA, 2015). Grain losses due to insect pest attacks could represent 15–50% of total production, depending on the country (Casini and Santajulia, 2015). In this sense, the post-harvest pest of stored maize *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) is considered to be extremely destructive because its larvae and adults feed and develop inside the stored grains (Oerke, 2006). This situation is aggravated by contribution to the dispersal of fungal spores by *S. zeamais*, through feeding damage, which provides entry points for fungal infections, favouring the production of mycotoxines by fungi (Ferreira-Castro et al., 2012). A large number of synthetic insecticides have been used in agriculture to reduce losses of stored products, particularly those due to *S. zeamais*. Nevertheless, their incorrect application, over-intensive use and the increasing amount of pesticides utilized have led to increasing human health risks, environmental damage and the emergence of pest resistance (Derera et al., 2010; Pimentel et al., 2009; Zhang et al., 2015).

During the last two decades, the silo bag technology has been implemented in more than 40 countries for grain storage in the field, reducing post harvest losses (Bartosik, 2012). Silo bags consist basically of a bilayered or trilayered polymeric film, generally made of polyethylene, which protects from UV radiation and acts as a gas barrier, providing hermetic storage. However, the modification of the internal atmosphere conditions may not be sufficient to generate a lethal environment against insects, which can affect these hermetic storage systems by direct attack or by infestation if the material is damaged. Thus, silo bags are usually fumigated (for

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example, with phosphine) in order to control insect pests (Ridley et al., 2011). Nevertheless, insect resistance to phosphine and control failures have been reported (Pimentel et al., 2009). Alternatively, more innovate tools, such as polypropylene bags loaded with an insecticide (deltamethrin) (Wasala et al., 2016), have been designed to protect grains and seeds against destructive insect pest infestations during storage.

In this context, there is an increasing tendency to use different combinations of methods to reduce storage pest levels while minimizing environmental risk, with the use of natural compounds derived from plants —such as terpenes— as an interesting alternative for pest control (Boulogne et al., 2012). The insecticidal toxicity of several essential oils and/or some of their pure volatile components against *S. zeamais* has been reported by numerous authors (de Lira et al., 2015; Liu and Ho, 1999; Paes et al., 2012; Tapondjou et al., 2005). Particularly, previous studies carried out by our research group have shown that α,β-unsaturated terpene ketones present fumigant as well as contact toxicity against this insect pest (Herrera et al., 2015a, 2015b, 2014). Among the screened ketones, *R*-(+)-pulegone and thymoquinone showed the highest activity. These ketones are naturally produced by several plants. Thymoquinone is the main active constituent of *Nigella sativa* L. essential oil, and it is also present in other genera such as *Monarda* and *Juniperus*, while *R*-(+)-pulegone is a natural constituent of the essential oil of several species from the genus *Mentha* (mainly *Mentha piperita* L.).

Due to the generally high volatility of terpenic compounds, it is often advantageous to incorporate them into polymeric matrices in order to obtain controlled release systems (Nerio et al., 2010). Among these, polyethylene is a convenient alternative because it is a commercial, widely available and inexpensive polymer, with a broad range of properties, and as mentioned before it is the main constituent of silo bags. Among the different technologies for the incorporation of active compounds in polymers, impregnation using supercritical fluids as solvents —particularly carbon dioxide— is a novel technique with interesting advantages: it can be operated at low temperatures, thus preserving thermolabile compounds; terpenes are highly soluble in supercritical CO_2; and solvent-free products are obtained (Cocero et al., 2009; Kikic and Vecchione, 2003; Kiran, 2016). Moreover, CO_2 is a non-toxic, non-inflammable and inexpensive solvent. Recently, some authors have investigated the impregnation of polyethylene films with organic active compounds, such as thymol (Torres et al., 2014), 2-nonanone (Rojas et al., 2015) and eugenol (Goñi et al., 2016) in order to develop active materials for food packaging and preservation, thus demonstrating the feasibility of this process. To our knowledge, although this technology has been studied in the case of films in contact with liquid food simulants, it has not been applied in the development of active materials for grain storage and protection.

The aim of this contribution was to evaluate the fumigant toxicity of a mixture of active ketones against *S. zeamais* and its effect on maize grain germination. In addition, the potential effect of piperonyl butoxide (PBO), a cytochrome P450 inhibitor commonly used as synergistic agent of pesticides, on the toxicity of the active ketones was also investigated. To the best of our knowledge, only a few studies have focused on the effect of commercial synergistic agents such as PBO on the toxicity of terpene compounds or essential oils against *S. zeamais* (Khater, 2012; Koul et al., 2008). The effect of PBO on the metabolism of *S. zeamais* was also evaluated by analysis of the biotransformation products desorbed from weevils treated with terpene ketones in presence and absence of PBO. Finally, a mixture of the most active ketones was incorporated into low density polyethylene (LDPE) films by supercritical CO_2 impregnation in order to investigate the obtaining of an active material with potential application in grain storage. Moreover, the fumigant toxicity of these films against maize weevils as well as the release profiles of active ketones from the films to the air were evaluated under laboratory conditions.

2. Materials and methods

2.1. Chemicals

R-(+)-pulegone, thymoquinone, *R*-carvone, *S*-carvone, dihydrocarvone, PBO and menthofuran were purchased from Sigma-Aldrich (Steinheim, Germany). The chemical structure of these compounds is shown in Fig. 1. HPLC grade methanol and acetone were obtained from Sintorgan (Argentina). Industrial extra-dry carbon dioxide (Linde, Argentina) was used as the impregnation solvent. LDPE films (150 μm thickness, M_n: 2250, M_w: 229300 Da; Dow-Polisur, Argentina) were used as impregnation matrix.

2.2. Insects

Adults of *S. zeamais* Motschulsky (Coleoptera: Curculionidae) were collected from Metán, Salta, Argentina, and this colony was maintained in the laboratory for three years without exposure to insecticides. Weevils were reared on sterilized maize grains in sealed containers at 28 °C under a 12:12 h light-dark cycle (FAO, 1974) and 70% humidity. Adults of *S. zeamais* of both sexes and different ages were used in the experiments.

2.3. Fumigation bioassays

The fumigant toxicity of five ketones (*R*-(+)-pulegone, thymoquinone, *R*-carvone, *S*-carvone, dihydrocarvone) in combination with PBO against *S. zeamais* was evaluated following the methodology described by Herrera et al. (2015a), with some modifications. Briefly, two hours before treatment with ketones, weevils were placed in 30 mL glass vials (ten insects in each one) containing sublethal doses of PBO (0.5 μL/insect or 5 μL/glass vial) and sealed with a screw cap. Then, different dosages of the pure ketones (0.6–18 μL) were incorporated into the vials. Mortality was determined after 24 h of exposure by counting the dead insects, and from these values the corresponding lethal concentrations (LC_{50} and LC_{95}) were calculated. Controls were assessed under the same conditions but without the addition of ketones. The toxicity of the pure ketones in the absence of PBO was also tested using the same methodology in order to confirm the LC_{50} values reported in previous studies (Herrera et al., 2015a, 2015b, 2014). All fumigant activity assays were replicated five times and performed in complete darkness.

A mixture of *R*-(+)-pulegone and thymoquinone (corresponding to the ketones with the highest toxicity values), in an approx. 1:1 mass ratio, was tested against *S. zeamais* using the same fumigant assay described above. The mixture doses ranged from 0.096 μL to 0.771 μL and controls were assayed under the same conditions without incorporation of the ketone mixture. Mortality was determined after 24 h of exposure, and these values were used to calculate lethal concentrations (LC_{50} and LC_{95}). The commercial insecticide dichlorvos (DDVP) was used as a positive control. Each assay was replicated at least five times. In order to establish the type of interaction that occurred in the mixture (synergism, additive effect or antagonism) the combination index (CI) was calculated using the software Compusyn (Chou and Martin, 2005). Thus, for each combination of terpene concentrations the CI was calculated according to Eq. (1):

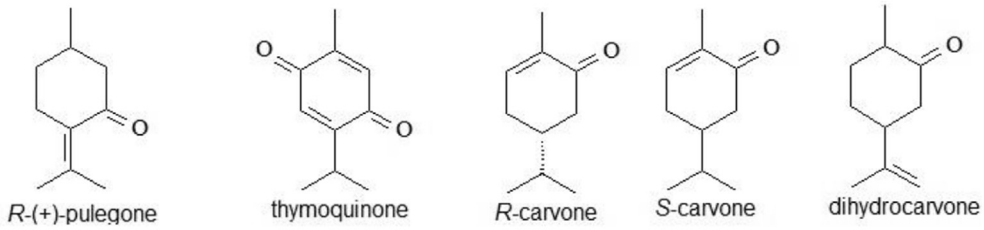

Fig. 1. Chemical structures of the ketone compounds used in the present work.

$$CI = \frac{T_{1x}}{T_1} + \frac{T_{2x}}{T_2} \quad (1)$$

where T_1 and T_2 are the LC_{50} values of each individual terpene, while T_{1x} and T_{2x} are the concentrations of each terpene that produce the same effect when applied in combination. The results were interpreted as: $CI < 1$: synergism; $CI = 1$: additive effect; $CI > 1$: antagonism (Chou, 2006).

2.4. SPME/GC analysis of volatiles absorbed and desorbed by S. zeamais

The analysis was performed using the methodology described by Rossi et al. (2012), with some modifications. Fumigation bioassays were carried out using the LC_{95} values of the pure ketones (with and without PBO) against *S. zeamais*. Then, with the aim of quantifying the terpenes absorbed and desorbed by the weevils, approx. 100 dead insects were collected in a 10 mL vial with septum. This vial was placed in a water bath at 60 °C for 30 min, after which, a solid phase micro extraction (SPME) microfiber (PDMS 100 μm, Supelco, Bellefonte) was placed into the vial for 15 min to capture the desorbed terpenes. Finally, the microfiber was injected in a Perkin-Elmer Clarus 600 gas chromatograph coupled with an ion trap mass detector (GC-MS) for terpene identification and quantitation. An ELITE 5-ms capillary column (60 m × 0.25 mm i.d. and 0.25 μm coating thickness) was used for the separation of the individual components. The chromatographic conditions were: injector at 250 °C; oven temperature programming: 50 °C (2 min) – 5 °C/min (1 min) – 240 °C (5 min), total run time 45 min; and constant detector temperature of 240 °C. Helium was used as the carrier gas (flow rate = 1.5 mL/min) and compounds were identified by comparing their retention indices and mass spectra with published data (Adams, 2007) and available libraries (NIST). The main compounds were confirmed by coinjection of pure standards (Sigma, USA).

2.5. Maize grain germination bioassay

The effect of the active mixture on the germination of maize grains was evaluated according to the method reported by Herrera et al. (2015a). Briefly, maize grains were sterilized with 2% sodium hypochlorite for 5 min and then rinsed with distilled water. Two filter papers (8 cm diameter) and 10 grains were placed in Petri dishes, after which, 5 mL of distilled water were added and an aluminium foil (2 cm diameter) was placed in the centre. The ketone mixture was then incorporated over this aluminium foil, at a concentration equal to its LC_{50}. A sample of grains with no addition of ketone mixture was used as a control. The Petri dishes were closed and sealed with parafilm to prevent evaporation of terpenes and then placed in a growth chamber at 27 ± 2 °C and a relative humidity of 60 ± 2% for 5 days. After 5 days, the number of germinated grains was counted, and the germination rate was calculated using Eq. (2):

$$Germination\ rate = \sum \left(\frac{n}{d}\right) \quad (2)$$

where n was the number of germinated grains on each day, and d was the number of days from the beginning of the test (Agrawal, 1980). Each treatment was replicated five times.

2.6. Supercritical CO_2 impregnation

The supercritical impregnation of the LDPE films with an active mixture of ketones was performed in a 50 mL high-pressure cell with magnetic stirring and temperature control, described elsewhere (Goñi et al., 2016). The initial mass of *R*-(+)-pulegone and thymoquinone to be loaded into the cell was determined in previous assays, by considering the estimated solubility of these compounds in supercritical CO_2 in order to ensure complete dissolution in the fluid phase. Briefly, 3 films of 5.4 cm^2 were placed into the cell using a metal support, and subsequently 100 μL *R*-(+)-pulegone and 0.12 g thymoquinone were added. The metal support prevented the contact between the films, thus ensuring a homogeneous impregnation through both sides of the films, and also maintained the films in a vertical position to avoid deposition of solutes on the film surfaces during depressurization. Impregnation was performed at a constant temperature and pressure conditions (45 °C and 15 MPa) for 4 h, under agitation, and depressurization was carried out at a constant rate of 0.5 MPa/min and controlled with a micrometering valve (Swagelok, USA). These operation conditions were selected from a previous work (Goñi et al., 2016) as well as other literature reports (Rojas et al., 2015; Torres et al., 2014), in order to enhance the impregnation loading. The mass of ketones incorporated into the films was determined gravimetrically, by measuring the film mass increase in a precision balance after gently cleaning the film surface with tissue paper, in order to remove residual amounts of deposited ketones. Despite their volatility, the evaporation of impregnated ketones was not fast enough to affect the measurement. The possible effect of moisture adsorption/desorption was checked using a control sample (non impregnated film), which was weighed with the samples before and after the impregnation process. Preliminary runs were also performed by subjecting film samples to high pressure CO_2 (without ketones) in order to check that the film weight was not affected by the pressurization-depressurization cycle itself, and that the CO_2 desorption was complete within few minutes before weighing.

2.7. Insecticidal activity of the impregnated films

The fumigant toxicity of the impregnated films against *S. zeamais* was tested using a methodology described by Herrera

Table 1
Fumigant toxicity of terpene ketones (with and without PBO) against *S. zeamais*, after 24 h of exposure.

Compounds	LC_{50} (μL/L)	95% CL (μL/L)	LC_{95} (μL/L)	95% CL (μL/L)	χ^2	Slope ± S.E.
pulegone + PBO	31.0	16.8–47.5	55.8	41.9–114.9	9.78	2.79 ± 0.465
pulegone[a]	11.9	11.2–12.6	14.2	13.2–16.9	–	–
thymoquinone + PBO	40.3	36.1–44.5	62.7	56.6–72.4	0.116	0.073 ± 0.010
thymoquinone[a]	13.8	12.7–14.9	19.2	17.5–22.4	–	–
R-carvone + PBO	225.2	163.7–444.5	409.8	292.5–1098.0	7.21	2.01 ± 0.231
R-carvone[a]	17.6	15.2–19.9	28.7	25.6–33.9	–	–
S-carvone + PBO	165.4	148.1–184.5	277.2	242.5–348.7	0.009	4.545 ± 0.633
S-carvone[a]	28.1	23.6–33.3	54.2	46.9–65.2	–	–
dihydrocarvone + PBO	266.6	234.9–305.7	480.2	418.8–580.2	8.48	3.41 ± 0.54
dihydrocarvone[a]	30.5	27.1–33.4	50.3	45.9–57.2	–	–

CL: confidence limits.
χ^2 (chi-square value, significant at $p < 0.05$) and slope are the parameters of the probit regression for calculating the LC values.
[a] LC_{50} values were reported by Herrera et al. (2015a, 2015b, 2014).

et al. (2015b), with modifications. Ten adults and some maize grains (1.3 ± 0.04 g) were placed in 30 mL glass vials sealed with screw caps. The impregnated films were placed in the inner side of the caps, supported by a metallic mesh to prevent the direct contact with the weevils. Controls were kept under the same conditions with non-impregnated LDPE films. After 24 h, the mortality was determined by counting the dead insects and the same screw caps containing the impregnated films were removed and placed in new vials (with new weevils and grains). This procedure was repeated at 48, 72, 96 and 168 h from the beginning of the assay in order to evaluate the residual toxicity of the same impregnated films at each step under headspace renewal conditions, over 8 days. Experiments were performed by triplicate.

2.8. Release profile of active compounds

The release profile of the active compounds from the films into air in a confined environment was assayed. For that purpose, impregnated films of 1 cm^2 were placed inside 100 mL flasks, hermetically sealed and stored in a dark place at room temperature for 24, 48 and 72 h. The residual mass of ketones in the samples after each period of storage was determined by liquid extraction with methanol, followed by GC-FID determination. The films were stirred in 5 mL methanol for 4 h, and the extracts were analyzed in a Perkin-Elmer Clarus 500 apparatus coupled with a flame ionization detector (GC-FID), and equipped with a capillary column (EC^{tm}; 30 m × 0.32 mm × 1 μm film; Altech). The extraction procedure was repeated under the same conditions until no further residue was observed in the films, thereby ensuring total extraction. The chromatographic conditions were as follows: injector at 240 °C; oven temperature programming: 60 °C (3 min) − 8 °C/min (1 min) − 170 °C (2 min), total run time 17 min; and constant detector temperature of 240 °C. Nitrogen was used as the carrier gas (flow rate = 0.8 mL/min) and standards of *R*-(+)-pulegone and thymoquinone were used for peak identification (Sigma, USA). A calibration curve was constructed using a concentration range of 0.32–38 μg/mL for both *R*-(+)-pulegone and thymoquinone, and a linear response was obtained (R^2 = 0.996 and 0.997, respectively). The amount of ketones released at different times was calculated according to Eq. (3):

$$\%\ Released = \frac{m_{t0} - m_{ti}}{m_{t0}} \times 100 \quad (3)$$

where, m_{t0} refers to the mass of ketone initially incorporated into the film by CO_2 impregnation (in g/g of film), and m_{ti} denotes the mass that remained in the film after time t_i (determined by methanol extraction and GC analysis). Release experiments as well as chromatographic determination were performed by duplicate.

2.9. Statistical analysis

Mortality data values (LC_{50} and LC_{95}) were subjected to probit regression analysis using POLO PLUS software (POLO-PLUS, LeOra Software, USA) with the LC values being considered to be significantly different if 95% confidence limits did not overlap. A regression analysis was performed for the release profile study. All statistical analyses were performed using the software Infostat Professional 2010p (Di Rienzo et al., 2016).

3. Results and discussion

3.1. Toxicity of terpene ketones and active mixture

The fumigant toxicity of the five terpene ketones studied (*R*-(+)-pulegone, thymoquinone, *S*- carvone, *R*-carvone and dihydrocarvone), applied alone and in combination with PBO, against *S. zeamais* is reported in Table 1 in terms of LC_{50} and LC_{95} values. In the presence of this cytochrome P450 inhibitor, the LC values of all these compounds increased (2, 1, 6, 9, 12 times, respectively) compared to those observed without PBO, indicating the occurrence of antagonistic effects. A similar antagonistic behaviour was also observed by Rossi et al. (2012) for pulegone and menthone against *Musca domestica* L.

It can be seen that the compounds that presented the highest toxicity levels were *R*-(+)-pulegone and thymoquinone, showing the lowest LC_{50} and LC_{95} values among the set of ketones studied.

On the other hand, Table 2 shows the quantitative results of the SPME analysis of the ketones absorbed by the weevils as well as the volatile compounds formed as a result of the insect metabolism mediated by the cytochrome P450. The chromatographic analysis revealed that in absence of PBO, *R*-(+)-pulegone was biotransformed to menthofuran (relative proportion of 42%), following an oxidative pathway. Although this conversion has been described previously in several animal and plant organisms (Bertea et al., 2001; Chen et al., 2001, 2011; Rossi et al., 2012), to the best of our knowledge it has not been reported for *S. zeamais*. On the contrary, in the presence of PBO this biotransformation is inhibited, as indicated by the lower proportion of menthofuran detected (relative percentage of 10%). Considering that menthofuran is approx. three times more toxic than *R*-(+)-pulegone, presenting a LC_{50} value of 3.4 μL/L (data not shown), we can ascribe the observed antagonistic effect of PBO to the inhibition of the biotransformation of *R*-(+)-pulegone into a more toxic derivative. In the absence of PBO, thymoquinone, *S*, *R*-carvones and dihydrocarvone were

Table 2
Percentage of menthofuran, thymol, carveol and dihydrocarveol recovery from dead weevils by SPME/GC analysis.

Sample	Relative percentage (%) [a]	
	R-(+)-pulegone	menthofuran
Weevils dead by action of *R*-(+)-pulegone	57.9 ± 1	42.1 ± 0.5
Weevils dead by action of *R*-(+)-pulegone + PBO	89.5 ± 4.95	10.5 ± 4.95
R-(+)-pulegone	99.9 ± 0.1	nd
	thymoquinone	**thymol**
Weevils dead by action of thymoquinone	97.6 ± 0.7	2.4 ± 0.3
Weevils dead by action of thymoquinone + PBO	98.76 ± 0.2	1.24 ± 0.1
thymoquinone	100	nd
	***R*-carvone**	**carveol**
Weevils dead by action of *R*-carvone	98.3 ± 1.15	3 ± 1.15
Weevils dead by action of *R*-carvone + PBO	100	nd
R-carvone	100	nd
	S-**carvone**	**carveol**
Weevils dead by action of *S*-carvone	97 ± 1.02	3 ± 0.5
Weevils dead by action of *S*-carvone + PBO	99.8 ± 0.2	nd
S-carvone	100	nd
	dihydrocarvone	**dihydrocarveol**
Weevils dead by action of dihydrocarvone	97.8 ± 1.62	2.2 ± 1.62
Weevils dead by action of dihydrocarvone + PBO	100	nd
dihydrocarvone	100	nd

nd: not detected.
[a] Mean value ± S.E.

metabolized to thymol, carveol and dihydrocarveol, respectively, at low relative percentages (<5%). Carveol and dihydrocarveol were not detected in insects treated with PBO, while thymol was present at a low concentration. To the best of our knowledge, this is the first study that shows the conversion of terpene ketones by *S. zeamais*.

Due to the fact that *R*-(+)-pulegone and thymoquinone showed the highest toxicity levels, these compounds were selected as components for the active mixture to be evaluated. The toxicity of this active mixture against *S. zeamais* was tested in terms of the LC_{50} after 24 h exposure. The fumigant assay revealed the occurrence of synergism between the two ketones, with a combination index (CI) of 0.48, and a LC_{50} value of 7.12 μL/L air. Accordingly, several studies have reported that mixtures of terpenes show higher toxicity than their individual components (Gallardo et al., 2012). The positive control (DDPV) showed a LC_{50} value lower than 0.06 μL/L air.

The percentage of seed germinated in the presence or absence of the active mixture during 5 days is shown in Fig. 2, and the germination rate (as a measure of the seed vigor) were calculated from these values according to Eq. (2). The kernels exposed to the active mixture showed a germination rate of 105± 3.25% relative to the germination rate of the control sample (considered as 100%). However, this difference was not statistically significant, indicating that the mixture did not have any effect on maize germination after 5 days exposure.

3.2. Insecticidal activity and release profile of impregnated films

The content of active mixture incorporated into the films by supercritical CO_2 impregnation was 41.2 ± 7.0 g/kg. Although *R*-(+)-pulegone and thymoquinone were loaded into the cell at approx. 1:1 mass ratio, the chromatographic analysis revealed that their mass ratio in the impregnated films was almost 2:1, indicating that the impregnation process showed some degree of selectivity for *R*-(+)-pulegone.

The observed total loading values are in agreement with data reported by other authors using the same technique and compounds with comparable volatility and molecular weight (Rojas et al., 2015; Torres et al., 2014). Due to the higher penetration and swelling ability of supercritical CO_2 compared to conventional liquid solvents, the ketones were trapped inside the polymer matrix, and not only superficially. Therefore, they can be gradually released by diffusion from the polymer bulk to the surface, and bioactivity could be extended to longer periods. The impregnation efficiency is affected by two distinct phenomena. On one side, the absorption of CO_2 at high pressure conditions promotes the swelling of the polymeric matrix, with an increase of the ketones diffusivity, which can penetrate more easily and more deeply into the films. On the other hand, the ultimate distribution of ketones between the polymer phase and the fluid phase is limited by the thermodynamic partition equilibrium. The observed selectivity of the process towards *R*-(+)-pulegone suggests that this compound has a higher affinity for the polymer phase than thymoquinone (or, inversely, that thymoquinone partitions preferentially in the supercritical fluid phase), in spite of their similar molecular weight and chemical structure. A possible explanation is based in the lower dipole moment of thymoquinone ($\mu = 0.12$ D versus $\mu = 2.88$ D for *R*-(+)-pulegone) (Herrera et al., 2015b), due to the oppositely located carbonyl groups that tend to cancel out. In this sense, the less polar compound (thymoquinone) may have a higher affinity for the supercritical CO_2 phase, which acts as a non-polar solvent, and be more easily dragged out of the polymeric matrix by CO_2 during the depressurization step.

The toxicity of the impregnated films against *S. zeamais* is shown in Fig. 3, in terms of mortality percentage, during the different stages of the experiment. In the first two steps (24 and 48 h), the experiment revealed that insects exposed to the impregnated films exhibited a constant and high mortality percentage (93± 0.7%), suggesting that under these experimental conditions the impregnated films released to the flask atmosphere a concentration of active compounds similar to the LC_{95} value. At the third step (72 h), the insect mortality decreased to 50%, and finally, in the last two

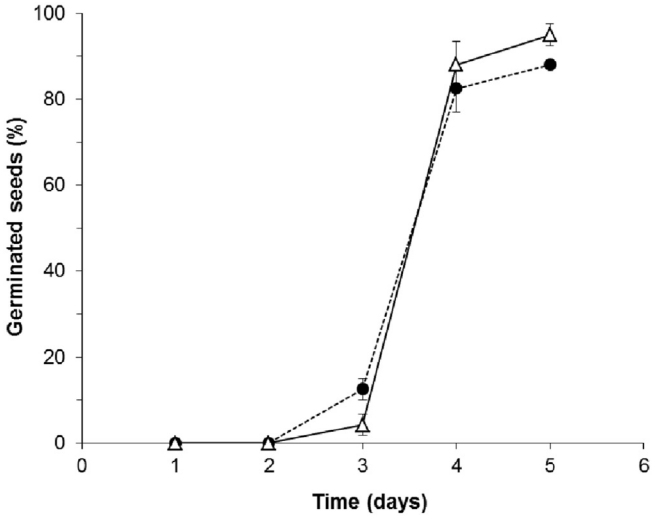

Fig. 2. Percentage of seed germinated when treated with (Δ) and without (●) the ketone mixture, during 5 days of exposure. Vertical bars indicate ± the standard error.

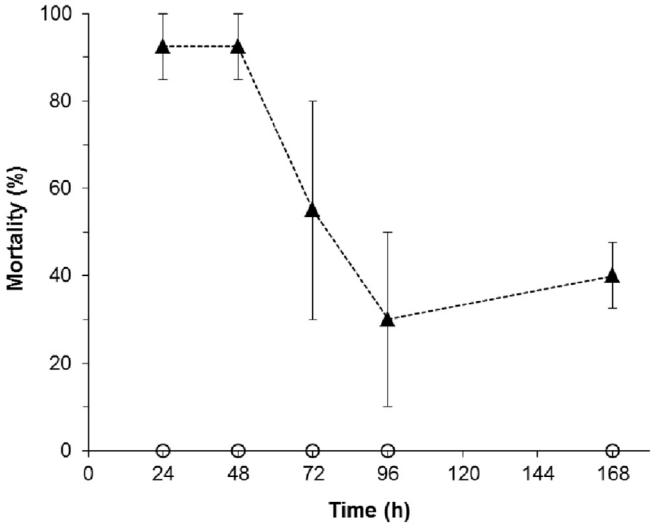

Fig. 3. Toxicity of impregnated films (▲) compared to untreated films (○), against *S. zeamais*, during 8 days. Vertical bars indicate ± the standard error.

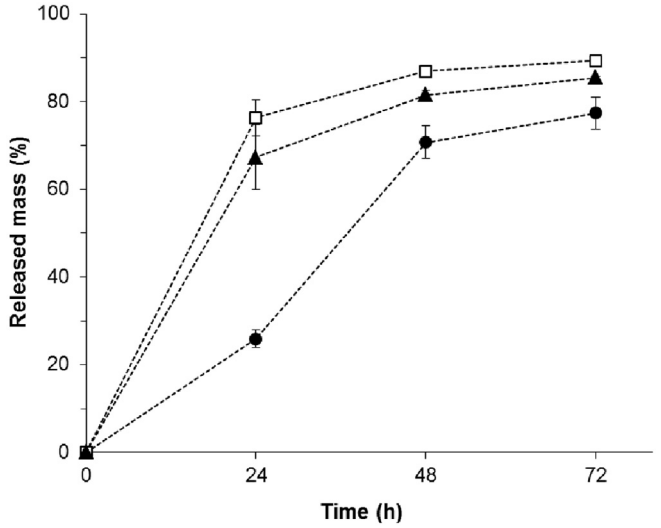

Fig. 4. Release profile of the ketone mixture (▲) and individual active components: (□) *R*-(+)-pulegone, (●) thymoquinone, into air. Vertical bars indicate ± the standard error.

stages (96 and 168 h), mortality decreased to approximately 30%.

The release profile of the mixture and its active components into air during 72 h of storage is shown in Fig. 4. The results showed that *R*-(+)-pulegone is released at a higher rate than thymoquinone: during the first 24 h, 75% of the total *R*-(+)-pulegone and only 25% of the total thymoquinone are released. At longer periods (48–72 h), the release rate decreased, with an almost constant headspace concentration in the last step, corresponding to a release of approx. 80% of the active mixture.

The release profiles of the mixture and the individual ketones can be compared with the toxicity bioassay results previously presented (Figs. 3 and 4). During the first 48 h, the high release rate of the active compounds –due to the higher initial concentration and the evaporation of the molecules located near the film surface– corresponded to the initially high mortality levels. Then, as the diffusion inside the film became the rate-controlling step, the decreasing release rates were reflected in moderate toxicity values. Results also showed an important difference in the release rate of each component of the active mixture, especially during the first 24 h. *R*-(+)-pulegone revealed a higher release rate in comparison with thymoquinone (0.31 and 0.06 g/kg·h, respectively), which may be explained in terms of the higher initial concentration of *R*-(+)-pulegone into the films as well as by differences in their physicochemical characteristics, such as vapor pressure, molecular geometry and physical state (*R*-(+)-pulegone is liquid at room temperature while thymoquinone is solid). All these aspects might also explain the lower diffusion rate of thymoquinone observed inside the films.

In conclusion, botanical insecticides have been proposed as an alternative to synthetic pesticides in an effort to reduce the environmental impact of current agricultural practices. The present study revealed that terpene ketones in the presence of PBO had lower toxicity values against *S. zeamais* than in absence of this P450 cytochrome inhibitor. The toxicity of an active mixture of *R*-(+)-pulegone and thymoquinone indicated the occurrence of synergistic effects between these ketones, with a combination index of 0.48. In addition, the toxicity of the impregnated LDPE films with this mixture on *S. zeamais* was evaluated and correlated with the release profile of the active ketones, showing initially high mortality levels and a fast release rate of the active components in the first two stages of the experiment (up to 48 h) and maintenance of moderate activity even after 8 days of exposure. Based on these results, and considering that *R*-(+)-pulegone and thymoquinone have low toxicities to mammals (LC_{50} values of 500 mg/kg and 90.3 mg/kg, respectively, when tested in rats (Mansour et al., 2001)), the incorporation of this synergistic mixture into polymeric materials, commonly used as storage bags, could be envisaged as a potential tool for controlling pests and so reduce the grain losses within hermetic bags, whilst ensuring food safety.

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