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Bioactivity of Essential Oil from *Citrus aurantium* Peel against the Pulse Beetle *Callosbruchus maculatus* F. on Chickpea

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Abstract: Plant essential oils (EOs) and their active compounds are recognized as sustainable tools for the management of arthropod pests. The bitter orange, *Citrus aurantium* L. (Rutaceae), is a widespread tree in the Mediterranean region that is used especially as a rootstock for other *Citrus*. Although most of this plant's parts are *accredited* with medicinal properties, its fruits are not consumable and generally considered as non-valued waste. The aim of this work was to assess the potential use of the EO extracted by hydrodistillation from *C. aurantium* peel in the fumigation of chickpea seeds against *Calloosbruchus maculatus*. Analysis of EO of the bitter orange peel by gas chromatography coupled with a mass spectrometer (GC-MS) identified twenty-two compounds with limonene as the major component (86%). EOs of *C. aurantium* peel exerted toxic effects, in a concentration-dependent manner, on eggs (LC_{50} = 62.7 µL/L air), larval stages inside the seed (LC_{50} = 62.8 µL/L air), and adults (females: LC_{50} = 148 µL/L air and males: LC_{50} = 109 µL/L air). The *C. aurantium* EO also negatively affected the biological and demographic performances of the weevil compared to the untreated control. Fecundity and the number of emerged adults were reduced by more than 57 and 71, respectively, while the net reproduction rate and the intrinsic rate of increase were respectively decreased by over 71% and 37%, resulting in the total extinction of the pest at a concentration equal to 100 µL/L air. Our findings suggest the possible valorization of bitter orange peel by using them as a source of bioinsecticide to be integrated within sustainable programs for the management of stored product pests. Further studies are needed to verify similar uses of essential oils extracted from solid wastes from citrus-processing industries.

Keywords: *Citrus* peel essential oil; botanical insecticides; alternative control method; *Callosobruchus maculatus*; stored product pests

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1. Introduction

Mitigation of ecotoxicological, environmental, and social consequences of synthetic pesticide use is a key pillar of any Integrated Pest Management strategy. In this framework, botanical-based biorational products (e.g., extracts and essential oils (EOs)) have been drawing considerable interest among researchers and consumers [1,2]. Indeed, a continuously increasing research body has highlighted the effectiveness of essential oils in the control of arthropod pests [1,3]. Among other essential oils studied, the insecticidal properties of *Citrus* EO peel have already been reported against several agricultural pests [4–8], which makes these compounds a promising alternative for pest management.

Additionally, sustainable food systems require the value maximization of the by-products of processed food production. For instance, in the citrus-processing industries, one of the main concerns is the management of solid wastes, usually consisting of peels, seeds, and leaf residues remaining after the juice extraction process. These wastes can be a

valuable source of secondary products such as EOs that are acclaimed as natural pesticides and repellents. In this context, the Moroccan citrus fruits industry constitutes a strategic economic sector, with over 57 thousand hectares of cultivated orange trees producing more than 1 million tons of fruits per year [9]. Furthermore, the bitter orange *Citrus aurantium* L. (Rutales: Rutaceae), popularly called "Ranj", is a very abundant tree in green spaces. The fruits are available at low cost and their pulp is used to macerate olives while their peel is directly discarded into nature, causing both the loss of valuable active compounds and pollution of the environment.

The bean weevil *Callosbruchus maculatus* F. (Coleoptera, Chrysomelidae: Bruchinae) is a very serious pest of pulses [10–13]. The larvae of this bruchid can cause, during its development, severe qualitative and quantitative losses due to its feeding activity on the internal content of the seeds. Currently, the management of *C. maculatus* is based exclusively on the use of synthetic insecticides with all the disadvantages inherent to these products including the development of resistance [14], as well as intoxication risks for consumers and non-target organisms. EOs of different plant species have been tested against the bean weevil with contrasting results [12,15–19].

Thus, this work aimed to extract and chemically characterize EO from the peel of *C. aurantium* fruits and subsequently test its toxicity and effects on the biological and demographic traits of the *C. maculatus.* Besides mitigating the undesirable effects of non-valued agricultural by-products on the environment, our study intended to promote citrus EOs as protectants of stored seeds against semenivorous pests.

2. Materials and Methods

2.1. Plant Material and EO Extraction

Ripe (orange-colored) fruits of *C. aurantium* were collected from 25-year-old trees in the National School of Agriculture farm (33.8435° N, 5.4775° W, Altitude: 550 m a.s.l.). The taxonomic confirmation of the species was kindly made by Professor M. Fanane, a botanist at the National Herbarium of the Scientific Institute, Mohammed V University of Rabat; a voucher has been kept in the herbarium of the same Institute under the reference 93,675; the name of the plant has also been checked against the database of plant lists "http://www.theplantlist.org (accessed on 15 June 2021)". The fruits' peel (epicarp + mesocarps) was dried in an oven at 30 °C until a constant weight was obtained. The ground dry peel (100 g) was subjected to hydrodistillation for 3 h using a Clevenger-type apparatus. The obtained EOs were separated by decantation, dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and stored at −20 °C in anairtight, amber-coloredd glass bottle for subsequent use. The EO yield is expressed as a percentage of the dry matter weight.

2.2. Analysis of EO

The chemical analysis of EO was carried out at the National Center for Scientific and Technical Research in Rabat "https://www.cnrst.ma/index.php/ (accessed on 17 September 2021)" using a gas chromatograph (Trace GC ULTRA, Milano, Italy) coupled to a Polaris Q MS type mass spectrometer (MS) (Milano, Italy) with ion trap and equipped with an apolar column of type VB5 (Methylpolysiloxane with 5% phenyl), 30 m length, ×0.25 mm i.d, ×0.25 µm film thickness (ValcoBond, Milan, Italie). The oven temperature varied from 50 °C to 250 °C at 5 °C/min and from 250 °C to 300 °C. Helium was used as a carrier gas at 1.4 mL/min. The injection temperature was 220 °C; 1 µL of EO diluted to 1/10 in hexane was injected manually according to the split mode. The MS interface was set at 200 °C and the mass spectra were recorded at 70 eV with a scan speed of 0.5"/scan and a range of 10–350 m/z. The retention indices were calculated with reference to the series of alkanes (C_9–C_{30}) co-injected according to the equation of Van Den Dool and Kratz (1963) [20]. The identification of the constituents of essential oils was carried out by comparing their retention times and indices as well as their mass spectra with those of the database of NIST Standard Reference Database Number 69 [21] and Adams (2007) [22].

Percentages of all constituents were calculated by electronic integration of peak areas without the use of response factor correction.

2.3. Strain of Callosobruchus Maculatus

The *C. maculatus* strain used in the bioassays was collected at the Meknes grain market (GPS coordinates: 33.895, −5.577) and reared on untreated chickpea seeds in 1 L glass jars aerated at the Zoology Laboratory (National School of Agriculture-Meknes). The rearing conditions were 28 ± 1 °C and r.h. 65 ± 5% with a photoperiod of 12 h:12 h (L: D).

2.4. Biological Tests

The bioassays were carried out in plastic Petri dishes (Ø = 9 cm, H = 1.5 cm), ventilated through a hole on the cover, and protected by transparent cotton tulle. Six Petri dishes were accommodated inside an airtight plastic box with a capacity of 1L (L × W × H: 20 × 10 × 5 cm). All tests were conducted at 27 ± 3 °C and r.h. 56 ± 8% under a natural photoperiod. Fifty healthy, untreated chickpea seeds (*Cicer arietinum var*. Rizki FLIP83-48C), weighing 28.4 ± 1.1 g and moisture content of 12%, and 10 pairs of bruchids (<24 h) were placed in each Petri dish. The concentrations used (i.e., 25, 50, 100, and 200 µL/L air) were determined on the basis of a preliminary test exposing adults to concentrations causing the mortality of 5–95% of the fumigated population for 24 h. The EO concentrations were deposited on a watch glass placed in the airtight plastic box. The negative control consisted of lots of 50 untreated chickpea seeds presented to 10 pairs while the positive control used lots of 50 chickpea seeds offered to 10 pairs of the weevil that were exposed to phosphine (Phostoxin tablets, Aluminum phosphide 56% CPCM; Supplier: DETIA DEGESH GMBH). The exposure to phosphine was carried out using the recommended dose (6 tablets/m^3 = 6 g a.i./m^3) under transparent polyethylene tarpaulin in a storage unit approved for the use of phosphine [23]. Six repetitions were used for each treatment (negative or positive controls and EO concentrations)

The longevity of the treated adults was assessed by daily counting, sexing, and removing the dead individuals. Females' fecundity was determined by counting under a stereomicroscope the number of eggs laid on each lot of 50 chickpeas after the death of the last female while females' fertility was obtained by computing the ratio between the number of larvae that entered the seed and the total number of eggs laid by treatment. The number of offspring of the first generation (F1) was determined by the daily counting of adult females and males who emerged from seeds starting from the 23rd day after treatment until the end of emergence. The population growth parameters of *C. maculatus* were calculated for each treatment as follows: Net reproduction rate: R_0 (Females/Female) = $\frac{Nt}{N0}$; where N_t: number of females emerged; N_0: number of females used at the start;

Average generation time: T (days) = $\frac{\sum(fixi)}{N}$ where f_i: Number of adults emerged, x_i: Date of the emergence, and N: Total number of adults emerged;

The intrinsic rate of natural increase: r (females/day) = $\frac{Ln\ (R0)}{T}$;

Finite growth rate: λ (Females/day) = e^r;

Time required for the population to double in size: DT (Days) = $\frac{Ln2}{r}$;

And the sex ratio = $\frac{\text{Number of males}}{\text{Number of females}}$.

2.5. Data Analysis

The data were synthesized in graphs or tables and expressed as means ± SEM. The longevity of the weevil was analyzed by constructing and comparing the survival curves using the Kaplan–Meier estimator followed by the log-rank test. The LC_{50} and LC_{99} and their confidence intervals were determined five days after fumigation for males and 6 days for females by the Probit's method. Using IBM SPSS Statistics version 21 software (IBM Corp., Armonk, NY, USA), mortality was corrected according to Abbott's formula. The LCs and LTs were compared based on the calculation of confidence intervals as described by Roberston et al. (2017) [24]. Adults' mortality over time was fitted to multilinear regression model using Statistica version 7 software (Statsoft Italia; Vigonza; Padova, ITALY).

For the other measured parameters (Fecundity, unhatched eggs, Individuals dead inside seeds, Offspring, R_0, r, T, DT, λ, Sex ratio), the homogeneity and normality of the variances were verified by the Levene and Shapiro–Wilk tests. For each biological parameter (Fecundity, Fertility, Offspring) or demographic one (R_0, r, T, DT, λ, Sex ratio), the data recorded were compared by one-way analysis of variance followed by the test of Tukey HSD at 5%. Data relative to unhatched eggs, individuals dead inside seeds, R_0, r, DT, λ, and sex ratio were transformed using Arc sine square root (Arcsin $\sqrt{}$). The numbers of daily emerged adults were compared by three-way ANOVA with three independent variables, i.e., =Duration * Gender * Concentrations followed by the Tukey HSD test at 5% and adjusted to nonlinear regressive models using Statistica 7 (Statsoft Italia; Vigonza; Padova, ITALY). In this work, the choice of regressive models was made based on the low value of the Akaike Information Criterion (AIC) and that of the standard error, as well as on the high values of R^2 and their significance by the variance analysis (F).

As no offspring were obtained for the 200 µL/L air concentration, it was excluded from the statistical analysis.

3. Results

3.1. Chemical Composition of EO

The average yield of essential oil, extracted from the peel of *C. aurantium*, was 1.25 ± 0.1% (N = 3) of the dry weight of the peel. Twenty-two compounds of monoterpenes and sesquiterpenes were identified (Figure 1, Table 1) and represented 98.95% of the identified constituents of the EO. Limonene (86.18%) was the major component in the EO of *C. aurantium* peel.

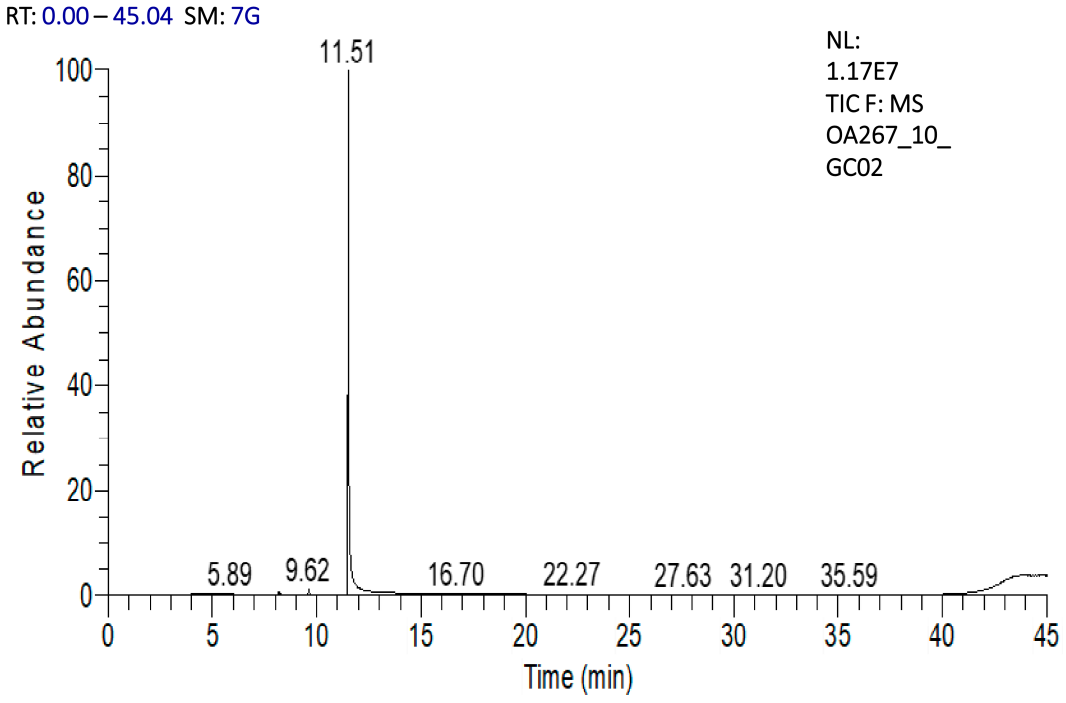

Figure 1. Gas chromatography profile of *Citrus aurantium* peel EO.

Table 1. Compounds of the EO of *Citrus aurantium* peel.

N°	Compounds *	RI **	RT ***	Formula	Content (%)
1	Octan-1-ol	925	5.89	$C_8H_{18}O$	0.63
2	α-Pinene	930	8.19	$C_{10}H_{16}$	1.01
3	Camphene	944	8.65	$C_{10}H_{16}$	0.03
4	Sabinene	971	9.62	$C_{10}H_{16}$	0.05
5	Limonene	1006	11.51	$C_{10}H_{16}$	86.18
7	α-Terpinene	1021	15.61	$C_{10}H_{16}$	0.06
8	7-Propylidene-bicyclo [4.1.0] heptane	1025	16.41	$C_{10}H_{16}$	0.08
6	*Trans*-Limonene-1.2 oxide	1056	22.27	$C_{10}H_{16}O$	0.06
9	Terpinen-4-ol	1083	24.87	$C_{10}H_{18}O$	0.08
10	3-Carene	1099	27.63	$C_{10}H_{16}$	0.08
11	Ocimene	1138	35.58	$C_{10}H_{16}$	0.07
12	Cyclobutane. 1.2-bis (1-methyle thenyl)-. trans-	1162	41.78	$C_{10}H_{16}$	0.03
13	Thymol	1174	42.18	$C_{10}H_{14}O$	0.03
14	Terpinen-1-ol	1189	42.45	$C_{10}H_{18}O$	3.81
15	Linalool	1196	42.59	$C_{10}H_{18}O$	1.73
16	Undecanal	1236	43.34	$C_{11}H_{22}O$	4.57
17	Geranyl acetate	1255	43.52	$C_{12}H_{20}0_2$	0.07
18	Neryl acetate	1265	43.59	$C_{12}H_{20}0_2$	0.08
19	δ-Elemene	1288	43.72	$C_{15}H_{24}$	0.08
20	CaryophylleneE	1335	44.57	$C_{15}H_{24}$	0.07
21	Geracrene-A or β-Bisabolene	1347	44.68	$C_{15}H_{24}$	0.09
22	Quercetin-7,3′,4′-trimethyl ether	1368	44.81	$C_{18}H_{16}O_7$	0.06
	Monoterpenes hydrocarbon				87.59
	Oxygenated monoterpenes				5.71
	Sesquiterpenes hydrocarbon				0.24
	Other				5.41
	Total				98.95
	Yield of EO (%)				1.25 ± 0.1

*: The components of the EO of the *Citrus aurantium*'s peel are classified in order of elution on an apolar column (DB-5). They all were identified by comparing their mass spectra with those of the NIST Standard Reference Database Number 69 [21] and those of Adams (2007) [22]; **: Retention time in min; ***: Retention index calculated using the Van den Dool and Kratz (1963) [20] equation for a homologous series of n-alkanes (C_9–C_{30}) using an apolar column DB-5.

3.2. Effects of Citrus aurantium Peel EOs on the Survival of Callosobruchus maculatus

3.2.1. Adult Mortality

The longevity of *C. maculatus* adults, fumigated with the *C. aurantium* peel EO, varies from around 1 to 8 days for males and from 1 to 9 days for females depending on the concentration (Figure 2). Compared to controls, longevity is shortened by approximately 5 to 7 days for males (log rang test: $\chi^2_{males} = 20.36$, $df = 5$, $p = 0.001$) and 3 to 7 days for females ($\chi^2_{females} = 18.28$, $df = 5$, $p = 0.003$) depending on the concentration. With regard to the reference product, phosphine, only 200 µL/L air exerts the same lethal effect ($\chi^2 = 1.21$, $p = 0.37$); with the other concentrations, the adults of *C. maculatus* treated take longer to die compared to phosphine ($\chi^2 = 5.91$–40; $p < 0.05$) (Figure 2). Regarding the comparison between the concentrations of EO tested, except for the males treated at 100 or 200 µL/L air, whose responses are statistically comparable ($\chi^2 = 2.50$; $p = 0.14$), all other concentrations gradually and differently affect the survival of the weevil adults, and the log-rank test values are significantly different ($\chi^2_{males} = 6.27$–27.17 and $\chi^2_{females} = 5.12$–47.51; $p < 0.05$). With regard to the sensitivity of the sexes to EO, compared to each other for the same concentration, 25 and 50 µL/L air were shown to be more toxic to males compared to their conspecific females (χ^2 (25 µL/L) = 11.90; χ^2 (50 µL/L) = 14.61; $p < 0.05$). With respect to 0, 100, or 200 µL/L air, the two sexes have comparable survival curves, and the χ^2 are equal to 0.70 ($p = 0.28$), 2.55 ($p = 0.13$), and 0.30 ($p = 0.64$), respectively.

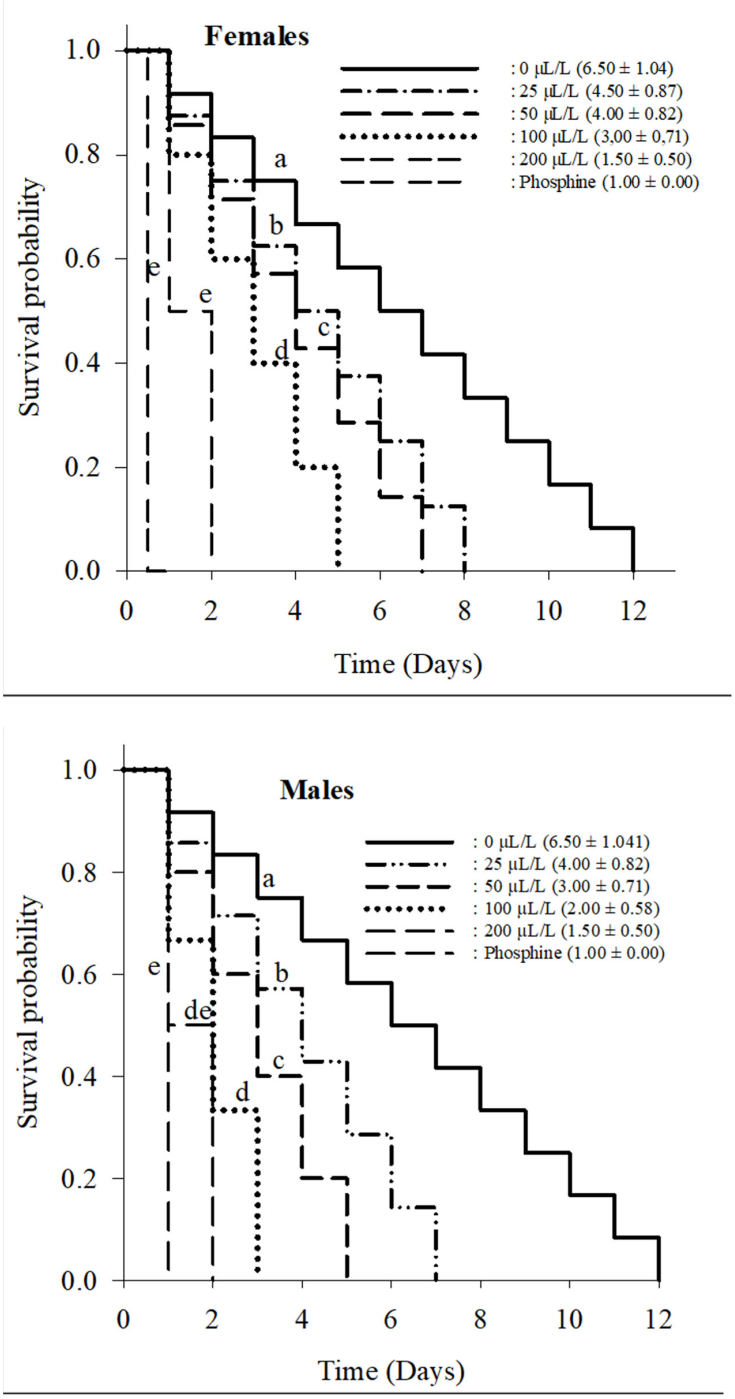

Figure 2. Effects of *Citrus aurantium* peel EO on the survival of adults of *Callosobruchus maculatus* (The values in parentheses represent the LT_{50}s with their SEs (Days). The concentrations affected by the same letter do not differ statistically between (Log-rank tests, $p \leq 0.05$); each curve represents the probability of survival of 60 adults).

In terms of lethal times (LT), i.e., the time required to kill the target organism, for each concentration, the survival of treated bruchids decreases with the duration of fumigation with *C. aurantium* peel EO according to a linear model (Supplementary Table S1). Peel's *C. aurantium* EO therefore significantly and rapidly shortens the longevity of *C. maculatus* adults in a concentration-dependent manner. In fact, the TL_{50}s decrease by about 8 in less than a day when the concentrations increase from 0 to 200 µL/L air, while TL_{99} ranges between about 13 and 2 days for females and between about 12 and 1 day for males when

concentrations increase (Supplementary Table S1). Moreover, and taking into account the slopes of the equations, the males were shown to be less tolerant to EO than the females, in particular at high concentrations (Supplementary Table S1). For both sexes, the times required to kill 50% (LT_{50}) or 99% (LT_{99}) of the treated population are negatively correlated with the concentrations of EO tested, and are inversely proportional to the increase in the concentration of EO (Figure 3). Moreover, the EO of *C. aurantium* peel is chronologically toxic to *C. maculatus*. Indeed, during the 5–6 days after the start of the test, the LC_{50} and LC_{99} decreased respectively from 109.26 to 10.32 and from 303.62 to 36.85 µL/L air for the males and from 147.59 to 12.39, and from 317.32 to 54.36 µL/L air for the females (Supplementary Table S2); the males have been shown to be more sensitive than their peers. However, it is noteworthy that during the first day after the start of the test, the LC_{99} values with their confidence intervals exceed the range of concentrations tested against *C. maculatus* (Supplementary Table S2). For both sexes, the LC_{50} and LC_{99} are negatively correlated with the duration of exposure to the fumigant—the higher the concentration, the shorter the duration of exposure (Figure 4). In addition, the estimation of the simultaneous influence of the concentration of EO and the duration of exposure made it possible to appraise their respective importance. Thus, the mortality due exclusively to the EO of *C. aurantium* peel tested positively depends both on the duration of exposure to these compounds and on their concentration with their interaction according to a multilinear model (Table 2).

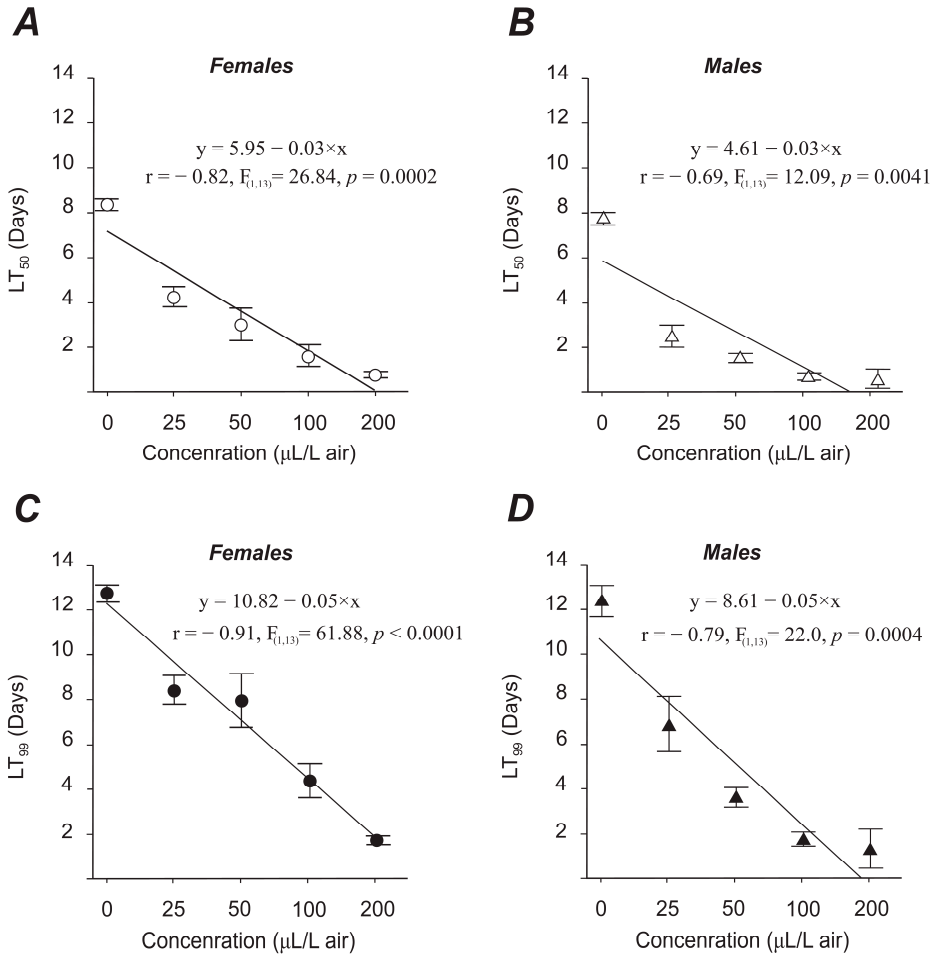

Figure 3. Toxicity relationships between lethal times LT_{50} (**A**,**B**) and LT_{99} (**C**,**D**) for exposed female (**A**,**C**) and male (**B**,**D**) adults of *Callosobruchus maculatus* and the tested concentrations of EO of *Citrus aurantium* peel (N = 60 adults for each concentration tested). LTs are shown as Mean ± lower and upper limits.

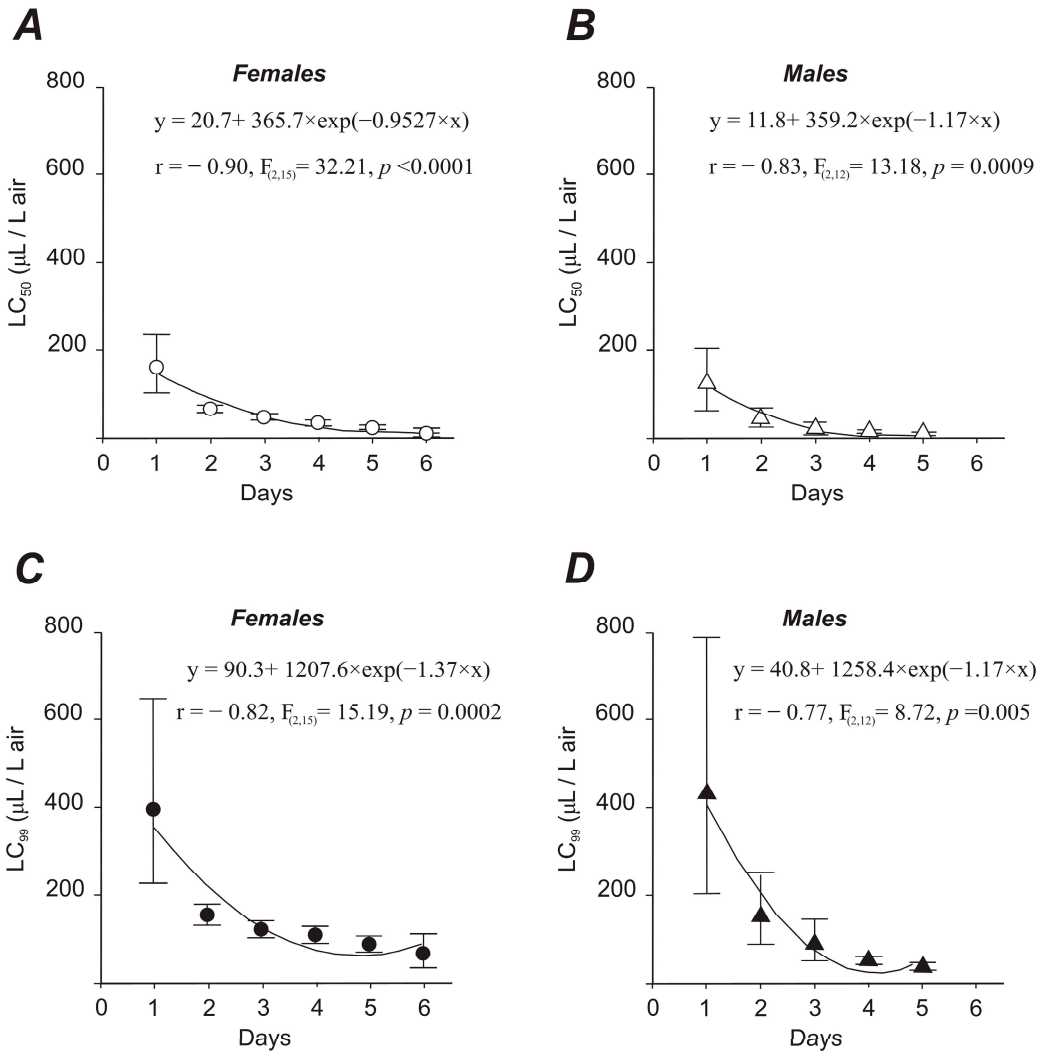

Figure 4. Toxicity relationships between the lethal concentrations LC_{50} (**A**,**B**) and LC_{99} (**C**,**D**) for exposed female (**A**,**C**) and male (**B**,**D**) adults of *Callosobruchus maculatus* and the duration of exposure to different concentrations of EO of *Citrus aurantium* peel (N = 60 adults for each concentration tested). LCs are shown as Mean ± lower and upper limits.

Table 2. Parameters for adjusting the concentration (C: (µL/L air)) and the duration of exposure (T: (Days)) to the corrected mortality (y (%)) of *Callosobruchus maculatus* due to the EO of peel's *Citrus aurantium* to the multilinear regression model.

Gender	Model	R^2	F	*df*	*p*
Females	y = −6.24 + 8.88T + 0.13C +0.14TC	0.92	89.08	3, 22	<0.0001
	T	0.66	31.93	1, 22	<0.0001
	C	0.54	4.53	1, 22	0.0448
	T × C	0.62	29.26	1, 22	<0.0001
Males	y = 10.62 + 2.98T + −0.05C + 0.47TC	0.95	94.95	3, 14	<0.0001
	T	0.69	171.87	1, 14	<0.0001
	C	0.46	113.40	1, 14	<0.0001
	T × C	0.59	90.30	1, 14	<0.0001

3.2.2. Non-Hatchability of Eggs and Mortality of Individuals Dead in Seeds

Percentages of the unhatched eggs and those of the individuals dead in seeds are statistically different ($F_{(1, 50)}$ = 11.95, $p < 0.001$), and both categories are affected by the concentration of EO tested ($F_{(4, 50)}$ = 471.17, $p < 0.001$). Indeed, not all of the eggs laid have

hatched and the larvae, having entered the seed of the chickpea, do not all reach the adult stage. In the fumigated lots, the numbers of unhatched eggs are much higher than those counted in the control lot; the percentages of unhatched eggs vary from 8.10 to 8.86, from 32.05 to 38.28, from 55.33 to 62.86, from 73.52 to 77.27, and from 86.67 to 88.89% of the eggs emitted in the fumigated lots with 0, 25, 50, 100, and 200 µL/L air, respectively (Figure 5A). Peel's *C. aurantium* EO inhibited eggs by approximately 29–86%. The proportion of unhatched eggs laid on treated seeds is therefore reduced by approximately 4 to 10 times depending on the concentration considered (Figure 5A). In this work, the EO of *C. aurantium* peel drastically inhibited the fertility of *C. maculatus* eggs, and the LC_{50} and LC_{99} with their confidence intervals are estimated at 62.66 [36.78–88.44] and at 202.71 [162.88–273.26] µL/L air, respectively. In the case of individuals that die inside the seeds, on average, about 8–100% of them die back in the seed depending on the concentration considered. Their percentages increased significantly with increasing concentration (Figure 5B). Thus, the EO from the peel of *C. aurantium* killed about 19 to 100% of the fumigated population depending on the concentration; with 200 µL/L air, no offspring was obtained (Figure 5B). The values of the LC_{50} and LC_{99} with their confidence intervals are 62.82 [51.66–74.44] and 174.99 [149.32–217.21] µL/L air, respectively. In addition, the comparison between unhatched eggs and individuals dead in the seeds reveals that the proportion of unhatched eggs exposed to 25, 50, or 100 µL/L air is statistically higher than that of individuals having withered in the seed ($p < 0.05$). With these concentrations, the eggs were shown to be more sensitive to the *C. aurantium* peel EO than the stages which are dead in the seeds. On the other hand, with 200 µL/L air, the percentage of individuals dead in the seed is significantly higher than that of unhatched eggs ($p < 0.05$). Related to each other, the percentages of individuals dead in the seed are positively correlated with those of unhatched eggs ($r = 0.92$, $F_{(1, 28)} = 157.80$, $p < 0.001$).

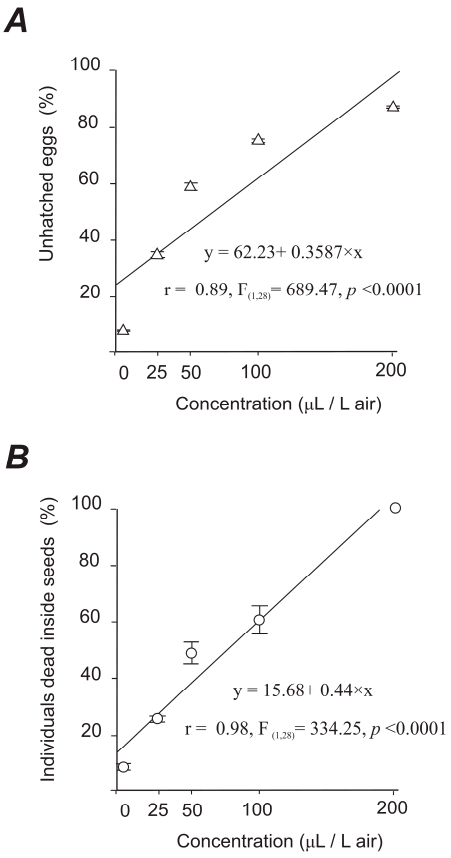

Figure 5. Mortality of embryos (**A**) and those of the pre-imaginal stages inside the seeds (**B**) of *Callosbruchus maculatus* fumigated with EO of the *Citrus aurantium* peel. Data are shown as Mean ± SE.

3.3. Effects of the Citrus aurantium Peel EO on Fecundity and Adult Emergence of Callosobruchus maculatus

The two measured biological parameters of *C. maculatus* are negatively affected by the *C. aurantium* peel EO, and they all depend on the concentration of EO (Table 3).

Table 3. Effects of the *Citrus aurantium* peel EO on fecundity and adult emergence of *Callosobruchus maculatus*.

Concentration (µL/L Air)	Eggs/10 Pairs	Adult Numbers/10 Pairs
0	507.83 ± 12.66 [a*]	426.17 ± 5.62 [a]
25	224.67 ± 4.61 [b]	109.33 ± 4.57 [b]
50	150.83 ± 3.36 [c]	30.50 ± 1.38 [c]
100	94.83 ± 3.24 [d]	9.17 ± 1.14 [d]
200	53.50 ± 2.29 [e]	0.00 ± 0.00 [e]
F	782.23	2879.43
df	4, 25	4, 25
p	<0.0001	<0.0001

*: At each column, the values assigned by the same letter do not differ statistically from each other (One-way ANOVA followed by THS Tukey at 5%). For each concentration, each parameter represents the mean ± standard error of 10 pairs (10 males and 10 females) with six repetitions, i.e., 60 males and 60 females.

3.3.1. Fecundity

The number of eggs laid by 10 females of *C. maculatus* is drastically affected by the EO of the *C. aurantium* peel ($F_{(4, 25)} = 782.23$, $p < 0.0001$), which goes from 209 to 239, from 138 to 157, from 87 to 108, and from 47 to 60 eggs/10 females for 25, 50, 100, and 200 µL/L air, respectively; it ranges from 467 to 535 eggs per 10 females in the non-fumigated lot. Fecundity is therefore inversely proportional to the increase in the concentration of EO, and it is statistically different between the concentrations (Tukey's HSD test at $p < 0.05$) (Table 3). Thus, compared to the control, the number of eggs emitted by 10 females is inhibited by approximately 47 to 90% depending on the concentration; it is negatively correlated with the concentrations tested ($r = -0.93$, $F_{(1, 3)} = 19.37$, $p = 0.022$). In addition, and in view of the longevity of the laying females, the number of eggs laid by females increases with their lifespan ($r = 0.98$; $F_{(1, 3)} = 90.741$; $p < 0.0001$). In this work, the response of the *C. maculatus* population treated with the EO of *C. aurantium* peel appears to be homogeneous, the variation coefficients vary from 5 to 10%.

3.3.2. Adult Emergence

The EO of the *C. aurantium* peel interfered with the growth and development of the young stages of *C. maculatus*, resulting in reduced emergence of adults. Indeed, as shown in Table 3, the number of adults insects that emerged from fumigated seeds was drastically decreased compared to untreated seeds ($F_{(4, 25)} = 2879.43$; $p < 0.0001$). It varies from 410 to 443 adults/10 females in the control, and from 94 to 122, 27 to 35, or 7 to 14 adults/10 females in the lots treated with 25, 50, or 100 µL/L air, respectively. The number of adults obtained is negatively correlated with the concentrations ($r = -0.91$, $df = 3$, $p \leq 0.05$). With 200 µL/L air, all individuals that entered the seeds died inside them. Compared to the control, the level of imaginal stages developed in the fumigated seeds was therefore reduced by approximately 74 to 100% depending on the concentration; the percentages of emerged adults are statistically different ($F_{(4, 25)} = 1098.17$, $p < 0.0001$). Compared to the number of eggs laid per 10 females (i.e., success rate), the proportion of adults that emerged from seeds varies on average from 84.05% of eggs laid on non-fumigated seeds to no emergence in the lots fumigated with 200 µL/L air (Table 3). In addition, the number of hatched eggs and that of adults emerging from the seeds are inversely proportional to the concentrations of EO ($r_{(eggs)} = -0.996$, $F_{(1, 28)} = 3069.9$, and $r_{(adults)} = -0.999$, $F_{(1.28)} = 10702.37$, $p < 0.0001$), while they are positively correlated with each other ($r = 0.998$, $p < 0.0001$).

Moreover, daily monitoring of the adult emergence of *C. maculatus* from seeds allows us to not only note the effect of the *C. aurantium* peel EO on population levels but also on the chronology of their emergence. Adult emergence fits the Gaussian model in all lots with three different parameters (Figure 6, Supplementary Table S3) and indicated that fumigation with EO from the peel of *C. aurantium* caused a reduction in the daily emergence of adults of *C. maculatus*. In terms of numbers, there is a significant difference between the days of emergence ($F_{(10, 440)} = 1229.71$, $p < 0.0001$), between the concentrations ($F_{(3, 440)} = 14477.24$, $p < 0.0001$), and between the sexes ($F_{(1,440)} = 12.24$, $p < 0.0001$) with interactions between Days × concentrations ($F_{(3,440)} = 551.93$, $p < 0.0001$), between concentrations * sexes ($F_{(3, 440)} = 6.73$, $p < 0.0001$) and between days × concentrations × sexes ($F_{(30, 440)} = 1.50$, $p = 0.05$). Thus, the number of offspring from negative control lots is approximately 4 to 57 times higher than that obtained in treated lots for females and approximately 4 to 33 times for males. In the fumigated lots, the number of adults decreases significantly with the concentration, and the females are more numerous than the males in the control lots and those fumigated with 25 or 50 µL/L air (Figure 6). Regarding the period of emergence, except for a lot of females treated with 50 µL/L air and that of males fumigated with 100 µL/L air, whose emergence began on the 25th day after fumigation, in all the others lots, the emergence of adults begins from the 24th day after treatment (Figure 6). The emergence period lasted 11 days in the control lots and 6–10 days in the treated ones. The peak emergence (≈50% of the imaginal population) occurred on the 3rd and 4th days after the onset of emergence in the non-fumigated lots and occurred on the 4th and 5th and in the lot treated with 25 µL/L air. In lots fumigated with 50 or 100 µL/L air, adults emerge at the same rate (Figure 6). The peaks of emergence were drastically reduced in the fumigated lots compared to the untreated ones and their magnitude decreased with the increase of EO concentrations. Finally, it should be noted that the concentration required to inhibit the emergence of 50% of adults is estimated at 68.09 (CI = 40.04, 111.12) µL/L air and that the percentages of F1 adults emerging from the seeds are inversely proportional to those of the dead stages within them ($r = -1$).

3.4. Effects of the Citrus aurantium Peel EO on the Population Growth Parameters of Callosobruchus maculatus

The demographic parameters of *C. maculatus* fumigated with the *C. aurantium* peel EO are summarized in Table 4. The duration of the generation of the weevil is statistically comparable between the control lot and the ones fumigated with 25, 50, or 100 µL/L air ($F_{(3, 20)} = 0.41$, $p = 0.75$). Under the conditions of the trial, *C. maculatus* takes 24 to 33 days to complete a generation. On the other hand, the sex ratio of adults emerging from untreated or fumigated seeds differs according to the concentration ($F_{(3, 20)} = 26.66$; $p < 0.0001$). In lots fumigated with 0, 25, or 50 µL/L air, the proportion of males is comparable to that of their congeners, whereas in the lot fumigated with 100 µL/L air, the number of males is greater than that of females. In this case, the numbers of adults produced are too low to judge the statistical significance (on average 5.33 ± 1.51 males and 3.83 ± 1.33 females). Regarding the net reproduction rate (R_0), intrinsic rate of increase (r), and finite rate of increase (λ), their respective values are clearly lower than those recorded in the control lot (R_0 ($F_{(3, 20)} = 1904.51$, $p < 0.0001$); r ($F_{(3, 20)} = 272.91$, $p < 0.0001$); λ ($F_{(3, 20)} = 245.00$, $p < 0.0001$); indeed, compared to the control, R_0, r and λ are reduced from 4 to 57, from 2 to 6, and from 1.05 to 1.2 times, respectively, depending on the concentration. The values of these three parameters are negatively correlated with the tested concentrations ($r_{R0} = -0.99$ ($F_{(1, 2)} = 74.97$, $p = 0.013$); $r_r = -0.99$ ($F_{(1, 2)} = 86.20$, $p = 0.011$); $r_\lambda = -0.99$ ($F_{(1, 2)} = 66.28$, $p = 0.015$)). In addition, it is noteworthy that with 100 µL/L air, the value of r is negative. This concentration causes the extinction of the population of *C. maculatus*. Besides, to double their numbers, the treated populations take approximately 2 to 7 times longer than the non-fumigated lot ($F_{(3, 20)} = 68.94$; $p < 0.0001$) (Table 4). With regard to concentrations 0, 25, and 50 µL/L air, the time required to double the number of *C. maculatus* populations is extended exponentially ($r = 0.97$ ($F_{(1, 1)} = 16.90$, $p = 0.015$)). The bruchid populations from

the control grow considerably and more rapidly than those obtained in the lots fumigated. Thus, the *C. aurantium* peel EO has not only been shown to be toxic to *C. maculatus*, but it also impacts the growth potential of its population.

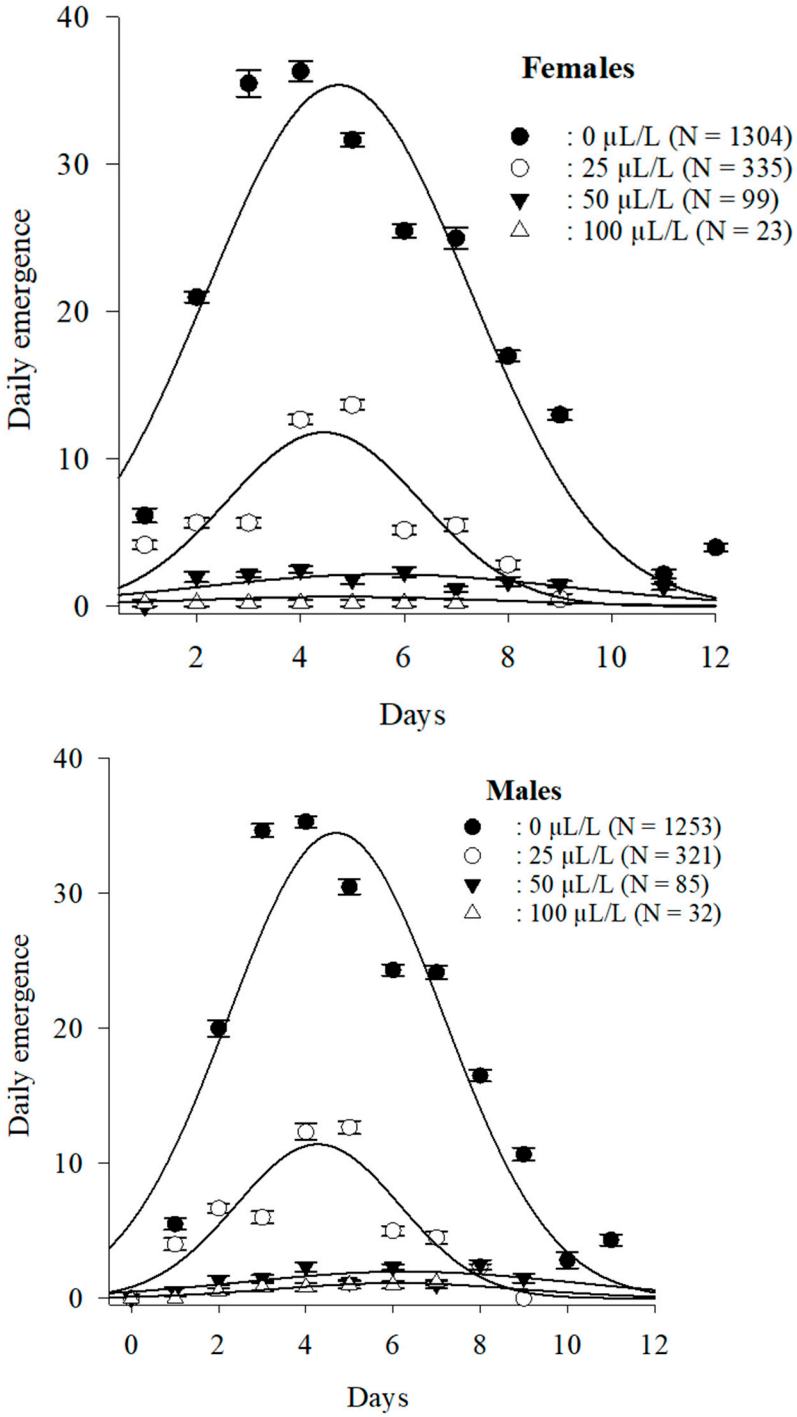

Figure 6. Daily emergence (Mean ± SE) of adults of *Callosobruchus maculatus* from seeds of *Cicer arietinum* fumigated with the *Citrus aurantium* peel EO (For concentrations of 50 and 100 µL/L of air, the emergence rate is constant throughout the period). For each concentration, 6 repetitions were used at the rate of 10 pairs each.

Table 4. Effects of *Citrus aurantium* peel EO on the demographic growth parameters * of *Callosobruchus maculatus*.

Concentration * (µL/L Air)	T (Days)	Sex Ratio	R_0 (Female/Female)	r (Female/Day)	λ E (Female/Day)	DT (Days)
0	28.17 ± 0.82 [a**]	0.97 ± 0.03 [a]	21.70 ± 0.58 [a]	0.110 ± 0.004 [a]	1.12 ± 0.004 [a]	6.36 ± 0.25 [a]
25	27.01 ± 0.78 [a]	0.97 ± 0.03 [a]	5.55 ± 0.24 [b]	0.064 ± 0.003 [b]	1.07 ± 0.004 [b]	11.02 ± 0.57 [b]
50	28.39 ± 0.77 [a]	0.87 ± 0.04 [a]	1.63 ± 0.08 [c]	0.017 ± 0.002 [c]	1.02 ± 0.002 [c]	42.52 ± 4.17 [c]
100	28.54 ± 1.10 [a]	1.42 ± 0.08 [b]	0.38 ± 0.05 [d]	−0.04 ± 0.01 [d]	0.96 ± 0.01 [d]	−22.70 ± 4.86 [d***]
F	0.41	26.66	1904.51	272.91	245.00	68.94
df	3, 20	3, 20	3, 20	3, 20	3, 20	3, 20
p	0.747	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

*: For each concentration, each parameter represents the mean ± standard error of 10 pairs (10 males and 10 females) with six repetitions, i.e., 60 males and 60 females. **: For each column, the values assigned by the same letter do not differ statistically from each other (One-way ANOVA followed by THS Tukey at 5%. ***: The negative value of DT indicates that all the individuals have been exterminated).

4. Discussion

EOs are complex natural mixtures that generally contain various compounds at different concentrations. In the present work, the EO was extracted from *C. aurantium* peel with a yield exceeding 1% of the dry matter. The chemical composition of the extracted EO revealed the presence of twenty-two compounds in different proportions and limonene accounted for more than 86% of the EO composition in concordance with previous studies [25,26]. EO obtained from the peel of *C. aurantium*, like most species of *Citrus*, is composed mainly of monoterpene hydrocarbons like limonene with concentrations ranging from 32 to 98% [7,26,27]. Generally, the yield of citrus-extracted EOs, as well as the diversity of their constituents, varies markedly according to genetic, phenological, climatic, and edaphic factors, as well as agronomic techniques and the extraction method [28–30].

The EO here extracted from *C. aurantium* peel demonstrated a remarkable fumigant activity against *C. maculatus*. The toxic action for both adults and pre-imaginal stages varied depending on the concentration, the duration of the exposure, and the sex of exposed insects. The fumigant toxicity to adults was inversely proportional to the concentration with males generally more sensitive to the EO action than females. At a concentration of 200 µL/L, its lethality was comparable to the widely used synthetic product (i.e., phosphine) applied at the recommended dose. The fumigant activity of citrus EOs has been reported not only for *C. maculatus* [5,31] but also for other pests associated with stored agricultural commodities, such as *Tribolium castaneum* [32], *T. confusum* [33], *Rhyzopertha dominica* [34], *Sitophilus oryzae* [35], and *S. zeamais* [36]. Similarly, the percentages of unhatched eggs and juvenile stages dead inside the seeds increased with increasing concentrations. The absence of hatching of the eggs can be due to the oil vapors diffused in the eggs and thus affecting the physiological and biochemical processes associated with embryonic development [37,38]. EOs can reach both the embryo in its chorion and the stages that have entered the seed. Their active molecules can even reach the interstitials in the grain, thus increasing the exposure and death of the pest to the treatment [39–41].

Because plant secondary metabolites often have more than one physiological or behavioral effect on insects, their bioactivity should always be assessed with more than one endpoint. Besides EOs inducing mortality, their oviposition deterrence, repellence activities, and consequently, their effects on demographic parameters should not be discounted, as they may be important in the control strategy of stored product pests. Thus, in addition to the high toxicity of the tested EO, adults of *C. maculatus* that survived fumigation showed significantly lower reproductive performances than non-fumigated adults. The number of eggs laid in the fumigated lots was drastically reduced compared to the untreated ones resulting in a similar trend in progeny production. The females' oviposition and offspring emergence were inversely proportional to the concentration. Negative effects on fecundity and fertility were mirrored in the population growth parameters. Indeed, apart from the generation time and the sex ratio, which were not significantly affected, R_0, r, λ, and DT were drastically reduced with increasing concentrations of EO. The decrease in fecundity

can be attributed to females' mortality, to inhibition of oviposition and/or reduction of eggs' hatching, as has been already reported for *C. maculatus* [5,42,43], *Acanathoseclides obtectus* [44], and *C. chinensis* [41] exposed to essential oils of various plant species. The reduction in fecundity can also be linked to disturbances during the vitellogenesis process as in the case of *C. chinensis* exposed to the EOs of *Artemisia herba-alba* (Asteraceae), *Salvia verbenaca* (Lamiaceae), or *Scilla maritima* (Amaryllidaceae) [45].

The constituents of EOs exert their neurotoxic insecticidal activities by inhibiting acetylcholinesterase [8,31,46–49] or disturbing the GABAergic [50–55] and aminergic [56–59] transmissions. They have been also reported to promote severe histological and structural alterations in the carbohydrate contents, muscle fiber, midgut epithelium, and fat droplets [60].

The lethality of EO extracted from *C. aurantium* peel to *C. maculatus* can be attributed to its major component, limonene, as reported for other *Citrus* EOs [7,61,62]. Actually, EO of *C. sinensis* was shown to inhibit cytochrome P450-dependent monooxygenases responsible for the detoxification of (4*R*)-(+)- limonene to carveol and carvone, non-toxic metabolites, glutathione S transferase, and reduce the total content of carbohydrates used in locomotion and flight, lipids, and proteins essential for the reproduction of insect pests [63–65]. In addition to inhibiting the above targets, EO from *C. sinensis* peel has also been suggested to inhibit ATPase N^+/K^+ (Na^+/K^+ -ATPase activity) activity of *C. maculatus* and *S. zeamais* adults [31,36]. However, although the biological properties of the EOs are generally attributed to a few major components, the synergistic and antagonistic interactions among all EO constituents should be always taken into account [66,67].

5. Conclusions

During the past decades, a large number of studies have described the biotechnological and commercial potential of plant-based products in pest insects' management but with only a few practical applications as new botanical insecticides. Some of the major limitations faced by the large-scale production and use of potential active plant-based compounds include the availability of raw materials and performance under field conditions. Hence, the recovery of by-products from wastes of fruit processing industries can improve the overall profitability of processing units and significantly reduce or avoid environmental pollution while being a sustainable source of plant-based products. In this context, and based on the biological activities of the EO of *C. aurantium* peel against *C. maculatus*, its use as a fumigant in the management of insects associated with stored agricultural commodities can be further explored in Morocco. On a large scale, EOs can be easily extracted by hydrodistillation from solid wastes of *Citrus*-processing units that are frequently rejected in nature. For small farmers for whom chemical insecticides are expensive and pose risks of poisoning due to the lack of adequate technical knowledge for safe use, the raw materials readily available from *C. aurantium* trees freely available in green areas can be used for EO extraction.

Overall, while these results are encouraging for promoting the inexpensive and environmentally friendly valorization of citrus peel, further studies are needed to test the applicability and efficacy of the EO of *C. aurantium* peel under broader stored products conditions with a special focus on assessing the effects on stored seeds' quality and on non-target organisms.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture13020232/s1, Table S1: TL_{50} and TL_{99} values (days) and associated parameters for adults of *Callosbruchus maculatus* fumigated with EO of peel's *Citrus aurantium*, Table S2: LC_{50} and LC_{99} with their 95% confidence intervals (CI) (µL/L air) and χ^2 of the EO of *Citrus aurantium* peel used against adults of *Callosobruchus maculatus*, Table S3: Parameters of non-linear regression analyses of *Callosobruchus maculatus* adults F1 emerged daily from chickpea grains fumigated with *Citrus aurantium* peel EO (shown in Figure 6) according to the model $y = a \times \exp(-0.5 \times ((x - x_0)/b)^2)$ [a (Number) =Peak of the daily emergence of adults, b (Days) = Standard deviation of x_0, x_0 (Days) = Location of peak emergence since the start of fumigation].

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