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Orijinal araştırma (Original article)

Vapor activity of essential oils extracted from fruit peels of two *Citrus* species against adults of *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Bruchidae)

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Summary

The present study investigated the effects of volatile fractions of *Citrus limon* (Linnaeus, 1753) and *Citrus reticulata* Blanco, 1837 (Rutaceae) peel essential oils on the cowpea adult bruchid, *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Bruchidae). The oils were extracted from the fruit peels using water steam distillation. The results indicated that the citrus peel oils had high fumigant activity against adult beetles. The mortality of 1 to 2-day-old adults increased with increasing oil concentration or exposure time. The oil of *C. reticulata* was significantly more toxic than that of *C. limon* based on both 24 h LC_{50} (33 and 45 µl L^{-1}, respectively) and 24 h LC_{90} (75 and 99 µl L^{-1}, respectively) estimates. The results suggested that citrus peel oils can be used as potential control measure against cowpea beetles.

Keywords: *Citrus limon*, *Citrus reticulata*, cowpea seed beetle, botanical insecticides, fumigant activity

Anahtar sözcükler: *Citrus limon*, *Citrus reticulata*, börülce tohumböceği, bitkisel insektisitler, fumigant etki

Introduction

The cowpea seed beetle, *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Bruchidae) is one of the most widespread and destructive insect

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pests of the stored legumes. According to Rees (2004), loss of seed material is considerable -each adult *Callosobruchus* emerging from a cowpea [*Vigna unguiculata* (L.) Walpers] would have consumed about 25% of the seed from which it emerged. Fumigation is still heavily relied upon to disinfest stored products. The fumigants, methyl bromide and phosphine, have been widely used for this purpose in many parts of the world. However, EPA (2001) had proposed elimination of the production of methyl bromide by 2005 because of its ozone depletion potential (Champ & Dyte, 1977). Additionally, some stored-product insects are found to have developed resistance to methyl bromide and phosphine (Subramanyam & Hagstrum, 1995). Sulphuryl fluoride (SO_2F_2) has received registration as a fumigant in stored product pest control in several countries. The compound has been considered as a potential substitute (MBTOC, 1998), and it has been recently registered by EPA as a fumigant (trade name Profutone®) for trading food commodity storage facilities, processing plants and flour mills (Rajendran & Hajira-Parveen, 2005). However, the egg stage of insects is the most difficult stage to control with this fumigant and strategies to overcome this problem are being investigated (Bell, 2000). The widespread use of this fumigant for stored food protection may be possible after receiving the successful outcome of these studies.

All these problems have initiated the development of new types of selective insect-control alternatives to conventional fumigants. The toxicity of essential oils extracted from aromatic plants to stored-product insects has been widely investigated during the last decade (Shaaya et al., 1993; Ho et al., 1997; Obeng-Ofori & Reichmuth, 1997; Obeng-Ofori et al., 1997; Huang et al., 2002; Moravvej & Abbar, 2008). The fruit peels of some *Citrus* species have been reported to have insecticidal properties against insect pests (Don-Pedro, 1985; Onu & Sulyman, 1997; Elhag, 2000). Don-Pedro (1985) demonstrated the toxicity of powdered sun-dried orange and grapefruit peels to *C. maculatus*. In other studies, the essential oils of citrus peels proved to reduce oviposition or larval emergence through parental adult mortality (Don-Pedro, 1996; Elhag, 2000). The fumigant activity of essential oil extracted from the fruit peels of *Citrus sinensis* Osbeck against the bruchid, *Acanthoscelides obtectus* Say, 1831 has been demonstrated (Papachristos & Stamopoulos, 2002). More recently, Moravvej & Abbar (2008) indicated that the essential oils extracted from the fruit peels of four *Citrus* species including *Citrus sinensis*, *Citrus aurantium* L., *Citrus limonium* Risso and *Citrus paradisi* Macfad showed high fumigant activity against the adults of *C. maculatus*. In the present study, the effects of volatile fractions of essential oils extracted from the fruit peels of two other *Citrus* species, *Citrus limon* (Linnaeus, 1753) and *Citrus reticulata* Blanco, 1837, on the cowpea adult bruchid, *Callosobruchus maculatus*, were investigated.

Materials and Methods

Insect culture and bioassay procedure

A culture of non-flight form of the cowpea seed beetle, *Callosobruchus maculatus* (Fabricius, 1775), was established on the seeds of *Vigna unguiculata* (L.) Walpers in wide-mouthed plastic jars under laboratory conditions (32 ± 6°C, 45±5% r.h. and a 12:12 h light-dark cycle). Adding new seeds and removing very old ones to and from the main culture monthly prevented crowding of the bruchids and so no flight form was developed. One to 2-day-old adults were used for all bioassays.

The citrus fruits were collected from the central local market of Mashhad-Iran during December 2006. The essential oils were extracted from fresh rind tissue (albedo and flavedo) of fruits of *Citrus limon* (Linnaeus, 1753) and *Citrus reticulata* (Blanco, 1837) by water steam distillation using a Clevenger-type apparatus. About 1.5 ml oil was extracted per 100 g fresh peel. Extracted oils were stored at 5°C until the onset of bioassays.

The bioassays were conducted as described previously (Moravvej & Abbar, 2008). The 2-cm-diameter pieces of filter paper (Whatman No. 1) were impregnated with six different oil concentrations (including 0 $\mu l\ L^{-1}$ as control) to give the equivalent concentrations in air of 19-110 $\mu l\ L^{-1}$. The range of concentrations had been chosen on the basis of a number of preliminary trials. The filter paper was attached to the undersurface of the screw cap of a glass vial (volume 27 ml). The cap was screwed tightly onto the vial containing five pairs of 1 to 2-day-old *C. maculatus* adults. Each bioassay was done with six replicates. The vials were placed randomly into the growth chamber and mortality was recorded after 3, 6, 9, 12 and 24 h from initial exposure to the essential oil; exposure periods were conducted on separate vials using different batches of insects. When no leg or antennal movements was observed, insects were considered dead.

Data analysis

Mortality data of adults at 24 h exposure time for each plant species were analysed with the probit model (Finney, 1971) using a Maximum Likelihood Program (POLO-PC, LeOra Software, Berkeley, California). The program POLO-PC calculates a theoretical 'natural response' for each experiment, based on the pattern of mortality at all concentration levels, including controls (Abbott, 1925). The results include estimate of the LC_{50} (and other LCs if required) and the 95% confidence limits, slope and intercept of probit mortality regression, and the relevant statistical tests (such as "t" ratio, 'g' factor and heterogeneity). For comparison of the probit mortality lines of treatments, the program also provides the likelihood ratio tests of equality and parallelism (Russel et al., 1977). Estimated median lethal concentration to kill 50% of insects was expressed as LC_{50} (µl oil per unit air liter). The resistance ratio and 95% confidence limits of this ratio were determined between data from different oil treatments, and comparisons were made based on the procedure described by Robertson & Preisler (1992). The estimates of parameters needed for computing confidence limits of the resistance ratio were provided by individual probit analysis in the POLO-PC output.

Results

The essential oil vapors of the peel of two *Citrus* species showed variable toxicity to adults of *C. maculatus*, depending on concentration and exposure period (Table 1). There was no mortality in the presence of 19 $\mu l\ L^{-1}$ of *C. limon* oil at 3 h exposure. At 3 h exposure, the maximum rates of mortality of 23% and 40% were achieved by *C. limon* and *C. reticulata* oils, respectively, in the presence of 110 $\mu l\ L^{-1}$. At 12 h exposure, the minimum rates of mortality of 6.7% and 11.7% in adults were attained by the presence of 19 $\mu l\ L^{-1}$ of *C. limon* and *C. reticulata* oils, respectively. The essential oils of both *Citrus* species achieved about 98% mortality in adults by the presence of 110 $\mu l\ L^{-1}$ at 24 h exposure. In all bioassays, the control mortality was zero.

The results showed that there were positive and linear significant relationships between percent mortality of adults and duration of exposure to the essential oil vapors within all concentration levels and plant species (P<0.05), although not significantly within concentrations of 110 $\mu l\ L^{-1}$ of *C. reticulata* (P=0.07). Coefficients of determination (R^2) indicated that between 64-98% of the variation in the rates of adult mortality was explained by duration of exposure to essential oils. Within each essential oil, the slopes of regressions of mortality rates on exposure times were smaller in low concentrations than those in high concentrations (Table 2).

More appropriate comparison between the fumigant toxicity of two essential oils could be obtained using probit analyses of data. The dose-mortality responses of *C. maculatus* adults to citrus essential oils were compared in terms of differences in slope and/or intercept of probit regressions, and LC_{50}s and LC_{90}s. The slope values of probit regressions were in the range of 3.6-3.8. The heterogeneity factor less than 1.0 for *C. reticulata* oil indicated that the result was found to be within the 95% confidence limits, so no correction factor (g) was required. The heterogeneity factor more than 1.0 for *C. limon* (2.01) required to use "g" factor for correcting of the respective LC_{50} value. For both essential oils, the regression tests ("t" ratio) were greater than 1.96 and the potency estimation tests ("g" factor) were less than 0.5 at all probability levels. The slopes of the two probit morality regressions for the essential oils were not significantly different, as revealed by accepting the likelihood ratio test of parallelism (X^2 = 0.1013, df =1, P = 0.75). However, the intercepts of probit morality regressions for two essential oils differed significantly, as revealed by rejecting the likelihood ratio test of equality (X^2 =16.76, df = 2, P < 0.001) (Table 3).

Table 1. Mean percent (±SE) mortality of the adults of *Callosobruchus maculatus* (Fabricius, 1775) exposed for various periods of time to essential oils from fruit peels of two *Citrus* species at different concentrations (N = 6) [a]

Concentration	Exposure period (h)				
(µl oil L^{-1} air)	3	6	9	12	24
			Citrus limon		
Control	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
19	0.00 (±0.00)	1.67 (±1.67)	5.00 (±2.24)	6.67 (±2.11)	10.00 (±2.58)
29	5.00 (±3.42)	11.67 (±4.77)	13.33 (±2.11)	20.00 (±3.65)	23.33 (±3.33)
44	11.67 (±3.07)	21.67 (±3.07)	28.33 (±3.07)	35.00 (±3.42)	43.33 (±3.33)
70	15.00 (±4.28)	30.00 (±5.16)	40.00 (±8.16)	46.67 (±7.60)	70.00 (±2.58)
110	23.33 (±2.72)	45.28 (±3.71)	65.83 (±3.38)	78.33 (±2.79)	98.33 (±1.67)
			Citrus reticulata		
Control	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)
19	3.33 (±2.11)	6.67 (±2.11)	10.00 (±2.58)	11.67 (±3.07)	20.00 (±2.58)
29	8.33 (±1.67)	15.00 (±3.42)	20.00 (±5.16)	25.00 (±3.42)	38.33 (±3.07)
44	11.67 (±3.07)	25.00 (±5.63)	36.67 (±3.33)	46.67 (±4.94)	68.33 (±3.07)
70	31.67 (±6.01)	46.67 (±4.94)	58.33 (±3.07)	63.33 (±4.22)	85.00 (±4.28)
110	40.23 (±4.46)	58.47 (±2.91)	82.75 (±3.83)	89.39 (±2.67)	98.81 (±1.19)

[a] Oils were applied to 2-cm filter papers attached to the undersurfaces of the 27- ml vials' lids.

Table 2. Analysis results of linear regressions of *Callosobruchus maculatus* (Fabricius, 1775) mortality data on exposure periods [a] in various concentrations of essential oils from fruit peels of two Citrus species [b]

Essential oil source	Concentration (µl oil L^{-1} air)	Slope (±*SE*)	R-squared (adjusted)	*F* statistic (*df* = 1, 3)	*P* value
		Citrus reticulata			
	19	0.769 (±0.056)	0.979	185.473	0.001
	29	1.381 (±0.123)	0.969	126.095	0.002
	44	2.591 (±0.346)	0.932	56.202	0.005
	70	2.367 (±0.360)	0.913	43.179	0.007
	110	2.542 (±0.881)	0.647	8.329	0.063
		Citrus limon			
	19	0.468 (±0.086)	0.879	29.958	0.012
	29	0.811 (±0.211)	0.774	14.738	0.031
	44	1.400 (±0.310)	0.829	20.395	0.020
	70	2.462 (±0.310)	0.939	62.869	0.004
	110	3.326 (±0.771)	0.815	18.628	0.023

[a] Exposure periods were 3, 6, 9, 12 and 24 h. Mortality data at each exposure period were a mean of six replicates.

[b] Oils were applied to 2-cm filter papers attached to the undersurfaces of the 27- ml vials' lids.

The above differences in parameters of the probit mortality regressions between experimental treatments were reflected in the LC_{90} or LC_{50} estimates. Comparisons of fumigant toxicity to *C. maculatus* adults between two essential oils using their LCs ratios and 95% confidence limits indicated that the toxicity of *C. reticulata* oil was significantly higher than that of *C. limon* oil, based on either the LC_{50} or LC_{90} values (Table 4).

Table 3. Fumigant toxicity of two *Citrus* fruit peel essential oils to *Callosobruchus maculatus* (Fabricius, 1775) adults [a]

Essential oil source	*N* [b]	Intercept (± *SE*)	Slope (± *SE*)	"*t*" ratio	Heterogeneity	g (0.95) factor	Lethal concentrations (µl L^{-1} air) (95% *CL*) [c]	
							LC_{50}	LC_{90}
Citrus limon	364	-6.30 (± 0.61)	3.80 (± 0.37)	10.35	2.01	0.19	45 (35-59)	99 (72-196)
Citrus reticulata	377	-5.54 (± 0.58)	3.64 (± 0.36)	9.98	0.58	0.04	33 (30-37)	75 (65-92)

[a] Oils were applied to 2-cm filter papers attached to the undersurfaces of the 27- ml vials' lids. Exposure period was 24 h.

[b] *N* = Total number of 1- to 2-day-old adult insects tested (including control).

[c] *CL*= Confidence limits.

Table 4. *Citrus limon* (Linnaeus, 1753), *Citrus reticulata* Blanco, 1830 ratios of LCs and their respective 95% confidence limits calculated [a] for comparing fumigant toxicity to the adults of *Callosobruchus maculates* (Fabricius, 1775)

	Ratio	95% *CL* of ratio
LC_{90}s	1.316	[1.021 – 1.695] *
LC_{50}s	1.363	[1.169- 1.588] *

[a] Lower and upper 95% *CL* calculated as described by Robertson & Preisler (1992).

* Significant difference at $P < 0.05$

Discussion

Over 120 plants and plant products have been shown to have insecticidal or deterrent activity against stored product pests (Dales, 1996). The mixing of plant oils is traditionally practiced in Asia and Africa. This type of pest control was abandoned with the advent of modern synthetic insecticides. However, due to the problems encountered with the use of the insecticides, interest in the use of biocides from plants has been renewed (Papachristos & Stamopoulos, 2002). Rutaceae is a large family containing 130 genera in seven subfamilies, with many important fruits and essential oil products. Analysis of the toxicity data in the present study showed that the essential oil vapors from the fruit peels of two *Citrus* species exhibited different toxicity to the adult stage of *Callosobruchus maculatus* (Fabricius, 1775), and that the toxicity depended on oil concentration and exposure period (Tables 1 and 2). Papachristos & Stamopoulos (2002) studied thirteen essential oils related to seven plant families and showed variable toxicities among different plant species to the bruchid, *Acanthoscelides obtectus* Say, 1831. A similar positive relationship between the rate of mortality and exposure time obtained in the present study has also been demonstrated in bioassays of many insects with various toxicants (Robertson & Preisler, 1992; Perry et al., 1998; Tunc et al., 2000; Sanon et al., 2002; Moravvej & Abbar, 2008). The essential oil vapor of *C. reticulata* exhibited higher toxicity to the cowpea adult beetles than that of *C. limon*, as indicated by lower 24 h LC_{50} and LC_{90} estimates of the former than the latter (Tables 3 and 4).

Studying on the effects of essential oils from various *Citrus* species on the bruchid, *C. maculatus* using the same methodology, Moravvej & Abbar (2008) reported that the fumigant LC_{50} values of *C. paradisi*, *C. aurantium*, *C. limonium* and *C. sinensis* at 24 h exposure were 125, 145, 235 and 269 µl L^{-1}, respectively. These values were much higher than the LC_{50} values of 33 and 45 µl L^{-1} obtained for *C. reticulata* and *C. limon*, respectively, in the present study. The observed difference between various *Citrus* species seems to be reasonable because of different plant species concerned, as shown in similar experiments with various stored product pests and essential oil vapors from different species of a plant genus. Don-Pedro (1996) attributed the mortality of *C. maculatus* adults on citrus peel-treated grains to the fumigant activity of the vapor released by peels. He indicated that grains treated by 7 ml oil kg^{-1} seed caused 100% mortality 1 h after application. However, the present study did not use essential oil admixed with cowpea grains.

In the present study, the slopes of probit mortality regressions were 3.80 for *C. limon* and 3.64 for *C. reticulata* oil. However, the likelihood ratio test showed that the slopes were not significantly different (Table 3). These slopes were much greater than those obtained in a similar bioassay for *C. aurantium* (2.88), *C. paradisi* (3.14) and *C. limonium* (3.22) (Moravvej & Abbar, 2008). A steep slope value indicates that there is a large increase in the mortality of insects with relatively small increase in the concentration of toxicant (Robertson & Preisler, 1992; Perry et al., 1998; Tiwari & Singh, 2004). The values of "t" ratio were greater than 1.96 in both essential oils, so the regressions were significant. Values of heterogeneity factor less than 1.0 denote that in the replicate tests of random samples, the concentration response line would fall within 95% confidence limits and thus the model fits the data adequately, the case that was not true for *C. limon* (Table 3). The index of significance of potency estimation "g" indicated that the value of the LC_{50} or LC_{90} was within the limits at 95% probability level (Table 3) as it was less than 0.5 (Robertson & Preisler, 1992; Tiwari & Singh, 2004).

According to Moravvej & Abbar (2008), citrus peel oils are preferred as grain protectant to conventional insecticides because of their low toxicity to mammals, cost-effective and easy preparation and application. However, aromatic plants contain, in general, essential oils at concentrations of 1-3% (Cakir, 1992), so large quantities of plant material have to be processed in order to obtain essential oils in quantities sufficient for commercial scale tests.

Conclusion

The present study suggested that the essential oils from *Citrus* species, particularly from *Citrus reticulata* Blanco, 1837, can be considered as promising control measure of stored cowpea bruchids. Nevertheless, this study examined the effects of essential oils only on the adult stage of *Callosobruchus maculatus* (Fabricius, 1775), so the effects on other developmental stages need to be determined.

Özet

Callosobruchus maculatus (Fabricius, 1775) (Coleoptera: Bruchidae) erginlerine karşı iki _Citrus_ türünün meyve kabuklarından ekstrakte edilen uçucu yağların etkinliği

Bu çalışmada *Citrus limon* (Linnaeus, 1753) ve *Citrus reticulata* Blanco, 1837 (Rutaceae) kabuklarındaki uçucu yağların, Börülce tohum böceği *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Bruchidae) üzerindeki etkisi araştırılmıştır. Uçucu yağlar, meyve kabuklarından su buharı destilasyonu ile ekstrakte edilmiştir. Araştırma sonuçları, *Citrus* kabuklarındaki yağların ergin böceklere yüksek fumigant etkiye sahip olduğunu göstermiştir. Artan yağ konsantrasyonu veya maruz kalma süresi ile 1-2 gün yaşındaki erginlerin ölüm oranlarının artığı tespit edilmiştir. *C. reticulata*'nın uçucu yağı, hem 24 saat LC_{50} (33 ve 45 µl L^{-1}, sırasıyla) hem de 24 saat LC_{90} (75 ve 99 µl L^{-1}, sırasıyla) hesaplamalarına göre *C. limon'*dan önemli derecede daha toksik olduğu görülmüştür. Bu çalışma sonuçlara göre, *Citrus* kabuklarından elde edilen uçucu yağların Börülce tohum böceklerinin erginlerinin kontrolünde potansiyel olarak kullanılabileceği ortaya konmuştur.

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