Lemon chemical resistance mechanisms to *Ceratitis capitata*

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Chapter VIII

INTRODUCTION

Citrus are considered by many authors as poor hosts for fruit flies. Field studies allowed proposing lemon trees (*Citrus limon* (L.) Burman f.) as a species practically immune to the Mediterranean fly, *Ceratitis capitata* (Wiedemann), (Quayle, 1914; Bodenheimer, 1951), its host status having been questioned.

Back and Pemberton (1915) suggested that citrus resistance was related to various components of the essential oil glands present in the rind (albedo and flabedo). When these glands break, they release toxic compounds for eggs and first instar larvae. The main components responsible for the mortality observed are the volatile ones, especially oxygenated monoterpenoids such as citral (Greany *et al.*, 1983). In the case of lemons, greater toxicity would be related to a higher concentration of these compounds. There are no studies assessing the toxicity of other compounds also present in the rind.

It has also been observed that the susceptibility of citrus to *C. capitata* varied according to the degree of senescence of the fruit and to the fruit species, the development of the fly being possible in overripe lemons (Quayle, 1914). Yet, there are no studies that can explain the decreased resistance of citrus.

The aim of this work was to identify the presence of toxic compounds in the essential oil of the lemon rind gland and to analyze the effect of the post harvest storage period on the resistance of this fruit to *C. capitata*.

MATERIALS AND METHODS

The toxicity of the compounds present in the essential oil of the lemon rind glands was determined through bioassays on eggs and larvae by extracting the oil with different solvents. Egg and larvae mortality was also assessed with the essential oil extract from the lemon rind with the greater biocide effect obtained from lemons with different storage periods. Then the percentage composition of certain compounds and their fluctuation during the post harvest period was determined. Finally, some of the compounds were added to the extracts obtained from lemons with different storage periods and the agent responsible for larvae mortality was determined.

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Extractions

In order to extract the essential oil from the lemon rind two methodologies were used. In the first, two fractions were obtained by steam hauling: a soluble and an insoluble fraction. The rind from 1 kg of recently collected fruit was removed, cut into small slices and placed in a distiller for two and a half hours, recovering oil and water. The oil was separated from the water with monodistilled ethylic ether and sodium anhydro sulphate salts. The separated oil plus ethylic ether was placed in a rotavapor to separate the oil from the solvent, which was then placed in a container with sodium anhydro sulphate salts until dehydration was completed and kept in a freezer. With respect to the second case, extractions were carried out by hauling with decreasing polarity solvents (exane, ethyl acetate and methanol), three phases being obtained, each corresponding to each solvent. One kg of fruit was collected and the rind was scraped and placed in exane for a week. After that time, the maceration was filtered. The maceration exane was recovered through a rotavapor and the fraction extracted was kept at -20 ºC. The maceration was then placed under a desiccation bell for 3 h to allow the evaporation of the remaining exane traces. Then ethyl acetate was added to it and it was allowed to macerate for a week. After this time, the product was filtered and the solvent was recovered and preserved. Finally, methanol was used to exhaust the sample, separating the chemical substances of greater molecular weight by following the same procedure.

Bioassays

Once the different extractions had been obtained, the biological activity of each was assessed by soaking lemon slices and placing on them eggs with 24 h of embryo development. A concentration of 250 ppm was used in the different extracts. The slices were individually placed in Petri dishes covered with perforated film paper that were placed in a desiccator for 30 min to evaporate the solvent. Twenty eggs were seeded in each slice. The control was treated only with the solvent and 20 eggs per slice were also seeded on it. The slices were then incubated at 25ºC for 6 days to promote embryo and larvae development. After that time the slices were observed through a stereoscope microscope and, using entomological pins, the number of eggs from which larvae failed to emerge, the number of chorions (i.e., eggs from which larvae emerged) and live and dead larvae were counted. Each lemon slice was considered as a replicate, ten replicates being performed for each extract and five for the control.

Effect of fruit storage on the toxicity of the essential oil

In order to determine the larvae killing effect of the ether lemon extract with different storage periods after harvest, lemons were collected and stored at 25 ºC. Every week for 2 months extractions were performed and the soluble fraction was used to assess toxicity through bioassays with the same procedure described above.

Identification of the compounds responsible for toxicity

For the purpose of determining the component/s of the essential oil biologically active in relation to the mortality of eggs and larvae, a GC-MS spectrometry analysis was performed on the soluble fractions of lemons with different storage periods. Different compounds were identified, the concentration of each was assessed and the fluctuation as a function of post-harvest time was determined. Then extractions were made from lemons with different storage periods and compounds present in the essential oil were added to them in order to determine whether the toxicity observed in the recently collected lemons was recovered.

Data analysis

The variables analyzed were egg viability and larvae mortality. The former was calculated as the number of eggs from which larvae emerged divided by the total number of seeded eggs per slice (20). Larvae mortality was determined as the number of dead larvae divided by the total number of larvae present per slice. The effect of the various fractions on both variables was determined by means of an analysis of variance (ANOVA). When ANOVA results were significant, the comparisons between groups were performed by Tukey's test. In order to comply with the assumption of variance homogeneity, data were transformed by the root of arcoseno.

RESULTS AND DISCUSSION

Effect of the various extracts from the essential oil gland on larvae emergence and viability

Table 1 shows *C. capitata* egg viability and larvae mortality resulting from the different extracts of the lemon essential oil compared to the control. In both cases significant differences were found between the different extracts and the control ($F = 8.0$; $gl = 5$; $P < 0.0001$ for egg viability and $F = 80.3$; $gl = 5$; $P < 0.0001$ for larvae mortality). Although both developmental stages were sensitive to the different extracts, mortality was much greater in larvae, it being above 95% in the soluble fraction.

Egg viability and larvae mortality in relation to the post harvest storage period of the lemons.

When egg and larvae mortality was assessed in the soluble fraction of lemons with different post harvest storage periods, the toxic effect on larvae was found to decrease as storage time increased (Table 2, Fig 1). Extracts from recently collected lemons showed 98.1% larvae mortality while extracts from lemons with 7 week storage periods showed 22.5%, larvae mortality, these differences being statistically significant ($F = 189.5$; $gl = 8$; $P < 0.0001$).

Fluctuation in the different compounds present in lemon essential oil as a function of the post harvest storage time of the fruit.

Certain compounds were found to decrease with time (Table 3). Neral, geranial and coumarin reached values below 50% of their initial concentration (Fig. 2) while the other compounds did not decrease so markedly. The decrease took place mainly between the 1st and the 2nd week of storage.

Analysis and verification of the compounds responsible for larvae mortality.

In order to verify whether neral, geranial and coumarin were responsible for the mortality observed in the larvae, extractions were made from lemons with different storage times. Then coumarin and citral (a natural mixture of the neral + geranial isomers) were added and toxicity was determined through bioassays as described above. In view of the potentially synergistic effect of linalool, the effect of this compound was also assessed together with coumarin. The addition of the compounds restored the larvae killing ability of the extracts in lemons with over 2 week post harvest storage periods (Table 4). In addition, linalool increased the effect of coumarin.

CONCLUSIONS

The results presented in this chapter allowed us to conclude that:

1. The components of the lemon rind exert a toxic effect on eggs and larvae.
2. This effect was stronger on larvae than on eggs.
3. In the extracts from lemons stored for several weeks after collection larvae mortality decreased.
4. Certain aldehydes such as citral (neral and geranial) and coumarin decrease their concentration as fruit storage time increases.
5. The addition of citral, coumarin and linalool restored the larvae killing effect in extracts performed in lemons with several weeks post harvest.
6. These compounds are the agents responsible for larvae mortality.
7. The above demonstrates that lemons have chemical resistance natural mechanisms against *C. capitata* attack.

Table 1. Effect of the different oil extracts from the lemon essential gland on the viability of *C. capitata* eggs and larvae

Extract	Egg viability (%)	Larval mortality (%)
Ethyl acetate	85.50 ± 1.57 a	93.08 ± 1.84 c
Hexane	84.00 ± 1.63 a	71.18 ± 6.60 b
Methanol	85.00 ± 1.29 a	94.11 ± 1.73 c
Non-soluble fraction	77.00 ± 3.67 a	93.78 ± 2.12 c
Soluble fraction	84.50 ± 2.03 a	97.49 ± 1.68 c
Control	94.00 ± 1.63 b	2.64 ± 1.15 a

Mean ± Standard error. For each extract 10 replicates were analyzed with 20 eggs in each. Values followed by the same letter in each column are not statistically different (Tukey's test, $P < 0.05$).

Table 2. Effect of ether extract from lemons with different post harvest storage periods on *C. capitata* eggs and larvae.

Storage (weeks)	Egg viability (%)	Larval mortality (%)
0	92.33 ± 1.45 ab	98.95 ± 0.56 f
1	93.33 ± 1.16 ab	95.77 ± 1.38 f
2	88.00 ± 1.36 a	81.91 ± 2.44 e
3	88.67 ± 2.21 a	68.54 ± 2.42 d
4	88.00 ± 2.06 a	74.75 ± 2.30 de
5	92.33 ± 0.67 ab	61.66 ± 3.27 d
6	90.33 ± 1.03 a	36.18 ± 2.24 c
7	90.33 ± 1.03 a	22.50 ± 2.78 b
Control	95.33 ± 1.14 b	3.46 ± 1.21 a

Mean ± Standard error. For each extract 10 replicates were analyzed with 20 eggs in each. Values followed by the same letter in each column are not statistically different (Tukey's test, $P < 0.05$).

Table 3. Components of lemon essential oil and their concentrations in lemons with different post harvest storage periods.

Compound	Postharvest weeks							
	0	1	2	3	4	5	6	7
α-bergamotene	0.56	0.67	0.45	0.54	0.51	0.43	0.61	0.48
α-pinene	1.02	0.92	1.16	1.03	1.31	1.42	1.08	1.30
α-terpineol	0.33	0.36	0.24	0.28	0.27	0.25	0.30	0.25
β-bisabolene	0.71	0.84	0.52	0.63	0.61	0.51	0.79	0.60
β-myrcene	0.97	0.97	0.98	1.01	1.01	0.98	0.95	0.96
β-pinene	8.72	8.68	9.25	8.74	10.53	12.12	9.93	11.28
3-carene	8.43	8.34	7.42	7.88	8.28	8.35	9.05	8.24
Caryophylene	0.36	0.37	0.22	0.28	0.28	0.24	0.36	0.24
Coumarin	0.22	0.21	0.04	0.05	0.05	0.05	0.11	0.09
Geranial	1.49	1.28	0.60	0.78	0.78	0.78	0.74	0.78
Geraniol	0.09	0.16	0.06	0.10	0.09	0.07	0.14	0.08
Limonene	74.28	73.93	76.73	75.89	73.50	72.02	72.98	72.97
Linalol	0.09	0.10	0.07	0.08	0.09	0.07	0.11	0.07
Neral	1.11	0.90	0.47	0.59	0.63	0.59	0.54	0.63
Nerol	0.45	0.62	0.41	0.48	0.45	0.40	0.66	0.45

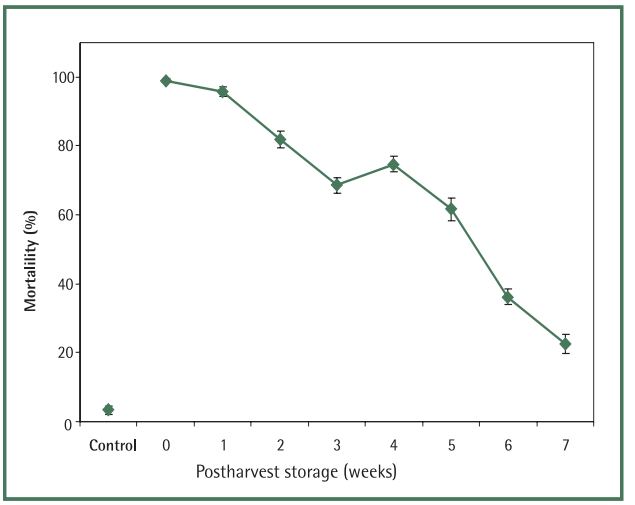

Figure 1. Larvae mortality assessed from the soluble extract from the lemon essential gland from lemons with different post harvest storage periods on the viability of *C. capitata* eggs and larvae.

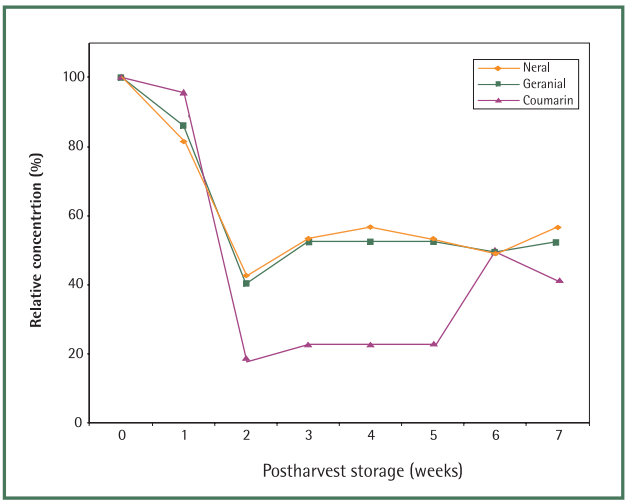

Figure 2. Percent concentration of neral, geranial and coumarin in lemon oil as a function of post harvest storage period.

Table 4. Effects of the addition of the different compounds in the ether extract from lemons with different times of postharvest storage in larval mortality of *Ceratitis capitata*.

Postharvest Storage (weeks)	Larval mortality (%)					
	EE	EE + coumarin	EE + coumarin + linalol	EE + citral	EE + coumarin + citral (T5)	Control
0	97.9 ± 0.7 aA	96.2 ± 1.1 aA	98.9 ± 0.6 aA	95.3 ± 1.1 aA	98.2 ± 0.6 aA	3.3 ± 1.0 g
1	95.8 ± 1.4 aA	93.6 ± 1.8 aA	97.5 ± 0.9 aA	93.5 ± 1.4 aA	96.5 ± 0.7 aA	2.4 ± 0.7 g
2	79.9 ± 2.7 bB	93.0 ± 1.2 aA	98.2 ± 1.0 aA	93.1 ± 1.0 aA	98.2 ± 0.6 aA	2.8 ± 0.9 g
3	63.9 ± 2.6 cD	88.5 ± 1.9 aC	98.2 ± 0.7 aA	92.3 ± 1.8 aA	98.5 ± 0.6 aA	2.3 ± 0.9 g
4	66.7 ± 2.2 cC	78.6 ± 2.3 bB	98.9 ± 0.6 aA	93.8 ± 1.4 aA	97.5 ± 0.6 aA	2.7 ± 0.8 g
5	50.9 ± 3.2 dC	77.8 ± 2.1 bB	98.6 ± 0.8 aA	91.4 ± 1.9 aA	97.2 ± 0.8 aA	3.1 ± 0.7 g
6	36.2 ± 2.2 eC	68.6 ± 2.2 cB	98.5 ± 0.8 aA	92.9 ± 1.2 aA	96.1 ± 1.3 aA	3.1 ± 0.9 g
7	20.5 ± 1.7 fC	54.6 ± 2.3 dB	97.2 ± 1.0 aA	89.9 ± 1.3 aA	95.6 ± 1.1 aA	2.7 ± 0.8 g

Mean ± Standard error. N = 15 replicates with 20 eggs each.
EE: Ether extract.
Means followed by the same letter (lower case) are not significantly different (P > 0.05, Tukey test). For upper case letters, multiple comparisons were made for each particular extract (within columns).
Taken from Salvatore, A., S. Borkosky, E. Willink and A. Bardón. 2004. Toxic effects of lemon peel constituents on *Ceratitis capitata*. J. Chem Ecol. 30: 323-333.

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