Everything you can imagine, Nature has already created.

*Albert Einstein*

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NICOLETTA FARAONE - EFFECTS OF NATURAL COMPOUNDS FROM *Citrus limon* ON THE BEHAVIOR OF *Ceratitis capitata* AND *Grapholita molesta*

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UNIVERSITÀ DEGLI STUDI DI PALERMO
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XXIII CICLO - ANNO ACCADEMICO – 2011-2012

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Settore scientifico disciplinare AGR/11

TESI DI
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*Dedicated to my grandma Miranda*

# *Riassunto*

## Effetti di composti naturali da *Citrus limon* (L.) Burm. f. sul comportamento di *Ceratitis capitata* (Wiedemann) e *Grapholita molesta* (Busck).

La ricerca ha riguardato gli effetti che le sostanze naturali provenienti da *Citrus limon* (L.) Burm. f. hanno sul comportamento di due importanti fitofagi dei fruttiferi, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) e *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae).

Inizialmente si è proceduto all'estrazione di differenti sostanze dal flavedo di quattro cultivar di limone coltivate in Sicilia; in particolare sono stati estratti gli oli essenziali e grazie a tre solventi organici (etere di petrolio, diclorometano e metanolo) sono stati ottenuti tre differenti estratti per ciascuna cultivar. Le sostanze volatili ottenute (oli essenziali) sono state saggiate sulle antenne della mosca mediterranea della frutta, mentre gli estratti sono stati saggiati sui tarsi dello stesso insetto; in entrambi i casi la risposta elettrofisiologica è stata registrata nei laboratori del prof. Antonio De Cristofaro dell'Università del Molise. Inoltre sono state condotte prove elettroantennografiche su α-terpineolo, geranil acetato e citrale, composti presenti nei frutti di *C. limon*.

I menzionati estratti delle cultivar Lunario e Interdonato sono stati utilizzati per prove comportamentali di laboratorio volte all'individuazione del comportamento indotto su *C. capitata*; in queste prove femmine adulte del dittero provenienti da allevamenti in insettario avevano la possibilità di scegliere e ovideporre su sfere trattate o meno con gli estratti. Per gli oli essenziali sono stati individuati i componenti e loro quantità; poiché era già nota una loro attività repellente, per quelli di due cultivar sono state approntate delle prove di campo in un aranceto biologico.

Nelle prove sperimentali, per la prima volta è stato dimostrato che quasi tutte le sostanze saggiate suscitano una risposta elettrofisiologica, mostrando quindi la loro percezione da parte della mosca mediterranea della frutta. In particolare per quanto riguarda gli estratti di Lunario e Interdonato, cinque su sei hanno mostrato una significativa attività repellente e antiovideponente aprendo nuove prospettive sui prodotti di possibile utilizzo

in pieno campo, soprattutto nei frutteti biologici dove non è permesso l'utilizzo di composti sintetici.

E' interessante notare anche l'innovazione nella metodologia degli studi comportamentali in laboratorio, che per la prima volta hanno visto l'utilizzo di sfere inerti, ma forate, quindi adatte a valutare l'attività dei composti sull'ovideposizione.

Degli oli essenziali saggiati negli aranceti, quelli della cultivar Lunario si sono dimostrati efficaci nel ridurre l'infestazione di *C. capitata* su arance a maturazione precoce, cv. Navelina, indicando con ciò che si è molto vicini alla formulazione di un preparato da utilizzare in campo nel prossimo futuro.

Un'altra parte del progetto portata avanti durante il dottorato di ricerca è stata svolta in Canada nei laboratori del dott. Robert Mitch Trimble, Agriculture and Agri-Food Canada di Vineland Station (Ontario). Qui si è sviluppata una tematica legata all'applicazione dei feromoni sessuali dei lepidotteri tortricidi; in particolare si è studiato l'effetto del citrale, composto comune a molti frutti di *Citrus* spp., su *G. molesta*, importante lepidottero fitofago del pesco e altre rosacee. Sono state condotte delle prove di adattamento sensoriale al citrale e al feromone, sulle antenne dei maschi mediante analisi elettrofisiologiche, e su maschi adulti in tunnel del vento. In entrambi i casi si è utilizzato il "piezo-electric microsprayer" che permette il rilascio di quantità note di sostanze volatili, e nel caso dei feromoni tale rilascio è molto vicino alle modalità di emissione delle femmine vergini.

E' stata messa in luce per la prima volta la capacità del citrale di stimolare le antenne dei maschi, esaltare la percezione del feromone sessuale e quindi di avere un effetto sinergico a causa del processo di adattamento sensoriale; nelle prove di comportamento si è anche evidenziata la capacità del citrale di ostacolare l'aggancio della scia feromonale da parte del maschio. Ciò potrebbe permettere un utilizzo del citrale in pieno campo nella tecnica della confusione sessuale da solo o in sinergia con il più costoso feromone.

# *Declaration*

I do hereby declare that the thesis entitled "**Effects of natural compounds from *Citrus limon* (L.) Burm. f. on the behavior of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) and *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae)**" submitted to the University of Palermo for the award of the Degree of Doctor of Philosophy in Plant Health Management of Eco-compatible Agro-forestry and Urban Environments, is a record of original and independent research work done by me during 2009 to 2011 under the supervision and guidance of *Prof. Dr. Virgilio Caleca*, Sr. Entomologist, Department DEMETRA, Entomological, Acarology and Zoology Section, University of Palermo, Viale delle Scienze, Edificio 5, 90128 Palermo, Italy Palermo, and of *Prof. Dr. Maurizio Bruno*, Sr. Chemist, Department STEMBIO, Molecular and Biomolecular Science and Technology, University of Palermo, Viale delle Scienze, Edificio 17, 90128 Palermo, Italy Palermo, and it has not previously the basis for the award of any other Degree or Diploma or Associateship or Fellowship or other similar title to any candidate of this or any other University.

**Nicoletta Faraone**

Nicoletta Faraone

# *Publications*

During the course of this project, public presentations have been made which are based on the work presented in this thesis. They are listed here for references.

- **N. Faraone**, V. Caleca, A. De Cristofaro, M. Bruno, R. M. Trimble; Potential for using the monoterpenoid citral alone and in combination with sex pheromone for managing the oriental fruit moth by mating disruption; **poster**
  Convegno SCI (Società Chimica Italiana) – "Sezioni Sicilia e Calabria" 2011;

- **N. Faraone**, V. Caleca, M. Bruno, S. Vitagliano, A. De Cristofaro; Biological Activity of *Citrus* spp. Metabolites on *Ceratitis Capitata* (Wiedemann); **oral communication**
  IOBC Working Group "Integrated Protection of Fruit Crops" 2010;

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The research presented was possible thanks to a lot of people with which I shared time and work.
First and foremost I want to thank Prof. Dr. Virgilio Caleca, for his continuous support, words of encouragement, quite urgings and carefully reading of all of my writing in the PhD program will never be forgotten. He argued my desire to know and my enthusiasm.
I'm grateful to Prof. Maurizio Bruno who has made possible this wonderful experience and has supported me.
This project would not have been possible without the expert guidance of Prof. Dr. Antonio De Cristofaro. He taught me a lot and made me grow professionally. He showed me different ways to approach a research problem and the need to be persistent to accomplish any goal. His direction has been invaluable to pursue everyday and longterm results.
Dr. Robert Mitch Trimble gave me the opportunity to spend one of the most fruitful, interesting and greatest periods in his lab. A great group leader and professor, he made me feel at home and relaxed every day of my stay. His great advices and his being keen on listening to people and guiding them towards their goals are incredibly valuable qualities.
I must acknowledge my friends, colleagues and research scholars who assisted, advised and supported my research and writing efforts over the years. Especially, I need to express my gratitude and deep appreciation to Dr. Silvia Vitagliano, Dr. Matteo Maltese and Mr. Erik Glemser whose friendship, hospitality, knowledge, and wisdom have supported, enlightened, and entertained me over this adventure.

Palermo, February 2012

Nicoletta Faraone

# *Preface*

The work presented in this PhD thesis has been carried out at Department DEMETRA - University of Palermo (Italy), under the direction of Prof. Virgilio Caleca, and at Department SAVA – University of Molise (Italy), under the direction of Prof. Antonio De Cristofaro, with the part devoted to the Mediterranean fruit fly. Nearly at third of PhD, research was conducted at the Agricolture and Agri-Food Canada, Vineland (ON, Canada), under the supervision of Dr. R.M. Trimble.

The whole PhD was devoted to investigate the electrophysiological and behavioral interactions and effects of natural compounds from Citrus spp. against two important pests, *Ceratitis capitata* (Wiedemann) and *Grapholita molesta* (Busck).

The thesis is organized in two main chapters. A brief introduction presents the concept of biological activities of secondary metabolites extracted from *Citrus* spp. to control some pests. Soon after, effects of natural compounds from *Citrus limon* on the ovipositional behavior of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) are discussed in the first chapter. The second chapert is about potential for using the monoterpenoid citral alone and in combination with sex pheromone for managing the oriental fruit moth *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) by mating disruption.

# *Contents*

**Effects of natural compounds from *Citrus limon* (L.) Burm. f. on the behavior of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) and *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae)**

**3. *Results***

**1. *Introduction***

**2. *Material & Methods***

**3. *Results***

# Effects of natural compounds from *Citrus limon* (L.) Burm. f. on the behavior of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) and *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae).

# General introduction

During the last years interesting study were carried out on effects of natural substances like plant extracts and essential oils on different types of phytophagous activity. The main activities of phytophagous insects are related of plants where they often meet, court and mate and then their sexual behaviour may occur principally on host plants. Host selection in phytophagous insects consists a sequence of behavioural responses to an array of stimuli associated with host and non-host plants. The insects are equipped with sensory receptors enabling them to perceive these stimuli. Plant stimuli involved include in varying proportions visual, mechanical, gustatory, and olfactory characteristics (Visser, 1986).

Plant essential oils, volatile natural complex secondary metabolites, are an important natural source of pesticides and an alternative means to controlling many insects and their rapid degradation in the environment have developed for a wide range of human and animal uses, including pest control (Regnault-Roger, 1997; Tripathi *et al.*, 2009). Recent research have demonstrated their interesting properties like deterrent effects on oviposition (Naumann *et al.*, 1995; Oyedele *et al.*, 2000) and repellent action (Landolt *et al.*, 1999) but despite these most promising properties, problems related to their volatility, poor water solubility and aptitude for oxidation have to be resolved before they are used as an alternative pest control system (Moretti *et al.*, 2002). Also vegetable extracts from aromatic plants were examined in detail, aimed to pest control (Pascual-Villalobos and Robledo, 1998; Kim *et al.*, 2003).

*Citrus* spp. have been reported as a source of botanical insecticides and have been shown interesting properties and secondary metabolites presents in peel (Siskos *et al.*, 2009), juice (Caristi *et al.*, 2003) and essential oils (Koul *et al.*, 2008), provide a wide variety of active compounds.

The first studies reported that lemon was not attacked by *C. capitata* until the fruit was overripe or partially decay (Back and Pemberton, 1918; Quayle, 1914, 1929). *C. limon* resistance to medfly attack has been attributed in part to chemical characteristics of the peel (i.e. essential oils, 5,7-dimethoxy-coumarin, linalool in the flavedo) (Greany *et al.*, 1983; Salvatore *et al.*, 2004) and to physical properties such as peel thickness (principally flavedo thickness) and peel resistance to puncture (Greany, 1989; Leyva *et al.*, 1991). Laboratory studies on commercial varieties of lemon confirmed that lemons should not be considered as host-fruits for *C. capitata* (Spitler *et al.*, 1984).

Da Silva Branco *et al.* (2000) found that oxygenated monoterpene aldehydes from *Citrus limon* (L.) Burm. f., like citral, are responsible for the chemical resistence of lemons to attack by medfly. In a study about the evaluation of the toxicity of essentials oils, citral has been shown to be both toxic and phototoxic against developing *Trichoplusia ni* (Lepidoptera: Noctuidae) larvae (Green and Berenbaum, 1994), and de Kramer *et al.* (2002) reported that terpenes reduced the effect of pheromone on Lepidoptera, and citral has shown to be particularly effective. Limonoids, extremely bitter chemicals present in citrus seeds, act as antifeedants or antagonize ecdysone action in many lepidopteran species (Klocke and Kubo, 1982; Jayaprakaha *et al.*, 1997).

On the basis of this reports and continuing with research of natural insecticides, peel extracts and essential oils and their natural compounds of *Citrus limon* were tested to evaluate the effects on behaviour of two important pest, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) and *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae).

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# Section 1

# Effects of natural compounds from *Citrus limon* on the ovipositional behavior of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae).

# 1. *Introduction*

## 1.1 *Role of host plant compounds in ovipositional behavior.*

Oviposition behavior has been at the centre of many debates on the ecology and evolution of interactions between insects and plants: the causes of host specificity, the origins of host shifts, the modes of coevolution, and the pattern of attack on host plants within local populations. The selectivity of ovipositing females may often provide the initial basis for divergence of insect populations onto different plant species, and it may drive the evolution of some plant defences (Bernays, 2003).

Factors that affect the oviposition behavior of some tephritid fruit flies include host-plant odour as well as the physical and chemical features of the oviposition substrate, such a size, shape, colour, and presence of some deterrent chemicals (Bateman, 1972; Fletcher and Prokopy, 1991).

Plant chemicals play an important role, together with visual cues, in attracting phytophagous insects to their chosen host plants for both feeding and oviposition (Harborne, 2001). Host plant compounds affect ovipositional behavior in various ways: orientation and landing are primarily guided by plant volatile constituents, whereas assessment of a surface depends on contact stimuli.

Less is known about the chemical factors that guide ovipositing female insects away from unsuitable host plants, since only few deterrents have been identified. For example, a chemical defense mechanism is represented by plant constituents released as a result of a previous damage (Hilker and Meiners, 2002). Perception of chemical cues that affecting oviposition involves receptors on antennae, tarsi, mouthparts or the ovipositor (Renwick, 1989). The influence of chemical agents on insects' ovipositional behavior can be used to control pests as well as vector insects. Some natural products display oviposition-deterrence activity against different insects (Yu-Tong Qiu *et al.*, 1998). Essential oils from medicinal plants have been successfully used as oviposition deterrents on *Thrips tabaci* (Lindeman) (Thysanoptera: Thripidae) (Koschier and Sedy, 2003) and mosquitos species (Prajapati *et al.*, 2005), and an aqueous extract of *Tanacetum vulgare* L. (Compositae) has shown antifeedant and oviposition deterrent activities on two cabbage pests (Hough-Goldstein and Hahn, 1992). A styrene glycoside isolated from methanol extract of *Elaphoglossum piloselloides* (Dryopteridaceae) partially inhibited the oviposition of *Ceratitis capitata* (Wiedemann) when incorporated onto the surface of artificial fruit (Socolsky *et al.*, 2003).

## 1.2 *General information on Mediterranean fruit fly.*

The Mediterranean fruit fly (medfly), *C. capitata* (Diptera, Tephritidae), is one of the most injurious pests worldwide (McPheron and Steck, 1996; Aluja and Norrbom, 2000) because of its direct economic impact (*i.e.* female oviposition and larval feeding render fruits unmarketable) and the strict quarantine restrictions imposed by many Countries to curtail its entry (Follett and Neven, 2006). The species is spread in temperate regions and attacks a wide variety of hosts (Hegen *et al.*, 1981; Liquido *et al.*, 1991). It has become a major pest of *Citrus* spp., causing extensive fruit losses worldwide and in particularly in Mediterranean countries, as well as in similar climatic regions (Staub *et al.*, 2008; Aluja and Mangan, 2008); orange, clementines, mandarines, grape fruits, satsumas, are attached by *C. capitata*, while lemons are almost totally resistent to it (Sproul, 1976; Spitler *et al.*, 1984; Elekcioglu *et al.*, 2008); just in some cases, overripe lemons can be attacked by the Mediterranean fruit fly (Porras *et al.*, 2009).
During the last decade, the development of crop protection methods had to take into account the need of preserving the precarious equilibrium among pests and their natural enemies, mainly affected by the application of chemical pesticides, as well as the need to use alternative control measures. For these reasons, several studies have been carried out to investigate the role of plant volatile compounds on medfly behavior (Teranishi *et al.*, 1987; McInnis *et al.*, 1988; Warthen *et al.*, 1989) with the aim of identifying biologically active compounds that could be used in sustainable control techniques (Lai *et al.*, 2006; Peteu et al. 2010) and organic farming (Vincent et al. 2003).

**Figure 1:** Dorsal view of *Ceratitis capitata* (Wiedemann) adult male. Credits: Scott Bauer, USDA.

### 1.3 *Control of medfly and disrupting insect behavior using natural compounds from Citrus spp.*

Interesting studies have been carried out on the effects of host plant compounds, by electrophysiological analysis to investigate medfly olfactory response towards host plant volatiles (Light *et al.*, 1988; Light *et al.*, 1992); interesting results have been obtained by studying the effects of plant extracts and essential oils on *C. capitata* (as well as other Tephritid flies): these compound could influence medfly feeding activity, as well as its oviposition behavior, and could have an insecticidal activity, too (Sanna Passino *et al.*, 1999; Bado *et al.*, 2004; Siskos *et al.*, 2009). It also has been observed that lemons are not attacked by medfly, probably because of the peel oil, that is toxic to other fruit flies (Katsoyannos *et a*l., 1997; Salvatore *et al.*, 2004). This makes the lemon a candidate for the study of its interesting properties, which could be used as a control instrument against medfly.

In the present paper, the biological activity of peel oils and peel extracts of Sicilian cultivars of *Citrus limon* (L.) Burms.f. on medfly oviposition behavior has been investigated by electrophysiological recordings (SCR, EAG), behavioral bioassays (oviposition double-choice test) and field trials.

**Figure 2:** A female Mediterranean fuit fly, *Ceratitis capitata* (Wiedemann), during the oviposition. Credits: Scott Bauer, USDA.

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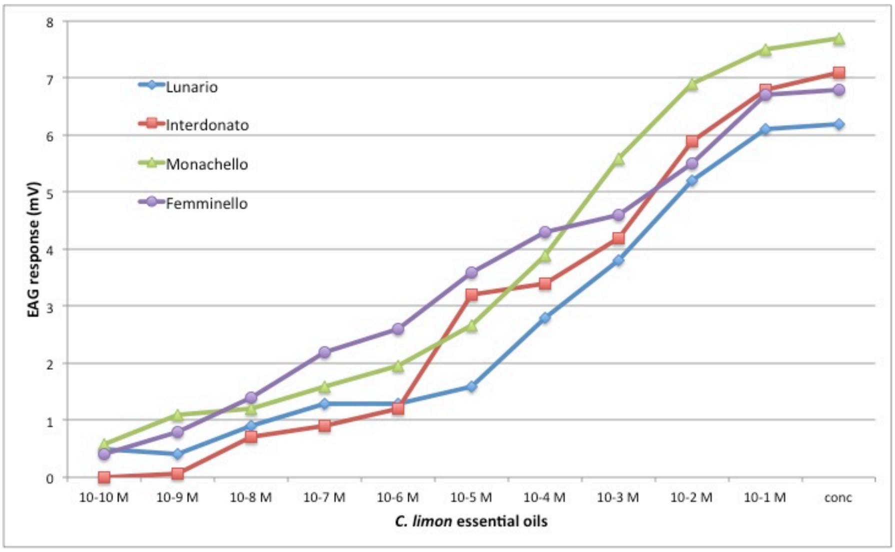
# 3. *Results*

## 3.1 *Electrophysiology.*

- ### 3.1.1 *EAG of peel essential oils of* C. limon *'Interdonato', 'Lunario', 'Monachello' and 'Femminello' cultivars.

The results are reported as mean±SE and in all statistical analysis $P > 0.05$ were considered not significant. The EAG data showed a high sensitivity (about -6.5/-7.0 mV) of the medfly antennae to the oils of *Citrus* spp. and a clear dose-response relationship (Fig. 13).

**Figure 13:** Mean (±*SE*) EAG responses of *C. capitata* female antennae after stimulation (n=10) with increasing doses of *C. limon* essential oils.



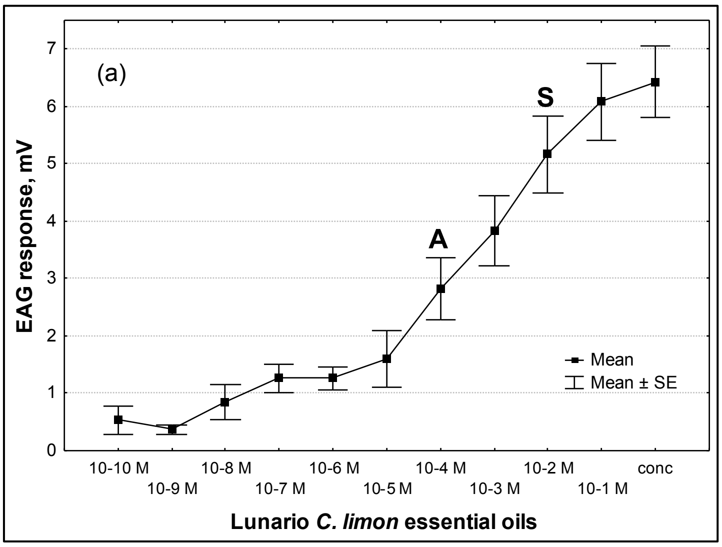
Stimulation with various concentrations of essential oil in hexane elicited a typical sigmoid-shaped dose-dependent response. The activation threshold dose were $10^{-4}$ M for 'Lunario', $10^{-5}$ M for 'Interdonato' (Fig. 14b) and 'Femminello' (Fig. 15b), $10^{-6}$ M for

‘Monachello’ (Fig. 15a); the saturations threshold doses were $10^{-2}$ M for ‘Lunario’ and ‘Monachello’, $10^{-3}$ M for ‘Interdonato’ and $10^{-4}$ M for ‘Femminello’.

The EAG responses to higher dose of pure essential oil were among 7.1 ± 0.8 mV (‘Interdonato’) and 7.7 ± 0.5 mV (‘Monachello’); the EAG response to the lower dose $10^{-10}$ M were among 0 mV (‘Interdonato’) and 0.58 ± 0.09 mV (‘Monachello’).

One-way ANOVA on each essential oil data indicated significant differences among the EAG responses to different concentrations.

**Figure 14:** Mean (±*SE*) EAG dose-responses of *C. capitata* antennae females after stimulation (n=10) to increasing doses of ‘Lunario’ (a) and ‘Interdonato’ (b) *C. limon* essential oils. Different letters indicate statistically significant differences. ANOVA followed by Tukey test. A= activation dose; S= saturation dose.



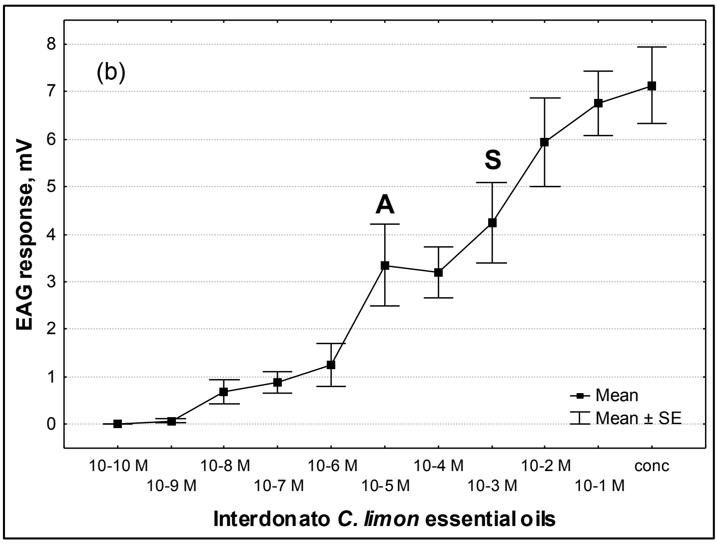


**Table 1:** Mean (±*SE*) EAG dose-responses (mV) of *C. capitata* antennae males after stimulation (n=10) to increasing doses of 'Lunario' and 'Interdonato' *C. limon* essential oils in hexane.

| | **'Lunario' *C. limon* Essential Oils** | | **'Interdonato' *C. limon* Essential Oils** | |
|---|---|---|---|---|
| **Dose** | Absolute mean EAG response in mV (mean ± *SE*)[a] | | Absolute mean EAG response in mV (mean ± *SE*)[a] | |
| **$10^{-10}$ M** | 0.5 ± 0.1 | a | 0.0 ± 0.0 | a |
| **$10^{-9}$ M** | 0.4 ± 0.1 | a | 0.07 ± 0.04 | a |
| **$10^{-8}$ M** | 0.9 ± 0.4 | ab | 0.7 ± 0.3 | ab |
| **$10^{-7}$ M** | 1.3 ± 0.4 | ab | 0.9 ± 0.2 | ab |
| **$10^{-6}$ M** | 1.3 ± 0.1 | ab | 1.2 ± 0.5 | ab |
| **$10^{-5}$ M** | 1.6 ± 0.4 | abc | 3.2 ± 0.9 | bc |
| **$10^{-4}$ M** | 2.8 ± 0.8 | bc | 3.4 ± 0.5 | bc |
| **$10^{-3}$ M** | 3.8 ± 0.8 | cd | 4.2 ± 0.8 | cd |
| **$10^{-2}$ M** | 5.2 ± 0.8 | de | 5.9 ± 0.9 | cd |
| **$10^{-1}$ M** | 6.1± 0.7 | e | 6.8± 0.6 | d |
| **conc** | 6.2 ± 0.9 | e | 7.1 ± 0.8 | d |

[a] Values followed by different letters are significantly different at $P = 0.05$ (Tukey-HSD test).

**Figure 15:** Mean (±*SE*) EAG dose-responses of *C. capitata* antennae females after stimulation (n=10) to increasing doses of 'Monachello' (a) and 'Femminello' (b) *C. limon* essential oils. Different letters indicate statistically significant differences. ANOVA followed by Tukey test. A= activation dose; S= saturation dose.

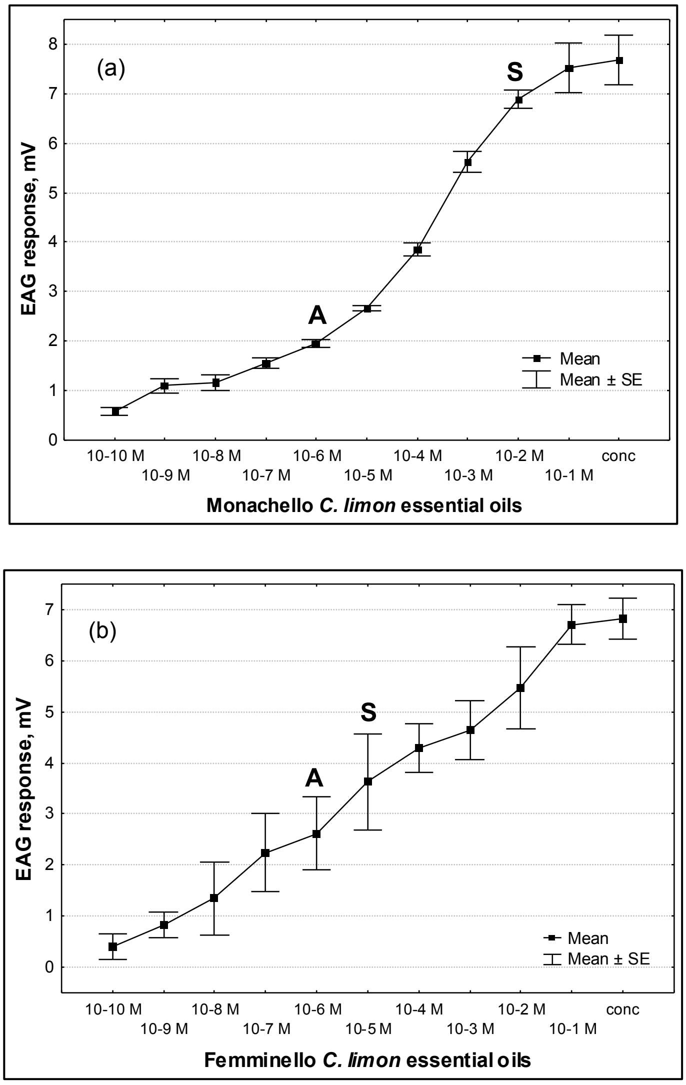


**Table 2:** Mean (±*SE*) EAG dose-responses (mV) of *C. capitata* antennae males after stimulation (n=10) to increasing doses of *C. limon* 'Monachello' and 'Femminello' essential oils in hexane.

| | **'Monachello' *C. limon* Essential Oils** | | **'Femminello' *C. limon* Essential Oils** | |
|---|---|---|---|---|
| **Dose** | Absolute mean EAG response in mV (mean ± *SE*)[a] | | Absolute mean EAG response in mV (mean ± *SE*)[a] | |
| **$10^{-10}$ M** | 0.58 ± 0.09 | a | 0.4 ± 0.3 | a |
| **$10^{-9}$ M** | 1.1 ± 0.1 | ab | 0.8 ± 0.3 | ab |
| **$10^{-8}$ M** | 1.2 ± 0.2 | ab | 1.4 ± 0.7 | abc |
| **$10^{-7}$ M** | 1.6 ± 0.1 | abc | 2.2 ± 0.8 | abcd |
| **$10^{-6}$ M** | 1.96 ± 0.09 | bc | 2.6 ± 0.7 | abcde |
| **$10^{-5}$ M** | 2.67 ± 0.06 | cd | 3.6 ± 0.9 | bcde |
| **$10^{-4}$ M** | 3.9 ± 0.1 | d | 4.3 ± 0.5 | cdef |
| **$10^{-3}$ M** | 5.6 ± 0.2 | e | 4.6 ± 0.6 | def |
| **$10^{-2}$ M** | 6.9 ± 0.2 | f | 5.5 ± 0.8 | df |
| **$10^{-1}$ M** | 7.5 ± 0.5 | f | 6.7 ± 0.4 | f |
| **conc** | 7.7 ± 0.5 | f | 6.8 ± 0.4 | f |

[a] Values followed by different letters are significantly different at $P = 0.05$ (Tukey-HSD test).

- 3.1.2 *EAG of single compounds.*

The EAG response of the other compounds presents in essential oils composition of citrus and also in Sicilian lemon cultivars has been investigated. The selected compounds were α-terpineol (Fig. 16), that showed toxic and repellent activities toward medfly (Sanna Passino *et al.* 1999; Salvatore *et al.* 2004), and geranyl acetate (Fig. 17), that elicited a strong response in medfly male antennae (Cossé *et al.* 1995) and also citral (Fig. 18), not present in these essential oils composition but common in *C. limon* essential oils.

The activation threshold doses were $10^{-3}$ M for α-terpineol (Tab. 3), $10^{-5}$ M for geranyl acetate (Tab. 4) and $10^{-6}$ M for citral (Tab. 5). The saturation threshold doses were $10^{-2}$ M for α-terpineol, $10^{-3}$ M for citral and geranyl acetate.

One-way ANOVA on each essential oil data indicated significant differences among the EAG responses to different concentrations.

**Figure 16:** Mean (±*SE*) EAG dose-responses of *C. capitata* antennae females after stimulation (n=10) to increasing doses of α -terpineol. A= activation dose; S= saturation dose.

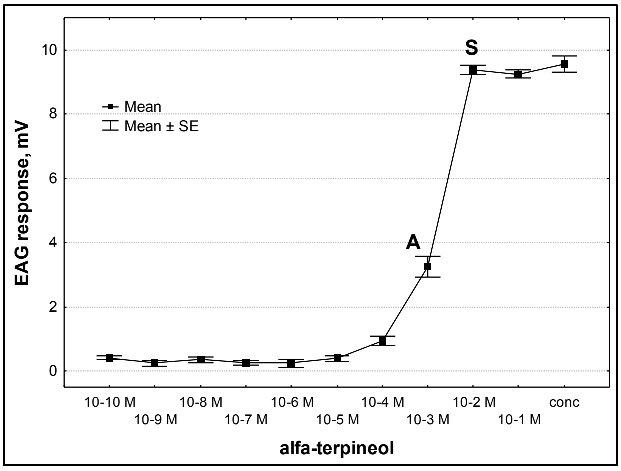


**Table 3:** Mean (±*SE*) EAG dose-responses (mV) of *C. capitata* antennae males after stimulation (n=10) to increasing doses of α-terpineol in hexane. Different letters indicate statistically significant differences. ANOVA followed by Tukey test.

| | **α-terpineol** | |
|---|---|---|
| **Dose** | Absolute mean EAG response in mV (mean ± *SE*)[a] | |
| **10$^{-10}$ M** | 0.41 ± 0.06 | a |
| **10$^{-9}$ M** | 0.3 ± 0.1 | a |
| **10$^{-8}$ M** | 0.4 ± 0.1 | a |
| **10$^{-7}$ M** | 0.25 ± 0.08 | a |
| **10$^{-6}$ M** | 0.2 ± 0.1 | a |
| **10$^{-5}$ M** | 0.4 ± 0.1 | a |
| **10$^{-4}$ M** | 1.0 ± 0.1 | a |
| **10$^{-3}$ M** | 3.3 ± 0.3 | b |
| **10$^{-2}$ M** | 9.4 ± 0.1 | c |
| **10$^{-1}$ M** | 9.2 ± 0.1 | c |
| **conc** | 9.6 ± 0.3 | c |

[a] Values followed by different letters are significantly different at $P = 0.05$ (Tukey-HSD test).

**Figure 17:** Mean (±*SE*) EAG dose-responses of *C. capitata* antennae females after stimulation (n=10) to increasing doses of geranyl acetate. A= activation dose; S= saturation dose.

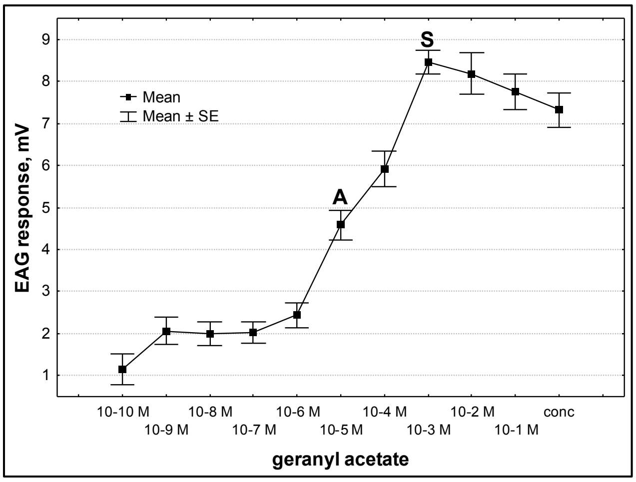


**Table 4:** Mean (±*SE*) EAG dose-responses (mV) of *C. capitata* antennae females after stimulation (n=10) to increasing doses of geranyl acetate in hexane. Different letters indicate statistically significant differences. ANOVA followed by Tukey test.

| geranyl acetate | | |
|---|---|---|
| **Dose** | Absolute mean EAG response in mV (mean ± *SE*)[a] | |
| **$10^{-10}$ M** | 1.2 ± 0.4 | a |
| **$10^{-9}$ M** | 2.1 ± 0.3 | a |
| **$10^{-8}$ M** | 2.0 ± 0.3 | a |
| **$10^{-7}$ M** | 2.0 ± 0.3 | a |
| **$10^{-6}$ M** | 2.4 ± 0.3 | a |
| **$10^{-5}$ M** | 4.6 ± 0.4 | b |
| **$10^{-4}$ M** | 5.9 ± 0.4 | bc |
| **$10^{-3}$ M** | 8.5 ± 0.3 | d |
| **$10^{-2}$ M** | 8.2 ± 0.5 | d |
| **$10^{-1}$ M** | 7.7 ± 0.4 | d |
| **conc** | 7.3 ± 0.4 | cd |

[a] Values followed by different letters are significantly different at $P = 0.05$ (Tukey-HSD test).

**Figure 18:** Mean (±*SE*) EAG dose-responses of *C. capitata* antennae males after stimulation (n=10) to increasing doses of citral. A= activation dose; S= saturation dose.

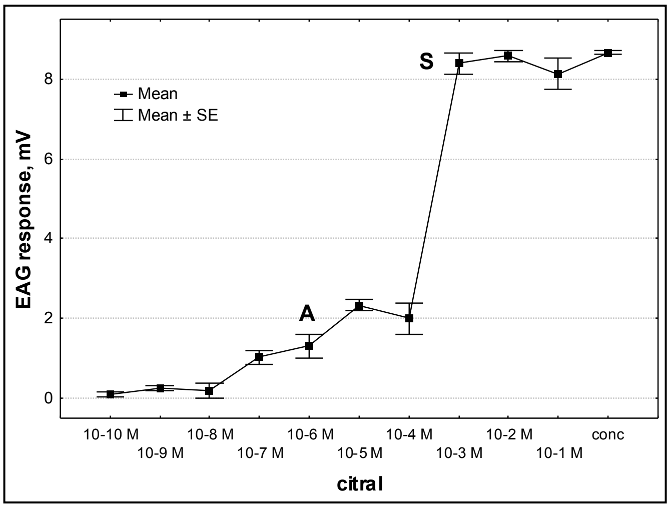


**Table 5:** Mean (±*SE*) EAG dose-responses (mV) of *C. capitata* antennae females after stimulation (n=10) to increasing doses of citral in hexane. Different letters indicate statistically significant differences. ANOVA followed by Tukey test.

| **citral** | | |
|---|---|---|
| **Dose** | Absolute mean EAG response in mV (mean ± *SE*)[a] | |
| **$10^{-10}$ M** | 0.10 ± 0.07 | a |
| **$10^{-9}$ M** | 0.25 ± 0.05 | ab |
| **$10^{-8}$ M** | 0.2 ± 0.2 | a |
| **$10^{-7}$ M** | 1.0 ± 0.2 | abc |
| **$10^{-6}$ M** | 1.3 ± 0.3 | bcd |
| **$10^{-5}$ M** | 2.3 ± 0.1 | d |
| **$10^{-4}$ M** | 2.0 ± 0.4 | cd |
| **$10^{-3}$ M** | 8.4 ± 0.3 | e |
| **$10^{-2}$ M** | 8.6 ± 0.1 | e |
| **$10^{-1}$ M** | 8.1 ± 0.4 | e |
| **conc** | 8.66 ± 0.05 | e |

[a] Values followed by different letters are significantly different at *P* = 0.05 (Tukey-HSD test).

- 3.2.3 *Single Cell Recording (SCR) of lemon peel extracts.*

Different gustatory tarsal cells responses to lemon peel extracts of two *C. limon* 'Interdonato' and 'Lunario' cultivars were recorded. The higher sensitivity was evoked by *C. limon* 'Interdonato', particularly to the methanol extract, which elicited significant increases in the spike frequency at increasing concentrations (Tab. 6).

**Table 6:** Responses (action potential) of *C. capitata* gustatory cells after stimulation (n=5) with increasing doses of *C. limon* 'Lunario' and 'Interdonato' peel extracts in three different solvents (petroleum ether, dichloromethane and methanol). Control: NaCl 0,1 M + Tween 80 solution.

| | NaCl 0,1 M | NaCl 0,1 M + Tween 80 | $10^{-5}$M | $10^{-4}$M | $10^{-3}$M | $10^{-2}$M | $10^{-1}$M |
|---|---|---|---|---|---|---|---|
| **Interdonato** (petroleum ether) | - | - | + | + | + | + | ++ |
| **Interdonato** (dichlorometane) | - | - | - | + | ++ | ++ | ++ |
| **Interdonato** (methanol) | - | - | + | + | ++ | +++ | +++ |
| **Lunario** (petroleum ether) | - | - | - | - | - | - | + |
| **Lunario** (dichlorometane) | - | - | - | - | + | + | ++ |
| **Lunario** (methanol) | - | - | - | + | + | + | + |

- = 25±2 spikes/s frequency; + = > 30-50 %; ++ = > 50-100 %; +++ = > 100 %.

### 3.2 *Behavioral bioassays.*

The interpretation of the SCR results was made through behavior essays in the laboratory, aimed at investigating the effects of these extracts of the two cultivars on oviposition behavior. The behavioral bioassay has shown an oviposition decrease in the treated spheres in comparison with the control ones (Fig. 19-20). In particular, a significantly lower number of eggs have been laid by medfly females when the sphere was treated with *C. limon* 'Lunario' dichlorometane extract (Fig. 19) and with *C. limon* 'Interdonato' petroleum ether extract (Fig. 20).

**Figure 19:** Mean (±*SE*) percentage of *C. capitata* eggs (6 replicates) laid on spheres treated with *C. limon* 'Lunario' extracts in different solvents (petroleum ether, dichloromethane and methanol). Different letters indicate significant differences (*t* –Test; P = 0,05).

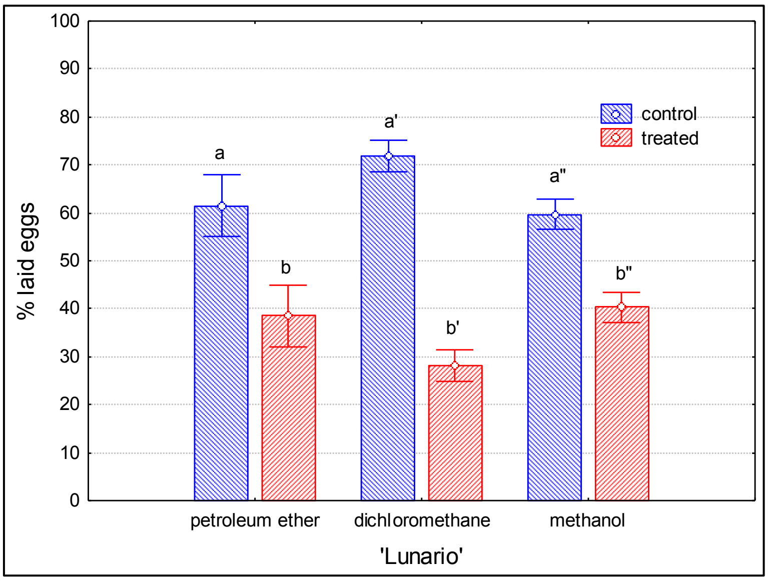
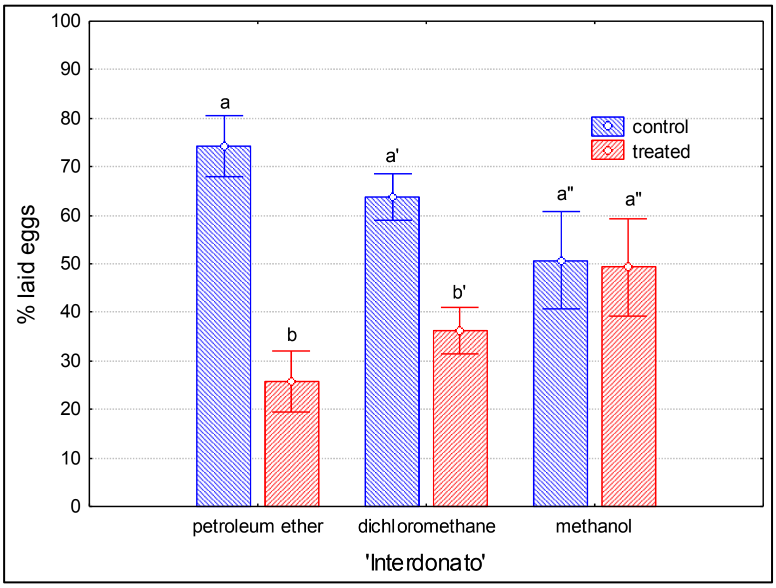


**Figure 20:** Mean (±*SE*) percentage of *C. capitata* laid eggs (6 replicates) on spheres treated with *C. limon* 'Interdonato' extracts in different solvents (petroleum ether, dichloromethane and methanol). Control: untreated spheres. Different letters indicate significant differences (*t* -Test; P = 0,05).



## 3.3 *Field trials.*

Field trials have been conducted in an organic orange orchard (*C. sinensis* 'Navelina'). During field observation (September 30-November 27), the number of males captured was an average of 13 males/trap, showing a low level of infestation. Two doses for each treatment were carried out (only one for kaolin) on 11 October and 27 October and two sampling procedures on 3 November and 18 November (Fig. 21-22). Visual analysis of fruits was carried out on 600 oranges for each sampling procedure.

**Figure 21:** Visible punctures by *C. capitata* on fruits as treated with 'Interdonato' and 'Lunario' essential oils, kaolin and bentonite treatments on different dates. Different letters indicate significant differences (*t* -Test; P = 0,05).

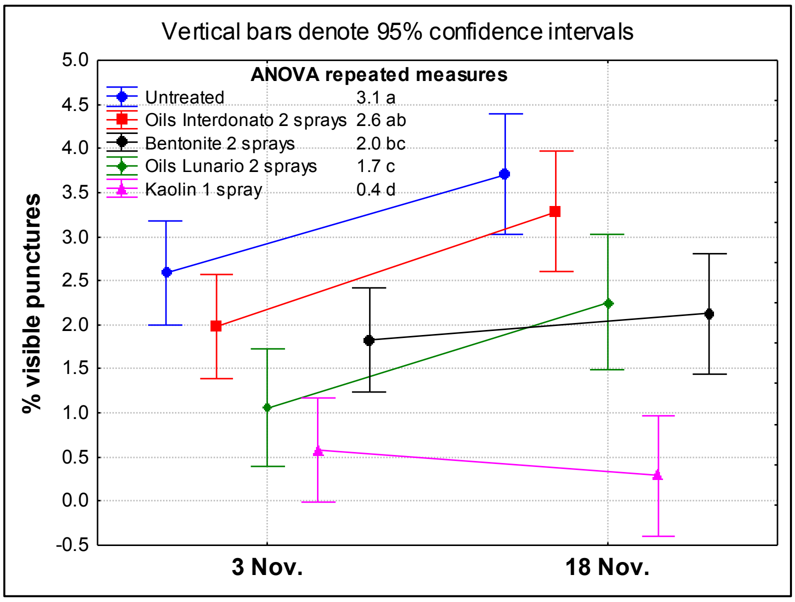
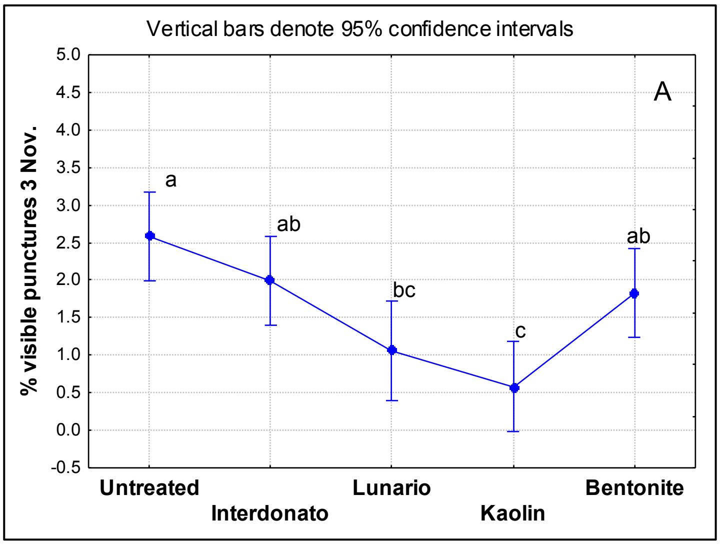
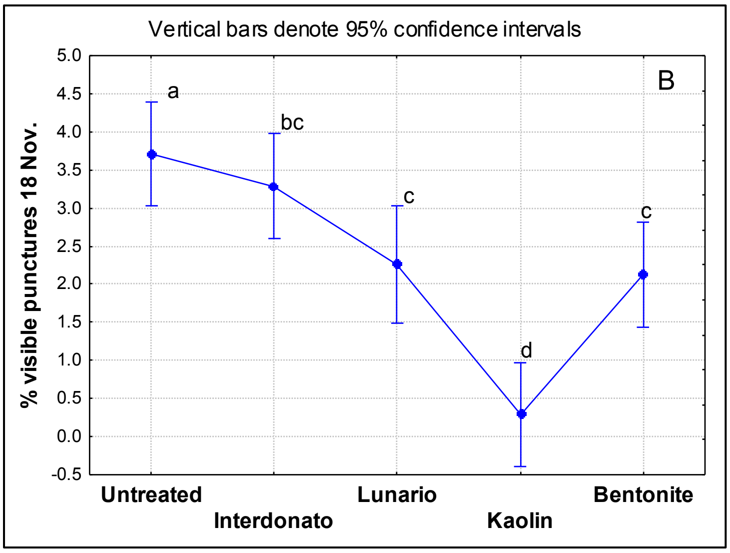


**Figure 22:** Visible punctures by *C. capitata* on fruits treated with 'Interdonato' and 'Lunario' essential oils, kaolin and bentonite treatments on 3 (A) and 18 (B) November. Different letters indicate significant differences (*t* -Test; P = 0,05).





# 3R *References*

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# 4. *Discussion*

The present study provides clear evidences that *C. capitata* females strongly respond to chemicals of the essential oils and peel extracts of Sicilian lemon cultivars; they have shown repellent activities in oviposition behavior and can be potentially used for the development of effective control of this important pest. Host status of citrus fruits against Mediterranean fruit fly was studied and it was demonstrated that lemons are very poor host for medfly (Staub *et al.*, 2008) and lemon cultivars appeared to be almost immune to its attack, unless overripe or suffering from physical damages (Spitler *et al.*, 1984). The main factors limiting the attack in lemons are considered skin thickness and penetrability (Rull and Prokopy, 2004), sugar content (the lowest in lemons and the highest in oranges: Bodenheimer, 1951) and peel oils, which were toxic to other fruit flies (Greany *et al.*, 1983). Salvatore *et al.* (2004) observed in bioassays experiments that the lemon peel extracts are toxic when incorporated into larval and adult medfly diet; furthermore the previous authors demonstrate that the addiction of other amounts of citral (present in the lemon flavedo: Rodov *et al.* 1995) increases the toxicity of the extract.

EAG and SCR techniques, applied to investigate olfactory and gustatory electrophysiological responses of *C. capitata* females, represent an innovative approach to study the biological activity of plants extracts and essential oils; they are useful to select compounds active for different sensilla before laboratory and field bioassays on larvae or adult insects usually carried out without this preliminary selection (Katsoyannos *et al.*, 1997; Salvatore *et al.*, 2004; Macedo *et al.*, 2008; Socolsky *et al.*, 2008).

In gustatory tarsal cell tests recorded by SCRs, petroleum ether peel extracts have not elicited a high level of response; in contrast we observed that the methanol extract of *C. limon* 'Interdonato' has elicited significant increases in the spike frequency at increasing concentrations (Tabb. 7-8) as well as the dichloromethane extracts of *C. limon* 'Interdonato' and 'Lunario', which also showed a high response. For the first time the sensitivity of medfly adult females to *C. limon* peel extracts has been quantified in a double-choice test, using plastic hollow spheres as oviposition devices.

Five out of six lemon peel extracts have resulted repellent, except the 'Interdonato' methanol extract which in SCR had elicited the highest response (Tab. 8). Since the recorded electrophysiological activity can be interpreted by the ability of tarsal sensilla to perceive substances, 'Interdonato' methanol extract contains molecules perceived by tarsal sensilla but not related to oviposition. In contrast, the 'Lunario' petroleum ether extract, which did not produce an increase of action potential, exerts repellent activity. The absence of considerable signal may be due to the presence in the petroleum ether extract of substances that exert an anti-perceptive action, with desensitization of tarsal sensilla and inability to perceive substances on fruit promoting the oviposition. Instead the better repellent action exerted by dichloromethane extracts of both cultivars may be due to the presence of non-volatile aromatic compounds as coumarins, that show toxic activity towards medfly larvae (Baldi *et al.*, 1995; Salvatore *et al.*, 2004).

The activity of these extracts on the oviposition behavior was explained but it would be interesting to investigate the chemical composition of these extracts by phytochemical analyses and subsequently to test main secondary metabolites. Therefore further study and tests on extracts are needed to improve the formulation and application in the field treatments.

**Table 8:** SCR response (action potential) of *C. capitata* tarsal cells after stimulation with *C. limon* 'Lunario' and 'Interdonato' peel extracts in three different solvents and evaluation of the behavioral bioassay results.

| **Tested extracts** | | **SCR (highest dose)** | **Bioassays results** |
|---|---|---|---|
| **'Interdonato'** | Petroleum ether extract | + | Repellent (*p*= 0.00) |
| | Dichloromethane extract | ++ | Repellent (*p*= 0.00) |
| | Methanol extract | +++ | (*p*= 0.92) |
| **'Lunario'** | Petroleum ether extract | - | Repellent (*p*= 0.02) |
| | Dichloromethane extract | ++ | Repellent (*p*= 0.00) |
| | Methanol extract | + | Repellent (*p*= 0.00) |

- = 25±2 spikes/s frequency; + = > 30-50 %; ++ = > 50-100 %; +++ = > 100 %.

*C. limon* essential oils of Sicilian cultivars (‘Interdonato’, ‘Lunario’, ‘Monachello’ and ‘Femminello’) and α-terpineol, geranyl acetate and citral have provided a large uniform degree of EAG response in *C. capitata* female, also at low concentrations; the activation threshold dose as defined by Sant’ana *et al.* (1998) was low (from $10^{-6}$ to $10^{-4}$ M), showing a high sensitivity by medfly female antenna (Figg. 14-18). The saturation threshold dose, as defined by Germinara et al. (2009), was close to the activation one in all essential oils and compounds, with the exception of ‘Monachello’ essential oils (Fig. 15a, Tab. 8). Furthermore the dose-response curves of α-terpineol, geranyl acetate and citral showed a sharp increase, after an initial flat electrophysiological response (Figg. 16-18). This specific response could be explained by considering that the sensory system of the insect could be tuned to a specific dose and very narrow range of doses; therefore it is useful to test intermediate doses, highlighting a gradual response, and to detect more precisely activation and saturation doses.

**Table 8:** Activation and saturation threshold doses of *C. capitata* female antenna after stimulation with *C. limon* essential oils and single compounds in EAG experiments and evaluation of the field trials results.

| Tested substances | EAG (activation dose) | EAG (saturation dose) | Field trials |
|---|---|---|---|
| **‘Interdonato’** essential oils | $10^{-5}$M | $10^{-3}$M | Same attacks as control |
| **‘Lunario’** essential oils | $10^{-4}$M | $10^{-2}$M | Less attacks than control |
| **‘Femminello’** essential oils | $10^{-5}$M | $10^{-4}$M | |
| **‘Monachello’** essential oils | $10^{-6}$M | $10^{-2}$M | |
| **α-terpineol** | $10^{-3}$M | $10^{-2}$M | |
| **geranyl acetate** | $10^{-5}$M | $10^{-3}$M | |
| **citral** | $10^{-6}$M | $10^{-3}$M | |

Though the sensitivity of medfly female antennae for ‘Interdonato’ and ‘Lunario’ essential oils is high, field trials on ‘Navelina’ oranges underline a strong difference in avoiding females attack in oranges; it’s really satisfying for this first field trial the repellence by ‘Lunario’ essential oils that showed a repellence close to the kaolin, known as an effective product (Lo Verde *et al.*, 2011). The different activity exerted in the field by two essential oils may be due to the different composition of ‘Lunario’ oils, then it would be interesting to investigate properties of single compounds through bioassays and field tests and evaluate so if the repellent activity exerted by the essential oils is due to the presence of a particular compound or a synergism between the components of essential oils.

It is not yet possible to discriminate the type of response: whether the repellence is linked to gustatory sensilla, when female tarsi get in touch with treated fruits, or to olfactory sensilla due to interaction with semi-volatile compounds released by evaporation from treated fruits. Further studies on essential oils of lemon cultivars are needed, in laboratory and in the field, to develop the formulations of all products and to reach the best results. The innovative aspect of this formulations based on lemon essential oils is evident as they are easy to prepare and widely available. In addition, these formulations of natural origin are perfectly compatible with biological systems and it is possible to use in organic farming.

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