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Biological activity of metabolites extracted from *Citrus* spp. on *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae)

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Abstract: The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most injurious pest at global level. During the last years, several electrophysiological and behavioural studies have been carried out in order to investigate plant volatile compound-insect interactions with the aim to use this knowledge in sustainable control techniques.

It has been observed that lemons are not attacked by medfly, probably because of the peel oil, that is toxic to other fruit flies. In the present paper electrophysiological recordings were conducted to evaluate the insect sensitivity to peel extract and peel oil of two Sicilian cultivars (Interdonato and Lunario) of *Citrus* x *limon* (L.) Burm.f. on *C. capitata* females. Behavioural bioassays were also performed to show their possible biological activity (repellent, antioviposition, insecticidal). *C. limon* peel extracts in different solvents (petroleum ether, dichloromethane and methanol) were investigated at various concentrations using a single cell recording technique (stimulation of tarsal taste chemosensilla). Different tarsal taste cell responses to the two 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. The peel oil of the same cultivars as well as that ones of other two *C. limon* varieties (Monachello and Femminello) have been tested by EAG techniques. The EAG data showed a high sensitivity (about -8.0/8.5mV) of the medfly antennae to the oils of *Citrus* spp. and a clear dose-response relationship. Responses of adult females (virgin and mated) to *Citrus* spp. peel extract were quantified in a double-choice test using yellow spheres (diameter 7.0cm) housed in field cages. Preliminary tests conducted on three extracts of *C. limon* Interdonato and Lunario have provided interesting results. It was recorded a general decrease of the oviposition on treated spheres compared to control and in the case of the cultivar Lunario, a mortality of insects.

Key words: medfly, *Citrus limon*, EAG, SCR, oviposition behaviour

Introduction

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann) (Diptera, Tephritidae), is one of the most injurious pest at global level. The species is spread in temperate regions and attacks a wide variety of hosts (Hagen *et al*., 1981; Liquido *et al*., 1991). It has become a serious pest of cultivated *Citrus* fruits in Mediterranean countries, as well as in similar climatic regions.

During the last years, the development of crop protection methods had to take into account the need of preserving the precarious equilibrium between 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 behaviour (Teranishi *et al*., 1987; McInnis *et al*., 1988; Warthen *et al*., 1989) with the aim to identify biologically active compounds that could be used in sustainable control techniques (Lai *et al*., 2006).

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 compounds could influence medfly feeding activity, as well as its oviposition behaviour, 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 al.,* 1997; Salvatore *et al*., 2004).

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

Material and methods

Insects

C. capitata adults were obtained from artificially-reared colonies maintained, for many generations, in the insectarium of the IAM-B Istituto Agronomico Mediterraneo (Bari, Italy).

The electrophysiological recordings were carried out using *C. capitata* females*,* 15-20 days old, kept in plexiglas cages (30x30x30cm) at 22±3°C, 60±10% R.H., 12:12 L:D photoperiod and fed with a mixture of sucrose, casein and yeast (ratio 4:3:3) supplied on a wet cotton ball.

The behavioural bioassays were carried out using *C. capitata* females (8-10 days old) in a controlled environment (T: 22 ± 3°C; 60±10% R.H.; 12:12 L:D photoperiod) in cylindric cages (50cm length, 30cm diameter) and fed with a mixture of sucrose, casein and yeast (ratio 4:3:3), supplied on a wet cotton ball.

Plant extracts

The following *C. limon* varieties, collected in the north of Sicily Island and provided by Botanic Garden of Palermo, were used: Interdonato, Lunario, Monachello and Femminello.

Fifteen ripe lemons for each variety were collected in early autumn 2009; their peels were removed from the fruits and some of these (Interdonato and Lunario varieties) air-dried for about two weeks. The dried peels were chopped and extracted with different solvents: petroleum ether, dichloromethane and methanol. Peels were left to soak for a week. After filtering, the solvent was evaporated to dryness at reduced pressure.

The peel oils of the Lunario, Interdonato, Monachello and Femminello varieties were obtained from fresh peels (all the fruits had been collected 24 hours before the extraction) by steam distillation in Clevenger-type apparatus: water ratio was 2. At the end of each distillation, which lasted about 4 hours, the oils were dissolved in 1ml of *n*-pentane and were separated from the aqueous solution, dried by treating with anhydrous Na_2SO_4 (the solvent was evaporated by N_2). The oils were then transferred into dark glass flasks and kept at a temperature of 4°C.

Electrophysiological bioassays

Electrophysiological responses were recorded combining different equipments and techniques previously used to study single chemosensory (gustatory or olfactory) sensilla (Den Otter, 1992; Den Otter *et al*., 1996, Solinas *et al*., 2001).

The tarsal recording bioassay has been carried out testing the peel extracts of Lunario and Interdonato at increasing concentrations (from 10^{-5}M to 10^{-1}M in NaCl 0.1M with Tween-80®). Once the insect was immobilized, the indifferent electrode, a glass micropipette (tip diameter: 2-4µm), filled with a saline solution (NaCl 0.1M with Tween-80®), was placed inside the insect's chest. The recording electrode, a glass micropipette (tip diameter: 1-2µm), containing one of the test stimuli or the control (NaCl 0.1M with Tween-80®), was put in contact with tarsal chemosensilla. The electrical connection was obtained by silver wires inserted into the glass micropipettes and connected to an electrophysiological equipment (INR®, Syntech®, The Netherlands).

Responses from single tarsal cells were recorded for 20 seconds after stimulus onset and 5 minutes were allowed to elapse before the presentation of the next stimulus to the same sensillum. Stimuli were applied at increasing concentration, and the control was applied at the beginning and at the end of each experiment. Action potentials (spikes) were recorded on a magnetic tape by a double channel recorder (Sony® TC-D5M) and then analysed with the programme AutoSpike™ 3.1 (Syntech®, The Netherlands).

For each stimulus, SCRs were recorded from 5 female flies. Responses of the sensory cells were evaluated as spike frequency (spikes/s) during the first second of stimulation, 100ms after stimulus onset.

EAG responses were recorded as described in a previous paper (De Cristofaro *et al*., 2003). The essential oils were dissolved in spectrometric grade hexane at increasing concentrations (from 10^{-10}M to pure oil) and, from these solutions, odour cartridges were prepared for each compound, by absorbing 10µl aliquots onto 1x2cm pieces of filter paper, inserted into individual Pasteur pipettes.

Once the insect was immobilized, the indifferent electrode, a glass micropipette (tip diameter: 2-4µm), filled with Kaissling solution, was placed inside the insect's chest. The recording electrode, a glass micropipette (tip diameter: 2-4µm) containing Kaissling solution, was put in contact with the distal region of the terminal antennal segment.

A constant flow (1.0l/min) of charcoal-filtered and humidified compressed air was passed-over the antenna through a tube, connected with the primed cartridge and positioned ca. 1cm from the antenna. When activated, the system diverted the purified air through the stimulus cartridge (inserted in the tube), where evaporating volatiles were carried onto the antenna. Stimulation lasted 1.0sec and it was followed by an interval of ca. 1 minute of clean air.

For each stimulus, EAGs were recorded from 10 female flies. Control stimulus (10µl of the hexane solvent) was interspersed at the beginning and at the end of each experiment. EAG responses to the tested compounds were evaluated by measuring the amplitude of negative deflection (mV) elicited by a given stimulus and then subtracting the amplitude of the response to the hexane control.

Behavioural bioassay

Responses of adult medfly females to *Citrus* spp. peel extracts (Interdonato and Lunario) were evaluated in an oviposition double-choice test (McInnis, 1989; Prokopy *et al*., 1990). The experiments were performed when the females reached sexual maturity; before the bioassays the flies had never been exposed to fruits or to oviposition devices. For each extract in different solvent (petroleum ether, dichloromethane and methanol), an aqueous solution (10% ethanol with Tween-80®) at 0.1% w/w was prepared.

The flies were separated into 6 cages (20 females and 20 males per cages). Coloured, low-density-polyethylene plastic spheres (Ø = 70mm) were modified to serve as oviposition device; on the basis of previous experiments, which showed that yellow and blue spheres

have a greater attractive effect (Katsoyannos *et al*., 1986; McInnis, 1989), the yellow colour was finally chosen, to get the sphere more similar to an artificial fruit (orange or peach).

Each sphere was tied to a cotton yarn of c.a. 30cm and suspended within a Potter Tower; on each sphere, 8ml of solution 0.1% w/w were sprayed (pressure 9-10 psi). After the treatment, the spheres were allowed to dry for 30 minutes.

Twenty holes (Ø = 0.5-1.0mm) were drilled into each sphere (treated and control) in a 3-4cm wide band around the middle. The top of each sphere was cut to make a c.a. 3cm long incision, to insert 2ml of attractive liquid (a mixture of sucrose, casein and yeast) with a syringe. In each cage, a treated sphere and the untreated control were suspended, letting the flies lay their eggs on the spheres and in its holes; after 3 days the eggs laid on both spheres were counted. For each tested stimulus, 6 replicates have been carried out.

The mean percentage of laid eggs on the treated spheres has been compared with those laid on the control spheres (*t* -Test).

Results

Different tarsal taste cell responses to the two 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 (Fig. 1).

	NaCl 0.1 M	NaCl 0.1 M + Tween 80	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
Citrus limon Interdonato (petroleum ether)	-	-	+	+	+	+	++
Citrus limon Interdonato (dichlorometane)	-	-	-	+	++	++	++
Citrus limon Interdonato (methanol)	-	-	+	+	++	+++	+++
Citrus limon Lunario (petroleum ether)	-	-	-	-	-	-	+
Citrus limon Lunario (dichlorometane)	-	-	-	-	+	+	++
Citrus limon Lunario (methanol)	-	-	-	+	+	+	+

Figure 1: Responses (action potentials) of gustative cells of *C. capitata* 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. - = 25±2 spikes/s, + = frequency > 30-50 %, ++ = > 50-100 %, +++ = > 100 %

The EAG data showed a high sensitivity (about -8.0/8.5mV) of the medfly antennae to the oils of *Citrus* spp. and a clear dose-response relationship (Fig. 2).

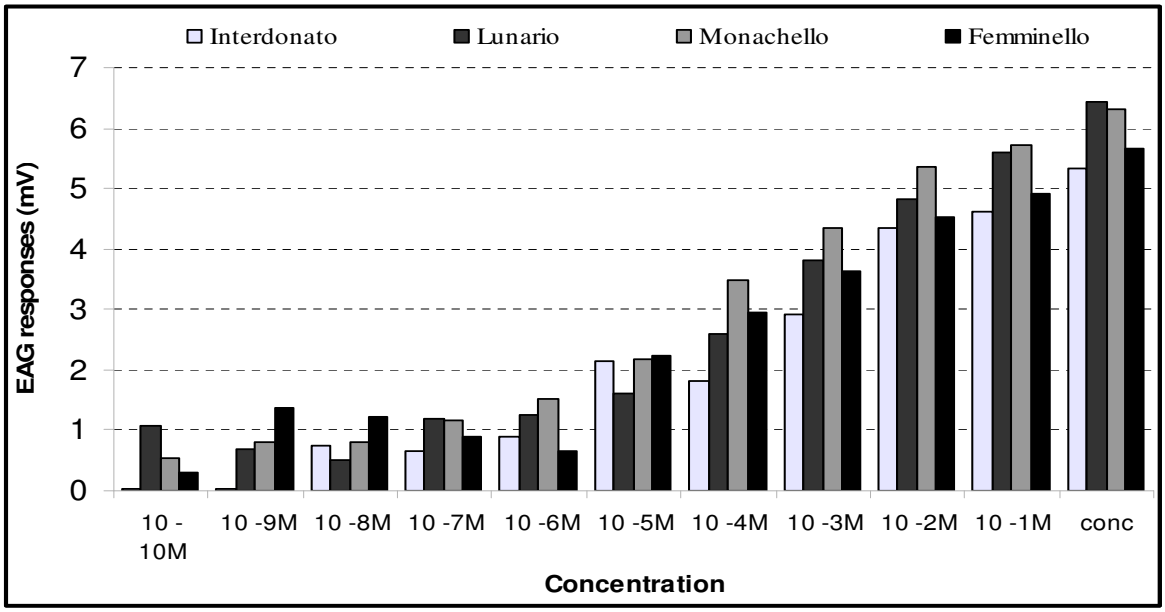

Figure 2: EAG mean responses of *C. capitata* antennae (n=10) after stimulation with increasing doses of *C. limon* essential oils. Control: hexane.

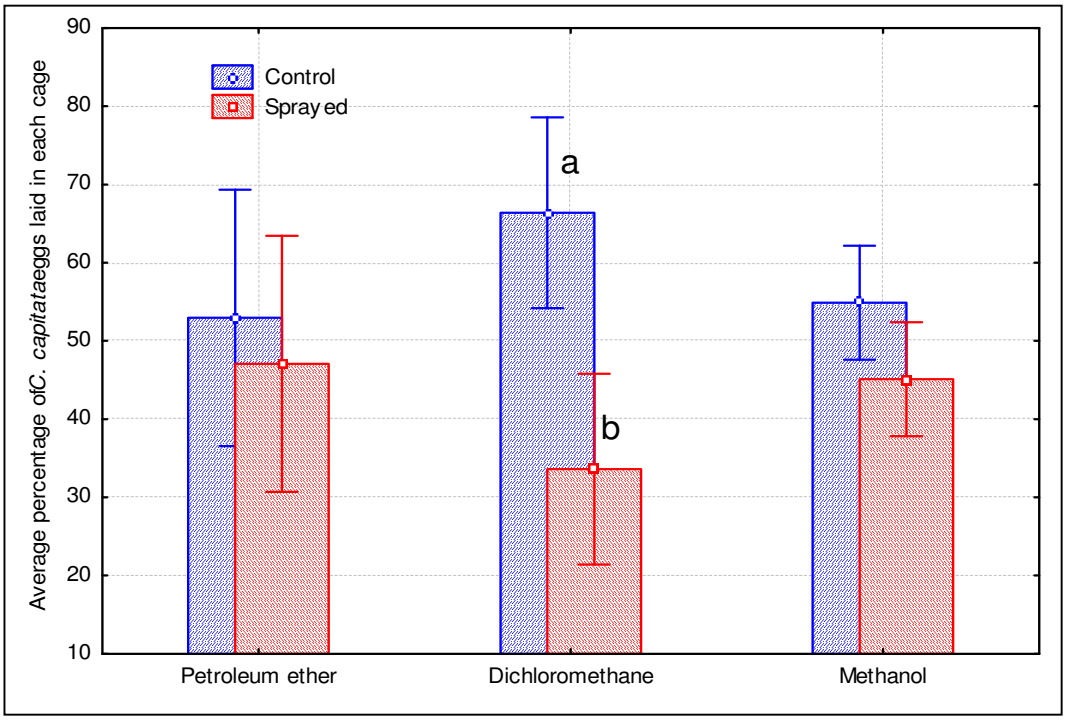

Figure 3: Mean percentage of *C. capitata* female oviposition (6 replicates) on spheres treated with *C. limon* "Lunario" extracts in different solvents (petroleum ether, dichloromethane and methanol). Control: non treated spheres. Different letters indicate significant differences (*t* - Test).

The behavioural bioassay has showed an oviposition decreasing in the treated spheres in comparison with the control ones (Figs. 3-4). In particular, a significantly lower number of eggs has been laid by medfly females when the sphere was treated with *C. limon* "Lunario" dichlorometane extract (Fig. 3) and with *C. limon* "Interdonato" petroleum ether extract (Fig. 4).

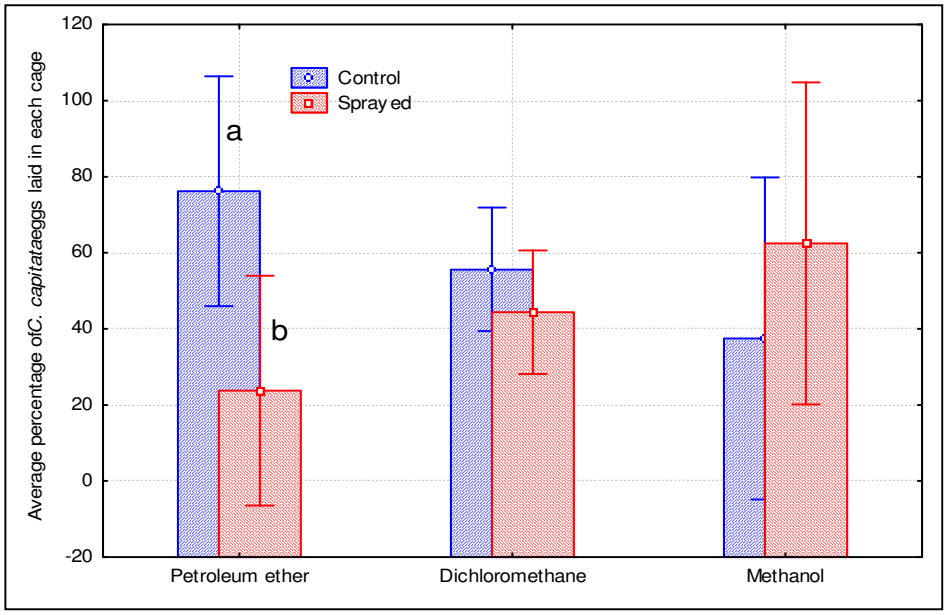

Figure 4: Mean percentage of *C. capitata* female oviposition (6 replicates) on spheres treated with *C. limon* "Interdonato" extracts in different solvents (petroleum ether, dichloromethane and methanol). Control: non treated spheres. Different letters indicate significant differences (*t* -Test).

Conclusions

The electrophysiological recordings show that *C. capitata* females are able to perceive metabolites extracted from different *C. limon* varieties both at olfactory (antennae) and gustative (tarsi) level. Lemon essential oils in hexane have elicited high antennal responses, which depend on the supplied dose.

Lemon peel extracts have induced an increase of action potential frequency in single gustative tarsal cells, which depends on the supplied dose but also on the kind of solvent. The solvent also influence the percentage of eggs laid by females on the spheres, when treated with lemon peel extract solutions, the percentage of laid eggs is strongly reduced.

More studies are needed, but all the results encourage us to keep on investigate, with the aim of evaluate the lemon extracts activity in semi-field and field conditions, too.

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