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Efficacy of selected volatile compounds for organic vine mealybug control

M. L. Peschiutta[1), 2)], V. D. Brito[1)], M. Ordano[3)] and J. A. Zygadlo[1), 2)]

[1)] Universidad Nacional de Córdoba, Facultad de Ciencias Exactas, Físicas y Naturales, Departamento de Química, Cátedra de Química Orgánica, Córdoba, Argentina
[2)] Instituto Multidisciplinario de Biología Vegetal - Consejo Nacional de Investigaciones Científicas y Técnicas, Córdoba, Argentina
[3)] Fundación Miguel Lillo and Unidad Ejecutora Lillo, Consejo Nacional de Investigaciones Científicas y Técnicas, San Miguel de Tucumán, Argentina

Summary

***Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) constitutes a high impact pest in vineyards. Synthetic insecticides are inefficient to control the mealybug population and they affect human health and the environment. The insecticidal properties of eight volatile compounds against vine mealybugs and their grapevine leaf phytotoxicity in laboratory conditions were evaluated. 2-decanone, 3-decanone, α-methyl cinnamaldehyde and cinnamaldehyde produced a higher percentage of mealybug mortality in relation to control at a fumigant dose of 300 µL·L^{-1} air. The 3-octanone, cinnamyl chloride, 1-octen-3-ol and 3-octanol were not effective against *P. ficus*. Cinnamaldehyde and α-methyl cinnamaldehyde produced a low acetylcholinesterase inhibition (IC_{50}= 2.67 µL·L^{-1} and 9.10 µL·L^{-1}, respectively), whereas 2-decanone and 3-decanone did not cause enzyme inhibition. Cinnamaldehyde was not phytotoxic for grapevine leaves; therefore, this compound was selected for a contact application to improve its effectiveness, resulting in a LC_{50} of 394.36 µL·L^{-1} solution. The results demonstrated the potential of cinnamaldehyde to be developed as a non-phytotoxic natural insecticide for the control of vine mealybugs in vineyards.**

K e y w o r d s : *Vitis vinifera*; *Planococcus ficus*; biopesticides; vineyard protection; natural products.

Introduction

During the past years the vine mealybug, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) has become one of the main pests of grapevine worldwide (Godfrey 2002, Daane *et al.* 2012). This insect is more harmful than other mealybugs because of its higher reproductive rate, faster development time and its ability to be easily dispersed in the immature stages (Daane *et al.* 2006). Mealybugs may cause direct damage to the host vine such as tissue necrosis, nutrient depletion, branch deformation and indirect damages, such as the transmission of several viral diseases, mainly Grapevine leafroll associated virus type 3 (GLRaV-3) (Tsai *et al.* 2010, Bertin *et al.* 2016). Wines produced from virus-infected plants or made with high percentages of infested clusters possess undesirable organoleptic characteristics (Cabaleiro *et al.* 1999, Bordeu *et al.* 2012). The continued use of pesticides to control them, leads to resistant populations of pests and inefficiencies in control with serious economic consequences (Mansour *et al.* 2018).

Inefficiency of synthetic insecticide on the mealybug population is possibly caused by the cryptic nature of vine mealybugs (Walton *et al.* 2004), frequent use of non-selective insecticides against other grapevine pests (Franco *et al.* 2004) and difficulty of traditional insecticides to get into the mealybug bodies since they are covered with a waxy substance (Dale 2017). Hence it is necessary to find new and more effective compounds against mealybugs and at the same time being more environmentally friendly. In this perspective, sustainable and insecticide-free control strategies have been tested against the vine mealybug (Karamaouna *et al.* 2013, Muscas *et al.* 2017, Cocco *et al.* 2018, Tacoli *et al.* 2018). For the study, 2-decanone, 3-decanone (Zunino *et al.* 2015), 3-octanone, 1-octen-3-ol, 3-octanol (Zhao *et al.* 2011, Herrera *et al.* 2015), cinnamaldehyde (Cheng *et al.* 2009, Kim *et al.* 2015, Saad *et al.* 2018) and α-methyl cinnamaldehyde (Cheng *et al.* 2009) were selected, because these volatile compounds have been reported as insecticides. The cinnamyl chloride was an interesting choice because it is a chlorinated compound such as the reference synthetic insecticides, dichlorvos and chlorpyrifos. In order to be used in field applications, the tested natural products should be effective against *P. ficus* and of little or no phytotoxicity to grapevines. The phenolic, terpenic and volatile compounds act on the outer membrane, increasing its permeability (Di Pasqua *et al.* 2007). The cell leakage leads to dispersion of the desaturase enzymes in the suspension, promoting their action on the membrane fatty acids, and consequently resulting in significant leaf phytotoxic effects. Currently, there are no studies showing the insecticidal activity of these volatile compounds against *P. ficus* nor their phytotoxic effects

Correspondence to: Dr. M. L. Peschiutta, Universidad Nacional de Córdoba, Facultad de Ciencias Exactas, Físicas y Naturales, Departamento de Química, Cátedra de Química Orgánica, Avenida Vélez Sarsfield 1611, X5016GCA, Córdoba, Argentina. E-mail: mlaurapeschiutta@gmail.com)

on grapevine leaves. Therefore, the present study evaluated the insecticidal properties of eight volatile compounds against vine mealybugs and their phytotoxicity on vine leaves. To reach this goal, it has been performed fumigation and contact tests against *P. ficus* pre-ovipositing adult females in laboratory conditions.

Material and Methods

Chemical products: The 2-decanone, 3-decanone, 3-octanone, cinnamaldehyde, cinnamyl chloride, α-methyl cinnamaldehyde, 1-octen-3-ol, 3-octanol and TWEEN® 20 (non-ionic surfactant) were supplied by Sigma Aldrich (Buenos Aires, Argentina). Chlorpyrifos (containing 48 % of active material, Química Dalton, Argentina) and dichlorvos or 2,2-dichlorovinyl dimethyl phosphate (DDVP, containing 98 % of active material, Chemotecnica, Buenos Aires, Argentina) were used as reference insecticides. Chemical structures of these compounds are shown in Fig. 1.

Insect rearing: *Planococcus ficus* adults were obtained from Colonia Caroya vineyards (31°2'0"S, 64°5'36"W), Córdoba, Argentina. The species was identified in the Facultad de Ciencias Naturales e Instituto Miguel Lillo (UNT) and Instituto Superior de Entomología "Dr. Abraham Willink" (INSUE) by professionals specialized in the study and identification of mealybugs (Contact: Dra. Patricia González; Peschiutta *et al.* 2017). Insects were maintained in boxes under controlled conditions and reared on sprouted potatoes as described in Peschiutta *et al.* (2017). The colony was maintained in our laboratory without any exposure to insecticides. In all experiments were used *P. ficus* pre-ovipositing adult females and bioassays were carried out under these same conditions and in complete darkness. Pre-ovipositing adult females were chosen for the tests, because this developmental stage was considered to represent the most waxy life stage and therefore potentially the most challenging for organic products to penetrate the cuticle and cause insect death (Hollingsworth and Hamnett 2009).

Fumigant toxicity assays: For the trials with *P. ficus*, an artificial diet was performed using NESTUM (30 g- cereal NESTUM® - vegetable pumpkin with carrot), glucose (1 g) and agar-agar (6.66 g) added to distilled water (1000 mL) and autoclaved (120 °C for 20 min) before cooling at 45 °C. Finally, the medium was placed in Petri dishes (90 mm).

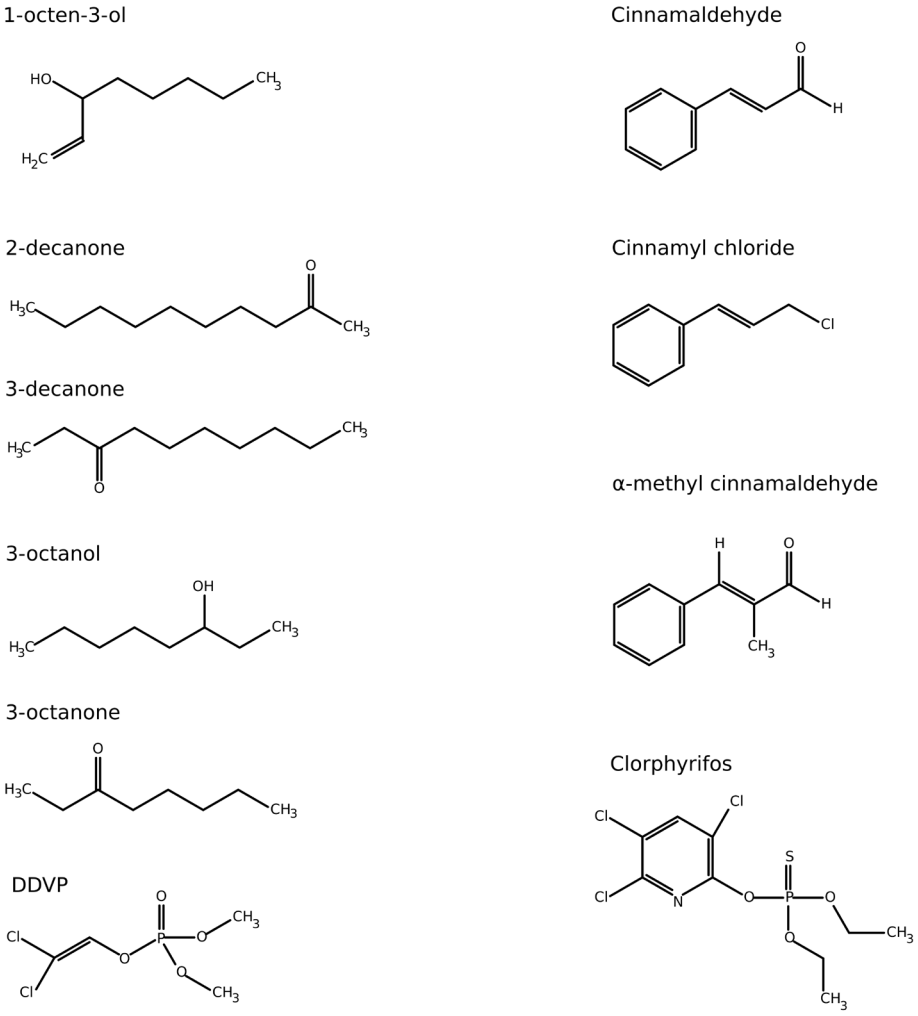

Fig 1: Chemical structure of volatile compounds used in the trials.

A fumigant toxicity test proposed by Peschiutta *et al.* (2017), was carried out to study the susceptibility of adults *P. ficus* to volatile compounds 2-decanone, 3-decanone, 3-octanone, cinnamaldehyde, cinnamyl chloride, α-methyl cinnamaldehyde, 1-octen-3-ol and 3-octanol. Different doses (20, 50, 150, 200 and 300 $\mu L \cdot L^{-1}$ air) of pure compounds (treatment) were placed on Whatman filter paper disks fixed to the underside of the Petri dish cap (37 mL air fumigation chamber) with artificial diet (27 mL). DDVP was used as a positive control. Twenty-four hours after application, insect mortality was recorded and the mortality percentages were calculated. Lethal concentration 50 (LC_{50}) and lethal concentration 95 (LC_{95}) values were calculated for volatile compounds that showed more than 50 % insect mortality at 300 $\mu L \cdot L^{-1}$. Insects were considered to be dead if appendages did not move when prodded with a fine hair brush, while observed under the light stereo-microscope (Peschiutta *et al.* 2017).

Contact toxicity assay: The insecticidal activity of cinnamaldehyde by a direct contact application assay was evaluated (Karamaouna *et al.* 2013). This compound was chosen because it showed the higher mortality as a fumigant against mealybug (see fumigant toxicity assay results, Fig. 2) and low phytotoxicity against grapevine leaf (see phytotoxicity results, Fig. 3). The Petri dishes (90 mm) containing filter paper disks (Whatman number 1) with 10 *P. ficus* pre-ovipositing adult females were sprayed with aqueous solution (0.5 mL; 0.079 $L \cdot m^{2-1}$) of cinnamaldehyde (using 0.2 % TWEEN® 20 as an emulsifier). Spraying was carried out using a small volume vessel of pharmaceutical use with a spray vaporizer. The excess run off solution was removed from the Petri dishes immediately after spraying. Five concentrations (70, 300, 600, 750 and 900 µL of cinnamaldehyde$\cdot L^{-1}$ aqueous TWEEN® 20) were tested. All treatments were replicated five times. The same procedure was followed to the control group, which consisted of (a) water, (b) water with TWEEN® 20 (0.2 %) (Karamaouna *et al.* 2013) and (c) a reference product (chlorpyrifos) (Zunino *et al.* 2012). Insect mortality was recorded after 24 h and mortality percentages and LC_{50} and LC_{95} values were calculated.

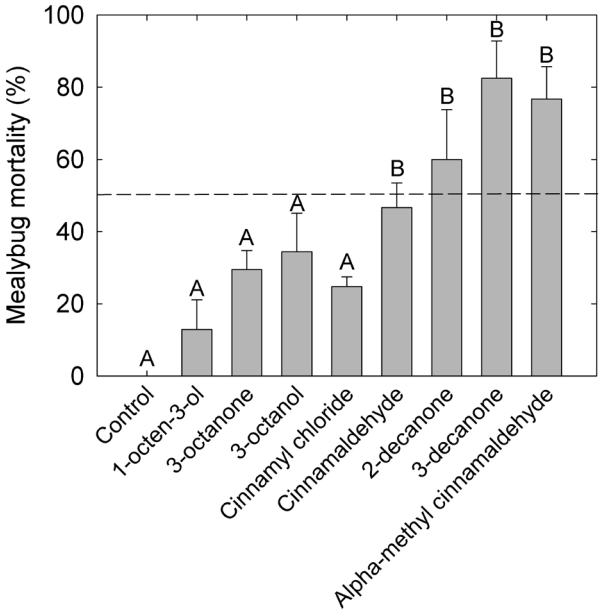

Fig 2: Percentage of *Planococcus ficus* mortality at the highest fumigant dose tested (300 $\mu L \cdot L^{-1}$ air) of 2-decanone, 3-decanone, 3-octanone, cinnamaldehyde, cinnamyl chloride, α-methyl cinnamaldehyde, 1-octen-3-ol and 3-octanol after 24 h of exposure. Bars represent the mean value + SE (n = 5) for each pure compound and negative control (without compound). Different letters above bars indicate significant differences among means (DGC test, $P < 0.01$). The dotted line represents 50 % mortality.

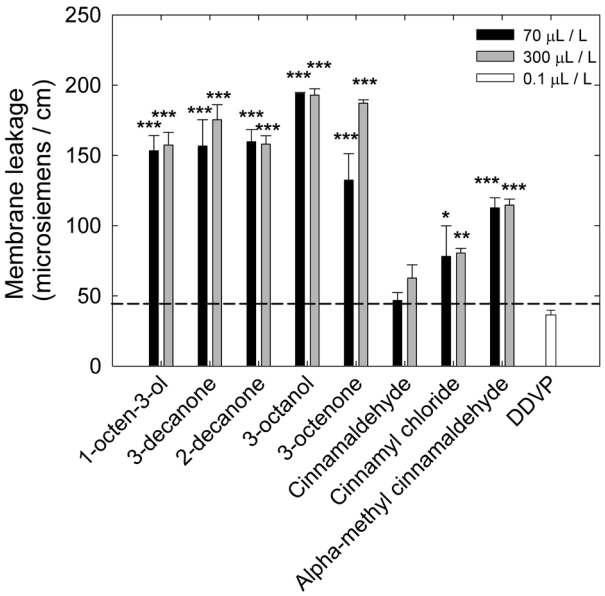

Fig 3: Phytotoxicity of 2-decanone, 3-decanone, 3-octanone, cinnamaldehyde, cinnamyl chloride, α-methyl cinnamaldehyde, 1-octen-3-ol, 3-octanol and DDVP applied by fumigant method on grapevine leaf. Significant differences between each treatment/dose with control are indicated as $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. The dotted line indicates the value of the control group.

Phytotoxicity on grapevine: Phytotoxicity on grapevine was measured according to Tworkoski (2002) with modifications. To measure phytotoxicity by fumigant effect, healthy grapevine leaves cut in the field were used. Leaf disks of 2.5 cm in diameter (0.3 g) were cut in the laboratory and then introduced in closed fumigation chambers (50 mL-glass vials). Doses of 70 and 300 $\mu L \cdot L^{-1}$ air of products (2-decanone, 3-decanone, 3-octanone, cinnamaldehyde, cinnamyl chloride, α-methyl cinnamaldehyde, 1-octen-3-ol and 3-octanol) were applied to Whatman filter paper disks (2 cm diameter) placed on the underside of the screw cap of the chambers. A Whatman filter paper disk (2 cm diameter) moistened with distilled water (600 µL) was placed in the bottom of fumigation chambers to keep the leaf hydrated. After 24 h of exposure, leaf disks were removed and placed in a falcon tube with distilled water (40 mL) and allowed to equilibrate for 24 h with shaking (300 rpm). Finally, electrical conductivity of the solution was measured using a water conductivity tester (Hanna, model HI98308). Each treatment was repeated three times and untreated and positive (DDVP) controls were used.

In vitro acetylcholinesterase (AChE) inhibition tests: The effects of 2-decanone, 3-decanone, cinnamaldehyde and α-methyl cinnamaldehyde on acetylcholinesterase (AChE) activities were examined

at different concentrations (0.5, 1, 10 mM). Inhibition of AChE was then determined by the colorimetric method of Ellman *et al.* (1961) using acetylthiocholine iodide (2.5 mM ATChI) (Sigma Aldrich Co., St. Louis, MO USA) as a substrate. Acetylcholinesterase from *Electrophorus electricus* aliquots (20 µL) and 5,5-dithio-bis (2-nitrobenzoic) acid (DTNB) (20 µL of 4 mM) were added to a phosphate buffer (0.1M, pH 7.4; 120 µL). The enzyme from *E. electricus* was used because the mealybug extract displays an orange coloration similar to DTNB, therefore inhibition of AChE using mealybug extract could not be determined by the colorimetric method of Ellman *et al.* (1961). Control treatments had the addition of absolute ethanol (20 µL) instead of an active compound. All mixtures were incubated (35 °C for 15 min) and the reactions were started by adding ATChI (20 µL), with absorbance being measured at 412 nm using a spectrophotometer (Model 680 Microplate Reader, Bio-Rad).

Each treatment was corrected by blanks for nonenzymic hydrolysis. The inhibition percentage of AChE activity was calculated as follows: AChE inhibition% = (ODC-ODT)/ODC x 100, where ODC is the optical density of control and ODT is the optical density of the treatment. Three replicates were performed, and the mid-point inhibitive concentration (IC_{50}) values were determined graphically from the inhibition curves (log inhibitor concentration in function of percentage of inhibition) (Mohammadi-Farani *et al.* 2013).

Statistical analysis: The LC_{50} and LC_{95} values were obtained using a Probit analysis (Finney 1971). For the analysis of mortality percentages, a General Linear Mixed Model with fixed-effects factors (treatments) was conducted and a DGC posteriori test ($\alpha < 0.01$) (Di Rienzo J.A. *et al.* 2017) was used. Residuals from this model were normally distributed (Kolmogorov-Smirnov test) and the variances were homogeneous (Levene test). Student's t-test was used for mean comparisons between leaf phytotoxicity treatment within a dose (70 and 300 $\mu L \cdot L^{-1}$) with the control. All analyses were performed using the InfoStat v. 2017 software (Di Rienzo *et al.* 2017).

Results and Discussion

Volatile organic compounds can be important managers of vine mealybug populations. The insecticidal efficacy of eight selected volatile organic compound against *P. ficus* was evaluated. The fumigant toxicity assay results revealed that 2-decanone, 3-decanone, cinnamaldehyde and α-methyl cinnamaldehyde produced a higher mealybug mortality percentage in relation to the negative control at a fumigant dose of 300 $\mu L \cdot L^{-1}$ air ($F = 10.16$, $P < 0.0001$, Fig. 2). These compounds had a LC_{50} very similar between them (Table). Cinnamaldehyde fumigant LC_{50} was not determined because at dose 300 $\mu L \cdot L^{-1}$ air did not exceed 50 % mealybug mortality. The DDVP positive control showed 100 % mortality at doses lower than 0.2 $\mu L \cdot L^{-1}$. Other studies found that 2-decanone and 3-decanone ($LC_{50} \leq 54.6$ $\mu L \cdot L^{-1}$) (Zunino *et al.* 2015) and trans-cinnamaldehyde ($LC_{50} = 0.01$ $mg \cdot cm^{2-1}$) (Saad *et al.* 2018) were good insecticides against insects of stored foods. Although in our study 2-decanone, 3-decanone and α-methyl cinnamaldehyde caused a mortality above 60 % at the concentration of 300 $\mu L \cdot L^{-1}$ air, they were all phytotoxic for the grapevine leaves, surpassing the control value (45 microsiemens$\cdot cm^{-1}$) by 155 to 290 %. Therefore, they are harmful for its application in vineyards. Conversely, cinnamaldehyde was not phytotoxic for grapevine leaves (Fig. 3).

In general, the oxygenated compounds have shown higher contact toxicities (Saad *et al.* 2018), then, the insecticidal effect of cinnamaldehyde by applying it by contact method was improved, resulting in a LC_{50} of 394.36 $\mu L \cdot L^{-1}$ solution (0.41 $mg \cdot mL^{-1}$) (Table). The other compounds tested in our study (3-octanone, cinnamyl chloride, 1-octen-3-ol and 3-octanol) were not effective against *P. ficus*. Kim *et al.* (2015) coincided that cinnamyl chloride did not present insecticidal activity when it was tested against *Metcalfa pruinosa* (Say) (Hemiptera: Flatidae). Conversely, Herrera *et al.* (2015) found that the most active fumigant compound against the maize grain pest, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), was 1-octen-3-ol, followed by 3-octanol and 3-octanone.

Different susceptibility of species to the compounds might be attributable to differences in one or more of physiological or biochemical characteristics: penetration, detoxifying enzyme activity, and the relative sensitivity to the toxic lesion at the target site (Graham-Bryce 1987). Mealybug control is difficult to achieve mainly because of the cryptic behaviour of the pest, as they are often located in concealed locations (e.g. under the bark), with a consequent reduced effectiveness of non-systemic insecticides (Lo and Walker 2011). A very important barrier that protects these insects from chemical products is a layer of wax, then mealybug control is very difficult to attain (Copland *et al.* 1985). Because of the hydrophobic character of the volatile compounds, the cytoplasmic membrane appears to be a suitable site of their action, influencing the percentage of unsaturated fatty acids and altering its structure (Di Pasqua *et al.* 2007). Thus, compounds such as cinnamaldehyde or α-methyl cinnamaldehyde, that have an aldehyde group, a conjugated double bond and a long CH chain outside the ring, have a much stronger insecticidal activity than others (Cheng *et al.* 2009). Cinnamaldehyde can act on the membrane, altering its lipid profile, increasing the surface area of the membrane, and altering its structure. However, it is also able to penetrate to the deeper part of the cell, leading them to death (Di Pasqua *et al.* 2007). Cinnamaldehyde and α-methyl cinnamaldehyde produced a low acetylcholinesterase inhibition ($IC_{50} = 2.67$ $\mu L \cdot L^{-1}$ and 9.10 $\mu L \cdot L^{-1}$ respectively), whereas 2-decanone and 3-decanone did not produce enzyme inhibition, and plausibly the insecticidal activity occurred in other action sites. For example, the octopaminergic and γ-aminobutyric acid receptors have been suggested as novel target sites for some products (Priestley *et al.* 2003). The insecticidal mechanism of trans-cinnamaldehyde was presumed to have a role in the neuromuscular system (Shen *et al.* 2012). Detailed tests are needed to fully understand the modes of action of these compounds.

Table

LC_{50} and LC_{95} of 2-decanone, 3-decanone, α-methyl cinnamaldehyde and cinnamaldehyde against *Planococcus ficus*

Compounds	LC_{50} [a] ($\mu L \cdot L^{-1}$)	95% Confidence interval ($\mu L \cdot L^{-1}$)	LC_{95} [b] ($\mu L \cdot L^{-1}$)	95 % Confidence interval ($\mu L \cdot L^{-1}$)	Slope ± S.E.[c]	(χ^2)[d]
2-decanone[e]	205.86	137.79 - 401.25	4312.05	1370.63 - 75050.71	1.25 ± 0.28	18.51
3-decanone[e]	220.58	157.42 - 404.80	510.70	323.10 - 13957.67	4.51 ± 0.79	64.68*
α-methyl cinnamaldehyde[e]	155.00	101.56 - 311.77	2787.76	910.31 - 47449.05	1.31 ± 0.23	26.92
Cinnamaldehyde[f]	394.36	197.10 - 889.20	5162.30	1687.58 - 376947.97	1.47 ± 0.19	136.88*

All the experiments were performed in triplicate. [a]The lethal concentration causing 50 % mortality after 24 h. [b]The lethal concentration causing 95 % mortality after 24 h. [c]Slope of the concentration-mortality regression line ± standard error. [d]Chi-square value. [e]Assay by fumigation method ($\mu L \cdot L^{-1}$). [f]Assay by contact method ($\mu L \cdot L^{-1}$ solution). *Implies that the goodness-of-fit test is significant ($P < 0.05$) and therefore a heterogeneity factor is used in the calculation of the confidence interval.

Few studies evaluated the effect of natural products on *P. ficus*. Karamaouna *et al.* (2013) found high insecticidal activity and lack of any phytotoxic effect on grape vine by citrus oils suggest that lemon and orange peels are an attractive botanical source for the production of alternative plant protection products against *P. ficus*. Despite this, these essential oils (EOs) applied by contact were less effective against this mealybug species than cinnamaldehyde tested in our study. The EOs tested by Karamaouna *et al.* (2013) presented a LC_{50} between 7 and 114 times higher that LC_{50} values of cinnamaldehyde. In recent laboratory experiments developed by Tacoli *et al.* (2018) using orange oil emulsion on *P. ficus* nymphs was found a LC_{50} of 344 $mL \cdot L^{-1}$, 800 times higher than the LC_{50} of cinnamaldehyde tested in our study.

Cinnamaldehyde showed potential to be developed as a non-phytotoxic natural insecticide for the vine mealybug control in vineyards. This compound is generally classified as safe and is approved for use in foods (21 CFR 182.60) by the Food and Drug Administration. On the other hand, it is a nontoxic product and has little harmful effects over non-target organisms as well as the environment (Shen *et al.* 2012). However, to find valuable applications for *P. ficus* control strategies, future studies would focus on the development of natural insecticides based on cinnamaldehyde in the framework of integrated pest management programs against the vine mealybug, associated with adequate cultural practices, mating disruption and biological control (Daane *et al*. 2006, Walton *et al*. 2006, Cocco *et al*. 2014, 2015). Validation of the effectiveness of these formulations containing cinnamaldehyde in large-scale trials, including further research about the safety of the compound to humans and others animals is also important to realize.

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