

Laboratory evaluation of insecticidal activity of plant essential oils against the vine mealybug, *Planococcus ficus*

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Summary

Planococcus ficus is a principal mealybug pest of vineyards worldwide. *Minthostachys verticillata* and *Eucalyptus globulus* essential oils (EO) were evaluated as insecticidal products on *P. ficus*, and the main components of *M. verticillata* and *E. globulus* EO were also tested as insecticidal compounds against vine mealybug females under laboratory conditions. The results revealed that *M. verticillata* EO was more toxic than *E. globulus* EO, while pulegone (LC₅₀ 39.60 µL·L⁻¹) was more toxic than the other constituents of the EO studied. Menthofuran, an oxidation product of pulegone by cytochrome P450 enzymes, showed an LC₅₀ value of 63.97 µL·L⁻¹. Thus, the mechanism of insect detoxification did not reduce the toxic potential of the pulegone. In addition, 1,8-cineole had a higher insecticidal property than its isomer 1,4-cineole. Our studies suggest that the pulegone can be a useful fumigant botanical insecticide to protect crops against vine mealybug attacks.

Key words: *Vitis vinifera*; vine mealybug pest; biopesticides; vineyard protection.

Introduction

The vine mealybug, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae), is a key pest in grapevine growing areas, such as Mediterranean regions of Europe, Africa, the Middle East, California, Mexico and Argentina (BECERRA *et al.* 2006, VARIKOU *et al.* 2010). Economic losses by mealybug infestations have increased rapidly in recent years (BECERRA *et al.* 2006), resulting in a cosmopolitan effort to improve control programs. Historically, synthetic pesticides have been a large part of the vineyard mealybug control strategy (DAANE *et al.* 2012), but many of the foliar sprays are not able to wet the insects, cannot kill mealybugs in the most protected plant organs or

are broad spectrum and kill not only the targeted mealybugs. Other problems of applying these synthetic products include the development of insecticide resistance, toxic residual effects in the grape and environmental pollution (ISMAN 2006, FANTKE *et al.* 2012).

Essential oils (EO) are considered an interesting alternative to synthetic pesticide, because of their effectiveness and versatility. In fact, their volatility and chemical diversity make them excellent fumigants, insecticides, and repellents (GLEISER and ZYGADLO 2009, REGNAULT-ROGER *et al.* 2012, HERNÁNDEZ-LAMBRANO *et al.* 2014, SOUSA *et al.* 2015). Consumer demand for naturally active compounds such as EO has stimulated the search for new products that reduce or completely replace the use of synthetic insecticides, which are harmful to human health and the environment (ISMAN and GRIENEISEN 2014). This is particularly relevant for mealybug control, because their bodies are covered with a waxy substance that hinders the entry of traditional insecticides, whereas EO are lipophilic compounds that are able to enter the insect body and exert their toxic effects (PATIL *et al.* 2010). However, despite terpenes being very important compounds in the *Planococcus* life cycle as they are sex pheromones (HINKENS *et al.* 2001, TABATA *et al.* 2015), the effects of these natural compounds on the behavior or toxicity of vine mealybug have seldom been studied (KARAMAOUNA *et al.* 2013).

Mealybug control may be carried out using regional plants whose EO are widely available on the market. Some investigations have shown that plant extracts and EO from *Eucalyptus globulus* have repellency and insecticidal properties on mealybug and other related insects (ROONJHO *et al.* 2013, FANOU *et al.* 2014, WELDEMARIAM and WELDERUFAEL 2015, PUMNUAN and INSUNG 2016). In addition, *Minthostachys verticillata*, locally referred to as "peperina", is an aromatic bush that grows widely in the central and north-western region of Argentina (ZYGADLO 2011), with its insecticidal activity having been described for *M. verticillata* EO (BANCHIO *et al.* 2005, PALACIOS *et al.* 2009), but not specifically against vine mealybug (KARAMAOUNA *et al.* 2013, TASKIN *et al.* 2014). Therefore, the aim of the

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present study was to evaluate the insecticidal properties of *M. verticillata* and *E. globulus* EO and their main components against *P. ficus*. The results of this study may serve as the basis for the potential development of new healthier pesticides for mealybug control in vineyards.

Material and Methods

Plant material: *Minthostachys verticillata* (Griseb.) Epling (syn. *M. mollis* (Kunth) Griseb), common name "peperina" (Lamiales: Lamiaceae), and *Eucalyptus globulus* Labill (Myrtales: Myrtaceae) leaves were bought in the herbal markets of Córdoba (Argentina).

Essential oils: The *M. verticillata* and *E. globulus* samples were subjected to hydro-distillation for 2 h in a Clevenger's apparatus in order to extract their vaporizing EO, which were then dried over anhydrous sodium sulphate and stored in dark glass tubes under refrigeration (4 °C) until use.

Gas-chromatographic analysis of essential oils: *Minthostachys verticillata* and *E. globulus* EO were studied by gas chromatography-mass spectrometry (GC-MS) using a Perkin Elmer Clarus 600 gas chromatography system, equipped with a mass selective detector in the electron impact mode (70 eV). The injector and mass spectrometry temperature were set at 230 °C and 290 °C, respectively, and a DB-5 capillary column (30 m x 0.25 mm, film thickness 0.25 mm) was used. The oven temperature was programmed to increase from 40 °C to 240 °C at a rate of 4 °C·min⁻¹, with Helium being used as the carrier gas at a flow rate of 0.9 mL·min⁻¹ and samples of 1 µL (1/100 in n-heptane, v/v) injected manually in the split-less mode.

Quantitative data were obtained electronically from the FID detector without the use of correction factors. The components of the EO were identified by comparing relative retention times, with the mass spectra of NIST, Wiley and Adams libraries, and by the use of pure standards. The gas chromatography study was performed under the same conditions, as GC-MS, using a Perkin Elmer Clarus 600.

The components 1,8-cineole, 1,4-cineole, limonene, (R)-(+)-pulegone, (-)-menthone and (+)-menthofuran were purchased from Sigma Aldrich (Buenos Aires, Argentina).

Insect rearing: *Planococcus ficus* adults were obtained from Colonia Caroya vineyards, Córdoba province, Argentina, and maintained in boxes (17 x 11 x 5 cm, length x width x height) under controlled conditions (26 °C and 60 % relative humidity), at 12 h:12 h lighting regime (D:L) and reared on sprouted potatoes (KARAMAOUNA *et al.* 2013). All bioassays were carried out under these same conditions and in complete darkness. The colony was maintained in our laboratory without exposure to insecticides before testing, with *P. ficus* pre-ovipositing adult females being used in all the experiments. We chose pre-ovipositing adult females for the tests, because this instar was considered to represent the most waxy life stage and therefore potentially the most challenging for EO with respect to penetrating the cuticle and causing insect death (HOLLINGSWORTH and HAMNETT 2009).

Bioassay: A new artificial diet for *P. ficus* was developed for the experiments. This medium was made by first placing 15 g of *Vitis vinifera* leaves in 500 mL of distilled water. Five grams agar-agar were then added to 250 mL of the filtered plant extract and autoclaved at 120 °C for 20 min before cooling at 45 °C, with 0.5 g glucose being added to the rest of the *V. vinifera* leaf juice. Finally, both mixtures were homogenized and placed in Petri dishes (90 mm). The purpose of this medium was to standardize the study and to provide the mealybugs with optimal conditions of food and shelter.

Susceptibility of *P. ficus* adults to volatile compounds from *M. verticillata* EO, *E. globulus* EO, 1,8-cineole, limonene, (R)-(+)-pulegone, (-)-menthone, 1,4-cineole and (+)-menthofuran was evaluated using a fumigant toxicity assay, as described by HERRERA *et al.* (2015) with modifications. Ten pre-ovipositing adult females were placed in 90 mm-Petri dishes with artificial diet for 24 h before performing the assay to enable them to adapt to the environment. Different doses (10, 20, 50, 75, 150, 200, 300 and 600 µL·L⁻¹ air) of pure compounds and EO (treatment) were placed on a Whatman filter paper disk fixed to the underside of the cap of a 90 mm-Petri dish (37 mL air fumigation chamber) with 27 ml of artificial diet. These concentrations were selected after preliminary tests and the experiments were repeated five times/dose. Twenty-four hours after application, insect mortality was recorded, and the mortality percentages and LC₅₀ values were calculated. Insects were considered to be dead if appendages did not move when prodded with a fine hair brush, when observed under the light stereo-microscope (CHOI *et al.* 2003).

Biotransformation of puligone by insects: A fumigation bioassay with (R)-(+)-pulegone using a sublethal dose (20 µL·L⁻¹) was carried out. Then, the mealybugs survivors (n = 40) were collected in a vial (10 mL) with a septum. This vial was placed in a bath at 25 °C for 20 min, and the terpenes released by *P. ficus* to the headspace of the vial were captured using a SPME micro fiber (Supelco, Bellefonte, PA, USA; with polydimethylsiloxane, thickness 30 m, length 1 cm). Then these terpenes were injected in a GC-MS and a GC-FID for identification and quantification respectively. The injector temperature was set 250 °C, and oven temperature was programmed to increase from 50 °C (2 min) to 240 °C (5 min) at a rate of 5 °C·min⁻¹, with Helium being used as the carrier gas at a flow rate of 0.9 mL·min⁻¹. The samples were injected manually in the split-less mode and the released terpenes were identified, quantified and transformed into relative percentages (ROSSI *et al.* 2011).

Statistical analysis: The LC₅₀ and LC₉₅ values were obtained using a Probit analysis (FINNEY 1971). The ANOVA and Fisher's LSD posteriori test were used for insect mortality at 600 µL·L⁻¹.

Results

The composition of the *M. verticillata* and *E. globulus* EO is shown in Tab. 1. Among the components of *M. verticillata* EO, menthone (36.33 %) and puligone (57.04 %)

Table 1

Chemical composition of essential oils extracted from *Minthostachys verticillata* and *Eucalyptus globulus* leaves. RI, identification based on Retention Indices; GC-MS, identification based on mass spectra; Co, coinjection with standard. The compounds are listed by elution order in the DB-5 column. (*) limonene, β -phellandrene and 1,8-cineole were also separated on the Carbowax column. Relative contents are expressed as percentages. Principal compounds from *M. verticillata* and *E. globulus* EO are indicated in bold

Compound names	RI	Relative content (%) ¹		Methods of identification
		<i>M. verticillata</i>	<i>E. globulus</i>	
a-pinene	939	0.23	0.29	MS, RI
Camphene	954	--	0.01	MS, RI
Sabinene	976	0.14	0.03	MS, RI
β -Pinene	979	0.36	0.04	MS, RI
Myrcene	992	0.05	0.02	MS, RI
p-Cymene	1025	0.02	1.03	MS, RI
Limonene*	1031	0.86	18.91	MS, RI, Co
β -Phellandrene*	1031	--	1.72	MS, RI
1,8-Cineole*	1033	0.26	76.73	MS, RI, Co
Menthone	1163	36.33	--	MS, RI, Co
Isomenthone	1170	1.69	--	MS, RI
Pulegone	1237	57.04	--	MS, RI, Co
Piperitone	1253	0.56	--	MS, RI
Spathulenol	1576	0.64	0.02	MS, RI
Caryophyllene oxide	1581	0.17	0.04	MS, RI

showed the highest values. The components 1,8-cineole (76.73 %) and limonene (18.91 %) were the most important ones of *E. globulus* EO (Tab. 1). Our results indicated that *M. verticillata* EO (mainly pulegone + menthone) was more active than *E. globulus* EO (1,8-cineole/limonene) on pre-ovipositing adult females of *P. ficus* (61.31 % and 25 % insect mortality at 600 $\mu\text{L}\cdot\text{L}^{-1}$ respectively, Figure). The pure standard of the *M. verticillata* and *E. globulus* principal components were tested for their insecticidal activities on *P. ficus*, the results revealed that (R)-(+)-pulegone, (-)-menthone, 1,8-cineole and limonene produced 100 %, 35.24 %, 26.67 % and 15.33 % mortality of *P. ficus*, respectively, at 600 $\mu\text{L}/\text{L}$ air (Figure). In addition, (R)-(+)-pulegone and (+)-menthofuran had a LC_{50} against *Planococcus ficus* lower than *M. verticillata* EO (Tab. 2).

Discussion

Insecticidal activity of *M. verticillata* and *E. globulus* EO and its principal compounds against the most important pest of vineyards, *P. ficus*, was evaluated. The *M. verticillata* EO composition found in our study satisfied the norm IRAM SAIPA N 18606, with menthone and pulegone being the main constituents (IRAM SAIPA N 18606, URL: <http://www.iram.org.ar/>, the argentinian standard analogous to ISO). The fact that 1,8-cineole and limonene were the principal compounds from *Eucalyptus globulus* EO are in agreement with previous studies on *M. verticillata* and *E. globulus* EO composition (DE FEO *et al.* 1998, MORA *et al.* 2009, HARKAT-MADOURI *et al.* 2015, LUÍS *et al.* 2016). The results of KARAMAOUNA *et al.* (2013) showed that EO with a high content of limonene, such as the citrus peel

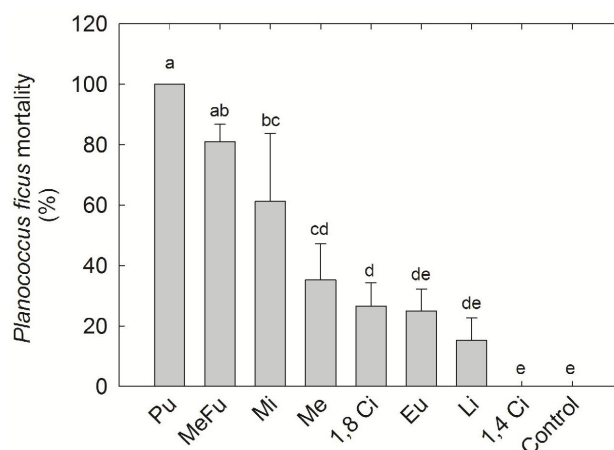


Figure: Percentage of *Planococcus ficus* mortality at highest dose tested (600 $\mu\text{L}\cdot\text{L}^{-1}$) of *Minthostachys verticillata* (Mi) and *Eucalyptus globulus* (Eu) essential oils and pure compounds: 1,8-cineole (1,8-Ci), 1,4-cineole (1,4-Ci), limonene (Li), (R)-(+)-pulegone (Pu), (-)-menthone (Me) and (+)-menthofuran (MeFu) after 24 h of exposure. Columns represent the mean value + SE (n = 5) for each essential oil and pure compounds. Different letters between bars indicate significant differences (Fisher's LSD test, $P < 0.05$).

EO, have a high toxic effect on *P. ficus* adults at spray applications. Previous comparative analyses of terpene insecticidal activity on the mealybug have shown limonene (HOLLINGSWORTH 2005, KARAMAOUNA *et al.* 2013), eugenol and citral (PUMNUAN and INSUNG 2016) to be highly toxic. Conversely, we found that limonene and *E. globulus* EO (containing limonene as a component) were less effective against *P. ficus* than other compounds such as pulegone and *M. verticillata* EO. Similarly, some other studies have

Table 2

LC₅₀ and LC₉₅ (µL·L⁻¹ air) of *Minthostachys verticillata* essential oil, (R)-(+)-pulegone and (+)-menthofuran against *Planococcus ficus*

Compounds	LC ₅₀ ^a (µL·L ⁻¹ air)	95 % Confidence interval (µL·L ⁻¹ air)	LC ₉₅ ^b (µL·L ⁻¹ air)	95 % Confidence interval (µL·L ⁻¹ air)	Slope ± S.E. ^c	(X ²) ^d
<i>M. verticillata</i> EO	127.72	73.89-236.65	2015.07	701.79-46903.21	1.37 ± 0.22	90.99*
(R)-(+)-pulegone	39.60	27.86-55.28	343.90	202.09-819.28	1.75 ± 0.25	23.52
(+)-menthofuran	63.97	40.33-103.98	2067.41	797.12-12147.48	1.09 ± 0.18	26.88

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

*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.

found that pulegone was the most bioactive terpene against *Sitophilus zeamais* and *Musca domestica* (PALACIOS *et al.* 2009, HERRERA *et al.* 2014).

Pulegone is known to be detoxified by the P450 enzymes of insects and mammals to menthofuran (GUNDERSON *et al.* 1986, McCLANAHAN *et al.* 1989, ROSSI *et al.* 2011), and *P. ficus* was no exception to this with pulegone being biotransformed when the insect was exposed to ketone terpene vapour.

In some cases, an insect detoxification system contributes to enhancing the toxicity of pulegone rather than decreasing it (ROSSI *et al.* 2011). In our study we found (+)-menthofuran showed a similar insecticidal activity to (R)-(+)-pulegone. ROSSI *et al.* (2011) hypothesized that the presence of other terpenes in EO might block the active site of the P450 enzyme, and for this reason, the EO can be more active than isolated terpenes. However, we did not find that *M. verticillata* EO was more active than (R)-(+)-pulegone.

The principal compounds of *E. globulus* EO (1,8-cineole and limonene) showed insecticidal activity similar to pure EO, which indicated that 1,8-cineole or limonene alone were not more effective than *E. globulus* EO. Although 1,4-cineole and 1,8-cineole are both monoterpene cyclic ethers, the insecticidal activity of 1,8-cineole was higher than its isomer, but the insecticidal mechanism of both cineoles is still unknown.

In conclusion, our data further validate the importance of the use of EO and their principal compounds for mealybug control, as these lipophilic compounds are able to penetrate the waxy cuticle and may thus more effectively kill these insects. In fact, as botanical products impair lipid synthesis, they lower the lipid content of the insect cuticle, thereby making the mealybugs more sensitive to pesticides and chemical action (PATIL *et al.* 2010). The results obtained in our study showed the capacity of EO derived from *M. verticillata* and *E. globulus* and its principal compounds such as pulegone as a potential tool for *P. ficus* control, due to its insecticidal activity. Further studies are needed to evaluate phytotoxicity of these EO on *Vitis vinifera* and the efficacy of these compounds in the field. This research contributes to the search of potential novel active compounds to environmentally-friendly control of *P. ficus* in vineyards.

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