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 grapeyard 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 Grieben 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 (Rooshio 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...
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 libraries, and by the use of pure standards. The components of the EO were identified, quantified and transformed into relative percentages using a Probit analysis (Rossi et al. 2011).

**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 LC₉₀ 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 (Choit et al. 2003).

**Biotransformation of pulegone 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 LC₉₀ and LC₅₀ 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 pulegone (57.04 %)
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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 µL·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 µL/L air (Figure). In addition, (R)-(+)-pulegone and (+)-menthofuran had a LC₅₀ 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 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 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   | M. verticillata (%) | E. globulus (%) | Methods of identification |
|----------------|------|---------------------|-----------------|--------------------------|
| a-pinene       | 939  | 0.23                | 0.29            | MS, RI                   |
| Camphene       | 954  | --                  | 0.01            | MS, RI                   |
| Sabine   | 976  | 0.14                | 0.03            | MS, RI                   |
| β-Pinen   | 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                   |

Figure: Percentage of *Planococcus ficus* mortality at highest dose tested (600 µL·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).
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 (Palacios et al. 2012: Biology and management of mealybugs in vineyards. In: Aleyrodidae). J. Econ. Entomol. 2012: Health impact and damage cost assessment of pesticides in Europe. Environ. Int. 2012: Microsomal oxidase activity and the Universidad Nacional de Córdoba (SECyT).

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