Towards a development of novel bioinsecticides for organic control of *Planococcus ficus* in vineyards

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Summary

The mealybug species, *Planococcus ficus* Signoret (Hemiptera: Pseudococcidae) is a pest that mainly affects grapevine plantations (*Vitis vinifera* L.), causing huge economic losses in the world. The numerous problems caused by the use of synthetic pesticides on the environment and human health have motivated the development and implementation of natural pesticides. The objective of this work was to develop effective and efficient contact bioinsecticide formulations for the control of *P. ficus* in vineyards. Anti-mealybug formulations were developed using (R)-(−)-pulegone as an active principle, TWEEN® 20 and soy lecithin powder as surfactants, and limonene and diatomaceous earth (DE) as insect waxy layer reducers. The insecticidal properties of formulations against vine mealybugs and their grapevine leaf phytotoxicity were evaluated in laboratory conditions. Pulegone+DE+Lecithin and Pulegone+Limonene+DE+Lecithin formulations showed the highest mortality rates of *P. ficus* (more than 70 % mortality) at 24 h and 48 h of treatment. This high mortality is probably due to an interaction between pulegone, DE and soy lecithin that allows more molecular mobility and increases the efficiency of the active substance, making it enter and act on the insect. In conclusion, the Pulegone+DE+Lecithin and Pulegone+Limonene+DE+Lecithin formulations can be an efficient tool for the organic control of *P. ficus* in vineyards.

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

Introduction

The development of the wine industry and viticulture constitutes an important economic engine in many rural regions worldwide (Torregrosa *et al.* 2015). The vine mealybug, *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) has become one of the main grapevine pests (Godfrey 2002). The misuse of synthetic insecticides such as carbamates, pyrethrins, and organophosphates against mealybugs carried risks in human health and the environment (Del Puerto Rodriguez *et al.* 2014). Recent research focused on the development and implementation of new highly selective pesticides less harmful for human health and more environmentally friendly (Robu *et al.* 2015). Many natural products can be effective bioinsecticides, because they are more selective towards pest target, thus avoiding toxicity to non-target organisms (Peschiutta *et al.* 2018). For example, pulegone is one of the most bioactive terpenes against mealybug (Peschiutta *et al.* 2017) due to the fact that it inhibits the acetylcholinesterase activity of the insects (Herrera *et al.* 2015). Pulegone is recognized as potentially toxic to the liver and lung of mice (Gordon *et al.* 1982); however toxic effect of this compound is produced at high concentrations and over a long exposure period only (Sanchez-Borzone *et al.* 2017). Pulegone in concentrations less to 1 % is widely used, in a safe way, in flavoring agents, perfumery and aromatherapy (Naar 2001, Dhangra *et al.* 2011). This compound has been given the Generally Recognized as Safe (GRAS) status by the United States Food and Drug Administration since 1965. It is approved by FDA for food use (21 CFR 172.515) and was included by the Council of Europe in 1974 to the list of artificial flavoring substances that may be added temporarily to foodstuffs without hazard to public health (Basar *et al.* 1998). Limonene is a lipophilic compound that has the potential to interact with mealybug cuticle wax dissolving it and producing mortality due to its high toxicity (Karamaouna *et al.* 2013). Diatomaceous earth (DE) is another product of natural origin that acts directly on the pest insect cuticle. This product is registered as an insecticide in different countries, mainly for the protection of stored grains, crop protection and domestic use (Quarles 1992, Subramanyam and Hagstrom 2012, Fusé *et al.* 2013). The use of compounds to remove the serous layer of insects, either by degradation and/or abrasion is especially important for the control of *P. ficus* because this insect is highly covered by secretions of hydrophobic wax that repels water-based insecticides (Walton and Pringle 2004).

Formulations with a mixture of natural products offer many possibilities in the search for better and more powerful uses of bioactive substances. Mixtures are not a simple addi-
tion of the effects produced by the components, for example, the action of one of them is increased by the presence others (synergism; Geiger et al. 2016). On the other hand, the addition of adjuvants in the formulations such as TWEEN® 20 or soy lecithin, modifies certain properties of the spray solution and/or increases its biological efficiency (Melo et al. 2019). These combinations allow the phytotoxicity reduction of natural products if they are applied alone in high doses and directly to the crop (Ibrahim et al. 2001). It is then of great interest to use this property to develop new products for pest control. Therefore, the present study evaluates the insecticidal properties of new formulations against Planococcus ficus and their phytotoxic effects on vine leaf. To reach this goal, we developed and evaluated contact bioinsecticides against pre-ovipository adult females of Planococcus ficus in laboratory conditions. The current need for viticulture focuses on the search for new formulations to allow the control of pests so that innovative tools for integrated management will build the enabling framework for economic, cultural, human health and environmental sustainability.

Material and Methods

Chemical products: Chlorpyrifos (containing 48 % active principle), used as a reference product, was obtained from Quimica Dalton, Argentina. Polysorbate 20 (TWEEN® 20), soy lecithin powder and diatomaceous earth (DE) were obtained from Todo Droga, Argentina. Limonene (97 %) and (R)-(+)-pulegone (97 %) were obtained from Sigma Aldrich, Argentina.

Insect rearing: Planococcus ficus adults were obtained from Colonia Caroya vineyards (31°20’S, 64°5’36"W), Córdoba province, Argentina. The insects were maintained in boxes under controlled conditions and reared on sprouted potatoes as described in Peschutta et al. (2017). Planococcus ficus pre-ovipositing adult females were kept free of insecticides before all experiments. We chose pre-ovipositing adult females for the tests because this instar was considered to represent the waxiest life stage and therefore potentially the most challenging for organic products in relation to penetrating the cuticle and causing insect death (Hollingsworth and Hamnett 2009).

Development of anti-mealybug formulations: Anti-mealybug formulations were prepared with (R)-(+)-pulegone, TWEEN® 20, soy lecithin powder, Limonene and DE as shown in Tab. 1.

(R)-(+)-pulegone was selected as the active ingredient for preparation of anti-mealybug formulations because it is a natural compound that presents the higher percentage of Planococcus ficus mortality (Peschutta et al. 2017).

TWEEN® 20 and soy lecithin powder were used as surfactants for anti-mealybug formulations. Limonene and DE were used as degraders of the insect outer waxy layer (Quarles 1992, Hollingsworth 2005). A mortar was used to decrease the size of DE particles before being incorporated into the formulation.

Contact toxicity assay: The insecticidal activity was measured at 24 and 48 h by a direct contact application test (Fig. 1; Peschutta et al. 2019a). Petri dishes (90 mm) containing filter paper disks (Whatman number 1) with 10 Planococcus ficus pre-ovipositing adult females were sprayed with 0.5 mL of each anti-mealybug formulation (7.86 µL·cm²). Bioinsecticides were applied using a low volume spray device consisting of a spray platform that held a pressurized spray bottle at the proper distance and angle to provide uniform and repeatable spray coverage to each petri dish. The spraying nozzle was held 10 cm away from Petri dishes so that the stream of spray insecticide would drift down on the mealybugs. The spray nozzle micropump was pressed 2 times for one full second for each dish (Forschler 1994).

The same procedure was followed for the control group, which consisted of a) water, b) water with DE, c) water with soy lecithin (5 g·L⁻¹), d) water with 0.2 % TWEEN® 20 (Karamaouna et al. 2013) and e) reference product (1,000 µL·L⁻¹ Chlorpyrifos) (Zunino et al. 2012). In accordance with the Argentine national register of plant protection products, Chlorpyrifos can be used in the field in cases of mealybug presence (SENASA 2019). All treatments were replicated five times and mortality percentages were calculated for all formulations. Insects were considered dead

Table 1

<table>
<thead>
<tr>
<th>Formulation name</th>
<th>Active ingredient: (R)-(+)-pulegone (mM)</th>
<th>Surfactant: TWEEN® 20 (% v/v)</th>
<th>Surfactant: soy lecithin powder (g·L⁻¹)</th>
<th>Limonene (mM)</th>
<th>DE (g·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulegone+Tween</td>
<td>7.37 (1200 µL·L⁻¹)</td>
<td>0.2</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Pulegone+Lecithin</td>
<td>7.37 (1200 µL·L⁻¹)</td>
<td>--</td>
<td>5</td>
<td>60</td>
<td>--</td>
</tr>
<tr>
<td>Pulegone+DE+Lecithin</td>
<td>7.37 (1200 µL·L⁻¹)</td>
<td>--</td>
<td>5</td>
<td>--</td>
<td>60</td>
</tr>
<tr>
<td>Pulegone+DE+Tween</td>
<td>7.37 (1200 µL·L⁻¹)</td>
<td>0.2</td>
<td>--</td>
<td>9.26 (1500 µL·L⁻¹)</td>
<td>--</td>
</tr>
<tr>
<td>Pulegone+Limonene+Tween</td>
<td>7.37 (1200 µL·L⁻¹)</td>
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<td>--</td>
<td>5</td>
<td>9.26 (1500 µL·L⁻¹)</td>
<td>60</td>
</tr>
</tbody>
</table>

1 The concentrations were selected according to previous experiences (see Appendix A: Fig. A1 in suppl. material).
2 The concentrations used were as indicated by Peschutta et al. (2019a).
3 The concentrations used were as specified in their respective containers.
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The lethal concentration value 50 (LC$_{50}$) at 24 h for the most efficient and economical anti-mealybug formulation was calculated using the formulation made with a series of concentrations (0, 400, 600, 800, 1,000 and 1,200 µL·L$^{-1}$) of (R)-(+) pulegone.

Phytotoxicity on grapevine: Phytotoxicity of anti-mealybug formulations on the grapevine was measured according to Peschiutta et al. (2019a; Fig. 1). We used healthy grapevine leaves, cut in the field. Leaf disks of 2.5 cm diameter (0.3 g) were cut in the laboratory and then were introduced in 30 mL glass vials. Each anti-mealybug formulation (0.5 mL) was applied directly to the leaf disk using the same spray system as for contact toxicity assay. A Whatman filter paper disk (2 cm diameter) moistened with 100 µL of distilled water was placed in the bottom of glass vials to keep the leaf hydrated. After 24 h of exposure, leaf disks were removed and placed in a Falcon tube with 40 mL of distilled water and allowed to equilibrate for 24 h in constant agitation (300 rpm). Finally, the electrical conductivity of the solution was measured using a pure water tester (Hanna Instruments, Woonsocket, Rhode Island, USA, model HI98308). Each treatment was compared with a control (water) and was repeated five times. The experiment was carried out at different times throughout the year; therefore, for the control leaf age was taken into account at the time of the experiment.

Statistical analysis: The normal distribution of the data was confirmed with the Kolmogorov-Smirnov test (Massey Jr. 1951) and Levene's test was used to assess the equality of variances (Levene 1961). Mortality percentages were analysed with general linear mixed models, with fixed effect factors (treatments) and a DGC a posteriori test ($P < 0.05$) was used (Di Rienzo et al. 2017). The student's t-test (Brown 1967) was used for comparisons of means between the phytotoxicity treatments with respect to the control. The LC$_{50}$ value and their 95% confidence intervals (CI) were calculated through the Probit method (Finney 1971). Statistical analyses were performed using the Infostat software (Di Rienzo et al. 2017) and SPSS v 21.0 (IBM 2012).

Results and Discussion

Pulegone+DE+Lecithin and Pulegone+Limonene+DE+Lecithin formulations showed the highest mortality rates of $P. ficus$ (more 70% mortality) at 24 h and 48 h ($F = 9.56; P < 0.01$ and $F = 11.63; P < 0.01$ respectively; Fig. 2 a, b). Pulegone+DE+Lecithin showed a LC$_{50}$ of

Fig. 1: Schematic diagram showing the test methods: Contact toxicity and phytotoxicity on grapevine using anti-mealybug formulations. The illustration shows the structural formula of (R)-(++)-pulegone (active principle of all formulations).

Fig. 2: Mortality (%) of Planococcus ficus in different anti-mealybug formulations after (a) 24 h and (b) 48 h of exposure. The bars represent the mean value ± SE (n = 5) for each formulation and control group. Different letters between bars indicate significant differences (DGC test, $P < 0.05$).
1,108.33 µL·L⁻¹ (CI = 1,017.24; 1,267.46 µL·L⁻¹). Puleg-
onge+Tween, Pulegone+Lecithin, Pulegone+DE+Tween, Pulegone+Limonene+Tween and Pulegone+Limonene+
- Lecithin showed significant differences in comparison to
the control group at 24 h (P < 0.05), however, the mortality
of P. ficus did not exceed 37 % (Fig. 2 a, b). The (R)-(+)-
pulegone was an important active principle of these formu-
lations because this compound produced mealybug mortality
by itself, similar to that found by Peschutta et al. (2017)
in fumigant toxicity tests. This terpene is also bioactive
against other insects such as Sitophilus zeamais (Herrera
et al. 2014, Peschutta et al. 2019b) and Musca domestica
(Palacios et al. 2009). This bioactivity may happen because
pulegone is metabolized to menthofuran by the oxidative
pathway cytochrome P₄₅₀ increasing its toxicity (Gunderson
et al. 1986, Peschutta et al. 2017). The Chlorpyrifos mor-
tality rate did not show significant differences in relation to
the control at 24 h and 48 h (Fig. 2 a, b), according to the
results obtained for other mealybug species (Phenacoccus
solenopsis) with high levels of resistance to synthetic in-
secticides (Azfal et al. 2015, Ahmad and Akhtar 2016).

Peschutta et al. (2017) found that limonene did not pro-
duce significant P. ficus mortality in relation to the con-
tral in fumigant trials. However, this compound applied by
contact, can be used to moisten and kill insects that have a
waxy cover, such as P. ficus (Hollingsworth 2005). These
dissimilar results show a different sensitivity to insects to
natural compounds in relation to their application way, by
contact or as fumigants. In fumigation tests, the volatile
compounds penetrate through the respiratory system, while
in contact experiments the toxic molecules pass through the
cuticle (Hubert et al. 2008). Application of limonene, due to
its lipophilic properties, has the ability to interact with cuticle
waxes and dissolve them (Karamouna et al. 2013). Despite
this, anti-mealybug formulations that presented limonene in
its composition were no more effective than the formulation
without this compound (Fig. 2 a, b), indicating that other
components such as DE and soy lecithin, could be more
effective in favouring the entry of the active product into the
insect body. In regard to this matter, its widely known that
the DE is an inorganic product that has the ability to affect
by abrasion the waxy layer that covers the cuticle, facilitating
the penetration of the formulation active component into
the insect (Quarles 1992, Subramanyam and Hagstrum
2012, Fuse et al. 2013). In this work, the DE did not cause
mortality by itself (Fig. 2 a, b) despite it has high residual
power and ability to produce insect dehydration (Quarles
1992). Regardless of species and dose, prolonged exposure
to DE particles is needed to obtain high levels of mortality
(Vayias and Athanassiou 2004). Also, under certain cir-
cumstances, insects could moderately lose water, which
increases survival, as in conditions of increased humidity,
or reduced mobility (Arthur 2000, Vayias and Athanassiou
2004). Surfactants such as TWEEN® 20 and soy lecithin
used in anti-mealybug formulations does not cause signifi-
cant differences with the control (water), indicating that
these substances do not cause mortality (Fig. 2 a, b). These
compounds could alter the physicochemical properties of
the spray solution, affecting the wettability, adhesion,
and dispersion of the spray drops, contributing to a better
retention and penetration of the active principle into the
insect body (Yu et al. 2009, Melo et al. 2019). The results
obtained in this test showed that the formulations with the
highest insecticidal action were those that used soy lecithin
as an emulsifier of the Pulegone-DE mixture. This could be
due to the fact that this mixture is better homogenized, has
greater stability and / or greater wettability with soy lecithin
than with TWEEN® 20, thus improving the physicochemical
properties of the spray (Melo et al. 2019). Soy lecithin is
a good surfactant and is used in foods for its null toxicity
(Čílek et al. 2006). The soy lecithin emulsion properties can
be associated with its phospholipid components, which are
emulsifying, wetting and dispersing agents (Nyankson
et al. 2015). A combination of several chemical compounds in a
formulation produces a larger overall effect than the effect of
each of them separately (Scalrand et al. 2018). We found
that the anti-mealybug formulations that had DE-lecithin
mixture in its composition doubled the mortality caused by
pulegone applied in isolation (Fig. 2 a, b). This was prob-
dably due to an interaction between pulegone, DE and soy
lecithin that allows more molecular mobility and increases
the efficiency of the active substance, making it express its
total insecticidal potential.

In addition, all anti-mealybug formulations were no
phytotoxic for vine leaves (P > 0.05, Tab. 2), mainly be-
cause all its components have little or no toxicity (Quarles
1992, Čílek et al. 2006, Nyankson et al. 2015, Peschutta
et al. 2019b).

Gordon et al. (1982) found that pulegone caused acute
hepatic and lung damage in mice at doses of 400 mg kg⁻¹,
far superior to doses that in our studies was applied, which
are not expected to cause negative effects in humans. Also,
pulegone concentrations are expected to decrease over time,
as a consequence of its volatility (Golden et al. 2018). An-
ti-mealybug formulations could be applied to the vineyards
after grape harvest. Regarding the consumption of treated
grapes; it is expected that in a few days, even without pro-
cessing, the pulegone content in the treated grape will be
reduced to sufficiently low levels. However, to confirm this,
comprehensive studies will have to be conducted. In general,
although pulegone is obtained from a natural source, tox-
icological studies, such as its effect on non-target organisms
should be carried out before the widespread application of
anti-mealybug formulation as methods for mealybug control.

The results of the present study demonstrate that the
Pulegone+DE+Lecithin and Pulegone+Limonene+DE+Lec-
ithin formulations could become environmentally friendly
products for the organic control of P. ficus in V. vinifera due
to its high efficiency and quick action. Both formulations
were non-phytotoxic for the vine and had similar insecti-
cidal activity, being the Pulegone+DE-lcithin mixture easier
to be manufactured and less expensive, so it would be
most suitable for the application. However, further studies
in field conditions are needed to evaluate the possible use
in vineyards. It should also be examined whether the use of
the product in open air has an effect on the fermentation of
the must and the quality of the wine.

The implementation of these novel bioinsecticides con-
stitutes a great step towards the generation of organic food
free of pesticides and other polluting products, also avoiding
Table 2

Electrical conductivity in fresh vine leaves after 24 hours treatment for each formulation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean ± SE</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos</td>
<td>12.38 ± 0.88</td>
<td>0.27</td>
<td>0.79</td>
</tr>
<tr>
<td>DE+Water</td>
<td>42.20 ± 10.42</td>
<td>-1.40</td>
<td>0.23</td>
</tr>
<tr>
<td>Lecithin+Water</td>
<td>24.23 ± 4.16</td>
<td>0.68</td>
<td>0.53</td>
</tr>
<tr>
<td>¹Tween+Water</td>
<td>11.40 ± 0.52</td>
<td>-0.44</td>
<td>0.68</td>
</tr>
<tr>
<td>Pulegone+Tween</td>
<td>34.27 ± 7.08</td>
<td>-0.94</td>
<td>0.39</td>
</tr>
<tr>
<td>Pulegone+Lecithin</td>
<td>30.91 ± 1.37</td>
<td>-1.50</td>
<td>0.21</td>
</tr>
<tr>
<td>²Pulegone+DE+Lecithin</td>
<td>69.42 ± 3.89</td>
<td>-1.39</td>
<td>0.19</td>
</tr>
<tr>
<td>Pulegone+DE+Tween</td>
<td>30.30 ± 4.45</td>
<td>-0.61</td>
<td>0.57</td>
</tr>
<tr>
<td>Pulegone+Limonene+Tween</td>
<td>32.57 ± 1.98</td>
<td>-1.89</td>
<td>0.13</td>
</tr>
<tr>
<td>Pulegone+Limonene+Lecithin</td>
<td>31.87 ± 5.83</td>
<td>-0.68</td>
<td>0.53</td>
</tr>
<tr>
<td>Pulegone+Limonene+DE+Lecithin</td>
<td>50.10 ± 10.26</td>
<td>-2.18</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Controls were leaves sprayed with distilled water and the leaf age at the time of the experience was always taken into account. Means are compared with the control (27.37 ± 1.92) with a t-test ($P < 0.05$).

1 Means are compared with the control (10.73 ± 1.25) with a t-test ($P < 0.05$).

2 Means are compared with the control (63.43 ± 8.51) with a t-test ($P < 0.05$).

* Indicates phytotoxicity.

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the phytotoxicity caused by the application of insecticides in the plantations.

Acknowledgements

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