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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.

K e y w o r d s : *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, pyrethroids, and organophosphates against mealybugs carried risks in human health and the environment (Del Puerto Rodríguez *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 (Sánchez-Borzone *et al.* 2017). Pulegone in concentrations less to 1 % is widely used, in a safe way, in flavoring agents, perfumery and aromatherapy (Nair 2001, Dhingra *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 (Baser *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 Hagstrum 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-

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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 *P. ficus* and their phytotoxic effects on vine leaf. To reach this goal, we developed and evaluated contact bioinsecticides against pre-ovipository adult females of *P. 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 Química 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°2'0"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 Peschiutta *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 *P. ficus* mortality (Peschiutta *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; Peschiutta *et al.* 2019a). Petri dishes (90 mm) containing filter paper disks (Whatman number 1) with 10 *P. ficus* pre-ovipositing adult females were sprayed with 0.5 mL of each anti-mealybug formulation (7.86 $\mu L \cdot cm^2$). 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 $g \cdot L^{-1}$), d) water with 0.2 % TWEEN® 20 (Karamaouna *et al.* 2013) and e) reference product (1,000 $\mu L \cdot L^{-1}$ 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

List and composition of anti-mealybug formulations

Formulation name	Active ingredient: (R)-(+)-pulegone mM)[1]	Surfactant: TWEEN® 20 (% v/v)[2]	Surfactant: soy lecithin powder ($g \cdot L^{-1}$)[3]	Limonene (mM)[1]	DE ($g \cdot L^{-1}$)[3]
Pulegone+Tween	7.37 (1200 $\mu L \cdot L^{-1}$)	0.2	--	--	--
Pulegone+Lecithin	7.37 (1200 $\mu L \cdot L^{-1}$)	--	5	--	--
Pulegone+DE+Lecithin	7.37 (1200 $\mu L \cdot L^{-1}$)	--	5	--	60
Pulegone+DE+Tween	7.37 (1200 $\mu L \cdot L^{-1}$)	0.2	--	--	60
Pulegone+Limonene+Tween	7.37 (1200 $\mu L \cdot L^{-1}$)	0.2	--	9.26 (1500 $\mu L \cdot L^{-1}$)	--
Pulegone+Limonene+Lecithin	7.37 (1200 $\mu L \cdot L^{-1}$)	--	5	9.26 (1500 $\mu L \cdot L^{-1}$)	--
Pulegone+Limonene+DE+Lecithin	7.37 (1200 $\mu L \cdot L^{-1}$)	--	5	9.26 (1500 $\mu L \cdot L^{-1}$)	60

[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 Peschiutta *et al.* (2019a).
[3] The concentrations used were as specified in their respective containers.

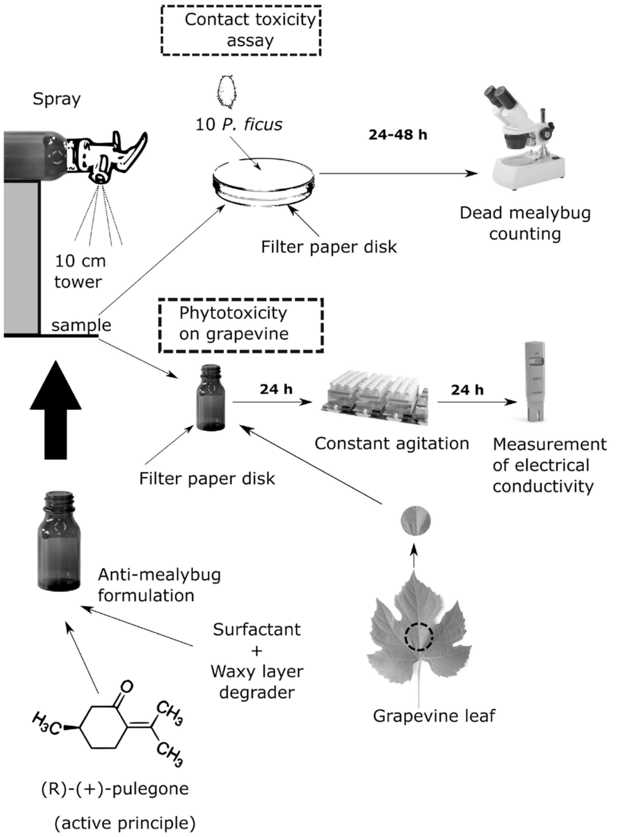

Fig. 1: Schematic diagram showing the test methods: Contact toxicity and phytotoxicity on grapevine using anti-mealybug formulations. The illustration shows the structural formula of (R)-(+)-pulegone (active principle of all formulations).

if appendages did not move when prodded with a fine hair brush (Peschiutta *et al.* 2017).

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 $\mu L \cdot L^{-1}$) 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

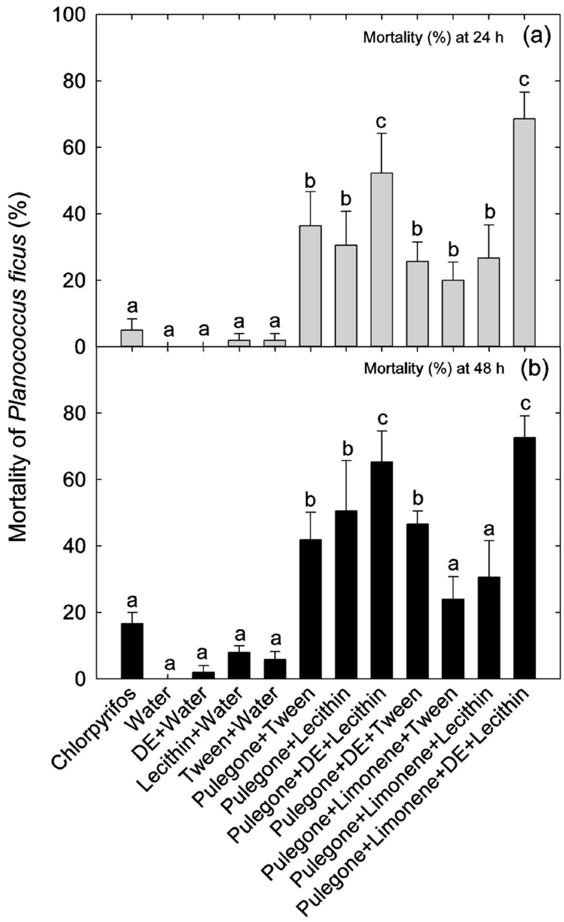

Fig. 2: Mortality (%) of *Planococcus ficus* in different anti-mealybug formulations after (**a**) 24 h and (**b**) 48 h of exposure. The bars represent the mean value + SE (n = 5) for each formulation and control group. Different letters between bars indicate significant differences (DGC test, $P < 0.05$).

1,108.33 µL·L^{-1} (CI = 1,017.24; 1,267.46 µL·L^{-1}). Pulegone+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 formulations because this compound produced mealybug mortality by itself, similar to that found by Peschiutta *et al.* (2017) in fumigant toxicity tests. This terpene is also bioactive against other insects such as *Sitophilus zeamais* (Herrera *et al.* 2014, Peschiutta *et al.* 2019b) and *Musca domestica* (Palacios *et al.* 2009). This bioactivity may happen because pulegone is metabolized to mentofuran by the oxidative pathway cytochrome P_{450} increasing its toxicity (Gunderson *et al.* 1986, Peschiutta *et al.* 2017). The Chlorpyrifos mortality 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 insecticides (Afzal *et al.* 2015, Ahmad and Akhtar 2016).

Peschiutta *et al.* (2017) found that limonene did not produce significant *P. ficus* mortality in relation to the control 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 of 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 (Karamaouna *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, Fusé *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 circumstances, 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 significant 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 (Čilek *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 (Scalerandi *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 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 express its total insecticidal potential.

In addition, all anti-mealybug formulations were no phytotoxic for vine leaves ($P > 0.05$, Tab. 2), mainly because all its components have little or no toxicity (Quarles 1992, Čilek *et al.* 2006, Nyankson *et al.* 2015, Peschiutta *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). Anti-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 processing, 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, toxicological 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+Lecithin 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 insecticidal activity, being the Pulegone+DE+Lecithin formulation 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 constitutes 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

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

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.

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

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