Research Article

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Efficacy of essential oil of *Piper aduncum* against nymphs and adults of *Diaphorina citri*

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Abstract

BACKGROUND: Insecticide application is the main way to control *Diaphorina citri*. However, it causes environmental contamination, has a negative impact on beneficial organisms and leads to psyllid resistance. The essential oil of *Piper aduncum* has low toxicity towards the environment and contains dillapiol, which has proven to be effective against several crop pests. Here, we studied its efficacy against nymphs and adults of *D. citri* under laboratory conditions. Oils with three concentrations of dillapiol (69.3, 79.9 and 85.4%) at 0.5, 0.75 and 1.0% dilutions plus 0.025% adjuvant were tested.

RESULTS: All treatments caused 90–100% mortality in nymphs. Topical treatments with oil containing 79.9 and 85.4% dillapiol at 0.75% and 1% dilutions were effective (mortality ≥80%) in adults. However, the essential oil showed no residual activity against adults (mortality ≤30%).

CONCLUSIONS: Dillapiol-rich oil is a promising compound for *D. citri* control.

Keywords: Asian citrus psyllid; HLB; botanical insecticide; active ingredient rotation; integrated pest management

1 INTRODUCTION

Pathogens spread by insect vectors are limiting factors for the cultivation of citrus. In particular, the phloem-infecting bacteria *Candidatus* Liberibacter asiaticus and *Ca.* Liberibacter americanus have been associated with the destructive citrus greening disease or huanglongbing (HLB), which affects commercial citrus varieties on the American and Asian continents.[1–3] The spread of HLB in orchards mainly occurs via the citrus psyllid *Diaphorina citri* Kuwayama, which has the ability to transmit both bacterial species.[4,5]

Management of HLB includes the use of citrus trees produced in screened vector-free nurseries, inspection and eradication of diseased plants in orchards and control of *D. citri* with applications of insecticides.[6] Chemical control, mostly by the active ingredient imidacloprid, is the primary method used for management of the insect vector.[7,8] However, insects have developed resistance against chemicals owing to their frequent use, which leads to a greater selective pressure. In Florida, *D. citri* was reported to have a resistance ratio higher than 30 for imidacloprid, followed by chlorpyriphosphos (17.9), thiamethoxam (15.0), malathion (5.4) and fenopropathrin (4.8).[9] The use of different control tactics can slow down the development of resistance and contribute to sustainable use of insecticides in the management of *D. citri*.[8]

One such alternative that requires additional studies is the use of botanical insecticides. Studies on these insecticides for control of *D. citri* are incipient, and most of them are focused on the use of extract and essential oil of neem (*Azadirachta indica* A. Juss.). Neem has been demonstrated to have an efficacy against *D. citri* nymphs of 92% in the greenhouse and approximately 30% in field conditions.[10,11] Khan *et al.*[12] reported 80% mortality of adult psyllids in the field using neem extract. Neem and *Datura alba* Nees extracts reduced the number of *D. citri* nymphs and adults by up to fourfold compared with untreated areas in field conditions.[13]

Piper aduncum L., a plant abundant in the Amazon region, displays insecticidal properties[14] because it contains secondary metabolites that show toxicity towards insects, especially monoterpenes,[15] sesquiterpenes[16] and phenylpropanoids,[17] with the phenylpropanoid dillapiol being the major compound.[18–20]

Dillapiol potentially inhibits the activity of cytochrome-P450-dependent monooxygenase, which transforms lipophilic compounds into more soluble (hydrophilic) and easily excretable products.[21] Through inhibition of monooxygenase, the ability of the herbivore insect to excrete xenobiotics present in the host plant is reduced, resulting in death owing to the accumulation of toxic substances in its digestive tract.[22,23] Bernard *et al.*[18] observed

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a 95% increased mortality of *Ostrinia nubilalis* (Hübner) using dillapiol extracted from *Piper cubeba* L. incorporated into an artificial diet at 100 μg g^{-1}. In a study on sucking insects, Silva *et al.*[24] reported mortalities of 72 and 80% for *Aetalion* sp. adults by using *P. aduncum* extracts from leaves and roots respectively, both at a concentration of 30 mg mL^{-1}. Castro *et al.*[25] observed 54.8% loss of viability in *Aleurocanthus woglumi* Ashby eggs after topical application of a 4% aqueous *P. aduncum* leaf extract. Previous studies have determined the insecticidal effect of essential oil of *P. aduncum* (OPA) on defoliating pests such as *Cerotoma tingomarianus* Bechyné,[26] flour pest *Tenebrio molitor* L.[27] and stored-grain pest *Sitophilus zeamais* Motschulsky.[28] However, currently there are no reports on the toxic activity of *P. aduncum* (dillapiol) towards *D. citri*.

The objective of the present study was to evaluate the efficacy of dillapiol-rich OPA on nymphs and adults of *D. citri* in our search for a new mode of action to be adopted in the rotation of active ingredients for controlling this insect vector.

2 EXPERIMENTAL METHODS

2.1 OPA extraction and quantification of chemical compounds

Three-year-old adult *P. aduncum* plants were collected in the production field of Embrapa Acre, Rio Branco, Acre, Brazil (10° 1′ 21.36″ S, 67° 42′ 31.70″ W) by cutting them at 0.4 m above the ground surface. Plants were harvested every 12 months for essential oil extraction. The leaves and fine stems were separated for processing. The plant mass was subjected to drying to achieve 20–30% moisture. Essential oil was extracted by steam distillation as described previously[29] with a yield of around 2–2.5%. The essential oil was redistilled through fractional rectification by using a heating mantle up to 150 °C and a 3000 mL flask. The flask was connected to an absorption tower consisting of a single glass column of 50 × 600 mm completely filled with 6–8 mm Raschig rings. The top of the column was connected to a 40 × 300 mm condenser for cooling water circulation to condense the volatiles. The condenser was connected to a fraction collector with a dispensing system under −760 mmHg pressure created by using a vacuum pump.

For the identification and quantification of chemical compounds, the OPA was analysed using a gas chromatograph (GC) coupled to a GCMS-QP2010 Plus mass spectrometer (MS) (Shimadzu, Tokyo, Japan) equipped with a capillary column (Restek Rxi-5MS, 10 m × 0.10 mm i.d. × 0.10 μm film thickness; Restek Corp., Bellefonte, USA). The GC temperature programme consisted of a start temperature of 40 °C, followed by a temperature ramp of 4 °C min^{-1} to 190 °C, followed by another ramp of 47 °C min^{-1} to 250 °C, and then holding for 1.10 min. This gave a total GC run time of 40 min. The injector and detector interface temperatures were 250 °C, and the ion source temperature was 200 °C; the carrier gas was He (column flow 0.64 mL min^{-1}, split ratio 1:500), and the samples were diluted in methanol (injection of 0.5 µL). Mass spectra were recorded at 70 eV, with a mass range from *m/z* 40 to 350. Chemical characterisation was performed by comparison of the obtained mass spectra with those available in the GC-MS spectra database from the National Institute of Standards of Technology (NIST), data from the literature and Kovats retention indices.[30] For determination of Kovats retention rates, a mixture of linear alkanes (C_8 to C_{20}) was injected into the chromatograph.[31] Component relative percentages were calculated on the basis of GC-MS peak areas.

Table 1. Treatments, dilutions and percentage active ingredient for each concentration of mix sprayed on sweet orange (*C. sinensis*) plants

Treatments	Dilution (v/v)	% a.i. L^{-1}
1. Control (water)[a,b,c]	–	–
2. Control (water + adjuvant)[a,b,c]	0.025	0.025
3. OPA 69.3% dillapiol[a,b]	0.5	0.3260
4. OPA 69.3% dillapiol[a,b]	0.75	0.4890
5. OPA 69.3% dillapiol[a,b,c]	1.00	0.6520
6. OPA 79.9% dillapiol[a,b]	0.5	0.383
7. OPA 79.9% dillapiol[a,b]	0.75	0.5745
8. OPA 79.9% dillapiol[a,b,c]	1.00	0.766
9. OPA 85.4% dillapiol[a,b]	0.5	0.408
10. OPA 85.4% dillapiol[a,b]	0.75	0.6120
11. OPA 85.4% dillapiol[a,b,c]	1.0	0.8160
12. Dillapiol 99.5%[a]	0.5	0.5
13. Dillapiol 99.5%[a]	0.75	0.75
14. Dillapiol 99.5%[a]	1.00	1.00
15. Imidacloprid[b,c]	0.004	20.0

[a] Phytotoxicity against *C. sinensis*.
[b] Topical application on nymphs and adults of *D. citri*.
[c] Residual application on adults of *D. citri*.

2.2 Phytotoxicity of the OPA against *Citrus sinensis* and definition of working concentrations

Before selecting the range of OPA concentrations to assess its efficacy against *D. citri* nymphs and adults, we evaluated the phytotoxic effect on sweet orange shoots using three dilutions with four different concentrations. The experiment was carried out at Fundecitrus (Fundo de Defesa da Citricultura), Araraquara, Sao Paulo, Brazil (21° 48′ 32.35″ S, 48° 9′ 50.82″ W) in a greenhouse (1.60 × 7.90 × 6.0 m) under ambient temperature (average temperature 27.13 °C) and relative humidity (average RH 71.62%) for the entire experimental period.

Forty-two nursery trees {one-year-old *Citrus sinensis* (L.) Osbeck var. Valencia grafted on Swingle citrumelo [*Citrus paradisi* Macf. × *Poncirus trifoliata* (L.) Raf.]} with three 15–18 cm young shoots per grafted tree were selected. The plants were grown in 20 L pots containing substrate (80% *Pinus* sp. bark, 15% vermiculite and 5% charcoal) (Multiplant Citrus®; Terra do Paraíso, Holambra, Sao Paulo, Brazil).

For the preparation of the insecticide sprays, OPA with different concentrations of dillapiol (69.3, 79.9 and 85.4%) and dillapiol 99.5% were diluted to 0.5, 0.75 and 1.0% v/v in water, with the addition of 0.025% Silwet® adjuvant (polyester copolymer and silicone at 100%) (Momentive, Itatiba, Sao Paulo, Brazil). The sprays were prepared by diluting the adjuvant in water and then adding the OPA according to the concentrations established for each treatment. In addition, two control treatments consisting of pure water and water with 0.025% adjuvant were included (Table 1). The young shoots were sprayed to a point just before run-off (7.0 mL) with the aid of a Brudden® S-600 manually operated sprayer (Brudden, Pompéia, Sao Paulo, Brazil).

Phytotoxicity was visually assessed in the young shoots at 1, 7 and 15 days after application (DAA) and scored as follows: score 0 (no toxicity), asymptomatic plants; score 1 (mild toxicity), plants with up to 1 mm necrotic spots (burning) on the leaves; score 2 (moderate toxicity), plants with 1–3 mm spots on the leaves and branches; score 3 (high toxicity), plants with necrotic spots

larger than 3 mm on the leaves and/or complete necrosis of young shoots. We used a randomised block experimental design with 14 treatments and nine replications. Each treatment consisted of three nursery citrus trees containing three young shoots each; each shoot was considered to be a replicate.

For efficacy studies on *D. citri*, doses that caused no or mild phytotoxicity (scores 0 and 1) or that induced moderate toxicity (score 2) in up to 30% of the plants were selected. The treatments that resulted in more than 30% of plants with score 2 and highly phytotoxic treatments (score 3) were excluded from these experiments.

2.3 Assessment of the efficacy of the OPA against *D. citri*

2.3.1 Insects, plants and testing conditions

The insects were obtained from a *D. citri* rearing established at Fundecitrus. The rearing was maintained on *Murraya paniculata* (L.) in a climatised room (temperature 25 ± 3 °C, photoperiod 14 h, relative humidity 65 ± 10%). To obtain eggs, plants with young shoots were transferred to acrylic cages (20 × 21 × 55 cm) with an anti-aphid screen and exposed to adults for 7 days. The plants containing eggs were kept in the cages until the emergence of adults. To evaluate the efficacy of the OPA, seedlings of *C. sinensis* var. Caipira, grown in tubes in screened nurseries, were used. All tests were conducted in the laboratory under the same temperature, photoperiod and relative humidity conditions as described for the rearing of psyllids.

2.3.2 Assessment of the efficacy of the OPA on D. citri nymphs and adults by topical application

The OPA treatments used in these experiments were selected on the basis of phytotoxicity test results (Section 2.2). We used treatment with imidacloprid (Provado® 200 SC; Bayer CropScience AG, Belford Roxo, Brazil) as a positive control (Table 1). To test the efficacy of OPA against nymphs, each seedling with one young shoot was infested with ten third-instar nymphs with the aid of a soft paintbrush. The infested plants were subjected to the various OPA spray treatments and maintained in a climatised room. To test the efficacy of OPA against adults, ten insects at 10 days after emergence were confined on each shoot by using sleeve cages that were pervious to spraying and that covered the whole shoot. The shoots were sprayed until product run-off. The same treatments as described for topical application on nymphs were used (Table 1).

The number of dead insects (nymphs or adults) was counted at 1, 3 and 7 DAA. Nymphs and adults were considered to be dead when they did not present mobility of legs, wings and antennae. For nymphs, the efficacy of the OPA was tested only for topical application on account of the fact that, after outbreak, the nymphs develop on the same branch until the emergence of adults, not justifying the testing of residual contact. For both tests, a completely randomised block design was used, and each seedling represented a replicate. Seven plants were used for the experiment with nymphs, and eight for the experiment with adults. Additionally, a 3 × 3 factorial design (concentrations of dillapiol × dilutions of OPA used) was used for the adult insects to verify whether there was an influence of increasing dillapiol/OPA and OPA/dillapiol concentration ratios on insect mortality.

2.3.3 Assessment of the efficacy of the OPA on adults of D. citri by residual contact

Ten adults at 10 days after emergence were placed on the shoot of each seedling on the dry residue (2 h after spraying), using the same insect confinement method and spray application as described above. The treatments used in this test were those classified as effective in the topical application test (mortality ≥80%) (Table 1), as described in Section 2.3.2. The assessments and experimental design were similar to those of the topical tests.

2.4 Data analysis and statistics

The results of the assessment of the phytotoxic effect were expressed as percentages calculated from the scores attributed to the damage in all young shoots per treatment. The number of dead insects for all efficacy tests was expressed as a percentage. All data were expressed as the mean ± standard error of the mean (SE). The data were transformed into arcsine $(x/100)^{0.5}$ prior to analysis to reduce heteroscedasticity and achieve normality. Means were subjected to analysis of variance (ANOVA) with repeated measures over time, and, in case of significance, compared by Tukey's test ($P \leq 0.05$). All analyses were performed using the AgroEstat software.[32]

3 RESULTS

3.1 Chemical constituents of essential oils

The compositions of the four OPAs obtained by fractional distillation used in the present experiments were determined by GC-MS, comparing their relative retention times and the mass spectra of the OPA components from a data library. We injected three samples from each OPA fraction to determine the average percentage ± SE for each fraction of oil. Characterised compounds of these oils with their relative percentages are listed in Table 2. A total of 40, 39, 30 and six components were identified in OPA 01, OPA 02, OPA 03 and OPA 04 respectively. Six compounds comprising myristicin, *z*-isoelemicin, caryophyllene oxide, globulol, dillapiol and apiol were present in all four OPAs. Dillapiol was the most abundant compound identified from OPAs obtained by fractional distillation, and the percentages were 69.3, 79.9, 85.4 and 99.5%. The different OPA fractions are termed OPA-69.3, OPA-79.9, OPA-85.4 and dillapiol-99.5 hereafter, for OPA 1, 2, 3 and 4 respectively, considering the percentage of dillapiol in the obtained fractions.

3.2 Phytotoxicity of OPA against *C. sinensis* and definition of working concentrations

The proportion of plants with the same degree of toxicity observed in the first assessment (1 DAA) remained constant during the entire experimental period.

Treatment of *C. sinensis* plants with OPA-69.3 at dilutions of 0.75 and 1.0%, with OPA-79.9 at 0.75% dilution and with OPA-85.4 at dilutions of 0.5, 0.75 and 1.0% caused no phytotoxic effect in 100% of the plants. OPA-69.3 at 0.5% was non-toxic to 88.88% of the plants, and OPA-79.9 at 0.5 and 1.0% was non-toxic for 66.6% and 33.33% of the plants respectively.

OPA-69.3 at 0.5% caused mild toxicity to 11.11% of the plants, and OPA-79.9 at 0.5 and 1.0% caused mild toxicity to 22.22 and 33.33% of the plants respectively.

OPA-79.9 at 0.5 and 1.0% was moderately toxic to 11.18 and 33.33% of the plants respectively, while dillapiol-99.5 at 0.5 and 1.0% caused moderate toxicity to 33.66% of the plants. Dillapiol-99.5 at 0.75% caused moderate toxicity to 66.66% of the plants.

Only dillapiol-99.5 was highly toxic to plants. The 0.5 and 1.0% dilutions were highly toxic to 66.6% of the plants, while the 0.75% dilution caused high toxicity to 33.33% of the plants. The two

Table 2. The composition of the essential oils of *Piper aduncum*

Compound	RT[a] (min)	RI[b]	Relative area (% ± SE)[c]			
			OPA 1	OPA 2	OPA 3	OPA 4
α-Pinene	3.123	917	1.08 ± 0.07	0.55 ± 0.04	tr	–
Camphene	3.385	931	tr[d]	tr	–	–
β-Pinene	3.957	962	1.92 ± 0.09	1.01 ± 0.06	tr	–
Myrcene	4.422	986	tr	tr	–	–
α-Phellandrene	4.634	997	0.90 ± 0.04	0.30 ± 0.03	–	–
p-Cymene	5.149	1018	tr	tr	–	–
Limonene	5.241	1021	0.48 ± 0.02	0.17 ± 0.00	–	–
(*Z*)-β-Ocimene	5.616	1035	0.79 ± 0.02	0.21 ± 0.01	–	–
(*E*)-β-Ocimene	5.878	1045	1.87 ± 0.05	0.46 ± 0.03	–	–
Terpinolene	6.886	1083	tr	tr	–	–
α-Cubebene	14.907	1342	0.10 ± 0.00	tr	tr	–
α-Longipinene	15.193	1351	tr	tr	tr	–
Cyclosativene	15.297	1354	0.17 ± 0.01	0.12 ± 0.00	tr	–
α-Copaene	15.634	1365	0.98 ± 0.01	0.73 ± 0.01	0.63 ± 0.01	–
β-Cubebene	16.127	1381	tr	tr	–	–
β-Elemene	16.203	1383	0.27 ± 0.00	tr	0.10 ± 0.01	–
α-Gurjunene	16.620	1397	0.19 ± 0.00	tr	tr	–
(*E*)-Caryophyllene	16.879	1405	10.44 ± 0.12	7.14 ± 0.08	4.85 ± 0.06	–
α-Santalene	17.058	1411	0.19 ± 0.01	0.14 ± 0.00	0.10 ± 0.01	–
β-Copaene	17.196	1416	tr	tr	tr	–
Aromadendrene	17.441	1424	0.24 ± 0.00	0.10 ± 0.00	tr	–
α-Humulene	17.872	1439	1.40 ± 0.02	0.93 ± 0.04	0.68 ± 0.00	–
allo-Aromadendrene	18.084	1446	0.24 ± 0.01	0.17 ± 0.00	0.13 ± 0.01	–
Dauca-5,8-diene	18.583	1463	0.10 ± 0.01	tr	tr	–
Germacrene D	18.720	1467	0.99 ± 0.01	0.80 ± 0.02	0.21 ± 0.01	–
Bicyclogermacrene	19.187	1483	1.31 ± 0.02	0.86 ± 0.01	0.28 ± 0.01	–
α-Muurolene	19.412	1490	0.34 ± 0.01	0.24 ± 0.01	0.15 ± 0.01	–
β-Himachalene	19.595	1496	0.72 ± 0.01	0.34 ± 0.01	0.13 ± 0.02	–
n-Pentadecane	19.718	1501	0.96 ± 0.03	1.34 ± 0.01	2.01 ± 0.10	–
δ-Amorphene	19.770	1496	0.24 ± 0.01	0.19 ± 0.01	0.46 ± 0.05	–
β-Curcumene	19.870	1506	0.26 ± 0.00	0.17 ± 0.00	0.18 ± 0.00	–
Myristicin	20.097	1514	2.68 ± 0.01	2.13 ± 0.02	2.06 ± 0.04	0.17 ± 0.02
α-Calacorene	20.534	1529	0.15 ± 0.00	0.12 ± 0.02	0.30 ± 0.01	–
Germacrene B	20.835	1540	0.10 ± 0.00	tr	tr	–
z-Isoelemicin	21.333	1557	0.15 ± 0.00	0.13 ± 0.01	0.12 ± 0.01	tr
Spathulenol	21.459	1562	0.12 ± 0.01	–	–	–
Caryophyllene oxide	21.549	1565	0.29 ± 0.01	0.61 ± 0.03	0.77 ± 0.04	tr
Globulol	21.822	1575	0.47 ± 0.01	0.45 ± 0.01	0.72 ± 0.04	tr
Dillapiol	23.128	1622	69.3 ± 0.40	79.9 ± 0.10	85.4 ± 0.21	99.5 ± 0.03
Apiol	24.570	1675	0.15 ± 0.00	0.18 ± 0.02	0.23 ± 0.01	0.15 ± 0.00

[a] RT = retention time on the Rxi-5MS (10 m × 0.10 mm i.d. × 0.10 μm) column.
[b] RI = retention index as determined on an Rxi-5MS column using a homologous series of *n*-hydrocarbons (C_8 – C_{20}).
[c] Expressed as area % mean ± SE from GC-MS data.
[d] tr = traces (<0.1%).

control treatments, with or without adjuvant, were non-toxic to 100% of the *C. sinensis* plants. Because dillapiol-99.5 presented moderate toxicity to more than 35% of the plants and was the only treatment that caused high toxicity, it was excluded from the efficacy tests on *D. citri*.

3.3 Assessment of the efficacy of the OPA against nymphs and adults of *D. citri* by topical contact

The nymphs of *D. citri* displayed high sensitivity to all treatments containing OPA. The average mortality obtained by the treatments varied between 90.00 and 98.57% on the first day of assessment (1 DAA), between 91.42 and 100% at 3 DAA and between 97.14 and 100.0% in the final assessment (7 DAA) (Table 3). The high mortality (90.00–98.57%) observed at 1 DAA in all treatments indicates a knockdown effect of the OPA for third-instar nymphs of *D. citri*. The average mortality did not significantly differ among the treatments and compared with the positive control imidacloprid. Only the lowest concentration evaluated, OPA-69.3 at 0.5%, showed a significantly higher mortality at 7 DAA compared with 1 and 3 DAA. However, the average mortality obtained by the treatments significantly differed from the control treatment at all time points (1 DAA: $F = 44.10$, df = 11, $P < 0.0001$; 3 DAA: $F = 35.80$, df = 11, $P < 0.0001$;

Table 3. Average mortality (± SEM) of third instar nymphs of *Diaphorina citri* in topical application of different concentrations of dillapiol and dilutions of the essential oil of *Piper aduncum*[a]

Treatments	Dilution (v/v)	*n*	Mortality (%)		
			1 DAA	3 DAA	7 DAA
Control (water)	–	7	1.42 ± 1.42 Cb	10.00 ± 5.34 Ca	10.00 ± 5.34 Ca
Control (adj)	0.025	7	35.71 ± 10.43 Bb	45.71 ± 10.87 Ba	47.14 ± 10.62 Ba
OPA 69.3% dillapiol	0.5	7	90.00 ± 5.34 Ab	91.42 ± 5.53 Ab	97.14 ± 1.84 Aa
OPA 69.3% dillapiol	0.75	7	100.00 ± 0.00 Aa	100.00 ± 0.00 Aa	100.00 ± 0.00 Aa
OPA 69.3% dillapiol	1.00	7	95.71 ± 2.97 Aa	97.14 ± 1.84 Aa	98.57 ± 0.00 Aa
OPA 79.9% dillapiol	0.5	7	94.28 ± 5.71 Aa	97.14 ± 2.85 Aa	97.14 ± 2.85 Aa
OPA 79.9% dillapiol	0.75	7	98.57 ± 1.42 Aa	100.00 ± 0.00 Aa	100.00 ± 0.00 Aa
OPA 79.9% dillapiol	1.00	7	97.14 ± 2.85 Aa	97.14 ± 2.85 Aa	97.14 ± 2.85 Aa
OPA 85.4% dillapiol	0.5	7	97.14 ± 2.85 Aa	97.14 ± 2.85 Aa	97.14 ± 2.85 Aa
OPA 85.4% dillapiol	0.75	7	91.42 ± 8.57 Aa	91.42 ± 8.57 Aa	91.42 ± 8.57 Aa
OPA 85.4% dillapiol	1.00	7	98.57 ± 1.42 Aa	100.00 ± 0.00 Aa	100.00 ± 0.00 Aa
Imidacloprid	0.004	7	100.00 ± 0.00 Aa	100.00 ± 0.00 Aa	100.00 ± 1.42 Aa

[a] Means followed by the same upper-case letter in a column and by the same lower-case letter in a row do not differ according to Tukey's test ($P \leq 0.05$).

Table 4. Average mortality (± SEM) of adults of *Diaphorina citri* in topical application of different concentrations of dillapiol and dilutions of the essential oil of *Piper aduncum*[a]

Treatments	Concentration (v/v)	*n*	Mortality (%)		
			1 DAA	3 DAA	7 DAA
Control (water)	–	8	1.25 ± 1.25 Da	2.50 ± 1.63 Ea	6.25 ± 2.63 Da
Control (adj)	0.025	8	5.00 ± 2.67 Da	11.25 ± 4.79 Ea	13.75 ± 4.60 Da
OPA 69.3% dillapiol	0.5	8	33.75 ± 12.94 BCDa	36.25 ± 13.22 DEa	43.75 ± 12.94 BCDa
OPA 69.3% dillapiol	0.75	8	16.25 ± 7.54 CDa	17.50 ± 7.73 DEa	21.25 ± 7.42 Da
OPA 69.3% dillapiol	1.00	8	46.25 ± 14.99 BCb	55.00 ± 14.26 CDb	70.00 ± 10.00 ABa
OPA 79.9% dillapiol	0.5	8	20.00 ± 9.25 CDb	25.00 ± 9.06 DEab	33.75 ± 9.43 BCDa
OPA 79.9% dillapiol	0.75	8	72.50 ± 11.76 ABb	78.75 ± 10.92 ABCab	86.25 ± 9.80 Aa
OPA 79.9% dillapiol	1.00	8	88.75 ± 6.39 Aa	95.00 ± 3.77 ABCa	96.25 ± 2.63 Aa
OPA 85.4% dillapiol	0.5	8	5.00 ± 3.77 Db	13.75 ± 7.05 Eab	22.50 ± 8.60 CDa
OPA 85.4% dillapiol	0.75	8	36.25 ± 10.84 BCDb	57.50 ± 13.59 BCDa	62.50 ± 13.59 ABCa
OPA 85.4% dillapiol	1.00	8	96.25 ± 3.75 Aa	97.50 ± 2.50 ABa	98.75 ± 1.25 Aa
Imidacloprid	0.004	8	95.00 ± 3.77 Aa	98.75 ± 1.25 Aa	100.00 ± 0.00 Aa

[a] Means followed by the same upper-case letter in a column and by the same lower-case letter in a row do not differ according to Tukey's test ($P \leq 0.05$).

7 DAA: $F = 35.87$, df = 11, $P < 0.0001$). The mortality induced by the control with adjuvant was significantly higher than that caused by the control containing water alone at all time points (Table 3).

In adults, OPA-79.9 at 0.75 and 1.0% dilutions and 1.0% OPA-85.4 induced the highest mortality (>72.5%) at 1 DAA, indicating a knockdown effect. The effects did not differ from those obtained with imidacloprid, but were significantly different from those with the controls ($F = 18.21$, df = 11, $P < 0.0001$). In the adult insects, the effect of the control containing water alone did not differ significantly from that of the control containing adjuvant. Treatment with OPA-69.3 at a concentration of 1.0% showed intermediate efficacy; the mortality was significantly higher than that induced by the control (46.25%), but lower than that induced by imidacloprid and the higher OPA concentrations ($F = 18.21$, df = 11, $P < 0.0001$). The other treatments did not differ from the controls (Table 4). At 3 DAA, OPA-79.9 at 0.75 and 1.0% dilutions and the 1.0% OPA-85.4 dilution sustained the efficacy observed in the first assessment, displaying higher values of mortality (78.75–97.50%). Again, the effects did not differ from those obtained with imidacloprid, but were significantly different from those with the controls ($F = 18.19$, df = 11, $P < 0.0001$). The efficacies of OPA-69.3 at 1.0% and OPA-85.4 at 0.75% were intermediate, as they caused significantly higher mortality (55 and 57.50% respectively) compared with the controls (2.5%), but were less effective than imidacloprid (98.75%) (Table 4).

At 7 DAA, OPA-69.3 at 1.0%, OPA-79.9 at 0.75 and 1.0% and OPA-85.4 at 0.75 and 1.0% did not significantly differ from imidacloprid, with the average mortality ranging between 62.5 and 96.25%; however, they significantly differed from the controls ($F = 17.25$, df = 11, $P < 0.0001$) (Table 4). The other treatments did not significantly differ from the controls (Table 4).

Comparison of the mortality caused at different time points by each treatment revealed that the mortality did not significantly differ between the time points for 1% OPA-79.9 ($F = 1.75$, df = 2, $P = 0.1767$) and 1% OPA-85.4 ($F = 0.17$, df = 2, $P = 0.8443$). These treatments induced high mortality in *D. citri* adults (>88.75%) at 1 DAA, not significantly differing from the other time points of assessment. In contrast, the effects of OPA-69.3 at 1.0% and

Table 5. Influence of the concentration of dillapiol and dilution of the essential oil of *Piper aduncum* on the percentage mortality (± SEM) of *Diaphorina citri* adults in topical application[a]

		Essential oil (v/v)		
Dillapiol (%)	*n*	0.5	0.75	1.00
69.3	8	43.75 ± 12.94 Aab	21.25 ± 7.42 Bb	70.00 ± 10.00 Ba
79.9	8	33.75 ± 9.43 Ab	86.25 ± 9.80 Aa	96.25 ± 2.63 Aa
85.4	8	22.50 ± 8.60 Ac	62.50 ± 13.59 Ab	98.75 ± 1.25 Aa

[a] Means followed by the same upper-case letter in a column and by the same lower-case letter in a row do not differ according to Tukey's test ($P \leq 0.05$). Values in the table correspond to the final time point (7 DAA).

Table 6. Average mortality (± SEM) of *Diaphorina citri* adults after residual application of different concentrations of dillapiol and dilutions of the essential oil of *Piper aduncum*[a]

			Mortality (%)		
Treatments	Concentration (v/v)	*n*	1 DAA	3 DAA	7 DAA
Control (water)	–	8	10.00 ± 4.62 Ba	14.28 ± 4.16 BCa	17.14 ± 4.51 BCa
Control (adj)	0.025	8	0.00 ± 0.00 Ba	2.50 ± 1.63 Ca	5.00 ± 3.27 Ca
Dillapiol 69.3%	1.00	8	5.00 ± 1.88 Bb	10.00 ± 4.62 BCb	18.75 ± 5.49 BCa
Dillapiol 79.9%	1.00	8	3.75 ± 2.63 Bb	7.50 ± 4.11 BCb	17.50 ± 4.53 BCa
Dillapiol 85.4%	1.00	8	16.25 ± 5.95 Bb	22.50 ± 5.59 Bab	30.00 ± 8.01 Ba
Imidacloprid	0.004	8	87.50 ± 5.26 Ab	98.75 ± 1.25 Aa	100.00 ± 0.00 Aa

[a] Means followed by the same upper-case letter in a column and by the same lower-case letter in a row do not differ according to Tukey's test ($P \leq 0.05$).

OPA-79.9 at 0.75% significantly increased over the experimental period, as indicated by the increasing mortality ($F = 15.67$, df = 2, $P < 0.0001$ and $F = 5.14$, df = 2, $P = 0.0068$ respectively), indicating the occurrence of a lethal action after a longer period, which may be considered to be an intermediate effect in comparison with more effective treatments. The 0.5% OPA-79.9 and OPA-85.4 treatments also displayed a significant increase in efficacy, as indicated by the significantly increased mortality, during the experimental period ($F = 5.25$, df = 2, $P = 0.0061$ and $F = 8.30$, df = 2, $P = 0.004$); however, their efficacy remained low in the final assessment (mortality <50%) (Table 4).

Next, we tested the interactions between the concentration of dillapiol in the OPA and the dilutions used in the treatments. An increase in dillapiol content was reflected in a higher mortality of adults for the 0.75% dilution ($F = 11.05$, df = 2, $P < 0.0001$) and the 1% dilution ($F = 9.88$, df = 2, $P = 0.0001$). However, for the 0.5% dilution, no significant difference in mortality in *D. citri* adults was observed ($F = 2.81$, df = 2, $P = 0.0659$) with increasing dillapiol content in the diluted oil extracts (Table 5). When comparing the effects of different concentrations of dillapiol for each oil extract dilution, we observed that OPA-69.3 caused significantly higher mortality at dilutions of 0.5 and 1.0%, but no significant difference was noted between 0.5 and 0.75% ($F = 3.09$, df = 2, $P = 0.0509$). OPA-79.9 caused significantly higher mortality at dilutions of 0.75 and 1.0% than at 0.5% dilution ($F = 17.54$, df = 2, $P < 0.0001$). Finally, OPA-85.4 caused significantly increased mortality with each decrease in dilution ($F = 29.22$, df = 2, $P < 0.0001$) (Table 5).

3.4 Assessment of the residual efficacy of the OPA on *D. citri* adults

The residual efficacy of all the treatments against adult *D. citri*, regardless of the concentration of dillapiol in the OPA and the dilution used, was significantly lower than that of imidacloprid at 1, 3 and 7 DAA ($F = 60.20$, df = 5, $P < 0.0001$; $F = 71.40$, df = 5, $P < 0.0001$; $F = 64.56$, df = 5, $P < 0.0001$ respectively). The treatments with OPA showed a significant increase in mortality over the assessment period ($F = 9.34$, df = 2, $P = 0.0002$; $F = 9.74$, df = 2, $P = 0.0002$; $F = 9.14$, df = 2, $P = 0.0003$ for 1, 3 and 7 DAA respectively); however, the efficacy remained low, even at 7 DAA (average mortality ≤30%) (Table 6).

4 DISCUSSION

The phytotoxicity experiment indicated that dillapiol at 99.5% was highly toxic against *C. sinensis*, independently of the dilution used. Thus, to facilitate the use of dillapiol at a high purity (99.5%), the development of new formulations would be required to mitigate this problem.

The treatments with the OPA were highly effective (mortality >90% as soon as 1 DAA) for the control of *D. citri* nymphs in topical applications, presenting similar efficacy to imidacloprid, a widely used and effective active ingredient for control of *D. citri*. These promising results revealed its great potential for the management of this insect vector and were consistent with other studies showing that nymphs are generally sensitive to botanical insecticides.[10,33] The toxicity of the adjuvant (0.025%) to nymphs observed in this study (approximately 50% mortality) corroborated the results of Srinivasan *et al.*[34] Although these authors used a fivefold lower concentration than the one tested in our study (0.005%), the higher mortality can be attributed to the application method used by them, which involved immersion of nymph-infested branches in the adjuvant solution.

Regarding the efficacy of topical application against adults, we observed that various concentrations of dillapiol in OPA at different dilutions were effective for control of *D. citri* (mortality between 70 and 98%). Other studies showed that neem extract

at 1% dilution caused 80% mortality and reduced the number of adults on leaves by up to fourfold compared with untreated areas.[12,13] Similarly, a 1% dilution of *D. alba* extract reduced the number of *D. citri* adults on leaves by up to fourfold compared with untreated areas.[13] Efficacies of *P. aduncum* extract against adults of the sucking insects *Aetalion* sp. and *E. herus* of 80 and 100%, respectively, have been reported after topical application at a dose of 3 and 8% respectively.[24,33]

It is important to emphasise that in our study we used the essential oil obtained by fractional rectification, which allows more accurate qualitative and quantitative profiling and normally has greater stability than botanical extracts. The major compounds (terpenes and terpenoids, and aromatic and aliphatic constituents) usually determine the biological properties of this essential oil. However, the activity of the major components might be modulated by other smaller molecules,[35] such as apiol and myristicin, that occur in minor quantities and can exert additive or even synergistic insecticidal effects on known insecticidal compounds such as dillapiol.[18,22,36] Because *D. citri* is an insect vector, its management requires frequent foliar insecticide spraying and the use of a reduced number of active ingredients with different modes of action, which can lead to selection of a psyllid population resistant to the insecticides commonly used for their control.[37,38] Therefore, searching for new active ingredients to be used in rotation to control this insect vector is a constant need.

The OPA is mainly composed of dillapiol,[14,19,20] which has a potential inhibitory activity against the detoxifying enzymes responsible for the elimination of plant metabolites potentially toxic to insects.[21,22] Previous studies have demonstrated that elevated levels of esterases, glutathione *S*-transferase and cytochrome P450 enzymes are responsible for the detoxification of insecticides in *D. citri* nymphs and adults, and these enzymes are associated with lower susceptibility of this insect to insecticides frequently used for its control.[39–41]

Studies have demonstrated that dillapiol acts as a potent synergist of synthetic and botanical insecticides in agricultural pest control.[42,43] Liu *et al.*[43] observed that dillapiol in combination with pyrethrum extract purified from *Chrysanthemum cinerariifolium* was 9.1-fold more effective for the control of *Leptinotarsa decemlineata* (Say) larvae resistant to insecticides including pyrethrum. Mukerjee *et al.*[44] showed that acyl derivatives of dihydrodillapiol have a synergistic activity towards pyrethrum against *Tribolium castaneum* (Herbst.), with a synergism factor (LC_{50} for pyrethrum/LC_{50} for pyrethrum plus synergist) of 2.3–4.0. Shankarganesh *et al.*[45] reported that dihydrodillapiol combined with pyrethroids caused significant reduction in resistance of *Spodoptera litura* (F.), which is currently resistant to cypermethrin, lambda cyhalothrin and profenophos. Tomar *et al.*[46] observed that a mixture of pyrethrum and dillapiol synthesised by chemical transformation (1:5) showed a synergism factor varying from two- to fivefold when compared with pyrethrum alone against *T. castaneum*. Thus, essential oils rich in dillapiol might help to reduce resistance in *D. citri* populations, because this active ingredient can potentially inhibit the activity of detoxifying enzymes in insects. However, further studies are required to prove this hypothesis. The present study has clearly demonstrated the high efficacy of the OPA in the control of *D. citri*. As the mode of action is unlike that of insecticides commonly used in citriculture, it could be used in rotation for effective management of *D. citri*. The results of this study will contribute to the future adoption of dillapiol-rich oils as a control strategy of *D. citri*.

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