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Insecticidal Activity of Caffeine Aqueous Solutions and Caffeine Oleate Emulsions against *Drosophila melanogaster* and *Hypothenemus hampei*

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The bioactivity of caffeine aqueous solutions (0.20−2.00 wt %) and caffeine oleate emulsions (20 vol % oil, 2.00 wt % surfactant, 0.04 wt % caffeine, 0.05 wt % oleic acid) was assessed against two biological models: *Drosophila melanogaster* and *Hypothenemus hampei*. The caffeine aqueous solutions showed no insecticidal activity, whereas caffeine oleate emulsions had high bioactivity against both *D. melanogaster* and *H. hampei*. By preparing the caffeine oleate emulsions with anionic surfactants (i.e., sodium lauryl sulfate, sodium laureate, and sodium oleate), we obtained a lethal time 50 (LT_{50}) of 23 min. In the case of caffeine oleate emulsions prepared with nonionic surfactants (i.e., Tween 20 and Tween 80), a LT_{50} of approximately 17 min was observed. The high bioactivity of the caffeine oleate emulsion against *H. hampei* opens the possibility of using this insecticide formulation as an effective way to control this pest that greatly affects coffee plantations around the world.

KEYWORDS: Caffeine oleate; insecticide; O/W emulsion; *Drosophila melanogaster*; *Hypothenemus hampei*

INTRODUCTION

Caffeine, an alkaloid of the methylxanthine family, is naturally produced by a number of plant species, including coffee, tea, mate, and guaraná (*1*). Caffeine acts as a chemical defense in these plants, showing repellent or toxicant properties. The first direct evidence of caffeine acting as an insecticide was reported in tobacco hornworm (*Manduca sexta*) larvae (*2*) at caffeine concentrations similar to those found in coffee seeds (i.e., 0.8−1.8 wt %). Caffeine aqueous solutions (2.0 wt %) have also shown molluscicidal activity against slugs (*Veronicella cubensis*) and orchid snails (*Zonitoides arboreous*) (*3*, *4*). Other pesticide applications of caffeine include fungi control (*5*), bird repellent (*6*, *7*), and pest coyotes control (*8*).

Caffeine was suggested as an effective defense for young coffee plant leaves against insect herbivores such as the leaf miner *Perileucoptera coffeella* (*9*), which is a well-known coffee plantation pest. However, no correlation was found between the alkaloid content in leaves or fruits and their resistance to the *Perileucoptera coffeella* (*10*). A similar conclusion was obtained for the resistance of coffee seeds to the berry borer *Hypothenemus hampei* (*11*), a pest that could cause 100% losses of coffee yields, indicating that *H. hampei* might have evolved an adaptation against the toxic effect of caffeine. However, the lack of insecticidal activity of caffeine against *H. hampei* could also be related to the type of formulation used for the bioassay, that is, the use of caffeine aqueous solutions instead of caffeine containing emulsions. That was the case for nicotine, with its formulation as an oil-in-water (O/W) emulsion containing nicotine carboxylate showing a direct relationship between the amount of nicotine remaining in the emulsion oil droplets and its bioactivity against *Drosophila melanogaster* (*12*). Additionally, the study also showed that the hydrocarbon chain length of the fatty acid used to prepare the nicotine carboxylate complex affected the nicotine insecticidal activity; this effect was correlated with the strength of the acid−base interaction between the nicotine and the different fatty acids used to produce the nicotine carboxylate complexes. Considering that caffeine could also form complexes with mono- and dicarboxylic acids through the carboxylic acid···imidazole hydrogen-bonding interaction (*13*), it is feasible to expect a higher bioactivity of caffeine carboxylate emulsions in comparison with caffeine aqueous solutions.

Surfactants used in insecticide formulations affect insecticidal activity (*14*). For instance, Tween 80 enhanced the bioactivity of extracts of *Melia azedarach* against sweetpotato whitefly nymphs (*15*). Silwet L-77, an organosilicone surfactant, was required to facilitate *Pseudomonas syringae* pv. *tagetis* penetration into Canada thistle (*16*). The molecular structure of the caffeine oleate complex, selected as the caffeine carboxylate

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Figure 1. Bidimensional structure of caffeine oleate complex. The carboxylic acid···imidazole hydrogen bonding interaction of the caffeine oleate complex formation is indicated with dashed lines.

complex in the present work (**Figure 1**), shows, in terms of polarity, a hydrophilic moiety (associated with the caffeine−carboxylic acid group complex) and a hydrophobic moiety (associated with the C18:1 hydrocarbon chain), suggesting its role as a surfactant in the insecticide emulsion formulation. The presence of conventional surfactants in the insecticide formulation could affect both the formation and adsorption of the caffeine oleate complex at the emulsion oil−water interface, changing the insecticidal activity of the caffeine oleate emulsion.

In the present work, the bioactivity of O/W emulsions containing caffeine oleate, prepared with anionic and nonionic surfactants, was tested against two biological models, the *D. melanogaster* as a standard model and the *H. hampei* because of its catastrophic effects in coffee plantations. To asses the effect of the functional group present in the charged hydrophilic head of the anionic surfactant, we selected sodium laureate [$CH_3(CH_2)_{10}COONa$] and sodium lauryl sulfate [$CH_3(CH_2)_{11}OSO_3Na$] for emulsion preparation. Tween 20 and Tween 80 were chosen as the nonionic surfactants considering their similar hydrophilic head groups but different hydrophobic chains, C12 and C18:1, respectively. Sodium oleate ($CH_3(CH_2)_7CH{=}CH(CH_2)_7COONa$) was also selected as part of the anionic surfactant group to assess a molecule with the same unsaturated hydrocarbon chain of the Tween 80 molecule but with an anionic head group.

MATERIALS AND METHODS

Chemicals. Tween 20 (T20), Tween 80 (T80), sodium lauryl sulfate (SLS), sodium laureate (SL), sodium oleate (SO), oleic acid, and caffeine HPLC grade were provided by Sigma Chemical Co. (St. Louis, MO). Sunflower oil was purchased from a local supermarket and purified by being passed through a silica gel column.

Alkaloid Quantification. The caffeine concentration present in emulsion droplets was determined from the difference between the amount of caffeine detected in the emulsion aqueous phase after emulsification and the known amount of caffeine initially added to the emulsion oil phase. To determine the caffeine content after emulsification, we centrifuged the prepared emulsion at 12×10^4 *g* for 90 min at 5 °C. Afterward, the creamed emulsion layer was phase separated. The caffeine presented on the separated aqueous phase was quantified by measuring the absorbance at 280 nm and interpolating the reading value on a calibration curve of caffeine standard solutions.

Oil-Phase Preparation. The caffeine powder was added to the sunflower oil at room temperature until complete dissolution was reached. Thereafter, oleic acid was added to produce an oil phase at a 1:1 base-to-acid molar ratio with a caffeine concentration of 0.20 wt % for emulsion preparation and interfacial tension measurements. In the case of blank emulsions, the oil phase did not contain caffeine.

Interfacial Tension Measurements. Interfacial tension measurements at the oil phase (0.20 wt % caffeine, 0.29 wt % oleic acid)−Tween 80 (0.001 M) interface were carried out using a processor tensiometer Krüss K12 using a Wilhelmy plate as a measuring device. The temperature was set at 25 °C for all measurements.

Preparation and Characterization of Insecticide Formulation. Blank oleic acid emulsions (20 vol % oil, 0.05 wt % oleic acid, 2.00 wt % surfactant) and caffeine oleate emulsions (20 vol % oil, 0.04 wt % caffeine, 0.05 wt % oleic acid, 2.00 wt % surfactant) were prepared at 25 °C using a high-pressure jet homogenizer working at 300 bar. Droplet size distributions were carried out using a Malvern Mastersizer 2000. All emulsions were prepared in triplicate.

Bioassay for Insecticidal Activity. The bioassay for insecticidal activity against adults of *Drosophila melanogaster* and adult females of *H. hampei* was as follows: a filter paper disk (diameter = 4 cm) was impregnated with 0.20 mL of insecticide emulsion and placed inside glass vials (5 cm wide × 6 cm deep). A total of 30 individuals were introduced into a glass vial and the number of dead individuals was recorded every minute, or every 5 min, depending on the insecticide activity of the sample. Data were recorded until all individuals were dead or the observation time reached 5 h; some bioassays were monitored for a period of 24 h because of the lack of bioactivity of the tested formulation. Three replicates for each dispersion sample were carried out. Lethal time 50 (LT_{50}) values and regression analysis data were calculated with the software Statgraphics Plus 4.1.

Bioassay of *H. hampei* Attack to Coffee Seeds. A total of 54 coffee seeds were imbibed for 24 h in 200 mL of caffeine aqueous solution (2.00 wt %), blank oleic acid emulsion (20 vol % oil, 0.05 wt % oleic acid, 2.00 wt % surfactant), or caffeine oleate emulsion (20 vol % oil, 0.04 wt % caffeine, 0.05 wt % oleic acid, 2.00 wt % surfactant) and then removed from the flask and left to dry in filter paper. The treated coffee seeds were placed into a glass flask (20 cm wide × 20 cm deep) together with 54 *H. hampei* female individuals. After a period of 5 h, the number of dead individuals and coffee seeds infected by the *H. hampei* were recorded.

Statistical Analysis. The results were expressed as the mean ± SEM, when appropriate. Statistical comparisons were done using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons test to compare the differences between means; $p < 0.05$ indicated significance.

RESULTS AND DISCUSSION

Formation of Caffeine Oleate at the Oil−Water Interface. Caffeine complexes with mono- and dicarboxylic acids, promoted by the carboxylic acid···imidazole hydrogen-bonding interaction, resemble the structure of surfactants. Therefore, the formation of the caffeine oleate complex could be assessed by interfacial tension measurements following the procedure used to study nicotine carboxylate complexes (*12*).

The interfacial tension for the interface formed between sunflower oil (0.29 wt % oleic acid) and water showed a value of 14.98 ± 0.02 mN m^{-1}, a figure that decreased to 13.37 ± 0.02 mN m^{-1} after the addition of 0.20 wt % caffeine to the oil phase. This interfacial tension value remained constant for at least 5 h, indicating that a compositional equilibrium at the interface was obtained. The reduction in the interfacial tension after adding caffeine to the system indicates the formation of the caffeine oleate at the oil−water interface. To determine the possible interactions between the surfactant and the caffeine oleate at the oil−water interface, we measured the interfacial tension for two systems: (i) Tween 80 aqueous solution (0.10 wt %)−sunflower oil (0.29 wt % oleic acid); (ii) Tween 80 aqueous solution (0.10 wt %)−sunflower oil phase (0.29 wt % oleic acid, 0.20 wt % caffeine). The concentration of Tween 80 used for these measurements was well above its critical micelle concentration (i.e., 0.00155 wt %) to guarantee full surfactant coverage of the oil−water interface. The interfacial tension value obtained for system (i) was 2.22 ± 0.02 mN m^{-1}, this being one-sixth of the figure obtained for the system with no Tween 80 present, therefore confirming the high surface activity of the Tween 80 molecule. The interfacial tension value of system (i) is also lower than that observed for the caffeine oleate complex, which suggests that if both Tween 80 and caffeine oleate molecules are present in the system, the Tween 80 molecule would have a preferential thermodynamic adsorption at the oil−water interface. Therefore, the addition of

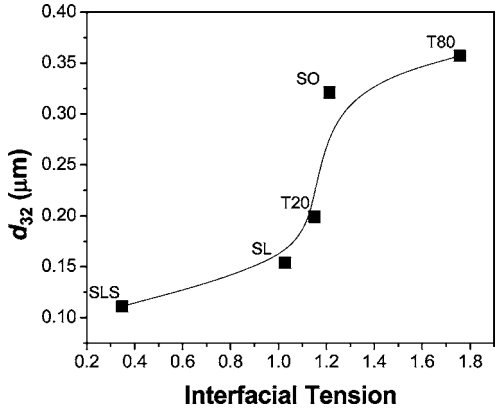

Figure 2. Effect of interfacial tension on the surface average particle size of caffeine oleate emulsions (20 vol % oil, 0.04 wt % caffeine, 0.05 wt % oleic acid, 2.00 wt % surfactant) prepared with different surfactants: sodium lauryl sulfate (SLS), sodium laureate (SL), sodium oleate (SO), Tween 20 (T20), and Tween 80 (T80). Solid line serves as a guide to the eye.

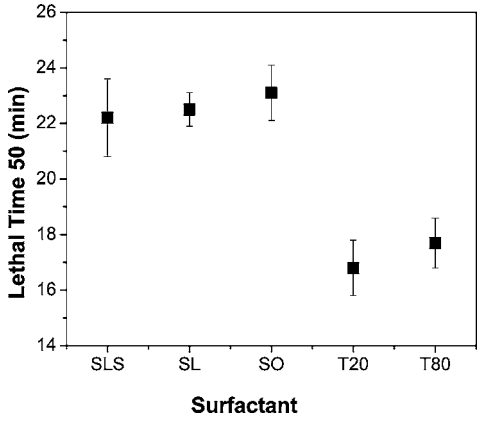

Figure 3. Effect of the surfactant used in emulsion preparation on the lethal time 50 (LT_{50}) bioactivity of caffeine oleate emulsions (20 vol % oil, 0.04 wt % caffeine, 0.05 wt % oleic acid, 2.00 wt % surfactant) against *D. melanogaster*.

caffeine to the system should not change the interfacial tension of the system because the caffeine oleate complex is less surface active than the Tween 80 molecule. However, the interfacial tension of system (ii) had a value of 1.73 ± 0.02 mN m^{-1}, indicating a further reduction in the interfacial tension due to the presence of the caffeine oleate complex at the interface. This result suggests that both Tween 80 and caffeine oleate are present at the interface working synergistically to reduce the interfacial tension. The tensiometric analysis of Tween 20, SLS, SL, and SO showed trends similar to those obtained for Tween 80.

Effect of the Type of Surfactant on Emulsion Particle Size. Emulsion particle size is among the physicochemical parameters that could affect the bioactivity of insecticide formulations (*18*). Because the type of surfactant affects the oil−water interfacial tension and the particle size is dependent on interfacial tension, particle size analysis was carried out for the emulsion prepared with the set of surfactants selected in the present study. **Figure 2** shows the effect of interfacial tension on the studied surfactants based on the average particle size of caffeine oleate emulsions. Here, the average particle size (d_{32}) increased with the rise in interfacial tension, following the trend of a sigmoid function, with the surfactants containing the short C12 hydrocarbon chain (i.e., SLS, SL and T20) at the lower end of the plot, whereas they contained the long C18:1 hydrocarbon chain (i.e., OS and T80) at the upper end of the plot. This effect of hydrocarbon chain length indicates a better interaction of the hydrophobic moiety of the surfactant with the oil phase of the droplet emulsions. The type of anionic surfactant head group also shows a trend, with carboxylate surfactants (i.e., SL and SO) having similar and higher interfacial tension than sodium lauryl sulfate. This effect can be explained in terms of the higher repulsion between carboxylate groups at the oil−water interface in comparison to that observed for the sulfate group.

Bioactivity against *Drosophila melanogaster*. The contact bioactivity test against *D. melanogaster* was carried out for aqueous caffeine solutions (0.20−2.00 wt %) and O/W caffeine-oleate-containing emulsions (20 vol % oil, 0.04 wt % caffeine, 0.05 wt % oleic acid, 2.00 wt % surfactant). In the case of the caffeine aqueous solutions, no individuals were killed during a 5 h period of experimental observation, indicating a lack of caffeine insecticidal activity. This result contrasts with the bioactivity of caffeine against leaf miner (*Perileucoptera coffeella*) (*10*) and tobacco hornworm (*Manduca sexta*) larvae (*2*) after its ingestion from feeding substrates. Therefore, the lack of insecticide activity of caffeine aqueous solutions is related to the contact bioassay carried out in the present work, which apparently reduces caffeine bioavailability. The dependence of bioactivity on the method of exposure has been previously observed in pesticides such as pyriproxyfen against *Chrysoperla carnea* adults (*19*) and indoxacarb against the tarnished plant bug *Lygus lineolaris* (*20*). Moreover, Hollingsworth and co-workers (*4*) showed differences in caffeine antifeeding and contact toxicity properties on slugs and snails.

The effect of the type of surfactant used to prepare the O/W caffeine oleate emulsions on lethal time 50 (LT_{50}) for bioassays with *D. melanogaster* is shown in **Figure 3**. The blank emulsion (i.e., no added caffeine) had an $LT_{50} = 50 \pm 2$ min, showing some bioactivity that is due to the oleic acid present in the emulsion (*21*). The caffeine oleate emulsion showed LT_{50} values <25 min for both anionic and nonionic surfactants, indicating an increase in caffeine bioactivity in comparison to the blank emulsion and the caffeine aqueous formulation. Molecule hydrophobicity has been shown to be an important factor influencing insect cuticular penetration and subsequent transport of bioactive molecules to targets (*22*, *23*). Therefore, the higher bioactivity of O/W emulsions containing caffeine oleate could be explained in terms of the higher hydrophobicity of the caffeine oleate complex present in the emulsion oil phase in comparison to the hydrate caffeine molecule in the aqueous formulation.

In addition to the effect of caffeine emulsification on insecticidal bioactivity, the type of surfactant used during the emulsification process seems to influence caffeine bioactivity, as observed from the LT_{50} values seen in **Figure 3**. This is confirmed by the analysis of variance (ANOVA) of LT_{50} values for the five insecticide emulsions showing an overall difference between data ($p < 0.05$). Furthermore, statistical comparisons among formulations effectively showed that anionic stabilized emulsions have a significant difference ($p < 0.05$) from the nonionic formulations, with the last one being more effective as insecticide. The LT_{50} value of the emulsions prepared with nonionic surfactants is close to that previously observed for the nicotine oleate emulsions (i.e., $LT_{50} = 11$ min, 0.73 wt % nicotine) (*12*). However, the caffeine concentration used in the present bioassay is 20-fold lower than that used for the nicotine system, indicating a much higher caffeine formulation insecticidal activity against the *D. melanogaster* biological model. Caffeine studies in *D. melanogaster* neurons have shown that

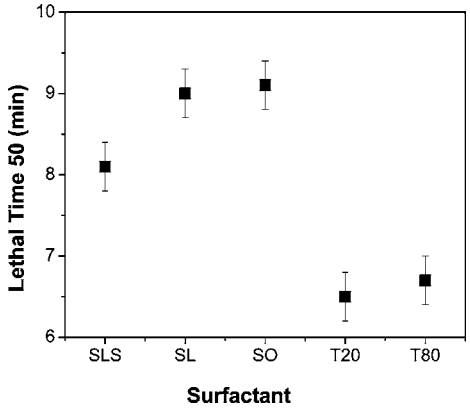

Figure 4. Effect of the surfactant used in emulsion preparation on the lethal time 50 (LT_{50}) of caffeine oleate emulsions (20 vol % oil, 0.04 wt % caffeine, 0.05 wt % oleic acid, 2.00 wt % surfactant) against *H. hampei*.

caffeine inhibits Ca^{2+} current, produces a weaker inhibition of Na^{+} current, and modulates the transient and sustained potassium current that could increase neuronal excitability (*24*). It has been proposed that insect calcium channels would offer an excellent pesticide target for commercial exploitation given their key role in multiple biological processes, including cell signaling and neurotransmitter release (*25*, *26*). Therefore, the much higher bioactivity of the caffeine-containing emulsions against *D. melanogaster* in comparison to the nicotine-containing emulsion could be related to its calcium inhibition role, whereas the nicotine molecule targets the acetylcholine receptor function.

The higher bioactivity of caffeine oleate emulsions prepared with nonionic surfactants is not related to the size of emulsion droplets because the Tween 80-stabilized emulsion has a d_{32} value nearly double that obtained for the Tween 20 (see **Figure 2**). Both emulsions have similar LT_{50} values. The same argument applies if the difference in bioactivity is attempted to be explained in terms of interfacial tension. The bioassays of nicotine carboxylate emulsions showed that bioactivity was mainly affected by the amount of nicotine remaining inside the emulsion oil droplets (*12*). The quantification of caffeine remaining in the emulsions stabilized by the anionic surfactants (i.e., SL, SLS, and SO) showed a value of 22 ± 2%, whereas the emulsions stabilized with nonionic surfactants had a value of 53 ± 2%. These results indicate that the difference in bioactivity between emulsions stabilized by anionic and nonionic surfactants is correlated to the amount of caffeine remaining in the emulsion oil droplets, supporting the hypothesis that the hydrophobicity of the caffeine oleate increases caffeine insecticidal activity.

Bioactivity against *Hypothenemus hampei*. The contact bioactivity test of aqueous caffeine solutions (0.20−2.00 wt %) against the biological model *H. hampei* showed that no individuals were killed within the first 24 h from the initial contact. On the other hand, carrying out the bioactivity test with the caffeine oleate emulsion (20 vol % oil, 2.00 wt % SLS, 0.04 wt % caffeine) induced the death of all individuals in less than 30 min with an associated $LT_{50} = 8.1 \pm 0.3$ min. The blank emulsion (20 vol % oil, 2.00 wt % SLS) did not kill any individual after 24 h. These results indicate that caffeine does not have bioactivity against *H. hampei* when formulated in aqueous solution, whereas the caffeine emulsion shows a high bioactivity with no effect seen from the oil emulsion phase.

Figure 4 shows the effect of the type of surfactant used to prepare the caffeine oleate emulsions on their bioactivity against *H. hampei*. Here, the caffeine oleate emulsions stabilized with anionic surfactants showed similar, although slightly higher, LT_{50} values than those observed for the emulsions prepared with nonionic surfactants, resembling the behavior shown in the *D. melanogaster* biological model.

The high bioactivity of caffeine oleate emulsions against *H. hampei* indicates that caffeine has toxic effects on this insect when applied as a contact insecticide. Caffeine loses its insecticidal activity when formulated as an aqueous solution for both the contact and ingestion bioassay tests. The lack of caffeine bioactivity in the ingestion test has been attributed to *H. hampei* evolution that handles the toxic effect of caffeine, metabolizing it by the digestive track of larva and adults (*11*). To determine if *H. hampei* is able to metabolize the caffeine oleate present in emulsions, a *H. hampei* attack bioassay was carried out using coffee seeds imbibed in caffeine aqueous solution (i.e., 2.00 wt %) and caffeine oleate emulsion (20 vol % oil, 0.04 wt % caffeine, 0.05 wt % oleic acid, 2.00 wt % SLS). Treating the coffee seeds with caffeine solution showed no insecticide activity against *H. hampei*, with zero individuals killed during the 5 h bioassay. However, caffeine aqueous solutions showed some repellent activity, reducing the infested seeds from 46% (untreated coffee seeds) to 26%. Guerreiro Filho and co-workers found neither insecticidal nor repellent activity (*11*). The repellent activity obtained in the present work could be related to the short time allowed for the bioassay (5 h) compared to the 6 months used by Guerreiro Filho and co-workers (*11*); therefore, it is possible that a longer exposure of coffee seeds to *H. hampei* could decrease caffeine repellent activity. The use of the caffeine oleate emulsion to imbibe the coffee seeds produced a dramatic change in both the number of insects killed and infested seeds. A third of the *H. hampei* individuals were killed, and no seeds were infested during the 5 h bioassay; the blank emulsion showed no insecticidal or repellent activity during the 5 h bioassay. Because a significant population of individuals were killed in the coffee seed attack bioassay, it indicates that the *H. hampei* were not able to handle the toxic effect of caffeine oleate in the digestive track. The difference in toxicity between the caffeine aqueous solution and the caffeine oleate emulsions in the ingestion test is probably due to the shorter absorption time of the caffeine oleate in the digestive track in comparison to the hydrated single caffeine molecule present in the aqueous formulation.

Caffeine formulated as a caffeine oleate emulsion has been shown to have high insecticidal activity against *H. hampei* in controlled laboratory bioassay conditions, providing the opportunity to test its bioactivity on field, while at the same time pursuing the control of this pest in coffee plantations. To achieve optimal pest control, further work is required to increase the caffeine remaining in the emulsion droplets and therefore its insecticidal activity. It is also of high importance to pursue a better understanding of the absorption mechanism of caffeine oleate in both the digestive track and the exoskeleton of insects.

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