

Original Article

INSECTICIDE SUSCEPTIBILITY AND DETOXIFYING ENZYME ACTIVITY IN MANAGED HONEY BEES IN A SOUTHERN MEXICO LANDSCAPE

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Abstract

Pesticide exposure in honey bees triggers a series of biochemical cascades, including the activation of detoxifying enzymes. Consequently, a specific pesticide susceptibility profile may correlate with a distinct enzymatic activity pattern. In this study, we analyzed pesticide residues in honey samples through gas chromatography, assessed the susceptibility profile by determining the median Lethal Time (LT_{50}) for commercial formulations of malathion, imidacloprid, and cypermethrin, and measured the activity of detoxifying enzymes - pNPA, α - and β -esterases P_{450} , GST, and AChE - in foragers from thirteen apiaries located in Chiapas, Mexico. We also conducted palynological analyses to identify pollen from crops in the bees' diets to corroborate the pesticide residue findings. No pesticide residues were detected above the method's detection limits in our samples; palynological analysis showed that foragers primarily collected pollen from wild plant species, suggesting low probability of exposure to insecticides and supporting the pesticide residues results. The malathion formulation caused the highest toxicity (lowest LT_{50}) across all sites, whereas cypermethrin was the least toxic (mortality < 20% at 12 h after exposure). Enzyme activities varied across study sites: foragers from the Tapachula apiary exhibited the lowest AChE activity, whereas those from Villa Comaltitlan showed the highest activity. No clear correlation between enzyme activity and LT_{50} for the different formulations was observed. Our results suggest that managed honey bee apiaries were exposed to xenobiotics other than insecticides, as shown by residue and palynological analyses, leading to distinct enzymatic and susceptibility profiles.

Keywords: *Apis mellifera*, detoxification, neonicotinoid, pesticide residues, pollen, pyrethroid

INTRODUCTION

Honey bees, *Apis mellifera* L. (Hymenoptera, Apidae), are highly social bees that contribute substantially to the maintenance of ecological networks by playing a role in the reproduction of many flowering plant species and as a food source for other animals (Roubik, 2020). In economic terms, their role in plant reproduction, or pollination, contributes roughly 9.5% of the commercial value of crops worldwide (Lara-Pulido et al., 2021).

Honey bees interact with environmental chemicals of both natural and human origin during everyday activities, some of which are

detrimental to them (Noi et al., 2021). For example, many synthetic insecticides still used in Latin America lack specificity toward pest insects and are often overapplied to address issues such as insecticide resistance, resulting in undesirable collateral effects on non-target species, including humans and honey bees (Zhao et al., 2022; Pervez & Manzoor, 2023). Since pollen provides the proteins needed for brood development and nectar represents the main caloric source for adult workers (Winston, 1991), most insecticides enter colonies mainly through the resources collected by foragers, making the forager itself the first point of contact and the vehicle to slowly poison the colony (Sponsler

& Johnson, 2017). Moreover, colonies require a continuous flow of food, and since honey bees are generalists, foragers collect food from several plant species, including ornamental plants and crops (Donkersley et al., 2017); therefore, it is common to observe bees foraging on flowering crops, despite the routine use of insecticides to control pests (Sanchez-Bayo & Goka, 2014). This creates a problem for growers: while pollination is known to improve fruit yield and quality, insecticides are still applied without regard for their impact on bees (James & Pitts-Singer, 2008; Tamburini et al., 2019). Therefore, honey bees in an agricultural, open-range context might experience continuous exposure to insecticides, which explains why residues have been detected in wax, honey, and pollen inside colonies (Sanchez-Bayo & Goka, 2014; Valdovinos-Flores et al., 2017). This situation allows the identification of the insecticides to which bees are most likely exposed by linking pollen analysis - used to determine the wild plants and crops from which they collect food - with information on the pesticides commonly applied to those plants (Danner et al., 2017; Malagnini et al., 2022). Consequently, insecticides may enter bee colonies depending on the surrounding vegetation (cultivated or wild), plant phenology, and the actual use of such compounds for pest control.

Pyrethroids, organophosphorus, and neonicotinoids are insecticides frequently reported in samples obtained from bee colonies (Chauzat & Faucon, 2007; Valdovinos-Flores et al., 2017; Calatayud-Vernich et al., 2018; Sabo et al., 2024). Insecticides' mode of action affects the central nervous system of honey bees, although their target molecules or sites within molecules are distinct among insecticides (Araújo et al., 2023). For example, pyrethroids bind to voltage-gated sodium channels on nerve cell membranes, while neonicotinoids target nicotinic acetylcholine receptors in the central nervous system of insects and deregulate the normal function of the nervous system (Soderlund, 2010; Thany, 2023). Acetylcholinesterase (AChE) is an enzyme that plays a crucial role in the proper transmission of electrical impulses between neurons at synaptic junctions under normal conditions and

is a common target for many such insecticides as organophosphorus malathion and carbamate propoxur (Fukuto, 1990; Jensen & Whatling, 2010). At high concentrations, insecticides cause death a few hours or even minutes after exposure; exposed bees show such signs of intoxication as tremors and paralysis before death (Serrão et al., 2022). At sublethal concentrations, the consequences of chronic exposure include effects at the individual level (e.g., loss of vision, impaired flight ability and spatial memory, disorientation while foraging, reduced appetite, and increased susceptibility to infections and diseases), which may result in the collapse of the colony (Desneux et al., 2007; Chakrabarti, 2019). The physiological response to insecticide exposure, which is also a defense mechanism, involves enzyme expression that help degrade extraneous molecules. Insecticide resistance in many insect species has been associated with high-level activity of such enzymes as carboxylesterases, GST, and P_{450} (Li et al., 2007; Ranganathan et al., 2022).

Similar to any other insect, bees exhibit three phases of detoxification: In phase I, the xenobiotic is chemically modified by enzymes, including P_{450} , by oxidation or reduction to facilitate the addition of further molecules. In phase II, the bioactivated molecule from phase I is conjugated with glutathione, glucuronic acid, or sulfate, resulting in the inactivation of the insecticide and a more hydrophilic conjugate; GST, UGT, and sulfotransferases are the enzymes responsible for such reactions. Finally, in phase III, the conjugated molecules are excreted (Li et al., 2007; Berenbaum & Johnson, 2015). Exposure to pesticides has been proposed to trigger specific patterns of enzymatic activity in bees. Carvalho et al. (2013) explored the changes in the activity of AChE, carboxyl esterases, catalase, GST, and alkaline phosphatase after exposure to deltamethrin, fipronil and spinosad, separately. These authors suggested that these enzymes could be used as biomarkers of honey bee health based upon alterations observed in their activities. In this regard, Christen and Fent (2017) found that the organophosphates chlorpyrifos and malathion,

at environmentally realistic concentrations, did not elicit higher expression of P_{450} genes compared to the control treatment; however, a significant increase in the activity of AChE was observed at the 95th centile field relevant concentration of a mixture of those organophosphates.

Insecticide susceptibility in honey bees results from historical exposure, (e.g. through contaminated pollen and nectar, and individual responses, e.g. changes in detoxifying enzyme activity. Comparing susceptibility among colonies in different locations could elucidate factors predisposing certain colonies to decline. Determining the lethal time to kill 50% of exposed individuals (LT_{50}) is a method used to assess susceptibility to insecticides, facilitating inter-colony comparisons (Ruiz-Toledo et al., 2018; Simon-Delso et al., 2018; Ruiz-Toledo & Sánchez, 2024). In this study, we addressed whether pesticide residue content in pollen and the activity of detoxifying enzymes (α - and β -esterases, P_{450} , GST, and AChE) correlate with the susceptibility - assessed as LT_{50} - to commercial formulations of these insecticides under the conditions of the agricultural landscape surrounding our colonies. In particular, we aimed to determine whether dominant crops commonly perceived by beekeepers as continuous sources of insecticide exposure (mango, rambutan, and soybean) could account for the observed patterns of enzyme activity and susceptibility. Our hypothesis is that enzyme activity closely tracks the LT_{50} for each insecticide formulation and is influenced by the presence of dominant crops in the study area.

MATERIAL AND METHODS

Study site, biological material, and insecticides

Outgoing flying *Apis mellifera* foragers 3-4 weeks old, and honey and pollen samples were collected between 08:00 to 10:00 h in June and July 2023 from three randomly selected colonies in each of thirteen apiaries located in agricultural areas from different municipalities (Tonala, Suchiate, Tuxtla Chico, Metapa, Tapachula, Huehuetan, Villa Comaltitlan, Acapetahua,

Arriaga, Mapastepec, Huixtla, Pijijiapan and Frontera Hidalgo) in the Soconusco region, in Chiapas, Mexico (Fig. 1). The selected colonies had 2-3 year-old queens and were considered healthy and free of diseases, as assessed by an experienced beekeeper. Palynological analyses and bioassays (LT_{50} determination and enzyme activity) were conducted in El Colegio de la Frontera Sur, Unidad Tapachula, in Chiapas, Mexico from 08:00 to 11:00 h. Pesticide residue was analyzed by a specialized third-party laboratory.



Fig. 1. Location of apiaries in the study region.

Pesticide residue analysis

A volume of 500 mL of honey was collected from each of the three selected colonies in each apiary, pooled and a 500 mL aliquot from each pool was analyzed. All thirteen samples were sent to the CIATEJ-Noreste laboratory, located in Nuevo Leon, Mexico and analyzed following the certified internal method INS-SM/NE-01. This method involves gas chromatography coupled to a triple quadrupole detector, with a quantification limit ranging from 0.001 to 0.049 mg/kg depending on the pesticide, as shown in Supplementary Material 1.

Acute contact LT_{50} determination

The following commercial pesticide formulations were used for LT_{50} assays: Malatión (83.6% a.i. malathion, organophosphorus; Agroquímica Tridente S.A. de C.V., Mexico), Rotaprid (30.2% a.i. imidacloprid, neonicotinoid; Rotam S.A. de R. L. de C.V., Mexico), and Isquimetrina (21.12% a.i. cypermethrin, pyrethroid; Agroquímica

Tridente S.A. de C.V., Mexico). At each apiary, we collected 160 foragers from the three selected colonies and transported them to the laboratory in groups of ten inside 500 mL ventilated plastic containers. Food was provided to each group through a cotton ball soaked in 5 mL of 2 M sucrose solution to prevent starvation. All formulations were diluted in a sterile aqueous solution containing 2% Tween 20 (Sigma-Aldrich, USA) to promote adhesion of the solution to the bee thorax, such that 5 μ L of the final solution delivered the 50% topical lethal dose of the active ingredient for *A. mellifera*, as determined in preliminary experiments: 0.035 μ g/bee for cypermethrin, 0.312 μ g/bee for malathion and 0.109 μ g/bee for imidacloprid.

Before exposure, each group was anesthetized with medical-grade CO₂ to facilitate solution application. The anesthetized bees from one container were temporarily placed in a Petri dish and 5 μ L of one treatment were applied to their thorax and taken back to the container; the same treatment was applied to the bees of two other containers, three replicates in total. The same procedure was applied to the other two formulations. Foragers from three groups each received 5 μ L of the sterile aqueous solution at 2% Tween 20 and served as the controls. After exposure, all bees were kept at 30 $\pm$ 1°C, 65% relative humidity, a 12 h light/12 h dark photoperiod, and mortality was assessed every 30 min for 12 h.

Enzyme assays

Enzymatic activity was determined according to the protocols established for mosquitoes (Solís-Santoyo et al., 2022), with slight modifications. The heads and abdomens of ten foragers from the selected colonies in each apiary were dissected and individually macerated on ice (room temperature 25°C) in 200 μ L of sterile water in a 500 μ L vial until a uniform suspension was obtained. The resulting twenty homogenates were subsequently distributed into two 96-well plates. Duplicates of 25 μ L of each head homogenate were placed into a 96-well microplate for the AChE assay. The abdomen homogenates were centrifuged at 4,000 rpm at 4°C for 30 min, and the supernatants were

individually distributed in duplicate into microplates placed on ice for α - and β -esterases (20 μ L), p-nitrophenyl acetate (pNPA)-esterases (10 μ L), GST (10 μ L), cytochrome P₄₅₀ (20 μ L), and proteins (10 μ L). Each microplate included two water-only control wells. All enzymatic reactions were measured using a Multiskan Spectrum microplate reader (Thermo Scientific, Finland).

For AChE determination, the first duplicate of each sample was added with 145 μ L of phosphate-Triton buffer (Triton X-100 at 1% in 0.1 M sodium phosphate buffer, pH 7.8), 10 μ L of a DTNB solution (dithiobis-2-nitrobenzoic acid 0.01 M in 0.1 M sodium phosphate buffer, pH 7.0), and 25 μ L of ASCHI substrate (acetylthiocholine iodide 0.01 M). The second duplicate of each sample was added with 25 μ L of ASCHI substrate containing 1 M of 0.2% propoxur 1 M. The two control wells received 25 μ L of sterile distilled water, 145 μ L of Triton buffer, 10 μ L of DTNB solution, and 25 μ L of ASCHI solution, with and without propoxur, respectively. The enzyme reaction kinetics were monitored for 5 min at 25°C with the use of a microplate reader set to 405 nm. AChE activity was expressed as μ mol/min/mg of protein. The percentage of AChE activity inhibition by propoxur was calculated by comparing the activities of the first and second duplicates.

For pNPA esterase assay, 200 μ L of the pNPA substrate solution (p-nitrophenyl acetate 100 mM in acetonitrile and 50 mM phosphate buffer, pH 7.4, 1:100) was added to each duplicate. pNPA activity of each specimen was recorded in μ mol/min/mg of protein with the use of an extinction coefficient of 6.53 mM⁻¹ (corrected for a path length of 0.6 cm) and a wavelength of 405 nm for 2 min.

For α - and β -esterase assay, 20 μ L of sodium α -naphthyl acetate (1-NA) solution (100 μ L of 30 mM 1-NA in acetone in 10 mL of 0.02 M phosphate buffer, pH 7.2) were added to the first duplicate, and 200 μ L of sodium β -naphthyl acetate (2-NA) solution (100 μ L of 30 mM 2-NA in acetone in 10 mL of 0.02 M phosphate buffer, pH 7.2) were added to the second duplicate. Both duplicates were incubated for 30 min at 25°C to allow the reaction to proceed. Then, 50 μ L of

the fast blue dye solution (22.5 mg of fast blue in 2.25 mL of distilled water plus 5.25 mL of 5% sodium lauryl sulfate in sodium phosphate buffer 0.1 M, pH 7.0) was added to each well to stop the reaction. The control duplicates contained 20 μ L distilled water, 200 μ L 1-NA or 2-NA solution, and 50 μ L dye. The enzyme activity was measured at 570 nm at a fixed point. The optical densities of the individual samples were compared to the standard concentration curves of 1-naphthol and 2-naphthol. Results are reported in nmol of product formed/min/mg of protein. This assay measures the activity of esterases using 1-NA and 2-NA as substrates.

For the glutathione S-transferase (GST) assay, 200 μ L of the GSH/CDNB working solution (10 mM reduced glutathione in 0.1 M phosphate buffer, pH 6.5, and 63 mM chlorodinitrobenzene diluted in methanol) was added to each duplicate. Two control wells were prepared with 10 μ L of distilled water and 200 μ L of GSH/CDNB working solution. Enzyme kinetics were measured at 340 nm for 5 min. GST activity per individual was reported in mmol of conjugated CDBN/min/mg of protein using an extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$, corrected for the path length of the solution in the well. This assay measures enzyme activity with the use of GSH as a substrate and conjugation of chlorodinitrobenzene.

For the cytochrome P₄₅₀ assay, 80 μ L of 0.0625 M pH 7.2 potassium phosphate buffer were added to each duplicate, followed by 200 μ L of TMB solution (0.01 g of 3,3',5,5'-tetramethylbenzidine diluted in 5 mL of methanol and mixed with 15 μ L of 0.25 M sodium acetate buffer, pH 5.0), and 25 μ L of the 5% hydrogen peroxide substrate. The reaction was allowed to proceed for 2 h at 25°C and the absorbance was read at a fixed point at 650 nm. The optical density of each reaction was compared to the standard curve of known concentrations of rabbit cytochrome P₄₅₀ 2B4. The results are reported in pmol of cytochrome P₄₅₀/mg protein.

For protein content determination, the concentrated dye-binding solution was diluted 1:4 with distilled water (Bradford, 1976). Then, 300 μ L of this solution was added to the crude

homogenate. Two blanks were prepared through the mixing of 10 μ L distilled water and 300 μ L diluted dye-binding solution. The absorbance was read at 570 nm after 5 min at 25°C temperature. Protein values were calculated for each sample and compared to a standard curve of known concentrations of bovine serum albumin. These values were used to convert the enzyme values into absolute units.

Palynological analysis

500 grams of pollen comb were collected from the selected colonies in each apiary. All pollen was carefully removed from the combs with a scoop and then pooled. One gram of this pool was processed with the use of Erdtman's acetolysis method (Erdtman, 1960). After centrifugation, the supernatant was removed, and the pellet was carefully mixed with glycerinated gelatin. A drop of this mixture was then placed on a glass slide and examined at 1000x magnification using a bright-field microscope (Axio Lab Model, Carl-Zeiss, Inc.). Pollen grains were identified with the use of various taxonomic keys (Palacios-Chávez & Ludlow-Wiechers, 1991; Roubik & Moreno, 1992; Cuadriello-Aguilar et al., 1993). In cases where a pollen grain could not be identified, a morphotype label was assigned. Six hundred pollen grains were counted per sample, and the percentage contribution of each pollen type was calculated.

Statistical analysis

Since none of the honey samples contained detectable pesticide residues, they were excluded from the statistical analysis. The percentage contribution of each pollen type was determined. Differences in pollen composition among samples were investigated with the use of principal component analysis in R with the stats v 4.3.1 package in R v 4.3.1 (R Core Team, 2020). Mortality data were analyzed with the use of survival analyses in R with the survival package v. 3.5-5 (Therneau, 2023), and differences among apiaries and formulations were estimated through the comparison of confidence intervals. The mean enzyme activity

among apiaries was compared with the use of analysis of variance (ANOVA) in R using the base package. To explore correlations among LT_{50} , enzyme activity, and pollen composition, a canonical correlation analysis was performed with the use of the CCA package (González & Déjean, 2021) with the CCP package for significance testing (Menzel, 2012). Additionally, principal component analysis was performed to compare the results with the principal component analysis based solely on pollen composition.

RESULTS

Pesticide residue analysis

No pesticide residues were detected above the method's detection limits in our samples. This finding suggests an absence of recent or direct pesticide exposure.

Acute LT_{50} estimation

At the applied dose, the cypermethrin formulation exhibited the lowest toxicity among bees from all apiaries, whereas the malathion formulation was the most toxic. Overall, foragers from the Huixtla apiary were the most susceptible to most formulations (Tab. 1).

Enzyme assays

Enzyme activity varied significantly among foragers. Those from the Tapachula apiary exhibited the lowest AChE levels, whereas those from Villa Comaltitlan showed the highest levels (Tab. 2). Overall, no consistent pattern of enzyme activity could be associated with the apiary of origin.

Pollen analysis

Thirteen pollen samples were analyzed, and sixty-four pollen morphotypes were identified: 17 at the family level, 15 at the genus level, and 32 at the species level (Supplementary Material 2). No pollen type was consistently detected across all samples, and twenty-seven pollen types occurred only once across all samples. On the other hand, Asteraceae sp. 1 was observed in twelve out of thirteen samples and had the highest average ($\pm$ SE) proportion of pollen across samples: 10.2 ± 1.8 . No pollen grain from the main crops surrounding the apiaries was found in the samples (Supplementary Material 2), which confirms the findings in the pesticide residue analysis. The sample from Frontera Hidalgo had the highest number of pollen types ($n=21$), whereas Arriaga had the lowest ($n=7$). Fig. 2 shows the principal component analysis based on pollen composition for each apiary.

Table 1.

Lethal Time 50 (LT_{50}) for each apiary, with 95% lower and upper limits in parentheses

Site	Imidacloprid	Malathion	Cypermethrin	Control
Acapetahua	8.75 (7.5, ND) - 18	4.5 (3.5, 7) - 28	ND (ND, ND) - 0	ND (ND, ND) - 0
Arriaga	ND (ND, ND) - 9	6.5 (4, 8) - 30	ND (ND, ND) - 0	ND (ND, ND) - 0
Frontera Hidalgo	9.25 (7.5, ND) - 16	4.5 (3.5, 5) - 30	ND (ND, ND) - 2	ND (ND, ND) - 0
Huehuetan	ND (ND, ND) - 9	5.5 (5, 7.5) - 29	ND (ND, ND) - 1	ND (ND, ND) - 0
Huixtla	4.5 (4.5, 5.5) - 30	4 (3.5, 4.5) - 30	ND (ND, ND) - 2	ND (ND, ND) - 0
Mapastepec	ND (ND, ND) - 3	6.75 (5, 11) - 25	ND (ND, ND) - 1	ND (ND, ND) - 0
Metapa	7.5 (.65, 11.5) - 23	6.5 (4.5, 9.5) - 23	ND (ND, ND) - 2	ND (ND, ND) - 0
Pijijiapan	ND (ND, ND) - 8	4.5 (4, 7.5) - 27	ND (ND, ND) - 1	ND (ND, ND) - 0
Suchiate	8.5 (5.5, ND) - 19	4 (3, 5.5) - 29	ND (ND, ND) - 2	ND (ND, ND) - 0
Tapachula	ND (2, 8.5) - 7	5.5 (4, 6) - 30	ND (ND, ND) - 0	ND (ND, ND) - 0
Tonala	ND (4.5, 6) - 9	6 (4.5, 9) - 24	ND (ND, ND) - 0	ND (ND, ND) - 0
Tuxtla Chico	ND (4.5, NA) - 6	5.5 (4.5, 5.5) - 30	ND (ND, ND) - 1	ND (ND, ND) - 0
Villa Comaltitlan	6.25 (6, 9.5) - 24	5.75 (4.5, 8.5) - 30	ND (ND, ND) - 5	ND (ND, ND) - 0

The number following the hyphen indicates mortality at 12 hours post-exposure (number 30 represents 100% mortality). ND: Insufficient data for estimation.

Table 2.

Mean enzyme activity ($\pm$ standard error) for each apiary

Sites	AChE	Glutathion S-transferases	Cytochromes P ₄₅₀	pNPA- esterases	α -esterases	β -esterases
Acapetahua	61.441 $\pm$ 3.513 ^{ab}	1.049 $\pm$ 0.036 ^{cde}	0.007 $\pm$ 0.001 ^b	0.546 $\pm$ 0.039 ^{bc}	11.757 $\pm$ 0.74 ^{bcd}	5.323 $\pm$ 0.883 ^{bcd}
Arriaga	42.822 $\pm$ 5.077 ^{bcd}	0.678 $\pm$ 0.103 ^{de}	0.008 $\pm$ 0.002 ^b	0.278 $\pm$ 0.083 ^c	13.136 $\pm$ 1.95 ^{abc}	2.579 $\pm$ 0.315 ^{cdef}
Frontera Hidalgo	37.13 $\pm$ 2.873 ^{cde}	1.016 $\pm$ 0.073 ^{cde}	0.007 $\pm$ 0.001 ^b	0.555 $\pm$ 0.043 ^{bc}	15.49 $\pm$ 1.643 ^{ab}	4.69 $\pm$ 0.674 ^{bcde}
Huehuetan	52.042 $\pm$ 3.4 ^{abc}	2.266 $\pm$ 0.225 ^a	0.021 $\pm$ 0.005 ^a	1.033 $\pm$ 0.125 ^a	3.446 $\pm$ 1.123 ^{cd}	0.555 $\pm$ 0.093 ^f
Huixtla	45.6 $\pm$ 3.005 ^{bcd}	0.656 $\pm$ 0.069 ^e	0.008 $\pm$ 0.001 ^b	0.496 $\pm$ 0.031 ^{bc}	3.274 $\pm$ 1.001 ^d	1.634 $\pm$ 0.391 ^{ef}
Mapastepec	53.531 $\pm$ 4.709 ^{abc}	0.941 $\pm$ 0.1 ^{cde}	0.008 $\pm$ 0.003 ^b	0.717 $\pm$ 0.063 ^{ab}	12.86 $\pm$ 1.499 ^{abcd}	2.476 $\pm$ 0.494 ^{cdef}
Metapa	45.202 $\pm$ 1.986 ^{bcd}	0.764 $\pm$ 0.084 ^{de}	0.004 $\pm$ 0.001 ^b	0.574 $\pm$ 0.033 ^{bc}	12.018 $\pm$ 1.634 ^{abcd}	2.797 $\pm$ 0.467 ^{bcdef}
Pijijiapan	28.735 $\pm$ 4.618 ^{de}	1.442 $\pm$ 0.127 ^{bc}	0.002 $\pm$ 0.001 ^b	0.469 $\pm$ 0.031 ^{bc}	20.58 $\pm$ 1.368 ^{ab}	6.366 $\pm$ 0.641 ^b
Suchiate	59.649 $\pm$ 2.483 ^{ab}	0.548 $\pm$ 0.086 ^e	0.006 $\pm$ 0.002 ^b	0.538 $\pm$ 0.088 ^{bc}	15.575 $\pm$ 2.367 ^{ab}	11.582 $\pm$ 1.4 ^a
Tapachula	18.531 $\pm$ 4.196 ^e	1.193 $\pm$ 0.091 ^{cd}	0.00036 $\pm$ 0.00012 ^b	0.47 $\pm$ 0.059 ^{bc}	12.909 $\pm$ 2.714 ^{abcd}	3.136 $\pm$ 0.335 ^{bcdef}
Tonalá	38.829 $\pm$ 6.384 ^{cde}	1.905 $\pm$ 0.071 ^{ab}	0.01 $\pm$ 0.003 ^{ab}	0.623 $\pm$ 0.033 ^{bc}	21.194 $\pm$ 4.398 ^a	6.035 $\pm$ 1.149 ^{bc}
Tuxtla Chico	45.483 $\pm$ 10.855 ^{bcd}	0.82 $\pm$ 0.131 ^{de}	0.009 $\pm$ 0.002 ^b	0.371 $\pm$ 0.158 ^{bc}	18.794 $\pm$ 1.327 ^{ab}	4.024 $\pm$ 1.125 ^{bcdef}
Villa Comaltitlan	66.853 $\pm$ 2.828 ^a	1.002 $\pm$ 0.123 ^{cde}	0.005 $\pm$ 0.002 ^b	0.365 $\pm$ 0.049 ^{bc}	4.34 $\pm$ 0.74 ^{cd}	2.194 $\pm$ 0.44 ^{def}

Different letters indicate statistically significant differences among apiaries.

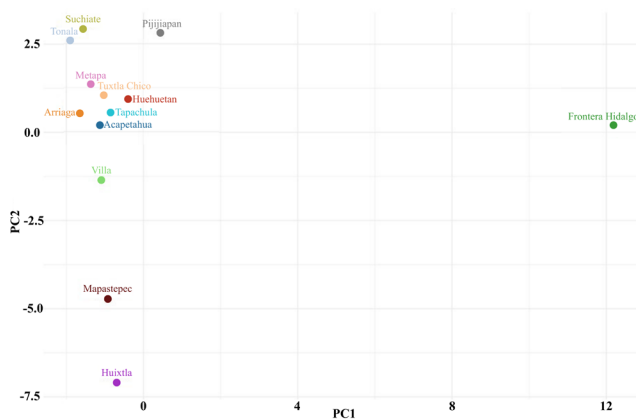
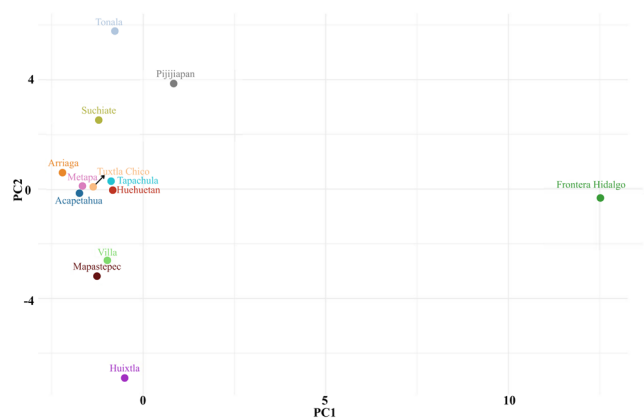


Fig. 2. Principal component analysis based on pollen data only.

Does LT₅₀ correlate with enzymatic activity and pollen diversity?

Canonical correlation analysis revealed a high correlation between enzyme activity and the LT₅₀ data ($r=0.81$), but this correlation was not significant (Wilks' Lambda=0.19, chi-squared =12.43, $p=0.64$). Moreover, no clear relationship was observed among pollen composition, LT₅₀, and enzyme activity as shown by principal component analysis (Fig. 3).

Fig. 3. Principal components analysis based on pollen, LT₅₀ and enzyme data.

DISCUSSION

In this study, we investigated the susceptibility to insecticide formulations and the activity of detoxifying enzymes in foraging honey bees from several locations and whether these variables could be related to the presence of pesticide residues and the pollen they collected. As expected, susceptibility and enzyme expression differed among foragers. Analyses showed that foragers did not collect pollen from the crops during the sampling period, which is consistent

with the pesticide chromatographic analysis. This explains why enzyme expression could not be associated with pollen diversity. Moreover, other potential pesticide sources, including surface water, fallen fruits, or guttation droplets likely contributed little to none, given the residue results. In fact, pollen diversity is an important factor in the diet of bees and potentially a trigger for detoxifying enzyme expression, especially if pollen comes from crops under pest or disease control, but it was not the case here. Moreover, this relationship may be influenced by other such factors as the presence of pathogens and climate change and not pesticide residues or the composition of secondary metabolites in the pollen sources available in the landscape.

According to the principal component analysis, pollen samples from Mapastepec, Huixtla, and Frontera Hidalgo exhibited distinct composition and could not be clearly assigned to any cluster, possibly because the dominant vegetation at these locations consisted of different grass species. These findings align with previous research highlighting the importance of these plant families as food sources for *Apis mellifera* (Ramírez-Arriaga et al., 2011; Córdova-Córdova et al., 2013; López-Roblero et al., 2021). No pollen from such agricultural crops of interest in the region as mango, soybean and rambutan, was detected, possibly because the flowering period of these crops generally occurs between November and March (Reyes-Moreno, 2017; Pérez-Barraza et al., 2018), so bees had to rely on other food sources. This finding suggests that bees rely on wild plant species when other options, particularly crops, are scarce, which may reduce their direct exposure to insecticides (Requier et al., 2015). Variation in pesticide exposure due to crop phenology may provide bees with recovery windows, during which appropriate apicultural management practices could enhance colony survival. Therefore, our results do not support the initial hypothesis that enzyme activity and LT_{50} values are driven by the presence of dominant crops perceived as continuous sources of insecticide exposure.

Regarding the LT_{50} bioassays, the malathion formulation was found to be more toxic to bees

than the imidacloprid and cypermethrin ones, reaching 100% mortality in some groups of foragers from six out of the thirteen apiaries within the first 12 h. Additionally, we observed mortality percentages over 65% during the exposure period in all exposed groups (except controls), confirming that the malathion formulation was highly toxic to bees across all evaluated sites. These findings are consistent with those of previous studies on *Plebeia emerina* and *Tetragonisca fiebrigi*, which demonstrated that malathion is even more lethal than other such insecticide classes as neonicotinoids (Villa et al., 2000; Padilha et al., 2020). The most susceptible foragers, with the highest lethality in the shortest time due to malathion and imidacloprid exposure, were from Huixtla, with an LT_{50} of only four hours, reaching 100% mortality in less than 12 h. In contrast, foragers from Mapastepec showed a lower mortality rate and a higher LT_{50} . The variation in susceptibility to these formulations in exposure tests could be linked to colony management, bee nutrition, and the availability of resources in each landscape (Blacquiere et al., 2012; Requier et al., 2015). However, these factors require further investigation.

Foragers from the apiary in Villa Comaltitlan exhibited the highest AChE inhibition levels, whereas individuals from Tapachula, Pijijiapan, and Tonalá showed the lowest sensitivities, suggesting a lower susceptibility to such insecticides as carbamates and organophosphates (Penilla et al., 1998). This could partially explain why foragers from Tonalá exhibited lower mortality in malathion contact exposure tests. However, in the case of Tapachula, the influence of carbamates may be stronger than that of organophosphates, given the high bee mortality caused by the malathion formulation at this apiary (Solís-Santoyo et al., 2022). The levels of α - and β -esterases and GST were lower than those reported previously. Organophosphates and pyrethroids are responsible for the low expression of GST. These findings could explain the results obtained in the exposure tests, where bees were more susceptible to the malathion formulation than

to the cypermethrin one (Penilla et al., 1998; Carvalho et al., 2013).

Formulation susceptibility and enzymatic expression observed in this study might have resulted from differences in pollen diets. Di Pasquale et al. (2013, 2016) reported that the diversity of pollen types in the diet directly affects honey bee health, longevity and the expression of certain enzymes. Some pollen types were even found to be inadequate for proper development. Similar results were described by Li et al. (2025), who concluded that pollen diets can be optimized to better support honey bee health. Thus, the complexity of the pollen diet may mask the effects of pesticide exposure, meaning that a seemingly high susceptibility to a particular pesticide could result from complex interactions among diet and pesticide exposure. This finding highlights the relevance of carrying out studies in which enzymatic activity, pesticide exposure and pollen diversity are included as potential bias of pesticide susceptibility.

Although we did not find a clear relationship among the factors evaluated in this study (LT_{50} , detoxifying enzyme expression, and pollen diversity), the lack of detectable pesticide residues and the absence of crop pollen in the samples suggests that crop phenology should be considered in this type of studies. Honey bee colony health and pesticide susceptibility may be the result of the quality of the resources that foragers collect. Other potential sources of pesticide exposure, such as home or public gardens, are unlikely to have influenced our results, since the landscape surrounding the apiaries was largely dominated by crops and grasslands, and any pesticide input from these sources would likely be negligible and remain undetected. We also cannot rule out the possibility that foragers are exposed to pesticides by collecting resources such as water and resins, but since we did not detect residues in our samples, we can discard this possibility. However, the detection threshold of the chromatographic method does not rule out low-level exposure, which could still result in sublethal, chronic effects. Moreover, exposure to other insecticides, herbicides, fungicides,

antibiotics, or additional compounds not included in the chromatographic analysis cannot be excluded and could influence our enzymatic and LT_{50} results. Since most pesticides in rural areas originate from crop pest and disease control practices, we consider chromatographic and melissopalynological analyses to be complementary, and potentially exchangeable. Finally, enzyme expression and LT_{50} would be attributable to exposure to xenobiotics other than pesticide formulations, and potentially to the quality of the pollen they collected. Further studies are required to confirm this hypothesis, though.

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Information on the residues included in the pesticide analysis

Determination	Limit of Quantification (LOQ)	Unit	Method
Chlorpyrifos-ethyl	0.005	(mg/kg)	Internal method INS-SM/NE-01
Fenvalerate II	0.001	(mg/kg)	Internal method INS-SM/NE-01
<i>cis</i> -Permethrin	0.003	(mg/kg)	Internal method INS-SM/NE-01
Fenvalerate I	0.004	(mg/kg)	Internal method INS-SM/NE-01
<i>trans</i> -Permethrin	0.004	(mg/kg)	Internal method INS-SM/NE-01
2,4'-D 2-ethylhexyl ester	0.005	(mg/kg)	Internal method INS-SM/NE-01
2,4'-D-isopropyl ester	0.005	(mg/kg)	Internal method INS-SM/NE-01
2,4'-DDT	0.005	(mg/kg)	Internal method INS-SM/NE-01
2-Phenylphenol	0.005	(mg/kg)	Internal method INS-SM/NE-01
Acrinathrin	0.005	(mg/kg)	Internal method INS-SM/NE-01
Aldrin	0.005	(mg/kg)	Internal method INS-SM/NE-01
Azoxystrobin	0.005	(mg/kg)	Internal method INS-SM/NE-01
Bifenthrin	0.005	(mg/kg)	Internal method INS-SM/NE-01
Boscalid	0.005	(mg/kg)	Internal method INS-SM/NE-01
Bromopropylate	0.005	(mg/kg)	Internal method INS-SM/NE-01
Carfentrazone-ethyl	0.005	(mg/kg)	Internal method INS-SM/NE-01
Gamma-cyhalothrin	0.005	(mg/kg)	Internal method INS-SM/NE-01
Lambda-cyhalothrin	0.005	(mg/kg)	Internal method INS-SM/NE-01
Chlorpyrifos-methyl	0.005	(mg/kg)	Internal method INS-SM/NE-01
Clothianidin	0.005	(mg/kg)	Internal method INS-SM/NE-01
Coumaphos	0.005	(mg/kg)	Internal method INS-SM/NE-01
Deltamethrin	0.005	(mg/kg)	Internal method INS-SM/NE-01
Diazinon	0.005	(mg/kg)	Internal method INS-SM/NE-01
Dichlofenthion	0.005	(mg/kg)	Internal method INS-SM/NE-01
Dicofol	0.005	(mg/kg)	Internal method INS-SM/NE-01
Dieldrin	0.005	(mg/kg)	Internal method INS-SM/NE-01
Endosulfan I	0.005	(mg/kg)	Internal method INS-SM/NE-01
Endosulfan II	0.005	(mg/kg)	Internal method INS-SM/NE-01
Spiromesifen	0.005	(mg/kg)	Internal method INS-SM/NE-01
Ethion	0.005	(mg/kg)	Internal method INS-SM/NE-01
Ethoxazole	0.005	(mg/kg)	Internal method INS-SM/NE-01
Fenclorophos	0.005	(mg/kg)	Internal method INS-SM/NE-01
Fenitrothion	0.005	(mg/kg)	Internal method INS-SM/NE-01
Fenthion	0.005	(mg/kg)	Internal method INS-SM/NE-01
Phentoate	0.004	(mg/kg)	Internal method INS-SM/NE-01
Fenvalerate	0.001	(mg/kg)	Internal method INS-SM/NE-01
Fipronil-sulfone	0.005	(mg/kg)	Internal method INS-SM/NE-01
Fipronil-sulfide	0.005	(mg/kg)	Internal method INS-SM/NE-01
Flubendiamide	0.005	(mg/kg)	Internal method INS-SM/NE-01
Phonofos	0.005	(mg/kg)	Internal method INS-SM/NE-01
Phorate	0.005	(mg/kg)	Internal method INS-SM/NE-01
Formothion	0.049	(mg/kg)	Internal method INS-SM/NE-01
Phthalimide	0.005	(mg/kg)	Internal method INS-SM/NE-01

HCH (α -isomer)	0.005	(mg/kg)	Internal method INS-SM/NE-01
Heptachlor epoxide (isomer B)	0.005	(mg/kg)	Internal method INS-SM/NE-01
Hexachlorobenzene	0.005	(mg/kg)	Internal method INS-SM/NE-01
Imidacloprid	0.005	(mg/kg)	Internal method INS-SM/NE-01
Malathion	0.005	(mg/kg)	Internal method INS-SM/NE-01
Oxyfluorfen	0.005	(mg/kg)	Internal method INS-SM/NE-01
Paclobutrazole	0.005	(mg/kg)	Internal method INS-SM/NE-01
Pentachloroaniline	0.005	(mg/kg)	Internal method INS-SM/NE-01
Pentachloroanisole	0.005	(mg/kg)	Internal method INS-SM/NE-01
Pentachlorophenol	0.005	(mg/kg)	Internal method INS-SM/NE-01
Perthane	0.010	(mg/kg)	Internal method INS-SM/NE-01
Pyridaben	0.010	(mg/kg)	Internal method INS-SM/NE-01
Pyridafenthion	0.005	(mg/kg)	Internal method INS-SM/NE-01
Pyrimethanil	0.005	(mg/kg)	Internal method INS-SM/NE-01
Pirimiphos-methyl	0.005	(mg/kg)	Internal method INS-SM/NE-01
Pyriproxyfen	0.003	(mg/kg)	Internal method INS-SM/NE-01
Profenofos	0.004	(mg/kg)	Internal method INS-SM/NE-01
Propetamphos	0.005	(mg/kg)	Internal method INS-SM/NE-01
Quinoxifen	0.005	(mg/kg)	Internal method INS-SM/NE-01
Sulfallate	0.005	(mg/kg)	Internal method INS-SM/NE-01
Tebuconazole	0.005	(mg/kg)	Internal method INS-SM/NE-01
Tecnazene	0.005	(mg/kg)	Internal method INS-SM/NE-01
Terbufos	0.005	(mg/kg)	Internal method INS-SM/NE-01
Tetradifon	0.005	(mg/kg)	Internal method INS-SM/NE-01
<i>cis</i> -1,2,3,6- Tetrahydrophthalimide	0.005	(mg/kg)	Internal method INS-SM/NE-01
Triazophos	0.005	(mg/kg)	Internal method INS-SM/NE-01
Trifloxystrobin	0.005	(mg/kg)	Internal method INS-SM/NE-01
Chlorfenapyr	0.010	(mg/kg)	Internal method INS-SM/NE-01
Dichloran	0.010	(mg/kg)	Internal method INS-SM/NE-01
Parathion-ethyl	0.010	(mg/kg)	Internal method INS-SM/NE-01
Pendimethalin	0.010	(mg/kg)	Internal method INS-SM/NE-01
Diphenylamine	0.026	(mg/kg)	Internal method INS-SM/NE-01
Folpet	0.049	(mg/kg)	Internal method INS-SM/NE-01

Supplementary material 2.

Pollen diversity in each location

Family	Species	Tonala	Suchiate	Tuxtla Chico	Metapa	Tapachula	Huehuetan	Villa	Acapetahua	Arriaga	Mapastepec	Huixtla	Pijijapan	Frontera Hidalgo
Aracaceae	<i>Acrocomia aculeata</i>	0	0	0	0	0	0	0	0	0	0	1.2	0	0
Asteraceae	Asteraceae sp. 1	4	6	14.9	14.9	22.2	10.4	11.4	6.7	0	5	5	22	10.5
Asteraceae	Asteraceae sp. 2	0	2	23	23	7.2	11	3.3	8.4	0	0	0	4	0
Asteraceae	Asteraceae sp. 3	0	3	18	18	3.6	12.2	0	0	0	0	0	0	0
Asteraceae	Asteraceae sp. 4	0	2	4.3	4.3	0	0	0	0	0	0	0	0	0
Asteraceae	Asteraceae sp. 5	0	0	5.9	0	0	0	0	0	0	0	0	0	0
Asteraceae	Asteraceae sp. 6	0	0	13	0	0	0	0	0	0	0	0	0	0
Boraginaceae	<i>Cordia alliodora</i>	13	0	0	0	0	1.7	0	3.3	11	0	0	5.1	0
Boraginaceae	<i>Cordia</i> sp.	10	0	0	0	0	0	0	1.8	0	0	0	0	3
Boraginaceae	<i>Cordia bicolor</i>	0	0	0	0	0	0	0	0	0	0	0	0	1.1
Burseraceae	<i>Bursera simarouba</i>	0	0	0	0	0	0	0	0	0	0	10	34.2	3.7
Burseraceae	Burseraceae sp. 1	0	0	0	0	0	0	0	0	0	0	0	5.3	7
Clethraceae	<i>Clethra mexicana</i>	5	0	0	0	0	0	0	0	0	0	0	0	0
Convolvulaceae	<i>Ipomoea purpurea</i>	0	0	0	0	0	0	0	0	0	4.3	9	0	0
Costaceae	<i>Costus</i> sp.	0	0	0	0	0	0	0	0	0	0	0	0	6.3
Cupresaceae	<i>Taxodium mucronatum</i>	0	0	0	0	0	0	0	0	0	1.4	0	0	0
Euphorbiaceae	<i>Hevea brasiliensis</i>	5	7	0	0	0	0	0	0	0	0	0	0	0
Euphorbiaceae	<i>Jatropha curcas</i>	1	0	0	0	6.7	0.3	9.9	0	0	0	0	0	0
Euphorbiaceae	<i>Cnidoscolus aconitifolius</i>	0	0	0	0	0	0	0	0	0	2.5	0	0	0
Fabaceae	<i>Lonchocarpus</i> sp.	5	4	0	0	0	0	0	0	21	0	0	0	0
Fabaceae	<i>Mimosa pigra</i>	6	5	0	0	0	9	0	0	0	0	0	2.2	0
Fabaceae	<i>Mimosa pudica</i>	8	0	10	0	0	0	3.1	0	0	15.3	13	4.3	4.6
Fabaceae	<i>Acacia mexicana</i>	8	0	0	0	6.6	0	0	13.8	16	2.8	0	0	0
Fabaceae	<i>Acacia collinsi</i>	2	0	0	0	0	0	0	0	15	7.3	1.7	0	0
Fabaceae	<i>Acacia macrantha</i>	3	0	0	0	0	0	0	0	18	0	0	0	0
Fabaceae	Fabaceae	0	0	0	0	7.2	0	0	0	0	0	0	0	0
Fabaceae	<i>Mimosa casta</i>	0	0	0	0	0	7.8	3.4	0	0	6.9	11	0	11
Fabaceae	<i>Tamarindus indica</i>	0	0	0	0	0	0	0	0	0	13.8	0.5	0	0
Fabaceae	<i>Enterolobium cyclorarpum</i>	0	0	0	0	0	0	0	0	0	0	14.6	0	0
Fabaceae	<i>Acacia acantophyla</i>	0	0	0	0	0	0	0	0	0	0	0	0	4.2
Fabaceae	<i>Acacia melanoceras</i>	0	0	0	0	0	0	0	0	0	0	0	0	5.3
Fabaceae	<i>Mimosa</i> sp. 1	0	0	0	0	0	0	0	0	0	0	0	1.8	6.4
Fabaceae	<i>Mimosa</i> sp. 2	0	0	0	0	0	0	0	0	0	0	0	0	2.7

Fabaceae	<i>Acacia</i> sp.	0	0	0	0	0	0	0	0	0	0	0	5.3	0
Heliconiaceae	<i>Heliconia</i> sp.	0	0	0	0	0	0	0	0	0	0	0	0	2.1
Lamiaceae	Lamiaceae	0	0	0	0	0.7	1.1	0	0	0	0	0	0	13
Lamiaceae	<i>Satureja</i> sp.	0	0	0	0	0	0	0	0	0	0	0	0	6.8
Lauraceae	<i>Litsea glaucescens</i>	3	0	0	0	0	0	0	0	0	0	0	0	0
Loranthaceae	Loranthaceae sp. 1	0	0	0	0	0	0	0	0	0	0	0	3.3	0.4
Malpighiaceae	Malpighiaceae sp. 1	7	0	0	0	0	0	5.7	0	0	0	0	0	0
Malpighiaceae	<i>Byrsonima crassifolia</i>	0	0	0	0	0	0	18.6	0	0	30.9	3	0	0
Malvaceae	Malvaceae sp. 1	7	0	0	0	0	0	1.9	0	0	0	0	2.8	0
Malvaceae	<i>Peudobombax ellipticum</i>	1	2.9	0	0	0	0	0	15.4	0	0	0	0	0
Malvaceae	<i>Ceiba pentandra</i>	0	3.4	0	0	0	0	0	0	0	0	0	5.6	0
Malvaceae	<i>Gossypium</i> sp.	0	24	0	5.2	0.5	0.3	0	0	0	0	0	0	0
Melastomataceae	<i>Miconia</i> sp.	0	0	0	0	0	0	13.6	0	0	9	8,5	0	0
Meliaceae	<i>Cedrela odorata</i>	4	0	0	9,9	0	0	0	0	0	0	0	0	0
Myrtaceae	<i>Eugenia</i> sp.	0	0.2	3	0	1.7	4.3	0	1.4	0	0	0	2	2.4
Myrtaceae	Myrtaceae sp. 1	0	0.4	0.9	5.8	0	2.1	21	0	0	0	0	0	0
Nyctaginaceae	Nyctaginaceae sp. 1	0	0	0	0	0	0	0	0	0	0	0	0	4
Papaveraceae	<i>Argemone mexicana</i>	0	0	0	0	0	0	0	0	0	0	9.4	0	0
Passifloraceae	<i>Passiflora edulis</i>	0	0	0	0	0	0	0	0	0	0	7.7	0	0
Pinaceae	<i>Pinus</i> sp.	0	0	0	9.7	0	0	0	0	9.7	0	0	0	0
Poaceae	Poaceae sp. 1	0	33	0	6	35	0.6	7.1	45.2	0	0	0	0	0
Poaceae	<i>Zea mays</i>	0	0	0	0	1.4	0	0,5	4	0	0.8	5.4	0	0
Polygalaceae	<i>Securidaca</i> sp.	0	0	0	0	0	0	0	0	0	0	0	0	3.5
Rubiaceae	Rubiaceae sp. 1	0	0.1	0	0	0	0	0	0	0	0	0	0	0
Rubiaceae	<i>Genipa americana</i>	0	0.2	0	0	0	0	0	0	0	0	0	0	0
Rubiaceae	<i>Borreria</i> sp.	0	0	0	0	0	0	0	0	0	0	0	0	1
Rutaceae	<i>Citrus limonia</i>	4	0	0	0	0	0	0	0	0	0	0	0	0
Rutaceae	<i>Citrus limon</i>	0	0	7	0	0	0	0	0	0	0	0	0	0
Sapindaceae	<i>Serjania mexicana</i>	4	5.7	0	3.1	7.2	39.2	0.5	0	9,3	0	0	0	1
Sapindaceae	Sapindaceae sp. 1	0	1.1	0	0	0	0	0	0	0	0	0	1	0
Sapindaceae	<i>Paullinia</i> sp.	0	0	0	0	0	0	0	0	0	0	0	1.1	0

