Abamectin and Difenoconazole Monitoring in Strawberry Flowers and Pollen Sampled from *Tetragonisca Angustula* (Latreille) (Hymenoptera: Apidae) Hives Located in Crop Vicinities

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Abstract

The increase of agricultural productivity associated with the emergence and the extensive use of agrochemicals is undeniable. However, the harmful effects, were laid aside in the first moment. Strong evidence indicates that the rising use of agrochemicals is one of the factors associated with the decreasing of pollinating insects, such as bees. The present work aims the determination of the insecticide abamectin (ABA) and the fungicide difenoconazole (DIF) in strawberry flowers (*Fragaria* x *ananassa* DUCH.) and pollen sampled from beehives of the stingless bee *Tetragonisca angustula* Latreille (Hymenoptera: Apidae) located nearby strawberry fields. For analysis, QuEChERS method was optimized, and analytical performance and determination of the two agrochemicals were verified using LC-MS/MS considering validation parameters as linearity (0.5 to 1500 μgL^{-1}), precision (> 80%), and accuracy (< 20). Thus, the method was applied in flowers and pollen sampled from field samples from three campaigns. While abamectin was not detected, the systemic fungicide difenoconazole was detected in almost all flowers and pollen samples. The results were then discussed mainly according to difenoconazole application and transport to colonies to estimate a preliminary environmental risk assessment considering exposure rates and toxicity data from the literature.

1. Introduction

In the environment, bees can act as important agents in the maintenance of diversity in vegetal communities and the increase in agricultural productivity (Braga et al. 2012). In Brazil, a study carried out on 75 Brazilian agricultural resources recorded the presence of 250 species of pollinating animals, 87% of which represented by bees (Giannini et al. 2015). Although bees are considered important pollinating agents, their population is recently decreasing in some areas worldwide. This massive disappearance of *Apis mellifera* bees in Europe and the USA is knowing as the Colony Collapse Disorder (CCD) and can be a result of many factors, including changes in temperature, habitat loss and fragmentation, deleterious pathogens, and, in most cases, due to the current pesticides applications in foraging crops (Van Oystaeyen et al. 2020). Besides *A. mellifera*, stingless bee species are also affected by continuous pesticide exposure, affecting the pollination process in nature and agricultural areas.

The stingless bee *Tetragonisca angustula* is broadly distributed in Brazil and its pollination can be considered as efficient in tropical flora (Braga et al. 2012). The pollination of strawberry flowers by the stingless bee *T. angustula* can be observed in studies conducted in the field, helping flowers in the process of cross-fertilization, and increasing the fruit quality; in closed systems, the number of visits can variate according to the number of hives located nearby the flowers and can reach about 17 visits $flower\ hour^{-1}$ in the presence of four hives (Antunes et al. 2007). The honey produced by these bees has greater economic value, as it is considered to have a peculiar flavor and aroma (Oliveira 2013), they are also well known, for adapting well to urban environments, often fix hives in cracks in buildings.

Besides the important role of bees in strawberry pollination, the frequent use of pesticides can directly affect this non-target insect. In Brazil, the region of Bom Repouso, located at Minas Gerais State is a great

strawberry provider for the southeast region and an ideal field study area. In this region, pesticides are commonly used in mostly strawberry crops and include insecticides, herbicides, and fungicides.

Abamectin is a nonsystemic insecticide/acaricide produced by the fermentation of the soil Actinomycete *Streptomyces avermetilis*, showing good effectivity against arthropods (Lasota and Dybas 1990). Is a mixture of 80% Avermectin B_{1a} and 20% of Avermectin B_{1b} (EFSA 2008) and commonly used in Brazil for the foliar spray treatment of a wide range of cultivars as tomato, citrus, soy, and others; for strawberry, the MRL of this commercial product is 0.02 mg kg^{-1} (ANVISA 2021a). In the environment, degradation rate used to be fast and includes process as hydroxylation, oxidation, demethylation, and photolytic cleavage (EFSA 2008).

Difenoconazole is a systemic fungicide that has demonstrated a good performance in the control of the black flower (*Colletotrichum acutatum* Simmonds) in strawberry plants (Domingues et al. 2001). The application of this fungicide is mainly by foliar spray and, in Brazil, the MRL level is 0.5 mg Kg^{-1} (ANVISA 2021b). In the environment, can be considered toxic and dangerous (Leite et al. 2018) and the degradation rate can be about 15.4 days in soils (Zhao et al. 2018) or 6.4 to 8.4 days in some cultivars (Mohapatra 2015) and 6.3 days for strawberry fruits after second application in a greenhouse condition (Sun et al. 2015).

Both pesticides have been pointed as toxic for bees alone or in the mixture, including lethal and sublethal effects (Mussen et al. 2004; Del Sarto et al. 2014; Ajedani 2016; Leite et al. 2018; Iverson et al. 2019; Ferreira et al. 2020; Prado et al. 2020). Considering the exposure through the application of these pesticides in strawberry fields coupled with the registered toxicity for a wide range of bee species can lead to the relevance of an estimated risk assessment for colonies. In this sense, the determination of pesticides in environmental matrices is one of these important steps for the estimation of risk for bees and other non-target biota. For this procedure, analytical procedures must be carried, considering methods with good accuracy and precision. For bee's related matrices, QuEChERS (Quick, Easy, Cheap, Effected, Rugged, and Safe) have been commonly used for those studies with good performance (Prado et al. 2020).

Thus, the discussion about the environmental rate of pesticides and bee's exposure can act as an interesting tool for the risk estimation, once the transport from the field for the hives can be measured. In this sense, the application and monitoring of abamectin and difenoconazole in flowers of strawberry and pollen from *T. angustula* displayed in the vicinities of the crop have proceeded and the discussion about the initial risk estimation is also described in the present work.

2. Materials And Methods

2.1. Reagents and Standards

Pesticides abamectin and difenoconazole with a high purity level (> 96%) were purchased from Sigma Aldrich (São Paulo, Brazil). Other reagents were supplied as follow: acetonitrile (HPLC grade) from Tedia (Fairfield, USA); sodium acetate and magnesium sulfate from J.T Baker (Xalostoc, Mexico); acetic acid from Synth (São Paulo, Brazil), formic acid from Honeyweel (Steinheim, Germany) and sodium chloride from Synth (São Paulo, Brazil); the dispersive salts primary and secondary amine (PSA) and C18 were purchased from Agilent Technologies (USA).

2.2. Samples for method development

The samples of strawberry flowers and pollen from *T. angustula* hives used for method development and validation were collected in a region in which the pesticides abamectin and difenoconazole were not applied, located at the Center for Water Resources and Environmental Studies (CRHEA - EESC) in São Carlos, Brazil. The pollen from *T. angustula* was removed from wax containers, homogenized, and stored at -20^{o}C until analysis, as well as the flowers.

2.3. Extraction

The extraction of abamectin and difenoconazole in strawberry flowers and pollen was done by the QuEChERS method, involving the use of organic solvent, extraction salts, and clean-up agents. The QuEChERS method was tested in three compositions: traditional, using magnesium sulfate and sodium chloride; acetate, using magnesium sulfate and sodium acetate; and finally, citrate, using magnesium sulfate, sodium chloride, and sodium citrate salts (Prestes et al. 2011). Three clean-up agents were also tested: magnesium sulfate, PSA, C18, and activated carbon in different amounts and combinations.

For the final extraction method, the following procedure was applied: 0.5 g of the matrix were placed in a 50 mL polypropylene tube and macerated with a glass stick; then, 10 mL of Acetonitrile (MeCN) was added in tubes; after, 0.40 g of magnesium sulfate and 0.10 g of sodium chloride were added and placed in the vortex for 1 min. The mixture was then centrifuged at 4400 rpm by 15 min and the supernatant was removed and placed in a new test tube containing clean-up salts: 150 mg magnesium sulfate and 50 mg PSA for the strawberry flower and 150 mg magnesium sulfate, 50 mg PSA, and 50 mg C18 for pollen. The mixture was vortexed for 1 min and then centrifuged by 5 min. All supernatant was removed and dried under N_2 moderated flow.

Finally, 1 mL of the mobile phase was used to transfer the final extract to a vial and was filtered

with regenerated cellulose (RC) filter 0.45 mm.

2.4. LC-MS/MS analysis and validation

The LC-MS/MS method parameters are described in Prado et al. (2020). A liquid chromatograph (LC-Agilent 1200) coupled with a mass spectrometer triple quadrupole (MS/MS QTRAP 3200- SCIEX), with electrospray ionization (ESI) was used for analysis. The column used was a C8 (250 × 4.6 mm, 5 µm, Macherey-Nagel) with a temperature of 25°C using water (A) and acetonitrile (B) acidified with formic acid 0.1%, operating in a gradient mode: 40% (A) for 0.5 min; increasing to 100% in 8 min and kept on this

percentage until 12 min; returning to the initial conditions until 12.5 min and kept in this percentage until 18 min. The runtime was 15 min and the retention time of involved analytes was: 9.4 min for difenoconazole and 10.2 min for abamectin.

The method validation was carried according to national and international guidelines (ANVISA 2017; MAPA 2011; SANTE 2017) considering the calibration curves in solvent and matrix (spiked before and after extraction), and the following parameters were evaluated: linearity (the concentration of the analytes varied from 0.5–1000 μg L^{-1}, containing 10 levels of concentrations in triplicate (n = 3), limits of detection (LOD) and quantification (LOQ), accuracy and precision (3 spiked levels and inter and intra-day injections) and matrix effect.

2.5. Sampling design and method application

Samples were collected in the city of Bom Repouso - MG, Brazil (22°27'57.01"S 46°08'57.04"W) in two strawberry fields named Area 1 and Area 2 (Fig. 1), located about 800 m of distance from each other. Three hives (n = 3) of *T. angustula* were placed nearby (100 m) of each strawberry field as depicted in Fig. 1. Strawberry flowers from 5 sampling points inside the fields P1 to P5 for Area 1 and P6 to P10 for Area 2, as well as the pollen from the respective hives H1 to H5, were sampled in three campaigns: November / 2018, January / 2019, and March / 2019. In this sense, a total of 30 samples of flowers and 15 samples of pollen were analyzed. The number of pollen analyses was lower than expected once in Area 1, we had only two healthy hives to collect the pollen during sampling campaigns.

Figure 1 Location of sampling areas (1 and 2) at the city of Bom Repouso, Minas Gerais State, Brazil, and general description of strawberry flowers sampling points in Area 1: P1 to P5 with the proximity of *T. angustula* hives (H1 and H2); and Area 2: P6 to P10 with the hives H3 to H5. *T. angustula* hives: structure (a) and location (b); strawberry field at Bom Repouso (c). Map source: adapted from IGAM (2020).

2.6. Risk Assessment

The procedure for the initial Environmental Risk Assessment related to the exposure of bees to pesticides has followed the protocol described by the Environmental Risk Assessment Manual for Pesticides for Bees from the American Environmental Agency (USEPA 2014; IBAMA 2017). In this protocol, the steps to characterize the exposure are included and predicted using the program BeeRex, followed by the refining step, where data from the determination of the pesticides in pollen samples were included. The toxicity endpoints (acute and chronic exposure) of abamectin and difenoconazole for bees is already described in the literature for *Apis mellifera* (EFSA 2011; SHARDA BRASIL 2019) and used to estimate the risk for *T. angustula* in this work. For the risk quotient (RQ), the level of concern (LOC) used for this calculation were 0.4 and 1 for acute and chronic exposure, respectively. In this sense, these calculations can help to assess the risk associated with the method of application of the pesticide in the strawberry crop, as well as determining the exposure of bees from their average consumption from environmental samples in all bee's life stages.

3. Results And Discussion

3.1. Method performance

The developed method for the analysis of abamectin and difenoconazole using LC-MS/MS showed good selectivity, once no interfering compounds were observed in the same retention time of analytes. Linearity was evaluated using analytical curves in the matrices (strawberry flower and pollen) with good response in the concentration range of 0.5–1,00 μg L^{-1}. Peak areas were used as responses, and the method was shown to be linear and determination coefficients (R^2) were greater than 0.98 for both pesticides, with deviation for each concentration ≤ 20%. The parameters of the analytical curves, the detection, and the quantification limits for the method and system are shown in Table 1. The matrix effect for abamectin and difenoconazole in the flower samples was less than 100%, indicating that there was a suppression of ionization. (Matuszewski et al. 2003; 2006). As for the pollen samples, both analytes showed values close to 100%, indicating that the response in the solvent and the matrix we're the same, and no effect was observed.

In all cases, the threshold (± 0.1) established by SANTE guidelines (SANTE/11813/ 2017) was achieved and ISO. The confirmation of the analytical parameters was carried out through the acquisition of the MS / MS transitions and a comparison of their intensity proportions, taking into account that the relative proportion between the transitions must be ≤ 30%. The selectivity of the proposed method was tested by the extraction and analysis of pure extracts from strawberry flowers and pollen-free from pesticides, to establish the absence of signs at the time of elution to target the pesticides and thus demonstrating that neither the matrix nor the compounds present in the sample gave false positives.

Table 1
Analytical parameters for LC-MS/MS analysis of abamectin (ABA) and difenoconazole (DIF) in strawberry flower and pollen.

Matrix		Linear equation	(r^2)	Linearity[a]	ME (%)	LC-MS/MS[a]		Method[b]	
						LOD	**LOQ**	**LOD**	**LOQ**
Flower	**ABA**	y = 73.66x-978.3	0.993	0.5 to 1000	80.48	0.15	0.50	0.30	1.00
	DIF	y = 5447.6x + 41872	0.995	0.5 to 1000	83.21	0.15	0.50	0.30	1.00
Pollen	**ABA**	y = 95.35x + 332.4	0.994	0.5 to 1000	100.0	0.15	0.50	0.30	1.00
	DIF	y = 6403.4x-147626	0.994	0.5 to 1000	97.80	0.15	0.50	0.30	1.00

ME = matrix effect

[a]$\mu g\ L^{-1}$

[b]$ng\ g^{-1}$

The optimization of the QuEChERS method (SM1) was done for the three versions of the method: original, acetate, and citrate, and the clean-up optimization was done using different salts, such as magnesium sulfate, PSA, C18, and activated carbon. Of the tested methods, for the flower matrix, the best performance was observed for the original QuEChERS method, which used as extraction salts 0.40 g of $MgSO_4$ + 0.10g NaCl and as clean-up salts 150 mg $MgSO_4$ + 50 mg PSA, in which recoveries were 99.7% for difenoconazole and 61.1% for abamectin. For the pollen matrix, the best performance was also observed for the original QuEChERS method, while for the clean-up, the “original + C18” method was the one that presented the best performance and used 150 mg $MgSO_4$ + 50 mg PSA + 50 mg C18, and recoveries were 109.1% for difenoconazole and 108.1% for abamectin and adequate RSD values were achieved (0.2-7%).

After selecting the method for extracting the analytes, the precision and accuracy for the two matrices were evaluated using three concentration levels: low (5 $\mu g\ L^{-1}$), medium (100 $\mu g\ L^{-1}$), and high (750 $\mu g\ L^{-1}$), and the results obtained are shown in Table 2.

Table 2
Accuracy and precision of the validated method for abamectin and difenoconazole determination in strawberry flower and pollen using the QuEChERS extraction method and LC-MS/MS analysis.

Matrix		Level	Accuracy (%)[a]	Precision (RSD %)	
				Intra-day[a]	**Inter-day[a]**
Flower	**Abamectin**	Low	80.84	3.78	6.19
		Medium	88.15	0.91	3.14
		High	92.82	0.25	0.22
	Difenoconazole	Low	100.99	1.29	1.80
		Medium	108.34	1.11	0.90
		High	103.32	1.09	2.28
Pollen	**Abamectin**	Low	108.71	7.31	6.55
		Medium	90.21	3.90	4.17
		High	90.43	1.14	1.03
	Difenoconazole	Low	96.65	0.72	1.33
		Medium	97.17	0.24	1.41
		High	105.23	0.17	1.11

[a] n = 5

Studies have used the modified QuEChERS method for detecting abamectin and difenoconazole in bee pollen samples. Friedle et al (2021) used the modified QuEChERS method to detect more than 260 pesticides in pollen samples and the method showed LOQ of 3 ng g^{-1} and recovery of 87% for difenoconazole. The maximum and minimum concentrations of difenoconazole detected in the samples were 48 and 1.5 ng g^{-1}, respectively. Other studies have determined difenoconazole in bee pollen samples using the modified QuEChERS method and evaluating different clean-up agents; the best method showed recoveries of 96 and 89% for spiking levels of 5 and 50 µg kg^{-1} of difenoconazole; accuracy less than 20%; LOQ of 5 µg kg^{-1} (Vázquez et al. 2015); Wiest et al (2011) used the citrate QuEChERS method to detect abamectin and other contaminants in pollen. For abamectin, the method presented LOD of 10.2 6 ng g^{-1} and LOQ of 30.6 ng g^{-1} and recoveries in the range of 81–112%, and abamectin was not detected in real samples. For flowers, there is still very little work on these and other pesticides in the literature, but there are some that corroborate with the extraction technique for identification and quantification in strawberries (Oshita et al. 2014) and validation of pesticides in processed fruit by UHPLC/MS-MS (Valera et al. 2020). Studies involving the determination of abamectin and difenoconazole in bees have been developed using different QuEChERS extraction salts tested in this

study. Prado et al (2020), using the acetate QuEChERS method, present abamectin recovery of 89.4% in the high-level spiking (100 ng g^{-1}) and for difenoconazole 95.5% using spiking levels from (1 to 100 ng g^{-1}) and LOQ of 0.01 ng g^{-1}.

Thus, in the present work, using the proposed method for pollen and strawberry flowers, was possible to detect concentrations below the MRL levels, with good linearity, quantitation limits as well as accuracy inside the recommended range of 80–120% and the precision below 20%. The application of this method is discussed below.

3.2. Monitoring

The commercial insecticide with abamectin (a.i) was not detected in any strawberry flower or pollen samples from the hives. The absence of this compound can be focused first, in the lower agronomic dose (Kraft@ 36), which corresponds to 13.5 g ha^{-1} in mass, indicated for strawberry fields. Besides this, for this crop, it is allowed only two applications in the period of 14 days; in contrast, the fungicide containing difenoconazole (a.i) mass is 20 g ha^{-1} with six possible applications over the same period (Syngenta, Score® CE). This information suggests, preliminarily, that the detection of fungicide is probably higher than for the insecticide considering the dose and application frequency.

However, an important factor to be considered is also the chemical properties and the persistence (1/2 life period) of each investigated pesticide. For difenoconazole, the half-life of the active ingredient in the terrestrial environment is about 85 days, whereas for abamectin is only 1 day. For abamectin, the behavior in plants is related to photolysis with no residues, where the avermectin B_{1a} component can be considered as more representative for environmental monitoring (EFSA 2008). In this sense, abamectin residues in the field can be considered as commonly low (below 0.025 ppm), with no persistence or accumulation observed in the environment (Lasota and Dybas 1990). Considering the bee's matrices, for pollen and bee samples (*A. mellifera*), Wiest et al (2011) have no abamectin detection in any sample in a multi-residue method. Besides the environmental matrices tested (pollen and flowers) have no residues of abamectin, for exposed stingless bees, Prado et al. (2020), have been observed the uptake of the commercial product (Kraft) via topic and oral exposure. This uptake can alert for the exposure of the commercial product via spray drift and toxicity related not only to abamectin but also the inactive ingredients of the commercial formulation over the native bees and other non-target insects.

As mentioned above, in opposition to abamectin, difenoconazole was observed in the majority of flowers and pollen samples. The results for difenoconazole in strawberry flowers are depicted in Fig. 2 in a concentration range of < LOQ (1 ng g^{-1}) to 7.53 ng g^{-1} demonstrating the capacity of the strawberry plant to take up this fungicide and accumulate. The sampling campaigns were proceeded during the wet season considering the Brazilian weather, where the fungi proliferation is pronounced. In this case, the application of consecutive treatments of difenoconazole can be evidenced. As observed in this figure, in November 2018, difenoconazole was detected in only three points (P1, P4, and P9), while for other

sampling campaigns this compound could be found in more sampling points. In January, difenoconazole was not detected only at P2.

The highest concentration found in the strawberry flowers was 7.53 ng g^{-1}, which occurred in March at P10 of area 2, almost equal to P4 of area 1, with 7.06 ng g^{-1} for the same period. In Area 1, is possible to note that the points located in the superior part of the strawberry field (P1 and P4) have presented higher concentrations of difenoconazole compared with other sampling points. An experimental design of the difenoconazole application in strawberry fields has detected a rapid dissipation of this fungicide after pulverization in leaves, but also an increase of the residual amounts in fruit after consecutive applications (250 g L^{-1}) with 14 days intervals (Heleno et al. 2014). This systemic effect can explain the general increase of concentrations through the sampling campaigns and mainly observed in P4. In another study, Sun et al. (2015) also have observed that the half-life of difenoconazole increases in consecutive applications from 3.65 (1 application) to 6.30 (2 applications) days in strawberry fields. When compared with other pesticides, in laboratory experiments, using a soil field rate application of difenoconazole in rice plants (*Oryza sativa* L.), Ge et al. (2017) have been observed that this fungicide has a greater half-life than thiamethoxam and imidacloprid, however with lower bioaccumulation factor (BCF) and translocation factor (TFs) compared with those neonicotinoids pesticides.

Figure 2 Concentration (ng g^{-1}) of difenoconazole in strawberry flowers sampled in Areas 1 (P1 to P5) and 2 (P6 to P10) in different sampling campaigns: November/18; January/19; and March/19. Missing data are below the limit of quantification (< LOQ).

In Brazil, the maximum residual limit for difenoconazole in strawberry fields is 0.5 µg g^{-1}, with foliar application with a security interval of 1 day (ANVISA 2021b). Converting the maximum value found in the strawberry flower (P10), we have 0.00706 µg g^{-1} of strawberry, which corresponds to an amount 70 times lower than the MRL level allowed for fruits. In this sense, for human health, the concentration levels found in the present study are well below the harmful maximum limits. In other countries, the MRL is also higher than the detected in flowers as in European Union (0.4 µg g^{-1}) (EFSA 2011).

For bees, the exposure and toxicity of pesticides applied in crops occur during the pollination process, but the magnitude of pesticides' risk must also consider the landscape and the diversity of visited plants (McCart et al. 2017). For strawberry fields, Antunes et al. (2007), has observed from 15.9 to 18.6 visits of *T. angustula* per flower, per hour, when the field is surrounded by 4 hives, the same number of hives displayed for the present investigation and that can contribute significantly for the results described below.

In a multi-residue method for the monitoring of 81 pesticides in pollen and bees (*A. mellifera*), Saibt (2017) has detected only difenoconazole (16 ng g^{-1}) in pollen samples from the Rio Grande do Sul State, Brazil. The exposure to difenoconazole (Score 250 EC 0.2 L ha^{-1}) in apple (*Malus domestica*) field has been also detected in bee (*A. mellifera*) products, where the pollen contamination was about 43 ng g^{-1}, with the detected concentration related to the capacity of fungicides to be fixed by sugars, amino acids or

proteins (Kubik et al. 2000). Other studies involving this fungicide detection in pollen (*A. mellifera*) has included: Friedle et al (2021) in a concentration range of 0.02 to 48 ng g^{-1}; and Vásquez et al (2015) with a concentration of 45 ng g^{-1}, levels below the mostly of detected concentrations observed in the present study.

Considering the hives arranged next to Area 1, pollen samples (Fig. 3) have presented impressing high concentration of difenoconazole, especially the H1 hive in January/19 (456 ng g^{-1}). The accumulation of this fungicide in the pollen collected by *T. angustula* can be associated with the visit of these stingless bees in many strawberry fields located in the Bom Repouso region reaching this bee flight range of 500 m (van Nieuwstadt & Iraheta 1996). Flight activity of *T. angustula* can also be dependent on temperature, where warm weather (above 19.6^{o}C) can allow a major activity and the collection of pollen to the hives (Marlebo-Souza and Halak 2016). This behavior can contribute to the major concentrations detected in January/19 samples which are observed as the greatest strawberry blossom in the field and bee's activity. Besides the increase of activity, the conditions inside the hives as protection against sunlight, temperature control, and anti-bactericide properties can contribute significantly to the accumulation and preservation of this pesticide.

Figure 3 Concentration (ng g^{-1}) of difenoconazole in pollen from *T. angustula* hives located nearby strawberry fields: Areas 1 (H1 and H2) and 2 (H3 to H5) in different sampling campaigns: November/18; January/19; and March/19. Missing data are below the limit of quantification (< LOQ).

In addition to strawberry fields, other plants can also be visited by *T. angustula* on area, where Asteraceae and Fabaceae are indicated in the literature as the favorite plant families for this species (Braga et al. 2012). A preliminary study (*not published*) in developing in our lab to investigate the diversity of pollen in samples from these hives and had demonstrated that about 90% of identified pollen is from strawberry plants.

3.3. Risk Assessment

Once the pollen is transported to the hive, the bee's contamination path reaches another configuration, changing from contact to oral. In this sense, it is important to highlight that pollen acts as a main food when worker bees are in their first two weeks of life, and as a supplement, after two weeks of their lifetime. So, while the honey supply is responsible for providing energy to bees, pollen is considered an important source of minerals, vitamins, and proteins (Vit, et al. 2004). All these exposures and further toxicity by the food supply can affect the colony's health, bringing sublethal effects and impact the maintenance of the colony through the effect over larvae development (Leite et al. 2018).

In this sense, for the risk assessment, once the exposure to difenoconazole was observed, the program BeeRex was used for estimated if the RQ exceeds the levels of concern (0.4 for acute risk). As mentioned above, for RQ calculations the variables considered were product application rate (Kg a.i. ha^{-1}), taken

into account the maximum recommended dose and the oral LD_{50} for 48h for *T. angustula* (µg a.i. bee^{-1}). At the same time, a calculation considering CAE/LD_{50} was also investigated.

For this calculation, once there is no *T. angustula* toxicity data for this compound in literature, we have consulted the literature data for *Apis mellifera*, where the oral toxicity endpoint (LD_{50}) registered is 177 µg a.i. bee^{-1} (EFSA 2011) and 33.48 µg a.i. bee^{-1} (SHARDA BRASIL 2019). Considering those two data, the LD50 resulting average was 105.24 µg a.i. bee^{-1}. However, due to the absence of toxicity data for this native bee, an adjustment of 10 times sensibility was made, considering the LD_{50} amount observed for *Apis*, (Arena and Sgolastra 2014), and applied for *T. angustula*, resulting in a final LD_{50} of 10.52 µg a.i. bee^{-1}.

Thus, considering the difenoconazole application rate of 20 g ha^{-1} by foliar spray and adding the empirical data of residual amount detected in pollen the risk quotient was estimated, corresponding to Tier 1. In this sense, including this estimated endpoint and the application rate in strawberry fields, the RQ for difenoconazole exposure was estimated at 6.8 (CAE/LD_{50}) and 194.03 (CAE – BeeREX), both surpassing the level of concern (< 0.4). However, when the levels detected in pollen samples are considered point by point, only the H1 hive sampled in January /19 has presented the risk (0.43).

Checking this scenario, we can verify that the ideal risk assessment must consider most complex analysis (Tier 2), including another aspects as behavior and physical aspects of *T. angustula*. When we consider the foraging efforts of pollen and nectar, bees can be exposed to pesticides by direct contact with contaminated flowers surface. This exposure route can be considered as lethal and break the colony balance, once foraging bees compose the majority of colony, representing 83% of the individuals in a hive of *T. angustula* (Prato et al. 2013); according to literature, this species makes about 40 daily flights (Vida Natural), touching an average of 40 thousand flowers, and that on each flight it carries, on average, 40 mg of pollen to the hive (Fujiyoshi, H. - Beekeeping Department Yamada). Using the highest concentration of difenoconazole detected in the strawberry flower (7.53 ng g^{-1}), and that the bee can carry, on average, 40 mg of pollen per flight, we have to, per flight, it “comes in contact” with 0.3012 ng of difenoconazole, still, considering the average of 40 daily flights, we have daily physical exposure to 12.048 ng bee day^{-1}. It is important to note that this value should be a little lower, since the pollen's contact area with the bee is not exactly the value of the mass it carries, therefore, it is an approximation.

Considering this previous risk assessment results, and, due to the absence of toxicity data for *T. angustula*, we cannot rule out the possible harm that the presence of this pesticide can cause in the bees’ lives, as behavioral changes, decreased birth rates, a lower expectation of life, and others. Therefore, a study of the real impacts for this stingless bee species is necessary, considering the uptake, lethal and sublethal effects under laboratory and field studies and involving adults and all brood (eggs, larvae, and pupae), mainly when the systemic pesticides are considered.

4. Conclusion

The monitoring of pesticides abamectin and difenoconazole in strawberry flowers and *T. angustula* colonies has demonstrated the accumulation of the fungicide in samples over the sampling campaigns, through the application of an optimized and validated method. The absence of the insecticide abamectin can be related to the degradation of the active compound, but also the lower application rate and frequency in strawberry fields. On other hand, Difenoconazole has demonstrated some persistence in the flower and pollen and can be transported to the hives by the studied stingless bee species. The detected concentration was considered for the risk assessment using the model discussed in the literature, however, due to the lack of data about the toxicity of difenoconazole for *T. angustula*, this risk analysis must be investigated in further studies.

Declarations

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Competing interests

Authors have no relevant financial or non-financial interests to disclose.

Author contributions

All authors contributed to the study conception and design as follow: **José Augusto Michelleti Burgarelli:** Conceptualization, Methodology, Investigation, Writting; **Dayana Moscardi dos Santos:** Investigation, writting and Edition; **Fernanda Scavassa Ribeiro do Prado**: Methodology, writting; **Waleria Ferreira Rabelo**: Discussion, writting; **Rafael Sardeli**: Methodology; **Janete Brigante**: conceptualization; methodology; investigation; **Michiel Adriaan Daam:** investigation, edition; **Eny Maria Vieira**: Conceptualization, Supervision. *All authors read and approved the final manuscript.*

Availability of data and materials

All data analyzed in this study are included in this manuscript and its supplementary information files

Ethical approval

According to University of São Paulo and Brazilian ethical guidelines for the use of animals in research, studies involving invertebrates do not require authorization from the committee.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

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Figures

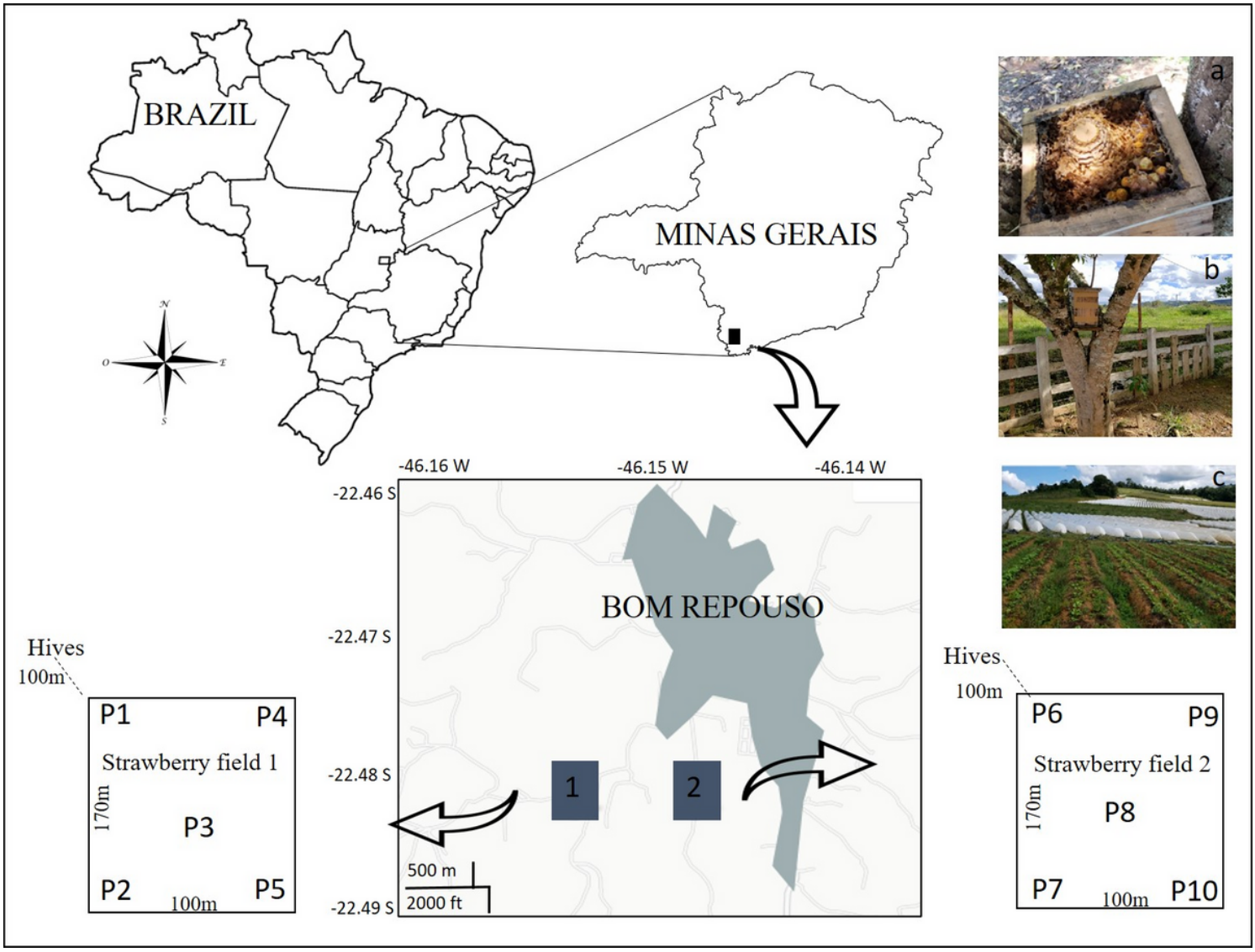

Figure 1

Location of sampling areas (1 and 2) at the city of Bom Repouso, Minas Gerais State, Brazil, and general description of strawberry flowers sampling points in Area 1: P1 to P5 with the proximity of *T. angustula* hives (H1 and H2); and Area 2: P6 to P10 with the hives H3 to H5. *T. angustula* hives: structure (a) and location (b); strawberry field at Bom Repouso (c). Map source: adapted from IGAM (2020).

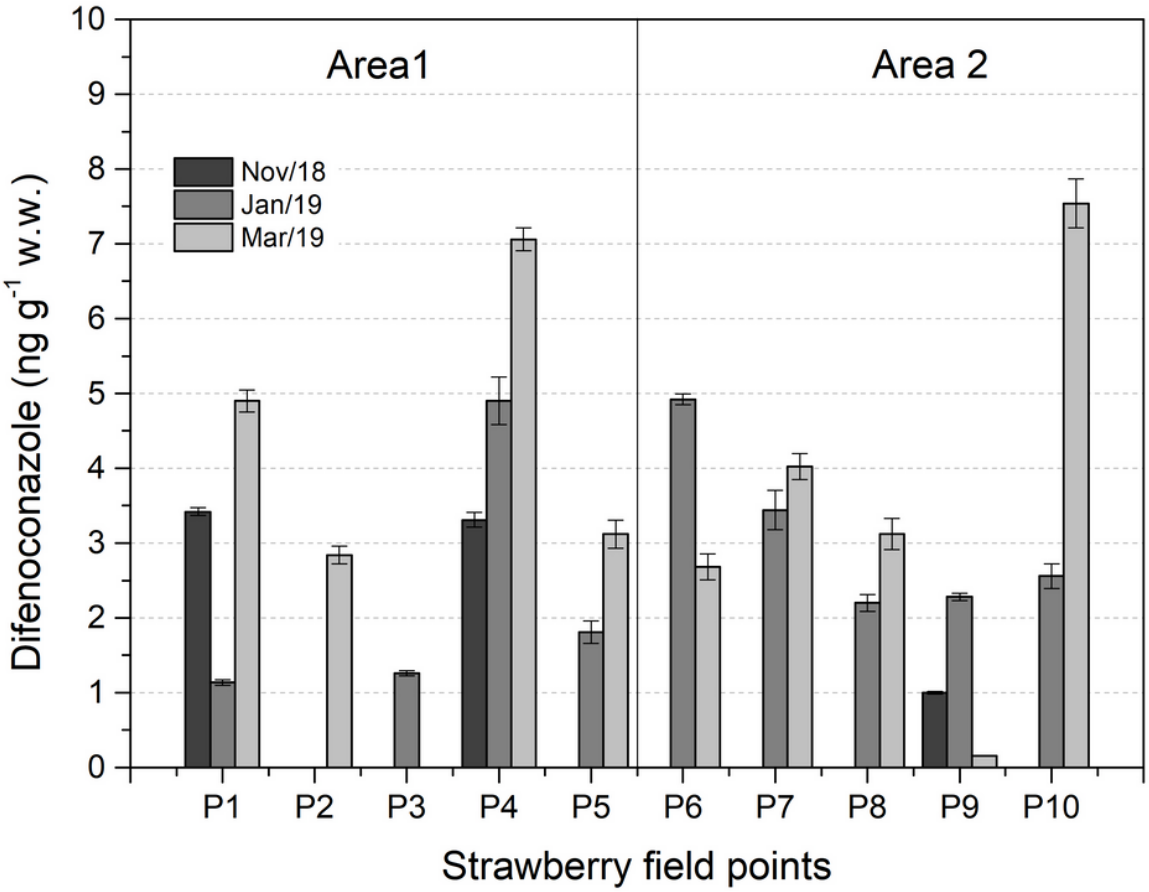

Figure 2

Concentration (ng g^{-1}) of difenoconazole in strawberry flowers sampled in Areas 1 (P1 to P5) and 2 (P6 to P10) in different sampling campaigns: November/18; January/19; and March/19. Missing data are below the limit of quantification (<LOQ).

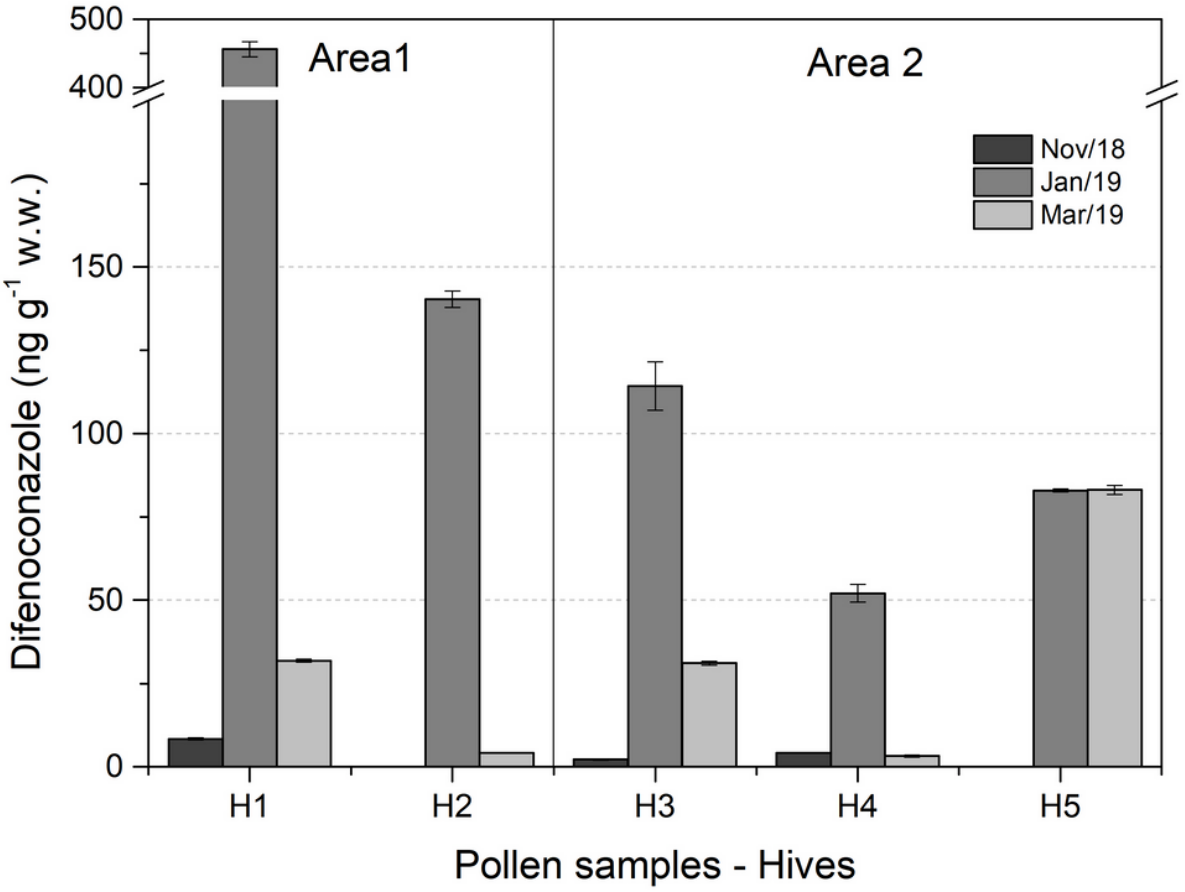

Figure 3

Concentration (ng g^{-1}) of difenoconazole in pollen from *T. angustula* hives located nearby strawberry fields: Areas 1 (H1 and H2) and 2 (H3 to H5) in different sampling campaigns: November/18; January/19; and March/19. Missing data are below the limit of quantification (<LOQ).

Supplementary Files

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