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STUDY ON IDENTIFICATION AND QUANTIFICATION OF PESTICIDE RESIDUES IN SOME HIVE PRODUCTS FROM RAPE (*BRASSICA NAPUS* SUBSP. *NAPUS*) AND SUNFLOWER (*HELIANTHUS ANNUUS*) CROPS IN THE ACTIVE SEASON 2022

STUDIU PRIVIND IDENTIFICAREA ȘI CUANTIFICAREA REZIDUURILOR DE PESTICIDE DIN UNELE PRODUSE ALE STUPULUI PROVENITE DIN CULTURILE DE RAPIŢĂ (*BRASSICA NAPUS SUBSP. NAPUS*) ȘI DE FLOAREA SOARELUI (*HELIANTHUS ANNUUS*) ÎN SEZONUL ACTIV 2022

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ABSTRACT

Intensive agriculture, especially the use of pesticides, is suspected of contributing to the decline of bee populations at the European level. The aim of this work was to establish the impact of some pesticides on bees and hive products in the bee year 2022 by monitoring a number of nine families of bees untreated with antiparasitics (clinically healthy bees). Six samples of honey/honeycomb and three samples of pollen/honey bread were selected from three experimental locations to determine the level of residues. It was found that the taken honey samples were negative in the toxicological analysis for neonicotinoid residues or other pesticides in rape and sunflower crops. The samples of rapeseed honey came from a crop adjacent to the experimental research station (Fundulea), which was calamitated, and the results showed the presence of the fungicide residues Azoxystrobin (0.081 mg/kg), *Boscalid* (0.042 mg/kg), *Ciproconazole* (0.042 mg/kg), *Dimoxystrobin* (0.028 mg/kg) and the insecticide *Acetamiprid* (0.052 mg/kg). However, this research offers a new perspective on researching the impact of agricultural intensification on bees. This research also provides a new perspective in researching the impact of intensive agriculture on bees. These results, obtained at an independent laboratory represented a step forward that could be complemented by future research linking beekeeping research with agricultural crop technology.

Keywords: bees, bee bread, honey, honeycomb, pollen, pesticides

REZUMAT

Agricultura intensivă și mai ales utilizarea pesticidelor este suspectată de scăderea populațiilor de albine la nivel european. Scopul acestei lucrări a fost stabilirea impactului unor pesticide asupra albinelor și produselor stupilor în anul apicol 2022, prin monitorizarea unui număr de nouă familii de albine netratate cu produse antiparazitare (albine sănătoase clinic). Șase probe de miere/fagure și trei probe de polen/păstură au fost selectate din trei locații experimentale pentru a determina nivelul de reziduuri. S-a constatat că probele de miere prelevate au fost negative în analiza toxicologică pentru reziduuri de neonicotinoide sau alte pesticide provenite de la culturile de rapiță și floarea soarelui. Probele de miere de rapiță au provenit de la o cultură învecinată stațiunii de cercetare experimentale (Fundulea) care a fost calamitată, iar rezultatele au arătat prezența reziduurilor de fungicide *Azoxystrobin* (0,081 mg/kg), *Boscalid* (0,042 mg/kg), *Ciproconazol* (0,042 mg/kg), *Dimoxistrobin* (0,028 mg/kg) și insecticidul *Acetamiprid* (0,052 mg/kg). De asemenea, această cercetare oferă o nouă perspectivă în cercetarea impactului agriculturii intensive asupra albinelor. Aceste rezultate obținute la un laborator independent au reprezentat un pas înainte care ar putea fi completat cu cercetări viitoare care leagă cercetarea apicolă cu tehnologia culturilor agricole.

Cuvinte cheie: albine, păstură, miere, fagure, polen, pesticide

Pollinators are an important link in maintaining plant biodiversity worldwide, of which 75% are bees (*Apis mellifera L*), as domestic pollinators (1, 2). The decreasing number of bees can have negative economic and ecological effects, with disastrous consequences for biodiversity, food security, and human well-being (2). The honeybee family is an important biological tool for understanding and even quantifying the impact of the various aggressor factors that intersect with its complex social life. The bee decreasing population and mortality in the last two decades have become very important problems worldwide. Although

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the causes of these problems are unclear, there have been numerous articles related to environmental factors and pesticide action (9, 10). The detection of neonicotinoid substances in honey and other beehive products has already been reported in the specialised literature (12, 13), highlighting the fact that these insecticides, by accumulating at sublethal levels in hive products, threaten the survival of pollinators, indirectly exposing the queen bees to these substances as well. A number of research studies have shown that under experimental and field conditions, exposure of bees to treated crops has recorded the presence of pesticide residues in pollen, honey (14, 15, 26), and beeswax (16). Another study highlighted the potential hazard of pesticides to bees through the consumption of contaminated pollen and nectar (2, 16), which can induce chronic bee poisoning during the inactive season. The pollen source is of particular importance because the flowering period overlaps with the period in which overwintering bee generations are reared, and this has a particular impact on brood growth for the next season, i.e., the next generation of bees. Following scientific data from numerous studies on the toxicity of neonicotinoid pesticides and their lethal and sublethal effects on bee populations, and based on scientific reports from the European Food Safety Authority (17), the European Union (EU) has banned the use of some neonicotinoids (imidacloprid, clothianidin, and thiamethoxam) for the treatment of certain agricultural crops (Reg. 2018/783/784/785/29.05.2018). In recent times, in Europe as well as in North America [34], there has been a decline in bee populations reported by researchers and beekeepers who report a high mortality situation in bees during the active period compared to the inactive period. Chemical poisonings are generally produced by plant protection products, and the most dramatic are those caused by insecticides/pesticides, which affect the nervous, respiratory, and digestive systems (18, 22). Pesticides can also cause sublethal poisoning, which reduces productive performance, prevents brood development, alters bee hygiene behaviour, and negatively influences bee immunity [4, 6, 14, 23].

MATERIALS AND METHODS

Placement of bee families in predetermined locations

To implement the protocol and carry out the analyses regarding the exposure of honeybee colonies to neonicotinoids, three fields were selected in different areas of intensive agriculture in the southeastern and eastern parts of Romania. They were located in Neamț county (Agricultural Development Research Station — S.C.D.A. Secuieni, 46◦51045″ N, 26◦49042″ E), Arges county (Agricultural Development Research Station — S.C.D.A. Albota, 44◦46054″ N, 24◦49031″ E), and Calarasi county (National Institute for Agricultural Development Research —I.N.C.D.A. Fundulea, 44◦27010″ N, 26◦30055″ E). In addition to these locations, a series of samples were collected from apiaries belonging to the Institute for Beekeeping Research and Development – I.C.D.A. Bucharest, apiaries being located in different areas in southeastern Romania (Baneasa-Bucuresti—44◦29033″ N, 26◦04045″ E).

Placement of bee families in rape and sunflower crops

The experimental group consisted of nine families of clinically healthy bees from the Beekeeping Research and Development Institute – I.C.D.A. Bucharest, three families of bees for each of the three locations, which were placed in the rape (*brassica napus subsp. napus*) and sunflower (*helianthus annuus*) at the beginning of the flowering of plants season, where they remained during the entire flowering period of the plants (respectively every two weeks/crop).

Monitoring bee colonies and collecting samples for analysis

After anamnesis, morpho-clinical, and laboratory examinations of the studied bee families, they were transported to monitor the impact of neonicotinoids and other pesticides on bees and/or hive products (honey, honeycomb, and pollen).

The families of bees transported and placed in the 3 experimental locations were monitored throughout

Table 1

Number of samples collected from experimental stations during the 2022 beekeeping season

Station	**Rape crop samples** (12)				**Sunflower crop samples** (12)			
	Dead/alive bees	Brood cells	Honey/ honeycomb	Pollen/ bee bread	Dead/alive bees	Brood cells	Honey/ honeycomb	Pollen/ bee bread
Albota	1	1	1	1	1	1	1	1
Fudulea	1	1	1	1	1	1	1	1
Secuieni	1	1	1	1	1	1	1	1
Total	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**

the experiment, with a particular focus on the behaviour of the worker bees, the prolificacy of the queens, brood development, morbidity and mortality status, and the productivity of the bee family, all of which were recorded on monitoring sheets. Monitoring of all basic parameters in the experiment was carried out in both oilseed rape and sunflower crops, before (T_0) and after the experiment (T_{final}), with a total of 6 monitoring sheets, one for each location and type of crop. In order to monitor throughout the experiment, the morpho-clinical evolution, morbidity and mortality status, and bee behaviour, 24 samples of bee biological material (live/dead bees, honey/honeycomb,pollen/bee bread) were collected (12 samples from the rape crop and 12 samples from the sunflower crop) for interim examinations according to OIE protocols (2018) (22), at the Pathology Laboratory I.C.D.A. (Table 1).

Out of the total number of 24 samples collected, the following samples were sent to determine the level of neonicotinoid and/or other pesticide residues: 6 samples of dead/alive bees (one sample from each location and each crop), 6 samples of brood cells (one sample from each location and each crop), 6 samples of honey/honeycomb (one sample from each location and each crop), and 3 samples of pollen/bee bread (one sample from each location, only from the rapeseed crop). Only the pollen/bee bread samples from the rapeseed crop were sent for analysis because, in the case of the sunflower crop, the temperatures were excessively high, which is why the bees did not collect pollen but consumed it from the hives. Therefore, no pollen or bee bread samples could be collected from the sunflower crop at all 3 locations. At the same time, we were particularly interested in collecting only sunflower honey samples because they constitute the food of bee colonies for the inactive season (wintering) and can induce chronic poisoning (population collapse). Out of the 21 analysed samples, only nine represent bee products, honey/honeycomb and pollen/bee bread, respectively (Table 2).

The present research was based only on morphoclinical observations and on the quantification of the chemical substances found in the samples. Besides this, the number of samples collected and analysed is small. Consequently, we consider this approach a pilot study, and the results cannot be generalised.

The MedCalc Software version 20.218 was used for statistical data analysis. One-way ANOVA for independent samples was chosen to identify significant differences for number of workers and mortality between experimental sites and types of crops, respectively, between the three locations (Albota, Fundulea, and Secuieni) and rape and sunflower crops. The samples were taken from all bee colonies, not only from those that showed signs of intoxication with morphopathological changes in the affected bees. The data were tested for normality using the Shapiro-Wilk test, and Levene's test was used to test for equality of variances. Because the rape culture from the Fundulea experimental location was affected by drought, the samples were collected from the bees that visited the rape cultures adjacent to the affected experimental lot. Standard treatments of the seeds with neonicotinoids were carried out in the rapeseed and sunflower crops in the

Table 2

Status of apiculture products sampling for neonicotinoid and/or other pesticide residues

No.	Experimental station	Crop	No. /Sample type
1	Albota-AG	rape	1. honey/honeycomb
			2. pollen/bee bread
	Fundulea-CL		3. honey/honeycomb
			4. pollen/bee bread
	Secuieni-NT		5. honey/honeycomb
			6. pollen/bee bread
2	Albota-AG	sun flower	7. honey/honeycomb
	Fundulea-CL		8. honey/honeycomb
	Secuieni-NT		9. honey/honeycomb

Table 3

Protocol for the sample collection of bee biological material

No.	Bee biological material	Amount/sample	Admitted recipients	Storage and transport of samples
1.	Alive/Dead bees	min. 200 g	Plastic or glass recipients, resistant to freezing (-20^{0}C), sealed.	Frozen samples, transported in maximum 1 day data [1]
2.	Honey/honey comb	250 g/ 15-20 cm^2 area	Plastic recipients	
3.	Pollen/Bee bread	150 g	Plastic recipients	

experimental lots according to the indications in the prospectus and the legislation in force. These substances were sought to be found in the beehive products collected from the experimental lots. We were not allowed to find out the concentration of substances applied to the rape field.

Sending samples for toxicological analysis to an accredited laboratory

Samples selected for toxicological analysis were placed in appropriate containers, coded, labelled, conditioned according to the established protocol, and sent to the ISO 17025 accredited laboratory, Primoris-Bulgaria, where they were analysed using GC-MSMS and LC-MSMS methodologies, with Reporting Limit (RL) = 0.01 mg/kg (25) (Table 3).

RESULTS AND DISCUSSION

In the 2022 active beekeeping season, suspected bee poisonings were evaluated by monitoring cases recorded according to the methodology for quantitative and qualitative analysis of bee exposure to various toxins (24, 25). Intermediate examinations (anamnesis, morpho-clinical, and laboratory examinations) of the studied bee colonies revealed clinical signs of suspected bee poisoning in foragers after rape harvesting, only at Fundulea station.

Following the One-way ANOVA test for independent samples to identify significant differences for the analysed parameters between experimental sites and types of crops, respectively between the three locations (Albota, Fundulea, and Secuieni) and rape and sunflower crops, we found out that the recorded data were not normally distributed, so we continued with the Kruskal-Walli's test. Highly different values at a 0.05 level of significance resulted. So, the results of morpho-clinical examinations performed on bee families after rape harvest, in which there was suspicion of acute poisoning (Fig. 1), could then be correlated with the identification of insecticides/pesticides in pollen and honey samples (Fundulea).

After the rape harvest in the active beekeeping season 2022, a survival of about 80% of bees was observed throughout the experimental period, depending on the number of bee intervals, starting at T_0 with a number of 9 intervals and at the end of the experiment T_{final} reaching a number of 7-8 bee intervals at all experimental locations (Fundulea, Secuieni, and Albota). The oilseed rape crop was totally (Fundulea) or mostly calamitated by the prolonged drought (Albota), when the plants did not secrete enough nectar or the bees were involved in the activity of maintaining ventilation in the brood or to avoid wax melting from the combs. At a distance of 3-5 km there were areas with well-developed rape fields (Fundulea station), and when examining the hives there were characteristic signs of acute intoxication in the unhatched bee in the hive (Fig. 1) (blackened heads and impossibility of hatching of bees from the comb cells) (Fig. 2) (4, 20), low number, agitated, and aggressive worker bees (3, 5, 6, 9, 19, 21-23, 28, 27], and evidence of dead bees at the hive entrance and especially in front of the hives (Fig. 3).

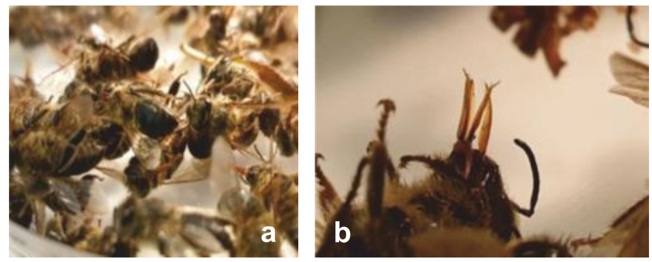

Fig. 1. (a) Affected foraging bee during rape harvest; (b) Characteristic clinical sign of chitin blackening and proboscis protrusion (Source: Pathology Laboratory I.C.D.A. Bucharest)

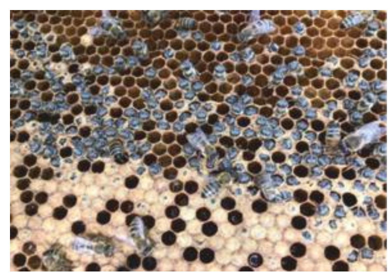

Fig. 2. Unhatched adult bee with signs of acute poisoning at rape harvest

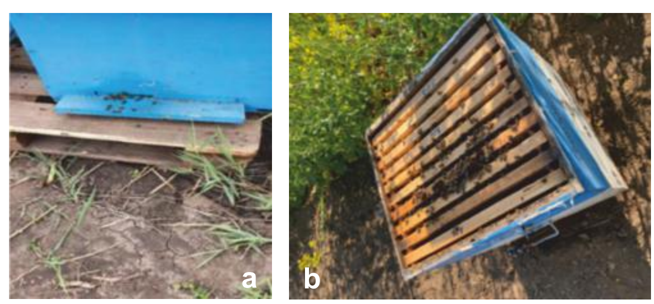

Fig. 3. (a) Dead worker bee at the hive entrance; (b) non-development of the bee family due to lack of sufficient food

Insufficient food due to the damaged rape and sunflower crops at Fundulea and Albota stations, may justify the non-development of bee families in these locations. Note that no queen losses were recorded at any experimental location in the rape crop.

In the sunflower harvest, based on the analysis of the data on the evolution of bee families during the active beekeeping season 2022, despite the prolonged drought in the southern part of the country, it was found that they survived throughout the experimental period, but with a progressive loss of bees from the hive. It was started with a number of 7 bee intervals at the T0 time, so that at the end of the experiment (T_{final}), at the locations of Fundulea and Albota, the bee population in the hives decreased to 4 intervals at Fundulea (57%), and 3 intervals at the location of Albota (43%). At Secuieni experimental station, the bee families had a good development in the sunflower crop, with the crop not being calamitated as at the other two stations. Due to the swarming phenomenon, there was a loss of bees in the sunflower crop at this experimental station. The results of the analysis of the samples taken during the 2022 active season for toxicological examination from the three experimental locations for the rape crop are presented in Table 4, while for the sunflower crop they are presented in Table 5.

After bringing together the results of the analysis of the samples collected from the experimental stations of rape and sunflower crops, the following was found: firstly, all dead/alive bees and brood cells samples, collected and toxicologically analysed, were negative for neonicotinoid/other pesticide residues; secondly, the samples (honey, pollen) taken from the rape crop from the Albota and Secuieni stations, toxicologically analysed, were negative for neonicotinoid/other pesticide residues, except for the rape crop from the Fundulea station, where the bee harvested from areas neighbouring the affected experimental lot; thirdly, honey samples taken from the sunflower crop from all three experimental stations were found negative in the toxicological analysis for neonicotinoid/other pesticide residues.

Although the Fundulea station crop was entirely calamitated by the prolonged draught, the bees collected on the cultivated areas at a distance of 3-5 km from the experimental area, and the samples showed the following residue load:

1. The neonicotinoid Acetamiprid was identified in the honey/honeycomb sample at 0.055 mg/kg, which is 5.5 times (550%) above the reporting limit (0.01 mg/g). Note that acetamiprid has a maximum limit of acceptability in honey and hive products EU-ML (Reg. EU 2019/88) = 0.05 mg/kg, slightly exceeding this limit as well (16, 20, 29);

2. In the pollen/bee bread sample, the following pesticides were identified: Acetamiprid, Azoxystrobin, Boscalid, Cyproconazole, and Dimoxystrobin. Acetamiprid (0.053 mg/kg), a neonicotinoid insecticide, has

Table 4

Toxicological examination results of samples taken after rape harvesting

No.	Origin of sample	Anamnesis	Sample type	Identified substances by toxicologic exam	Amount/ Measuring unit	Crop treatment substances
1.	Albota	-	honey/honey comb	-	-	standard treatment
			pollen/bee bread	-	-	
2.	Fundulea	Non-development, agitation aggressivity	honey/honey comb	Acetamiprid	0.055 mg/kg (Max. Limit EU-ML = 0.05)	
			pollen/bee bread	Fluvalinate (sum of isomers) resulting from the use of taufluvalinate (F)	0,054 mg/kg	
				Acetamiprid	0,053 mg/kg	
				Azoxystrobin	0,081 mg/kg	
				Boscalid	0,042 mg/kg	
				Cyproconazole	0,042 mg/kg	
3.	Secuieni	Good development, agitation, easy aggressivity	honey/honey comb	-	-	
			pollen/bee bread	-	-	

Table 5

Toxicological examination results of samples taken after sunflower harvesting

No.	Origin of sample	Anamnesis	Sample type	Identified substances by toxicologic exam	Amount/ Measuring unit	Crop treatment substances
1.	Albota	Non development, agitation, depopulation	honey/honey comb	Dimoxystrobin	-	standard treatment
2.	Fundulea			-	-	
3.	Secuieni	Good development		-	-	

a value exceeding the reporting limit (530%) by 5.3 times and the maximum limit of acceptability in apiculture products (0.05 mg/kg) by 6%, according to Reg. (EU) 2019/88 (7, 8, 29, 28). Azoxystrobin (0.081 mg/kg), a broad-spectrum systemic fungicide (Amistar, Abound, Heritage, Olympus, Ortiva, Priori Xtra, Scotts DiseaseEx, Azoxy 2SC), Haedes and Quadris), exceeds the reporting limit (810%) by 8.1 times and the maximum residue limit in apiculture products (0.05 mg/kg) by 62%, according to Reg. (EU) 2022/476. Azoxystrobin has low mammalian toxicity with an LD50 of over 5000 mg/kg (rats, oral) (24, 25). Boscalid (0.042 mg/kg), systemic fungicide, is 4.2 times above the reporting limit (420%) but below the maximum level of use in apiculture products (0.15 mg/kg) according to Reg. (EU) 2022/1324. *Boscalid* is classified with "suggestive evidence of carcinogenicity, but not sufficient to assess human carcinogenic potential", according to the US EPA. *Boscalid* is persistent, has low mobility in soil, but nevertheless, it can move to surface water via spray and runoff from soil and suspended sediment (30). Cyproconazole (0.042 mg/kg), a fungicide of the azole class, is 4.2 times above the reporting limit (420%) but below the maximum residue limit for apiculture products (0.05 mg/kg) according to Reg. (EU) 2018/70. *Cyproconazole* is used on cereal crops, coffee, sugar beet,fruit trees and grapes, on turf and golf courses, and on wood as a preservative. Cyproconazole inhibits demethylation, a special step in the synthesis of a fungal cell wall component called sterol. This means it affects fungal growth, but not fungal sporulation. This explains why it should be used when fungal growth is maximal, early in the infection, because in late infections fungal growth slows down and the agent is ineffective. The European Food Safety Authority recommended registration of cyproconazole in 2010 (31). According to Regulation (EU) 2015/1040, dimoxystrobin (0.028 mg/kg) is 2.8 times the reporting limit (280%), but less than the maximum amount of usage in apiculture products (0.05 mg/kg). Dimoxystrobin is primarily used as a fungicide in cereals. It is insoluble in water and has low volatility (32). It has the potential to persist in both soil and water systems. It is extremely harmful to earthworms and fish, but moderate to low in other species. Dimoxystrobin is an endocrine disruptor and has modest oral toxicity in animals (33). Groundwater contamination posed by persistent metabolites (11).

According to recent studies conducted in Romania, regarding the presence of pesticide residues in some products of the beehive, the level found in the analysed samples correlated with other scientific research carried out worldwide, emphasising the fact that chronic exposure has a cumulative effect, with an impact on the health of bees and implicitly on beekeeping, in general (36). Additionally, pesticide presence in hive matrices has been reported worldwide (13, 16, 18). In the USA, fluvalinate and coumaphos miticides and the insecticide chlorpyrifos were the most frequently detected pesticides, which had the highest concentrations in honey bees, beeswax, and pollen (1).

In France, surveys detected the widely used fungicide carbendazim and the acaricides amitraz and coumaphos, in honey bees and pollen (35). Italian beekeeping matrices were contaminated with the miticides clofenvinphos, coumapahos and amitraz, and the insecticide chlorpyrifos was detected in pollen samples (36). Taking into account the high level of resdue of pesticides found in different samples, it is important to show the risks that the European Food Safety Authority (EFSA) mentioned in its report published in 2019, such as “delayed effects or relevant sub-lethal effects on bees at relatively low concentrations cannot be excluded” (22).

The limitations of the research presented in this article require that the results describe a suspected poisoning of bee colonies. A first limitation consists in the fact that the number of samples was too small to allow generalisation of the research results. Samples were selected only from bee colonies that showed signs of intoxication with morpho-pathological changes in the affected bees (represented by groups of bees consisting of more than 100 dead individuals in front of the hive entrance). The second limitation consists in the fact that no definite information was obtained about the conditions of pesticide use in nearby crops. The third limitation comes from the fact that information about the application of pesticides was obtained from the directions in the leaflet and the legislation in force. For future research, we recommend representative investigations with a sufficient number of samples. These researches should be integrated across the two entities involved: agricultural farms and apiaries. This approach is necessary to correlate the pesticide administration schedule with the condition of bee colonies in apiaries located in different areas and under different climatic conditions.

The results could provide comprehensive information on bee colony maintenance technology and agricultural crop protection technology systems.

Consequently, statistical analysis would correlate low worker bee numbers and mortality with pesticides used in agricultural crops.

CONCLUSIONS

In conclusion, our toxicological examination of honey, honeycomb, and pollen samples from various experimental stations revealed concerning findings. While Albota and Secuieni stations showed negative results for neonicotinoids and pesticides, the Fundulea station exhibited the presence of neonicotinoid Acetamiprid, synthetic pyrethroid acaricide insecticide Fluvalinate, and several fungicides in bee products. This combination of chemicals is known to have detrimental effects on bees, leading to acute and chronic intoxication, neurological issues, and worker bee mortality.

Furthermore, clinical examinations of bee families in Fundulea suggested bee poisoning in areas adjacent to the compromised experimental plot due to prolonged drought. Bees exhibited characteristic signs of acute intoxication, including unhatched bees with blackened heads, aggressiveness, reduced worker bee numbers, and increased mortality. Bee colonies in Fundulea and Albota also suffered from developmental setbacks in rape and sunflower crops due to drought, resulting in a progressive loss of worker bees. In contrast, Secuieni's bee families fared better in the sunflower crop.

The presence of pesticides in hive products poses a significant risk through cumulative effects on bee health and population viability, especially during the inactive wintering period. Contaminated beehive products can affect subsequent generations of bees, leading to decreased immunity and population collapse due to chronic contamination.

While various factors have contributed to the decline in bee populations, including changes in habitat, pollution, biological changes, and climate shifts, the widespread exposure to pesticides remains a primary driver of bee colony disappearance. Addressing the uncontrolled use of pesticides and implementing standardized treatments in vegetation and seed phases is crucial for bee conservation and the preservation of our ecosystem.

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