THE PROPHYLAXIS OF CHALKBROOD IN BEES BY LABORATORY METHODS - MICROSCOPIC TESTING OF POLLEN

Ion RĂDOI[1], Gheorghe Florentin MILEA[1]*, Alexandros RĂDULEA[1], Vasilică SAVU[2], Agripina SAPCALIU[2], Maria MĂGDICI[2], Luiza BADIC[3]

[1]University of Agronomical Sciences and Veterinary Medicine Bucharest, Romania
[2]Beekeeping Research and Development Institute Bucharest, Romania, Bucharest, Romania
[3]Spiru Haret University Bucharest, Romania
E-mail address: florin.milea@gmail.com

Abstract

The purpose of this study was to monitor the load of Ascosphera apis spores in honey and pollen samples and to evaluate the pertinence of the method in the prophylaxis of Ascosphera apis infestation by eliminating contaminated sources used in bee fed. We investigated 8 apiaries for a period of 2 years at the end of the active bee season, the collected samples consisting in pollen (39 samples) and pollen supplimentary foods (7 samples). The samples were processed and tested for spores of Ascosphera apisthrough the method OIE/2008, adapted for samples of pollenandpollen supplimentary foods. The samples originated from apiaries suspected of nosema disease. Within the lot with pollen samples (46 samples), a number of 39 pollen samples belonging to the 4 dominant floral categories in Romania (polifloral, rape, sun-flower, linden) were chosen in order to determine the infestation level with Ascosphera apis spores. Most of the Ascosphera apis spores positive samples were represented by the rape and sunflower pollen samples. Tests evidenced the presence of Ascosphera apisspores in 22 samples of pollen and pollen supplimentary foods of the total of 45 examined samples during the monitoring process. The tests made on bee samples collected at the end of the beekeeping season, by comparison to the ones collected in the beginning of the following season, demonstrated a significant reduction in the infestation degree (30.43%) in bees by eliminating from consumption the sources of infestation (pollen supplimentary foods and pollen) in the winter season. Testing before the inactive season for Ascosphera apis spores in the reserve honey and pollen represents an important prophylactic method against Ascosphera apis infestation inbees.

Keywords: *Ascosphera apis, chalkbrood, microscopic testing, pollen, prophylaxis*

Introduction

Chalkbrood disease is an invasive disease producing important losses by a high mortality rate of bee larvae [1, 3, 20]. Caused by the fungus *Ascosphera apis,* who's able to infect brood of any caste (workers, drones and queens) [7, 21]. *Ascosphera apis* spores ingested by young bee larvae (4 days for age) with there food and germinate in the gute and after mycelium penetrates the gut wall [1, 9, 15]. The formation of spores cysts is on the outside of the dead larvae ad most of these spore cysts are ejected from the coliny by house-cleaning bees, but many will find the healthy larvae, in brood comb where they remain infective for many years [3, 15, 20]. *Ascosphera apis* rarely destroys the whole colony but it can cause substantial production loss [3, 22, 23]. Chalkbrood disease is common in most beekeeping countries in the temperate regions in Europe, in New Zealand, USA and Canada [18, 20, 23]. Any larvae killed by the fungus can then carry beetween 10^4-10^7 spores on their bodies [7, 9, 12]. The laboratory diagnosis is based on the demonstration of the causative agent (*Ascosphera apis)* in diseased material,the presence of spore cysts is usually sufficient to make a diagnosis [8, 20, 23]. These spore cysts which are about 60 µm in diameter, contain smaller round bodies known as spore balls (averange 12 µm in diameter) and it is in these spores (average 2.9 x 1.4 µm in diameter) that the most infective stage of the fungusis found [3, 20, 22].

Material and method

During a 2 year period a number of 8 apiaries were investigated at the end of the active beekeeping season [3, 6, 12]. 46 samples representing bee pollen (39 samples) and *pollen*

supplimentary foods (7 samples) were collected (Table 1) originating from apiaries suspected of *chalkbrood* disease. At the beginning and the end of the active season the infestation level with *Ascosphera apis* spores was investigated. The evolution of the disease within the monitored and studied honeybee colonies was also investigated [4, 6, 21].

Table 1.

Total number of investigated apiaries and samples

Number of examined apiaries	Number of bee pollen samples	Number of pollen supplimentary foods
8	39	7

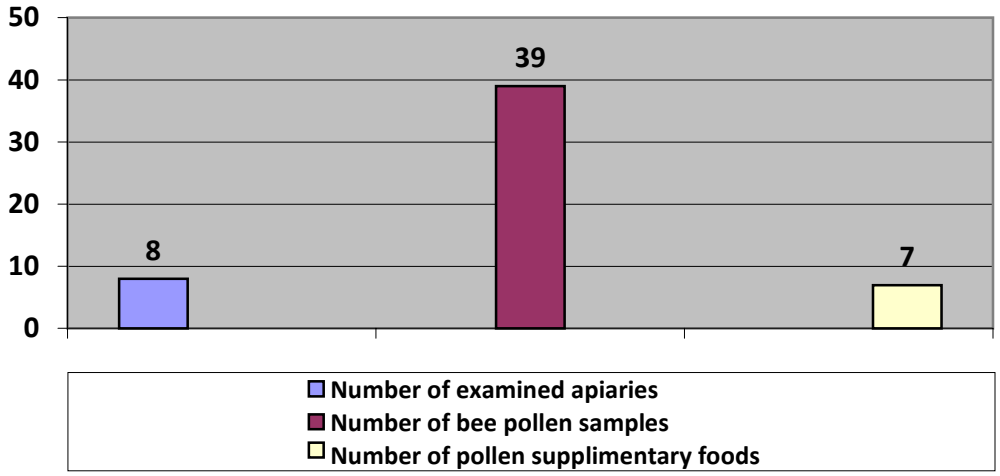


Figure 1 Repartization graphic of total number of investigated apiaries and samples

The collected samples were processed and analyzed to check the occurrence (presence) of the Ascosphera apisspores using the OIE/2008 [18, 22, 23] method that was adapted for the bee pollen and *pollen supplimentary foods* samples. At the beginning and the end of each active season the level of infestation with *Ascosphera apis* spores and the evolution of the disease within the monitored honeybee's colonies [3, 6, 13].

Within the lot with pollen samples (46 samples), a number of 39 pollen samples belonging to the 4 dominant floral categories in Romania (*polifloral pollen*, rape, sun-flower, linden) were chosen in order to determine the infestation level with *Ascosphera apis* spores. The potential variation of the infestation level correlated with the melliferous floral source was also studied (Table 2)

Table 2.

Pollen samples from various floral melliferous species in Romania examined

No. of samples *polifloral pollen*	No. of rape pollen samples	No. of sunflower pollen samples	No. of linden (Tillia spp.) pollen samples
17	8	8	6

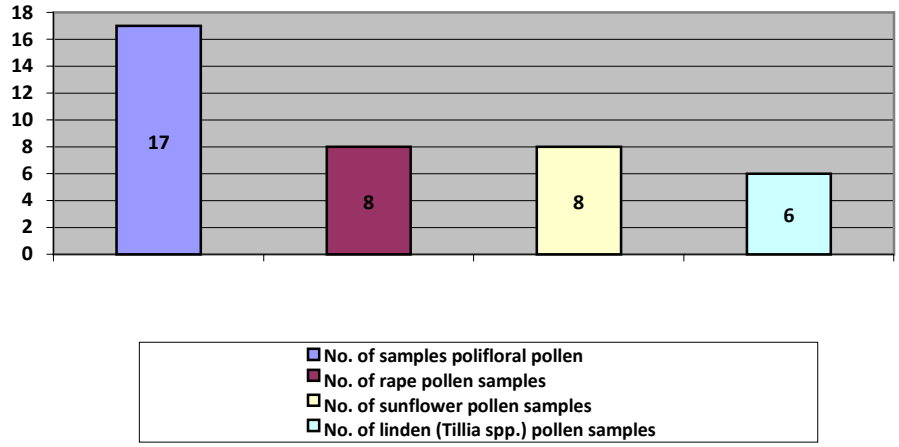


Figure 2 Representation Graphic of pollen samples from various floral melliferous species in Romania examined

Results and discussion

The direct microscopic examination [18, 21, 23] revealed the presence of the *Ascosphera apis*, spores in 20 samples of bee pollen and *pollen supplimentary foods* of the total number of 46 analyzed samples (table 3 and figure 1).

Table 3.

The samples analyzed for the presence of *Ascosphera apis* spores

Sample type	Number of examined samples	Total number of positive samples	Total number of negative samples
Bee pollen	39	15 (38.46%)	24 (61.54%)
Pollen supplimentary foods	7	5 (71.42%)	2 (28.58%)
Total number of examined samples	46 (100%)	20 (43.50%)	26 (56.50%)

The number of *Ascosphera apis* spores positive samples is shown in Figure 3.

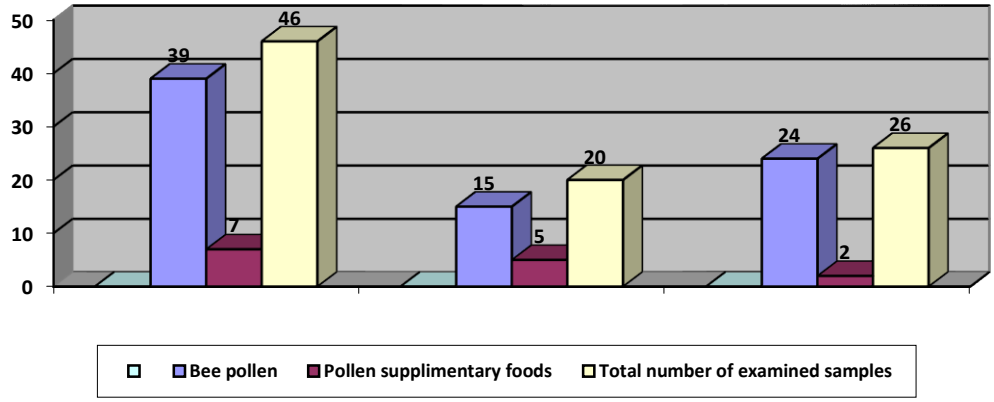


Figure 3. Graphic representation of the *Ascosphera apis* spores positive samples

Looking at the table above, we reach the conclusion that out of 39 pollen samples, 15 were positive (38.46%) for the presence of *Ascosphera apis* while out of the total number of 7 *pollen supplimentary foods*, 5 were positive for *Ascosphera apis*, spores (71.42%).

Analyzing the results of the study, it can be said that within the studied lots a high levelof contamination was noted for 3 categories of hive products which are used as food by the honeybees (pollen, *pollen supplimentary foods*).

The results made it possible to classify the level of *Ascosphera apis*sporesload in 3 categories: weak infestation (1-2 spores/microscopic field), medium (average) infestation (3-5 spores/microscopic field) and massive infestation (> 5 spores/field). (Table 4 and Fig.2,3,4).

Table 4.

The infestation level of the bee pollen and reserve honey combs

Sample type	No. of positive samples (%)	Infestation level			No. of negative sample (%)
		Weak (low) infestation (1-2 spores of *Ascosphera apis*./field) (%)	Medium infestation (3-5 spores of *Ascosphera apis*./field) (%)	Massive (high) infestation (> 5 spores of *Ascosphera apis*./field) (%)	
Bee pollen	15	9 (60%)	3 (20|%)	3 (20%)	24
Pollen supplimentary foods	5	2 (40%)	2 (40%)	1 (20%)	2

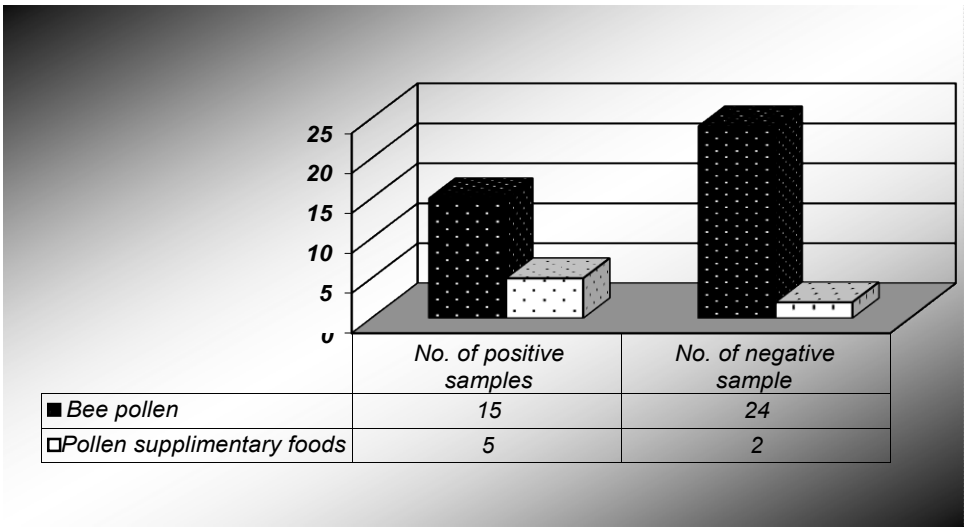


Figure 4. The number of *Ascosphera apis* spores positive samples from 8 apiary

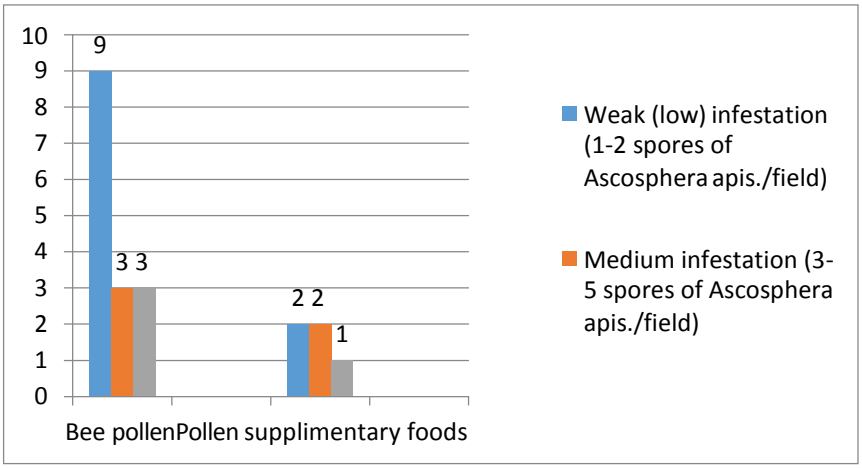


Figure 5. The infestation level of *Ascosphera apis* spores all samples (pollen and *pollen supplimentary foods)*

Followingthe direct microscopic examination of the pollen samples, the results made itpossible to classify the 3 categories for the Ascosphera apis spores load: weak infestation (1-2 spores/microscopic field, medium (average) infestation (3-5 spores/field) and massive (high) infestation >5 spores/field) (Table 5).

Table 5.

Ascosphera apis spores infestation level for the 39 pollen samples in correlation with the melliferous source

	Polifloral pollen				Rape pollen				Sunflower pollen				Linden pollen			
No. of examined samples	17				8				8				6			
No. of *Ascosphera apis* spores/field	1-2	3-5	> 5	Neg.	1-2	3-5	>5	Neg.	1 - 2	3 - 5	>5	Neg.	1-2	3-5	> 5	Neg.
No./percent of infestated samples	4 (10 %)	4 (10 %)	0	9 (60 %)	1 (12.5 %)	1 (12.5 %)	1 (12.5 %)	5 (62.5 %)	- %	- %	2 (25) %	6 (75 %)	1 (16.66 %)	1 (16.66 %)	-	4 (66.67 %)

According to the data shown in Table 5, from the total number of 17 pollen samples from polifloral pollen 8 samples (20%) were positive for *Ascosphera apis* spores. In the rest of the lot the positive values were as follows: 3 samples (37.5%) for the rape pollen, 2 samples (25%) for the sunflower pollen, 2 samples (33.66%) for linden pollen (Fig.5). Most of the *Ascosphera apis* spores positive samples were represented by the polifloral and rape pollen samples and according the data shown in fig. 5, a number of 5 samples were positive (71.43%).

The microscopic examinations of 46 samples of honeybees collected from 8 apiaries were analyzed during a 2 year interval (Fig. 7 and Fig. 8). The samples that were collected at the end of the beekeeping season (autumn), as compared to samples that were collected at the beginning of the next season (early spring), proved a significant decrease of the *Ascosphera apis* infestation level in the examined bees (the percentage was 28.50%) as a result of removing from consumption the main infestation sources during the inactive season, the infested honey andbee pollen that were given as wintering food. The level of infestation with *Ascosphera apis* spores at the end of the active beekeeping season (autumn) and at the beginning of the next active season (early spring) as well as the decrease of the infestation level is shown in table 6 and figure 6.

Table 6.

The *Ascosphera apis* infestation level at the end and the beginning of the beekeeping season

No. of pollen samples (8 examined colonies /2 years)	Level of infestation with *Ascosphera apis* spores at the end of the active beekeeping season	Level of infestation with *Ascosphera apis* at the beginning of the next active season	Decrease of infestation level with *Ascosphera apis* spores (%)
46	20 (43.5%)	3 (15%)	28.5%

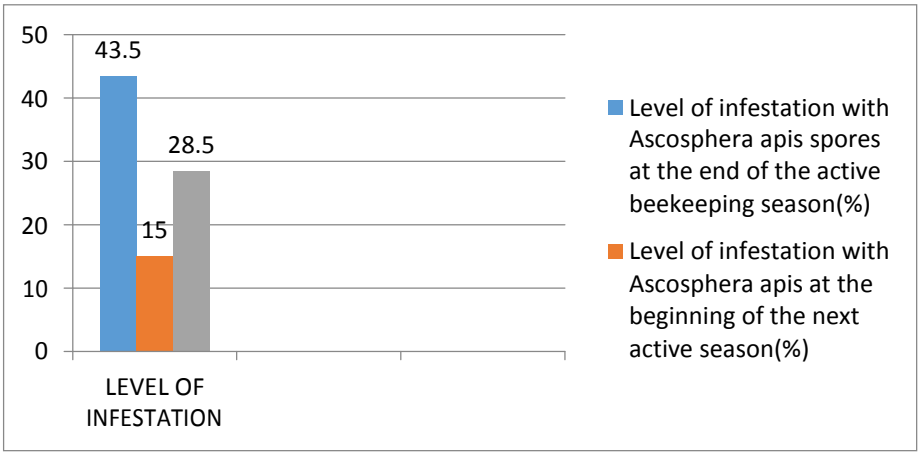


Figure 6. The *Ascosphera apis* infestation level at the end and the beginning of the beekeeping season

Removing the main contamination sources with *Ascosphera apis*, spores from the food ofthe honeybees during the inactive period (the wintering period) – contaminated pollen and pollen supplimentary foods is an undeniable proof of the significant decrease of the infestation level of the bees during the wintering season, an element that should be highly considered if the survival of the honeybees passing wintering period is wanted.

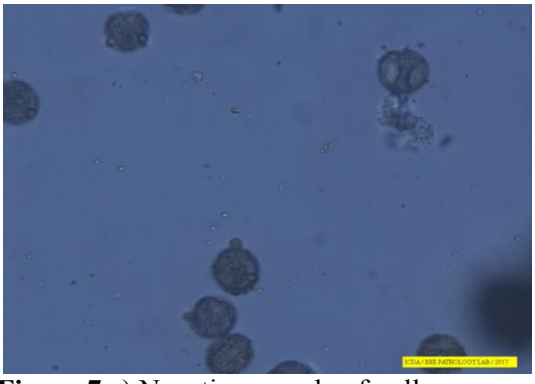
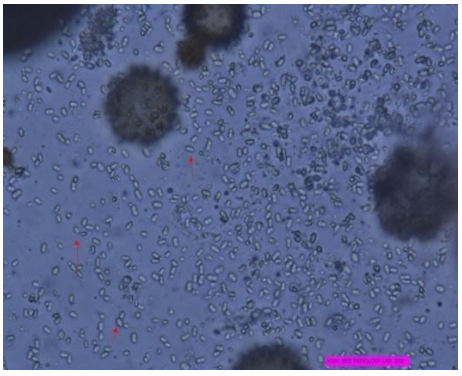


Figure 7 a) Negative sample of pollen sample (0 spores of *Ascosphera apis*./field)



b) Massive (high) infestation (> 5 spores of *Ascosphera apis*./field)

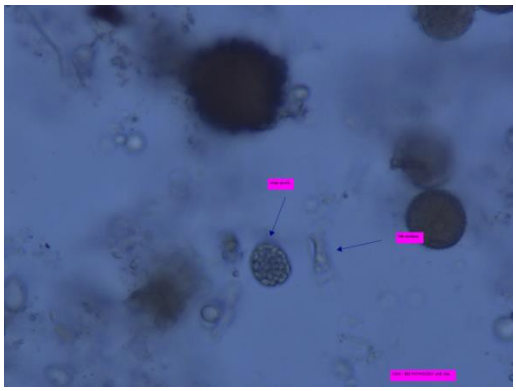
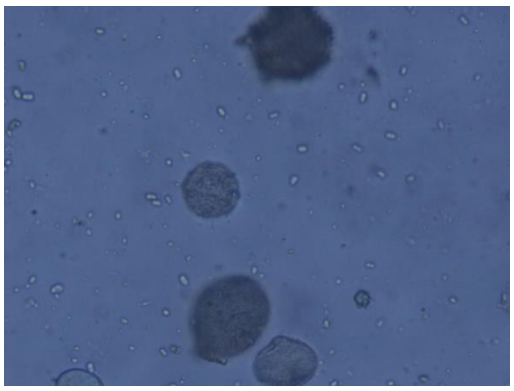



Figure 8. Figure showing: a) *A. apis* spore cyst (asoma)
b) mature ascospores (*Ascosphera apis*)

Conclusions

Out of a total number of 46 examined samples of pollen and *pollen supplimentary foods*, a number of 20 samples (43.5%) were positive for the presence of *Ascosphera apis* spores.

The infestation level with *Ascosphera apis* spores of the examined samples ranged from 1-2spores/microscopic field to more than 5 spores/microscopic field.

Most of the *Ascosphera apis* spores positive samples were represented by the rape pollen and polifloral pollen and pollen supplimentary foods samples.

The microscopic tests carried out on honeybees at the end of the beekeeping season (autumn) as compared to the honeybees that were checked at the beginning of the next beekeeping season (early spring), proved a significant decrease for the infestation level, 28.5% respectively, following the removal of the contaminated food.

The microscopic examination of pollen and pollen supplimentary foods for thepresence of the *Ascosphera apis* spores is an important prophylactic step against the infestation of thehoneybees during the wintering period.

The examination of the presence of *Ascosphera apis* spores in the food reserves beforewintering makes it possible toremove from consumption the reinfestation sources, afact which significantly decreases the morbidity and the mortality rate among honeybees.

Acknowledgements

„Preliminary results of PhD thesis".

References

1. ACA România, 2013. *Ghid de bune practici în Apicultură* Editura LVS Crepuscul, Ploieşti, Prahova. ISBN 978-973-7680-99-0.
2. Albo GN, Córdoba SB, Reynaldi FJ, 2017. *Chalkbrood: pathogenesis and the interaction with honeybee defenses,* International Journal of Environmental & Agriculture Research (IJOEAR) ISSN:[2454-1850] [Vol-3, Issue-1
3. Stelian A. Asiminei, autor coordonator, 2016 – *Patologia albinei melifere*. Editura Ion Ionescu de la Brad, Iasi 119-120
4. Annette BruunJensen1, Kathrine Aronstein, José Manuel Flores, SvjetlanaVojvodic, María Alejandra Palacio and Marla Spivak, 2013. *Standard methods for fungal brood disease research*, Journal of Apicultural Research 52(1): DOI 10.3896/IBRA.1.52.1.13

5. Aronstein and Murray, 2010. *Chalkbrood disease in honey bees*, J. Invertebr. Pathol., *Journal of Invertebrate Pathology*, 103 S20-S29, www.elsevier.com
6. B. Jensen, K. Aronstein, J. M. Flores, S. Vojvodic, M. A. Palacio and M. Spivak, 2013. "*Standard methods for fungal brood disease research",* J. Apicul. Res., vol. 52, pp. 1-20
7. Chorbiński P., 2004. *The Development of The Infection of Apis mellifera Larvae* By *Ascosphaera Apis*, Ejpau, 2004, Volume 7, Issue 2, Veterinary Medicine
8. Encarna Garrido-Bailón, et.al, 2013. *The prevalence of the honeybee brood pathogens Ascosphaeraapis, Paenibacillus larvae and Melissococcus plutonius in Spanish apiaries determined with a new multiplex PCRassay*, MicrobBiotechnol. 2013 Nov; 6(6): 731–739. Published online 2013 Aug 6. doi: 10.1111/1751-7915.12070
9. Flores J.M., Ruiz J.A., Ruz J.M., Puerta F., Bustos M., Padilla F., Campano F.1996. *Effect of temperature and humidity of sealed brood on chalkbrood development under controled conditions. Apidologie*, 27, 185-192.
10. Human H, Brodschneider R, Dietemann V, Dively G, Ellis J, Forsgren E, et al.2013. *Miscellaneous standard methods for Apis mellifera research*. In: Dietemann V, Ellis JD, Neumann P, editors. The COLOSS BEEBOOK, Volume I: standard methods for Apismellifera research. Journal of Apicultural Research, 52.
11. J. A. Yoder, A. J. Jajack, A. E. Rosselot, T. J. Smith, M. C. Yerke and D. Sammataro, 2013. "*Fungicide contamination reduces beneficial fungi in bee bread based on an area-wide field study in honey bee, Apis mellifera, colonies"*, J. Toxicol. Environ. Health A, vol. 76(10), pp. 587-600,
12. J. Danihlík, K. Aronstein and M. Petřivalský, 2015. "*Antimicrobial peptides: a key component of honey bee innate immunity*", J. Apicul. Res., vol. 54, pp. 123-136
13. Jensen AB, Aronstein K, Flores JM, Vojvodic S, Palacio MA, Spivak M.2013. *Standard methods for fungal brood disease research. J. Apic.* Res.52 (1):39
14. Kirsten Foley, 2013. *The ecology and evolution of Aspergillus spp. Fungal parasites in honey bees*, The University of Leeds, School of biology
15. MileaFlorentin Gheorghe, Ion RADOI, Agripina ŞAPCALIU,Vasilică SAVU, Ovidiu POPA – "A*scospherosis incidence in bees investigated for major bacteriosis in the beekeeping year 2016",*Scientific Works, Series C, vol. LIX (4), *2017,* ISSN 2065-1295, ISSN Online 2067-3663, ISSN I. 2065-1295, Veterinary Medicine *Bucuresti*
16. Nichita Ileana, 2007. Bulletin Usamv-Cj, 64 (1-2). Researches Regarding The Chalkbrood Disease (Ascosphaera Apis) Of The Honey Bees, Bulletin USAMV-CN, 64/2007 (1-2)
17. Ogradă I.,1986. *Bolile şi dăunătorii albineor*, Asociaţia Crescătorilor de albine din Romania, Redacţia publicaţiilor apicole, Ediţia a III a , Revizuită şi actualizată
18. OIE (World Organisation for Animal Health), 2009. Manual of *Diagnostic Tests and Vaccines for Terestrial Animals (mammals, birds and bees)*, vol.1, 2, ed. 25th Edition 2016
19. Sarah A. Maxfield-Taylor, Alija B. Mujic, Sujaya Rao,2015. First Detection of the Larval Chalkbrood Disease Pathogen *Ascosphaera apis* (Ascomycota: Eurotiomycetes: Ascosphaerales) in Adult Bumble Bees, https://doi.org/10.1371/journal.pone.0124868
20. Savu V., Agripina Şapcaliu, 2013. *Patologia albinelor*. Editura Fundaţiei România de Mâine.Bucureşti. ISBN 978-973-163-951-2. 31-38.
21. Wubie AJ, Xu S, Hu Y, Li W, Guo Z, Xue F, et al. 2013. *Current developments in Ascosphaera apis (Maasen ex Claussen) L. S. Olive &Spiltoir the causative agent of chalkbrood disease in honeybees (Hymenoptera: Apidae).* J Food Agric Enviro.; 11: 2190–2200
22. www.apimondia.org (2017)
23. www.oie.org (2017)