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STUDIES ON THE MORPHOLOGY OF STRAINS *ASCOSPHAERA APIS* ISOLATED FROM CHALKBROOD DISEASE OF THE HONEY BEES

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ABSTRACT

The *Ascosphaera* genus (*Plectomycetes: Ascosphaeraceae*) is found in the environment solely in populations of social and solitary bees. A typical feature of the genus *Ascosphaera* is production of spherical spore cysts abundant in ascospores forming spore balls. The species vary in the size of spore cysts, as well as in the shape and size of ascospores. The aim of this study was to determine morphological properties of strains *A. apis* isolated from field cases of chalkbrood diseased honeybees. The study material was inoculated onto Sabouarda's medium, underwent macroscopical and microscopical identification and was examined for morphological details by cryo-electron microscopy with a scanning electron microscope. The examination results and measurements obtained with the scanning microscope enable us to confirm the results received by means of commonly applied diagnostic techniques to determine whether the strains under study belong to the genus *Ascosphaera* and to the species *Ascosphaera apis*. Additionally, they prove that scanning microscopy can be useful in determining *Ascosphaera* species.

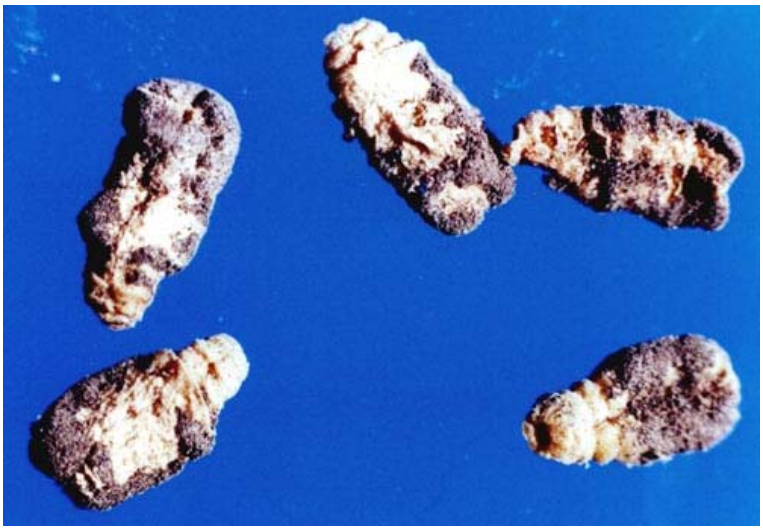
Key words: *Ascosphaera apis,* honey bee, brood cells, scanning electron microscope.

INTRODUCTION

The *Ascosphaera* genus (*Plectomycetes: Ascosphaerales*) is found in the environment solely in populations of social and solitary bees. So far, 21 species have been classified as the genus *Ascosphaera*, and the majority of them occurs in association with solitary bees. Four species *Ascosphaera apis* (chalkbrood disease), *Ascosphaera major, Ascosphaera atra, A. duoformis,* occur in honeybee colonies [1]. A typical feature of the genus *Ascosphaera* is production of spherical spore cysts abundant in ascospores forming spore balls. The species vary in the size of spore cysts, as well as in the shape and size of ascospores [3].

Ascosphaera apis (Maassen ex Claussen) Spiltoir and Olive (1955) is a heterothallic fungus causing a dangerous disease - chalkbrood of honey bee *Apis mellifera* L. larvae. The development and course of the disease in bee colonies vary as they are affected by many factors: infectiousness, individual immunity of bee colony, genetic potential of queen, environmental conditions, etc. [8, 10].

Fig.1. Honeybee larvae with chalkbrood disease, x 4



The brood cells are infected with *Ascosphaera apis* through the alimentary system. In the intestine of a larva, ascospores germinate and develop fungi. After the fungus has invaded the larva body, it defeats the immunological barrier of the body cover, spreading gradually a white mycelium over the whole of larva body. Along with the appearance of spore cysts, the mycelium turns dark, its coloration being irregular, and the dead larva becomes mummified (Fig.1). The course of the fungus invasion, as observed, can differ considerably, which is connected, on the one hand, the with seasonal growth of bee colony (changes in population proportions e.g. brood cells), the amount of food being collected and the free space in comb for brood rearing. On the other hand, it has also to do with the pathogen properties, e.g. the occurrence of fungus strains, which do not produce spore cysts and only develop light-coloured “mummies” [9].

MATERIAL AND METHODS

Laboratory identification of *A. apis* is quite easy provided that one succeeds in growing strains producing spore cysts. However, strain isolation is often a hard task, due to mixed fungal infections as well as occurrence of strains, which do not produce typical spore cysts. The great help in laboratory diagnose is not only morphological knowledge of examined genus of fungus but also ability to determine its biochemical functions (properties) with enzymatic activities. Enzymatic activity is closely related to the invasiveness and infectiousness of *A. apis* and thus it is good a fine diagnostic factor [4, 5, 6].

The aim of this study was to determine morphological properties of strains *A. apis* isolated from field cases of chalkbrood diseased honeybees. The examination involved ten strains: A1, A4, A 36, A 39, A 40, A 41, A 46, A 58, A 61, A 71, collected in Poland in 2001 - 2002. The study material consisted of dead bee larvae (mummies), which were inoculated onto Sabouarda’s medium (SDA-YE) with 0.2% yeast extract and 0.1% of chloramphenicol (BioMerieux, France) and incubated at 25 °C according Glinski’s procedure. [7]. Obtained pure cultures of the strains underwent first macroscopically and microscopically identification following their morphological traits, growth and ascospores production. For further examination strains producing ascospores and showing no pleomorphism properties in the succeeding inoculations were selected.

The isolates were examined for morphological details by cryo-electron microscopy with a scanning electron microscope (SEM, type LEO 435 VP). The samples were frozen in liquid nitrogen, and then ice crystals were removed. Next, they were coated with gold and observed at a cathodic voltage of 15 and 20 kV. Three-dimensional images of mycelia, spore cysts, spore balls and ascospores were compared with model description of *A. apis*. Also, morphometric measurements of the above mentioned anatomical elements were taken and spore cysts were analysed for their morphological structure. The measurements were analysed and compared with the species model after Anderson and Gibson, Bissett et al. [1, 3].

RESULTS AND DISCUSSION

In cultures grown on SDA-YE at 25 °C an average or moderately slow development was observed between 2 days and 8 days, as well as 4 and 9 days after inoculation. The colony diameter, after 8-day ascospores germination, was 5-8 cm, and ascospores growth was visible on 7-8 days after inoculation. The mycelium was white to light salmon-pink, compact or slightly floccose, aerial, mostly glossy, covered with droplets of "exudate" on the hyphae. While the spore cysts were produced, darkening or sometimes secreting a red stain into the medium, and a typical odour, were noticed.

Fig.2. Hyphae and spore cysts. SEM x 473

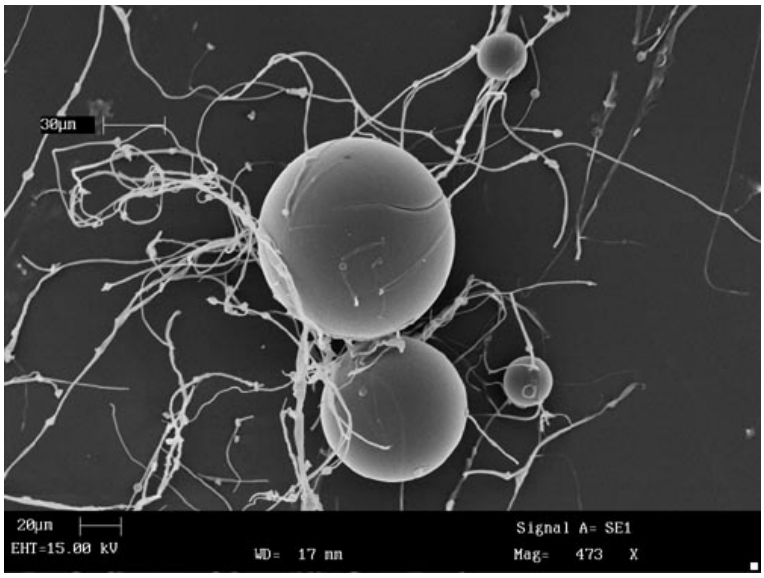
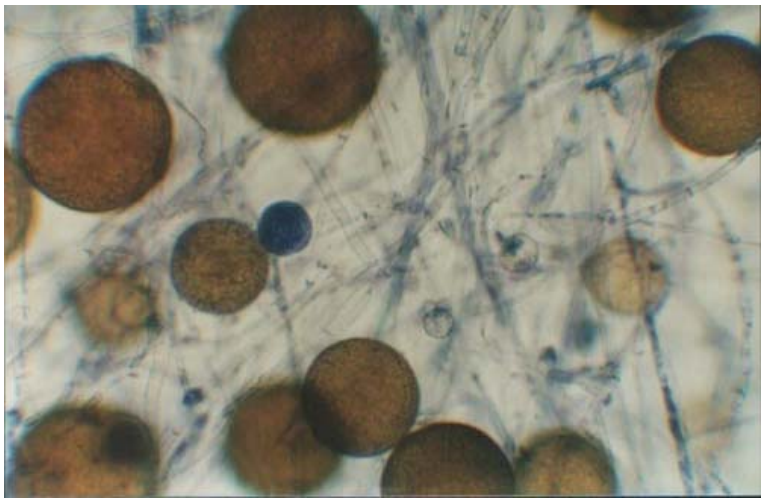


Fig.3. Spore ball, LM, bright field, x 350



In SEM images, the mycelium hyphae revealed smooth walls, with distinct septa (Fig. 5). The diameter of the hyphae differed considerably, from 2 to 7 µm, 5.1 µm on the average. Branching of the hyphae was rather scarce, sometimes dichotomous. The spore cysts were external or in aerial mycelium, plentiful, globose, the

diameter being 35-118 µm (84.5 &µm on the average) (Tab.1), dark brown or red, olivaceous shade in transmitted light (Figs. 2, 3). The wall of the ascomata was two-layered with a smooth outer surface (Figs. 4, 5, 6, 7). The inner wall was highly verrucate (Figs. 8, 9). Spore balls were full of ascospores, oval, yellow-green, with a diameter of 7-18 µm (12.5 µm on the average) (Tab.1) (Figs. 8, 10, 11). Ascospores are often ellipsoidal or fusiform, often bent, 2.7-3.5 µm long (2.9 µm on the average), with both ends rounded (Fig. 12).

Fig.4. Mature mycelia of *A. apis* with numerous spore cysts. SEM x 177

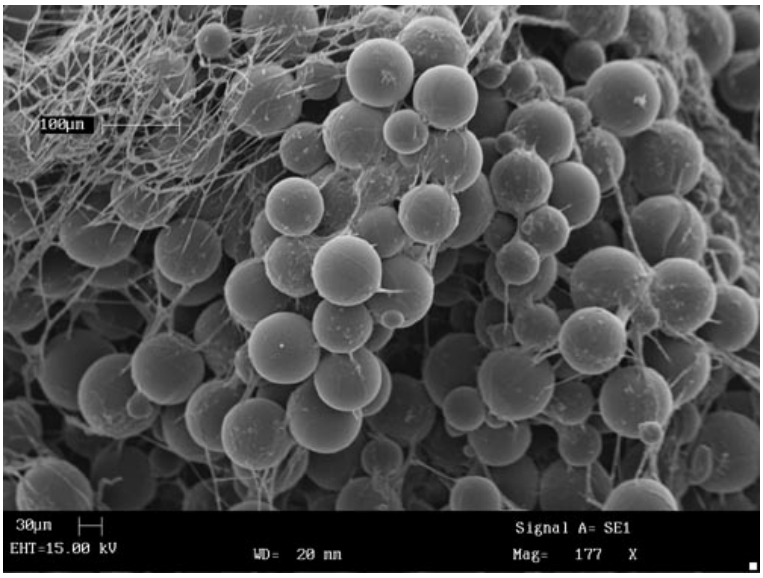


Fig.5. Mycelium from *A. apis* culture with spore cysts. SEM x 481

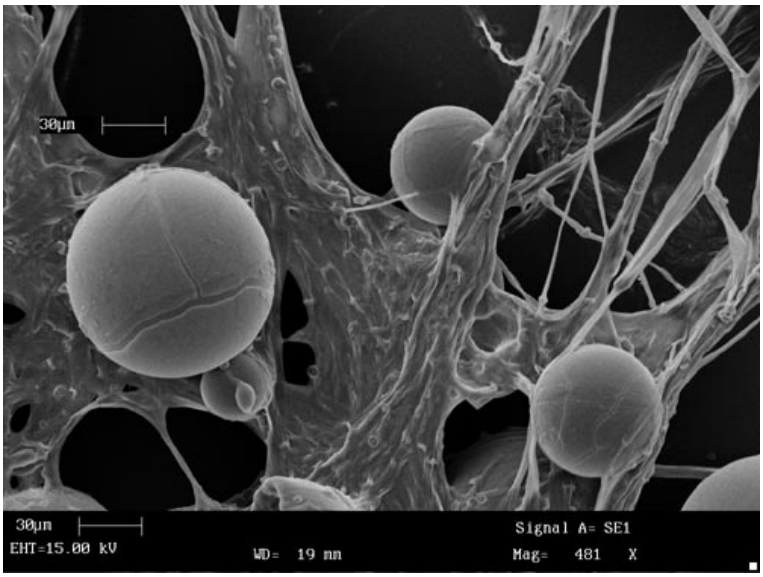


Fig.6. Single spore cyst. SEM x 1600

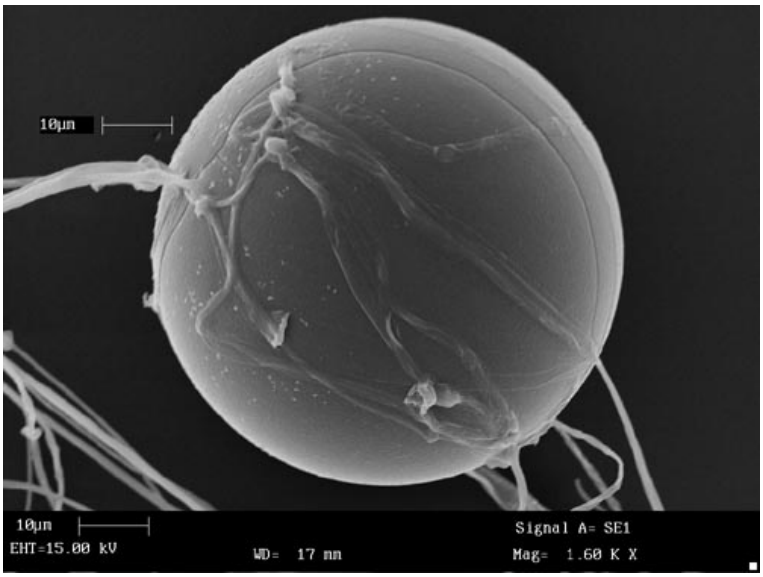


Fig.7. Young, formed spore cyst. SEM x 2260

Fig.8. Spore balls in cracked spore cyst. SEM x 1900

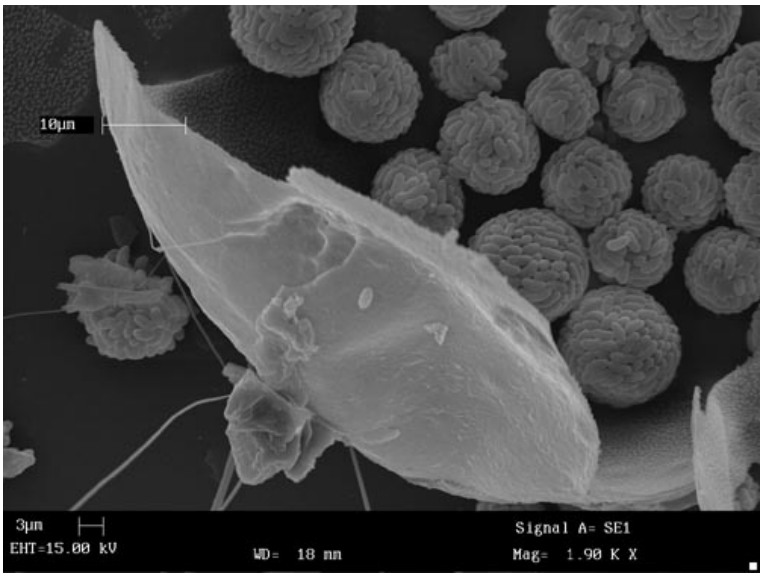


Fig.9. A typical verrucate inner wall of spore cyst. SEM x 5980

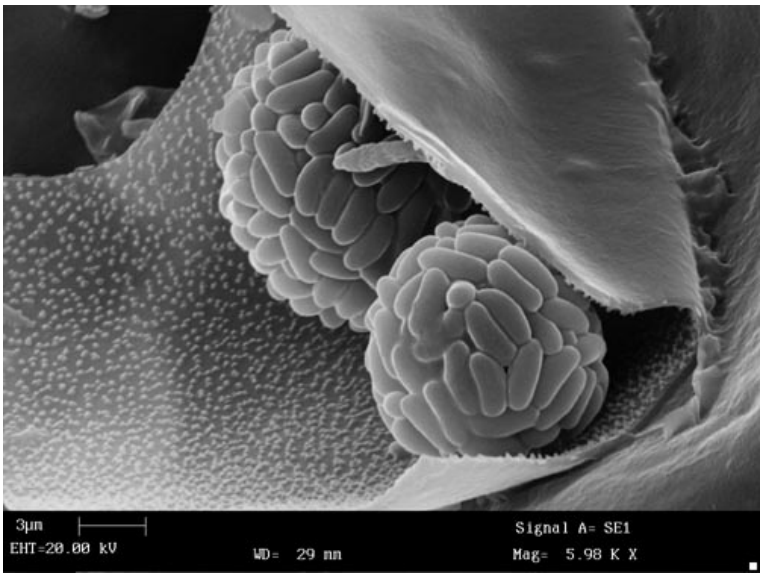


Fig.10. Spore balls. SEM x 3460

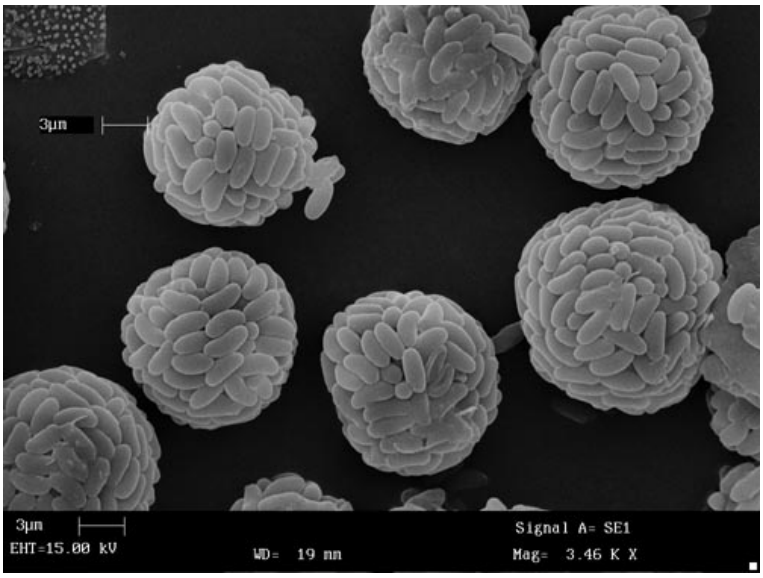


Fig.11. Single spore ball. SEM x 9000

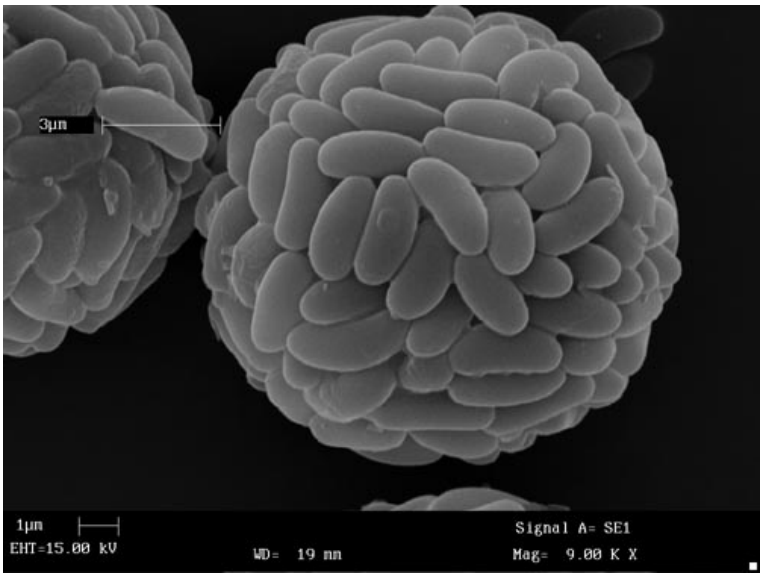


Fig.12. Ascospores. SEM x 10400

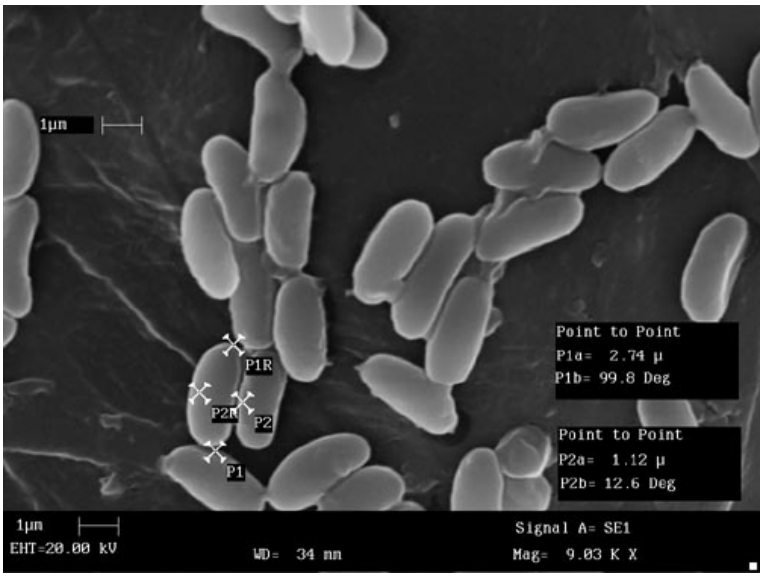


Table 1. Comparison of morphological traits in *Ascosphaera apis* strains

Strains	Spore cysts Diameter (µm)	Spore balls Diameter (µm)	Ascospores		Spore L:W Ratio
			Lengh (L) (µm)	Width (W) (µm)	
According to Anderson, Gibson (1998)	45.0-80.2-119.0	7.0-12.5-18.0	2.0-2.7-3.5	1.0-1.4-2.0	1.90
According to Bissett (1987)	40.0-70.0-110.0	7.0-12.0-20.0	2.1-2.9-3.9	1.1-1.4-1.7	2.04
Own material	35.0-84.5-118.0	6.8-12.1-15.3	2.7-2.9-3.5	1.1-1.5-1.8	1.92

Comparising morphological structures of ten examined stains *A.apis* shows that average dimensions of the spore cysts (84.5 µm) is bigger than Danish (80.2 µm) [1], American and Canadian (70.0 µm) (Tab.1) [2]. Diameters of spore balls of Polish strains (12.1 µm) seemed to be similar to the compared values obtained from Danish and North America strains (12.0 – 12.5 µm). Dimensions of the spore were also similar and counted L:W ratio of examined strains was 1.92 which shows that they are much more like European that North America strains (Tab.1). It should be said that fungus *A.apis* was transmitted to the New World with transferred honeybees, and primary comes from Europe.

Examination results and obtained values with the SEM allow confirming the results received by means of commonly applied diagnostic techniques. The results fully ensured that strains under study belong to the genus *Ascosphaera* and to the species *Ascosphaera apis*. In addition, they prove that scanning microscopy can be useful in determining *Ascosphaera* species.

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