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Zdzisław GLIŃSKI, Marek CHMIELEWSKI

Effects of Environmental Conditions on *Ascosphaera apis*. I. The Effect of Temperature and Relative Humidity on Growth and Sporulation of *A. apis*

Wpływ czynników środowiskowych na *Ascosphaera apis*. I. Wpływ temperatury i wilgotności względnej na wzrost i zarodnikowanie *A. apis*

Влияние среды на *Ascosphaera apis*. I. Влияние температуры и относительной влажности на рост и спорообразование *A. apis*

Gilliam et al., (1) and Gliński (2) have demonstrated that *Ascosphaera apis*, the causative agent of chalk brood disease of honey bee larvae causes death and replicates in various tissues of larvae under field and laboratory conditions, and that the fungus may remain viable in the honey bee larvae without causing disease. Population stress and unproper environmental conditions influencing both the host and the pathogen may start and enhance germination and growth of the fungus. As the result the disease reappears. In *A. apis* like in many entomophthoraceous fungi the typical mode of infection is through contact or ingestion, and subsequent penetration of the insect body by germinating spores (10). Besides intrinsic factors the efficacy of the pathogen largely depends on environmental conditions, among them relative humidity and temperature, which may greatly influence not only the viability and germination of spores but also their growth and sporulation.

Previous observations showed that *A. apis* sporulates easily on artificial solid and liquid media at 18°C and 25°C and that in appropriate medium good growth of mycelium can be obtained (6, 7). However, some investigators have suggested the need for favourable humidity and temperature for the successful infection of honey bee larvae by this pathogen (1, 4). The studies of Hale and Menapace (3) revealed that the disease agent stored dry or with pollen remained viable for at least 12 months regardless of storage temperature. Unfortunately, further experiments on the effects of environmental conditions on conidial germination, mycelial growth and sporulation of *A. apis* have not been performed. Therefore, experiments were undertaken to study the effect of temperature and relative humidity on growth and sporulation of several strains of *A. apis*. The results are described in the present paper.

MATERIAL AND METHODS

Strains. The investigations were carried out with 8 fertile strains of *A. apis* isolated from chalk brood diseased honey bee larvae in various regions of Poland. The strains were identified as *Ascosphaera apis* on the basis of morphological features, cultural characteristics and biochemical properties (6, 7). The isolates were maintained at 4°C on Sabouraud's dextrose agar medium plus 0.5% yeast extract (SDY, *pH* 7.2). This medium was also used for the estimation of mycelial growth and sporulation of the strains examined.

Humidity chamber. The humidity chamber consisted of metal containers which were capable of being tightly sealed with a glass closure. The stands positio-

Tab. 1. The effect of relative humidity on mycelial growth and sporulation of *Ascosphaera apis*

Wpływ wilgotności względnej na wzrost mycelium i zarodnikowanie *Ascosphaera apis*

No of strain	RH%	Growth after /h/ 24	36	48	60	Diameter of colony /mm/ 60h	at sporulation	Sporulation after /h/ 72	84	96	120
1	100		+			12	41				+
	98		+			14	42				+
	96		+			19	21	+			
	92.5		+			16	43				+
	89		+			15	40				+
	85			+		12	35			+	
	80				+	11	35				+
	75.5				+	11	35				+
2	100	+				22	42				+
	98		+			30	21			+	
	96		+			19	30			+	
	92.5		+			30	43			+	
	89		+			27	41				+
	85			+		22	40			+	
	80				+	22	41				+
3	100		+			12	43				+
	98		+			13	41				+
	96		+			12	43				+
	92.5		+			15	21			+	
	89		+			19	40				+
	85			+		12	40			+	
	80				+	11	40				+
4	100		+			9	42				+
	98		+			13	41				+
	96		+			14	41				+
	92.5		+			12	42				+
	89			+		13	40			+	
	85			+		10	14				
	80				+	9	14				+

ned upright within the chamber supported the glass slides covered by the growth medium. The relative humidities in the chambers were chemically controlled through the use of saturated solutions of chemicals according to Winston and Bates (9). The quantity of the appropriate salt solution was approximately 3 mm below the glass slide. Ten different solutions of chemicals were prepared to give the following relative humidities at 25°C: 100%, 98%, 96%, 92.5%, 89%, 85%, 80%, 75.5%, 71% and 55%.

Determination of the influence of relative humidities on mycelial growth and sporulation of *A. apis*. 0.1 ml of physiological salt saline suspended spores (about 5×10^4 spores per one ml) was plated on SDY agar coated slides. Spores before suspending in physiological salt saline were washed twice with a sterile distilled water. The culture slides after inoculation were inserted into the chamber on the stands, and then the chamber was sealed. In all tests humidity chambers were incubated at 25°C in darkness for 9 days. Time of the appearing of growth and sporulation were determined every 12 hrs for 9 days by direct and microscopic examinations (100×). The diameter of colonies was measured twice: at 60 hrs and after the appearing of spore cysts. All experiments were performed three times, and in the results the mean values from three observations are presented.

5	100	+		18	42		+
	98	+		17	43		+
	96	+		20	23	+	
	92.5	+		15	35	+	
	89		+	15	37		+
	85		+	14	42		+
	80		+	15	30		+
6	100	+		18	43		+
	98	+		16	43		+
	96	+		20	21	+	
	92.5	+		20	42		+
	89	+		20	41		+
	85		+	16	36		+
	80		+	15	36		+
7	100	+		13	41		+
	98	+		20	20	+	
	96	+		16	43	+	
	92.5	+		14	41	+	
	89	+		17	41		+
	85		+	14	40		+
	80		+	20	40		+
	75.5		+	13	42		+
8	100	+		12	43		+
	98	+		18	21	+	
	96	+		17	41	+	
	92.5	+		17	41	+	
	89		+	17	42		+
	85		+	12	43		+
	80		+	12	40		+
	75.5		+	12	40		+

RESULTS

The effect of relative humidities on appearing of mycelial growth, its abundance and time of sporulation are presented in Table 1. It is obvious that there is a clear correlation between relative humidity at 25°C and time is which the mycelial growth appears. As a rule, the growth in relative humidities at the range 100—92.5% is noted after 36 hrs since the time of inoculation of spores on SDY solid medium. It should be noted that the decrease of relative humidity retarded the germination process and appearing of mycelial growth of the fungus. Humidity of 71% or lower, did not permit mycelial growth. It was generally found that after 60 hrs of incubation in 96% relative humidity and 92.5% relative humidity the mycelial growth of *A. apis* was more abundant than that in higher and lower humidities. However, after 120 hrs of incubation more abundant growth was observed in higher that in lower humidities.

Moist conditions were also necessary for production of spore balls, fruiting bodies and sporulation. From 100% relative humidity to 75.5% relative humidity spores in spore balls developed within 72—120 h. In some strains sporulation required at least 84 hrs in 98%—92.5% relative humidities. Only two strains (no 1 and no 6) sporulated after 72 hrs of incubation at 96% relative humidity. However, the time of sporulation was not related to the time of appearing of growth. Retardation or even lack of growth at 75.5 relative humidity or below may explain some phenomena noted in pathogenesis of chalk brood disease in honey bee larvae.

DISCUSSION

The efficacy of entomopathogenic fungi in the field largely depends not only on internal factors of the host organism but also on environmental conditions. Especially relative humidity and extreme temperatures may greatly influence the infection, distribution of fungi and their persistence in nature (5). Therefore, numerous investigators suggested the need for favorable humidity and temperature for the successful infection of insects by pathogenic fungi (5, 10). In chalk brood disease of the honey bee larvae many factors have been postulated to contribute to the development of the disease in honey bee colonies. They include also poor ventilation of colony, high humidity and temperature and excessive hive moisture (1).

The estimation of the role of relative humidity on mycelial growth and sporulation of *A. apis*, the factors so important in the physiology of the fungus demands the appropriate methods of determinations. Among

numerous methods used for testing relative humidity in small closed spaces under controlled temperature, the method of Winston and Bates (9) is commonly recommended. It enables to obtain accurate and reproducible results. In this method saturated water salt solutions are used which enables to obtain constant relative humidities in the atmosphere over them. Furthemore, in closed containers the overgrowth of the tested fungus by saprophytes is eliminated.

The obtained results indicate that the moisture conditions suitable for good growth and sporulation of *A. apis* are at relative humidity above 92.5%. These conditions are present in honey bee colony. In soil where relative humidity is below 71% growth of the fungus is arrested. However, spores of *A. apis* may survive, then find the way into the food chain of honey bees and may be transmitted to larvae via contaminated larval food (3).

Other entomopathogenic fungi as *Beauveria bassiana, Metarrhizium anisopliae* and *Entomophthora* sp., require also high relative humidities for spore germination, mycelial growth and sporulation. Optimum germination, growth and sporulation of *B. bassiana* and *M. anisopliae* occurred at 100% relative humidity and at 25—30°C, but growth and sporulation were noted above 92.5% relative humidity at temperatures between 15 and 35°C (8, 11). Germination of *Entomophthora apiculata* and *E. virulenta* conidia was restricted to 100% relative humidity, when *E. coronata* germinated at both 100% and 95% relative humidities (10).

On the basis of the observed relationships between relative humidity, mycelial growth and sporulation of *A. apis* it is possible to explain why mycelial growth and sporulation of the fungus is arrested until the larva is sealed in the cell. After sealing, relative humidity inside the sealed cell with sick larvae increases almost up to 100%, and it is maintained on high level also after larval death. Lack of growth of *A. apis* mycelium on dead larvae which were infected after their death is probably also influenced by a low relative humidity in sealed cells containing these larvae. In this case mummification of larval body did not appear. It is also possible that some role in this process plays an environmental temperature. The relationhips between environmental temperatures, relative humidities and germination process of *A. apis* are presented in the second part of the article.

CONCLUSIONS

1. The moisture conditions suitable for good growth and sporulation of *Ascosphaera apis* are at relative humidity above 92.5% at 25°C. My-

celial growth is arrested and sporulation is absent at 71% relative humidity and below at 25°C.

2. The relationships between relative humidity, mycelial growth and sporulation of *A. apis* enable to explain some phenomena observed in the course of chalk brood, especially appearance of growth and sporulation of the fungus in infected brood after sealing the comb cells, retardation of lack of growth of the fungus in larvae which have been infected after death.

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STRESZCZENIE

W oparciu o 8 szczepów „płodnych" *Ascosphaera apis*, które wyizolowano z czerwia pszczoły miodnej chorego na grzybicę otorbielakową i metodę Winstona i Batesa uzyskiwania wilgotności względnej w ściśle określonych temperaturach wykazano, że obfity wzrost i zarodnikowanie *A. apis* ma miejsce przy wilgotności względnej 100—92,5% w 25°C. Wilgotność względna 71% i niższa w 25°C hamuje wzrost mycelium i zarodnikowanie. Zaobserwowane zależności między wilgotnością względną, wzrostem i zarodnikowaniem *A. apis* mogą przynajmniej w części wyjaśniać niektóre zjawiska występujące w przebiegu grzybicy otorbielakowej, zwła-

szcza przerastanie ciała chorego i martwego czerwia grzybnią dopiero po zasklepieniu komórek plastra oraz zahamowanie lub brak wzrostu grzybni w organizmie larw, które zakażono grzybicą otorbielakową dopiero po padnięciu.

РЕЗЮМЕ

На штаммах „плодовитых" *Ascosphaera apis*, изолированных из расплода медоносной пчелы, болеющего сумчатым микозом, методом Winstona и Batesa (получение относительной влажности в точно определенных температурах) показано, что большой рост и спорообразование *A. apis* достигаются при 100%—92,5% относительной влажности и 25°C. Относительная влажность (71% и ниже) и 25°C тормозит рост mycelim и спорообразование. Замеченные зависимости между относительной влажностью и ростом и спорообразованием *A. apis* частично объясняют некоторые явления выступающие в ходе болезни сумчатого микоза, особенно проникание микоза в тело больного и мертвого расплода после заклеивания клеток соты, а также заторможение или не выступление микоза в организме личинок, которые заражены были сумчатым микозом только после смерти.