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Effects of Environmental Conditions on *Ascosphaera apis*. II. Germination of *A. apis* Spores

Wpływ warunków środowiskowych na *Ascosphaera apis*. II. Kiełkowanie zarodników *A. apis*

Влияние условий внешней среды на *Ascosphaera apis*. Часть II. Прорастание споров *A. apis*

In infections caused by entomopathogenic fungi an essential role play routes of infection, mechanisms of pathogen penetration through the host cuticle, toxic action of fungus metabolites and the ability of the pathogen to utilise the own substances of the insect organism as a source of nutritients. The time of fungal spores germination in or on the body of the host is a factor which limits the appearance of the infection, especially when the fungus attacks individual stages of insect metamorphosis. In many cases metamorphosis is ceased before germination of the fungus and therefore the infection is absent. Two of the environmental factors greatly modify the process of spore germination, relative humidity and temperature (16). They also influence the host organism, especially its resistance to infection. Temperature influences not only the time of spore germination and penetration of mycelium in the organism, but also the viability of the pathogen in the environment. By modification of biochemical processes in insect host it may stimulate, retard or even arrest the fungal infection. Relative humidity like temperature influences the germination and the development of fungal infections.

This role is clearly evident in the first steps of infection when the temperature may accelerate both germination (1, 6) and postadaptative mechanisms of the host, enhancing or decreasing the susceptilibity of the insetc to infection (7, 10).

Studies performed with *Ascosphaera apis* have already established the optimal temperature and relative humidity for growth and sporulation of this fungus (2, 3, 9, 13). However, other aspects dealing with environmental effects on *A. apis* have not been examined. Therefore, experiments were performed to study: the effect of relative humidities and various tmperatures on spore germination of *A. apis*, the influence of high temperatures at optimal humidities on the time and per cent of spore germination. The results of these experiments are presented in this article.

MATERIALS AND METHODS

Strains. The examinations were carried out with 8 fertile strains of *Ascosphaera apis* (no 1—8) isolated in Poland from chalk brood diseased honey bee larvae. They were cultured on Sabouraud's dextrose agar plus 0.5% yeast extract (SDY), *pH* 7.2. and preserved at 4°C.

Spores were obtained from 7 days old cultures of individual strains of *A. apis* on SDY agar incubated at 25°C (5).

Determining amount of germination of spores at humidity chambers at various temperatures. Microscopic slides covered with SDY agar and slides without a nutritive medium were inoculated with 0.1 ml of spore suspension in sterile physiological salt saline (5×10^4 spores/ml). Then the inoculated slides were incubated at 5, 15, 25 and 35°C at 100 and 96% relative humidities and at 25°C at 92.5% relative humidity in humidity chambers. The relative humidities in the chambers were chemically controlled through the use of saturated solutions of chemicals according to Solomon (11). Distilled water was applied for developing the 100% relative humidity, saturated water solution of KH_2PO_4 for the 96% relative humidity and saturated water solution of KNO_3 for 92.5% relative humidity. Upon removal of the slides from the humidity chambers after 24, 36, 48, 60, 72, 84 and 96 h. percent of germinating spores was determined under phasecontrast microscope (400×). For this purpose 50 spores were selected at random from each three replicates of individual strains.

High temperature experiments. The germination of spores of *A. apis* at high temperatures was tested by exposure of dessicated spores (about 5×10^4) in small glass vials at humidity chambers at 100, 76 and 33% relative humidity at 25°C for 24 h. Saturated salt solutions (NaCl for 76% relative humidity, $MgCl_2$ $6H_2O$ for 33% relative humidity and distilled water for 100% relative humidity) were used to obtain an appropriate humidity in the chamber. The glass vials after removing from humidity chambers were immediately closed with robbers and sealed with strips of parafilm and then incubated in a water bath at 40, 50, 60, 70, 80 and 90°C for 30 minutes. After exposure to heat the spores were plated on SDY agar coated slides and incubated at dark at 25°C. Per cent of spore germination was determined by phasecontrast microscopical examination (400×) of 50 spores per each treatment and each strain after 24, 36, 48, 60 and 72 h. of incubation.

RESULTS

The relationships between moisture, temperature and per cent of spore germination are presented in Tables 1, 2 and 3 in which only the extremal values of per cent of germinating spores for 8 strains of *A. apis* is done. Moreover, the time of observations in Tables is shortened to 72 h because after this time per cent of germinating spores does not increase.

Data from Table 1 reveal that at the three relative humidities examined germination of *A. apis* spores both in the nutritive solid medium and on glass is arrested at 5°C. Generally, per cent of germination increases with an increase of relative humidity at individual range of temperature.

On SDY solid medium it is maximum at 25°C at 96% relative humidity and at 35°C at 100% relative humidity.

After 72 h. of incubation germinating spores appeared at 5°C both in SDY agar medium and on glass. However, on glass some strains do

Tab. 1. Percent of spore germination of 8 strains of *A. apis* (mean values) after 36 hrs incubation

Procent kiełkowania zarodników 8 szczepów *A. apis* (wartości średnie) po 36-godzinnej inkubacji

Relative humidity %	Percent of germination on							
	SDY-agar at				glass slide at			
	5°C	15°C	25°C	35°C	5°C	15°C	25°C	35°C
100	0	25-50	75-100	100	0	0-20	10-100	10-100
96	0	20-100	100	75-100	0	0-10	10-100	5-100
92,5	-	-	50-100	-	-	-	10-50	-

— not determined; nie ustalony
○ lack of germination; brak zarodnikowania

not germinate at this temperature (Table 2). As a rule, at 25°C or above 100% germination is noted on SDY solid medium irrespectively of the relative humidities of cultivation. On glass at the range of 92.5—100% relative humidity and the temperatures examined pronounced variations in the per cent of germinating spores are noted. Comparing germination

Tab. 2. Percent of spore germination of 8 strains of *A. apis* (mean values) after 72 hrs incubation

Procent kiełkowania zarodników 8 szczepów *A. apis* (wartości średnie) po 72-godzinnej inkubacji

Relative humidity %	Percent of germination on							
	SDY-agar at				glass slide at			
	5°C	15°C	25°C	35°C	5°C	15°C	25°C	35°C
100	10-50	50-100	100	100	0-50	0-50	10-100	50-100
96	20-50	100	100	100	0-20	10-20	20-100	20-100
92,5	-	-	100	-	-	-	50-100	-

of spores both after 36 h. and after 72 h. of incubation one can conclude that in every case the number of germinating spores is higher in the nutritive medium than that on glass.

The influence of short term action of temperatures on *A. apis* spore germination at three distinct humidities is summarized in Table 3. At 100% relative humidity temperatures 30 and 40°C do not disturb the per cent of germinating spores of the fungus. Exposition of spores to

50°C or above diminishes greatly the per cent of germinating spores. Exposition of spores to 90°C for 30 minutes arrests germination at all. The action of temperatures on per cent of germinating spores is more pronounced at 76% and 33% relative humidities, where a relatively smal-

Tab. 3. Thermal death points for *A. apis* spores at various humidites and temperatures

Punkty śmierci cieplnej dla *A. apis* przy różnych wilgotnościach i temperaturach

Relative humidity %	Temperature °C	Percent of spores germination at 25°C after /h/ 24	36	48	60	72
100	30	0-50	100	100	100	100
	40	0-75	100	100	100	100
	50	0-30	20-80	20-80	20-80	20-100
	60	0	0-80	0-80	0-80	0-80
	70	0	0-60	0-60	0-60	0-60
	80	0	0-40	0-40	0-40	0-40
	90	0	0	0	0	0
76	30	0	80-100	80-100	80-100	80-100
	40	0	80-100	80-100	80-100	80-100
	50	0	0-60	0-60	0-60	0-60
	60	0	0-50	0-50	0-50	0-50
	70	0	0-30	0-30	0-30	0-30
	80	0	0-10	0-10	0-10	0-10
	90	0	0	0	0	0
33	30	0	75-100	75-100	75-100	75-100
	40	0	75-100	75-100	75-100	75-100
	50	0	0-60	0-100	0-100	0-100
	60	0	0-30	0-100	0-100	0-100
	70	0	0-25	0-30	0-30	0-30
	80	0	0	0	0	0
	90	0	0	0	0	0

ler number of spores germinate or even germination is arrested. The obtained results point to the thermal death point which for *A. apis* spores at 100% and 76% relative humidities is 90°C, but at 33% relative humidity it is at 80°C.

DISCUSSION

Although many authors have demonstrated that the ability of *A. apis* to develop and sporulate on appropriate solid and liquid media (2, 4, 5, 14), the role of both temperatures and relative humidities in spore germination of this honey bee larvae pahtogen has not been quantitatively examined. The presented studies performed under strictly controlled conditions reveal that the moisture and temperature requirements of *A. apis* spores to germinate are similar to conditions that prevail when brood of honey bee is reared in natural conditions in summer. At this time the moisture and temperature of developing brood provide optimum conditions for spore germination, and hence chalk brood disease reaches its maximum development in our country (2, 12). The development and

metamorphosis of honey bee larvae is the most active when larvae develop at 35°C and at 100% relative humidity at the first 24 h, and then at 96% relative humidity at the same temperature. When the larvae pupate the relative humidity decreases from 96% to 80%. Also the filed observations on the course of chalk brood disease are in a close agreement with our observations. The most susceptible to infection caused by *A. apis* are larvae at the age of 3—6 days, prepupae and pupae are more resistant (2, 4, 5).

The fact that any decrease of the temperature of incubation of *A. apis* spores at the range of 25—35°C does not influence negatively the per cent of germinating spores, and in the contrary it influences negatively the growth and development of honey bee brood plays some role in pathogenesis of chalk brood disease (8). Most of the chilled larvae is weak and therefore they are more susceptible to infection. Consequently, even the low inoculum of *A. apis* spores causes infection in weakened larvae. Weiss (15) in studies on the influence of temperature and relative humidity on the development of honey bee larvae showed that larvae of workers died after 72 h. at 18—24°C and at 50—75% relative humidity.

The results of the examinations of heat resistance of spores of *A. apis* under various relative humidities reveal a clear correlation between temperature and moisture conditions. They also point to a relatively great resistance of *A. apis* spores to temperature both at high and at low humidity. Even under dry conditions (33% relative humidity) the temperatures of 30—70°C are not lethal to all spores of the fungus, and the spores may survive 30 min. exposition. The thermal death points in a moisture atmosphere (at 76%—100% relative humidity) are 90°C, and at 33% relative humidity it is 80°C. The obtained values are higher than these noted by Zimmermann (17) for conidia of *Metarrhizium anisopliae*. The medium lethal temperature for 30 min of exposure is 50.5°C at 100% relative humidity, 57.5°C at 76% relative humidity and 68.8°C at 33% relative humidity. The susceptibility of *A. apis* spores to 30 min. exposition to 90°C at 100% relative humidity points to the possibility to desinfection of utensils contamined by spores of this fungus. This distinfection may be performed in boiling water.

Germination of spores not only on artificial media but also on sterile glass suggests that spores can germinate on the walls of comb cells. Therefore, the formed germs may attack the larvae inside the cells just after their hatching.

The environmental conditions in apiaries are not lethal to spores of *A. apis*. At these conditions of temperature and relative humidity the spores may survive in the soil and on the beekeeping instruments and utensils.

CONCLUSIONS

1. The best conditions for germination of spores of *Ascosphaera apis* are at 92.5%—100% relative humidity at 25°C or above. These conditions appear in developing brood of the honey bee.

2. High resistance of *A. apis* spores to temperature at various relative humidities enables their survival in apiary. Thermal death point for *A. apis* spores is 90°C at 76%—100% relative humidity, and 80°C at 33% relative humidity.

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STRESZCZENIE

W badaniach przeprowadzonych z zarodnikami 8 szczepów „płodnych" *Ascosphaera apis* określono wpływ wilgotności względnej i temperatury na kiełkowanie zarodników oraz punkt śmierci cieplnej w trzech różnych wilgotnościach względnych. Stwierdzono, że odsetek kiełkowania zarodników wzrasta wraz ze wzrostem wilgotności względnej w temperaturze 15°, 25° i 35°C. W temperaturze 25°C i powyżej kiełkuje 100% zarodników przy wilgotnościach 92,5—100%. Punkty śmierci cieplnej zarodników wynoszą 90°C przy wilgotności względnej 76—100% i 80°C przy wilgotności 33%. W oparciu o uzyskane wyniki autorzy tłumaczą pewne zjawiska obserwowane w przebiegu grzybicy otorbielakowej czerwia pszczoły miodnej.

РЕЗЮМЕ

В опыт взяли споры 8 „плодородных" штаммов *Ascosphaera apis*, определяя влияние относительной влажности и температуры на прорастание споров и точку тепловой смерти в трех разных относительных влажностях. Определено, что процент прорастания споров возрастает с ростом относительной влажности при температуре 15°, 25° и 35°C. При температуре 25°C и выше прорастает 100% спор с влажностью 92,5—100%. Точка тепловой гибели спор равняется 90°C с относительной влажностью 76—100% и 80°C с влажностью 33%. На основе полученных результатов, авторы объясняют некоторые явления, выступающие при осумоальбиносном микозе расплода медоносной пчелы.