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Temperature requirements of four entomopathogenic fungi

RYSZARD MIĘTKIEWSKI[1], CEZARY TKACZUK[1], MIECZYSŁAW ŻUREK[1], L. P. S. van der GEEST[2]

[1]Agricultural and Pedagogical University, ul.Prusa 14, 08-110 Siedlce, Poland
[2]University of Amsterdam, Department of Pure and Applied Ecology, Kriuslaan 320, 1098 SM Amsterdam, The Netherlands

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Infection potential of *Beauvaria bassiana, Metarhizium anisopliae, Paecilomyces farinosus* and *P. fumosoroseus* to *G. mellonella* larvae in pine litter was established. The growth of these fungi on the Czapek's Dox medium exposed to different temperature was determined.

INTRODUCTION

Temperature is a fundamental factor in the development and activity of all organisms. When entomopathogenic fungi are isolated from soil by means of the insect bait method, temperature has an effect on both the fungus and the insects. Miętkiewski, Żurek, Geest (1992) exposed *Tribolium destructor* larvae in soil to different temperatures and showed that *Metarhizium anisopliae* can infect larvae at temperatures between 10°C and 30°C provided that only this particular fungus is present. However, the presence of not only *M. anisopliae*, but also of *Paecilomyces fumosoroseus*, an interaction between the fungus species becomes apparent (Miętkiewski, Tkaczuk, Badowska-Czubik, 1992). *M. anisopliae* can then only infect insect larvae at temperatures between 20°C and 30°C while *P. fumosoroseus* infects larvae at temperatures ranging from 10°C to 30°C. Miętkiewski et al. (1991) found four entomopathogenic hyphomycetes (*Beauvaria bassiana* (Bals.) Vuill., *Metarhizium anisopliae* (Metsch.) Sorok., *Paecilomyces fumoroseus* and *P. farinosus* (Dicks.: Fr.) Brown et Smith. in pine litter. In the present study, the effect of temperature was determined on the infection capacity of these four fungi in pine litter as substrate was assessed. In addition, the maximum, optimum and minimum temperatures for growth of these fungi on Czapek's Dox medium was determined. The results of these two experiments are of

value for the forecast of mycoses by these fungi under field conditions. The results may also be used for the development of the insect bait method for the isolation of entomopathogenic fungi from soil.

MATERIAL AND METHODS

Forest pine litter was collected in the same location where had been observed earlier previously (M i ę t k i e w s k i et al., 1991). Petri dishes were filled with litter and 20 larvae of *Galleria mellonella* L. were added. The petri dishes with the litter and larvae were kept in incubators at seven different temperatures, ranging from 5°C to 35°C with temperature intervals of 5°C. At each temperature 10 Petri dishes were placed each with 20 larvae. The first observation were made every 3 days until day 21. Diseased and mummified larvae were recovered and, in case of absence of external fungus, surface was sterilized by submersion in 1 % sodium hypochlorite for 3 minutes, followed by rinsing with sterile water. The cadavers were then incubated in a moist chamber until the presence of pathogens could be assessed and the species identified.

Isolates of *B. bassiana*, *P. farinosus* and *P. fumorososeus* obtained from pine litter with *G. mellonella* larvae as "bait" at 15°C (isolates A) and at 25°C (isolates B) were grown on Czapek's Dox medium. Only one isolate of *M. anisopliae* obtained at 25°C was cultured. Fungal cultures were kept in incubators at the same seven different temperatures in which they had been isolated from the pine litter. The diameter of the fungus colonies was measured every 5th day until day 20.

In a subsequent experiment the optimum temperature for growth of each fungal isolate was determined. Petri dishes with the fungal cultures were placed in incubators which were set for a range of temperatures in steps of 2.5°C. The temperature range for *P. farinosus* was 17.5°C to 25°C, for *B. bassiana* and *P. fumosoroseus* 20°C to 32.5°C and for *M. anisopliae* 25°C to 37.5°C.

RESULTS

Four entomopathogenic fungi were isolated from larvae of *Galleria mellonella* using the bait method (Tab. 1). In addition, some interesting fungus species were found on larvae kept at 5°C. These larvae became covered by white mycelium after exposure to a high humidity in the moist chamber, but no sporulation occurred, even at higher temperatures.

No entomopathogenic fungus appeared at 35°C. It should be emphasized that at this temperature, a considerable number of larvae 40.3 % were infected by *Aspergillus fumigatus*, but figure of dead larvae is no indication of the entomophatogenic properties of the fungus.

The highest percentage (40 %) of larvae infected by *B. bassiana* was observed at 25°C.

Table 1

Mortality (%) of *Galleria mellonella* larvae in pine litter at different levels of temperature after 21 days of incubation

Factors of mortality	Temperature						
	5°C	10°C	15°C	20°C	25°C	30°C	35°C
Entomopathogenic fungi							
Beauvaria bassiana	–	10.7	34.0	31.1	40.0	–	–
Metarhizium anisopliae	–	–	–	–	2.8	54.0	–
Paecilomyces farinosus	–	5.3	25.2	41.3	24.3	–	–
Paecilomyces fumosoroseus	–	–	2.1	0.7	0.7	–	–
Unidentified entomopathogenic species	50.0	–	–	–	–	–	–
Total	50.0	16.0	61.3	73.1	67.8	54.0	–
Fungi of unproved entomopathogenic abilities							
Aspergillus fumigatus	–	–	–	–	–	–	40.3
Mucor sp.	–	–	–	2.1	–	1.9	–
Non-sporulated mycelium	–	0.7	–	0.7	0.7	–	4.5
Total	–	0.7	–	2.8	–	1.9	44.8
Others							
Nematodes	–	25.0	29.3	19.9	15.6	19.0	12.0
Unidentified causes	50.0	57.3	8.2	2.8	12.1	25.1	43.2
Total	50.0	82.3	37.5	22.7	27.7	44.1	55.2
Total dead specimens	100.0	99.0	98.8	98.6	96.2	100.0	100.0

P. farinosus infected larvae at the same temperatures as *B. bassiana*. The highest number of infected larvae (41.3 %) was found at 20°C. *P. fumosoroseus* seemed to be present in low abundance and was able to infect larvae only at temperatures between 15°C and 25°C. *M. anisopliae* present in litter showed the greatest temperature requirements. Maximum mortality was reached at 30°C at which temperature none of the other fungi caused infection (Table 1).

Temperature had also an effect on the time of larval mortality (Fig. 1). The first dead larvae infected by *B. bassiana* appeared after 5 days when kept in pine litter at 10°C and after 8 days maintained at 15, 20, and 25°C. Maximum larval mortality was observed on day 8 at 20°C and on day 12 at 15°C and 25°C. The first dead larvae infected by *P. farinosus* were found after 5 days at 10°C and 20°C, after 8 days at 25°C and after 12 days at 15°C. The last dead larvae were noted on day 18, within the exception of 15°C at which temperature dead larvae were found up to day 21. Maximum mortality caused by this fungus was noted at 20°C and 25°C after 12 days. At 15°C, maximum mortality was retarded up to 15 days.

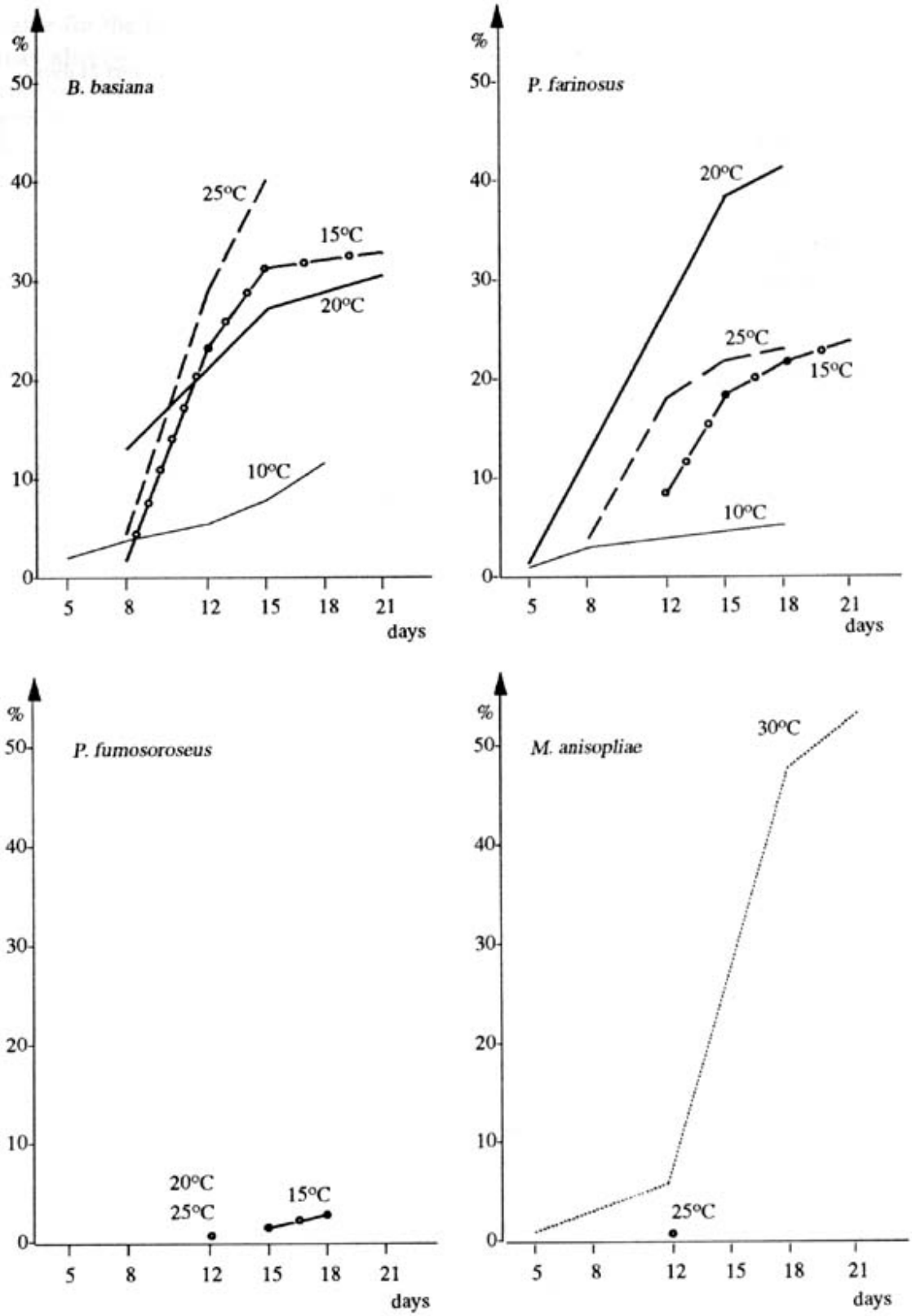

Fig. 1. Mortality of *Galleria mellonella* larvae caused by four fungi at different temperatures

The first dead larvae infected by *M. anisopliae* at 30°C were observed after 5 days and at 25°C after 12 days. The maximum number of dead larvae at 30°C was observed on day 15.

The dynamics of the larval mortality by the unidentified fungus at 5°C was peculiar: the first dead larvae were noted after 5 days and maximum mortality was reached after 8 days.

The results of the two experiments showed that there are differences in growth of the fungus species on Czapek's Dox medium at the temperatures investigated (Fig. 2-3). In the first experiment (a) the optimum growth of *P. farinosus* was at 20°C, for *B. bassiana* and *P. fumosoroseus* at 25°C and for *M. anisopliae* at 30°C. The diameter of the *M. anisopliae* colonies at 25°C was only slightly smaller and about 0.9 % of that at 30°C. It should be mentioned that in this experiment a temperature range was studied with 5°C intervals (Table 2).

In the second experiment (b) a range of temperatures was studied with intervals of 2.5°C. Again, 25°C was found to be the optimum temperature for growth of *B. bassiana* and *P. fumosoroseus*. The optimum temperatures for *P. farinosus* and *M. anisopliae* were 22.5°C and 27.5°C respectively. Colony diameter at optimum temperatures were 82.3 mm for *M. anisopliae*, 81.3 mm for *P. fumosoroseus* 69.0 mm, for *B. bassiana* and 65.6 mm for *P. farinosus*.

Table 2

Diameter of fungal colonies on Czapek's Dox medium at different temperatures (given in % maximum colonies at the optimum temperature)

Isolate	Temperature (°C)											
	5°C	10°C	15°C	17.5°C	20°C	22.5°C	25°C	27.5°C	30°C	32.5°C	35°C	37.5°C
	Beauveria bassiana											
A	11.0	37.6	61.8	–	82.0	87.8	100 (67.3)*	96.7	87.7	ng	–	–
B	8.5	37.8	63.6	–	86.7	99.2	100 (69.0)*	96.6	82.0	ng	–	–
$\bar{x}$	9.7	37.7	62.8	–	84.1	94.1	100 (70.7)*	96.7	84.8	ng	–	–
	Paecilomyces farinosus											
A	19.4	40.9	74.2	84.7	95.6	100 (64.0)*	77.5	54.7	32.2	ng	–	–
B	17.9	35.6	69.8	77.2	88.7	100 (65.6)*	83.2	58.0	29.8	ng	–	–
$\bar{x}$	18.6	38.2	72.0	80.7	92.1	100 (67.2)*	80.4	56.4	30.9	ng	–	–
	Paecilomyces fumosoroseus											
A	2.3	19.0	47.6	–	73.0	92.7	100 (82.0)*	84.5	59.5	ng	–	–
B	2.4	19.9	48.6	–	74.2	99.0	100 (81.3)*	85.4	52.1	ng	–	–
$\bar{x}$	2.3	19.4	48.1	–	73.6	95.8	100 (80.6)*	84.9	55.8	ng	–	–
	Metarhizium anisopliae											
B	0.7	2.3	24.5	–	54.4	77.7	89.7	100 (82.3)*	90.6	67.3	39.1 %	ng

Values in bracket indicate the colony diameter (mm) at the optimum temperature for growth of each fungus.

* – mm; ng – no growth; isolate A – obtained from pine litter at 15°C; isolate B – obtained from pine litter at 25°C

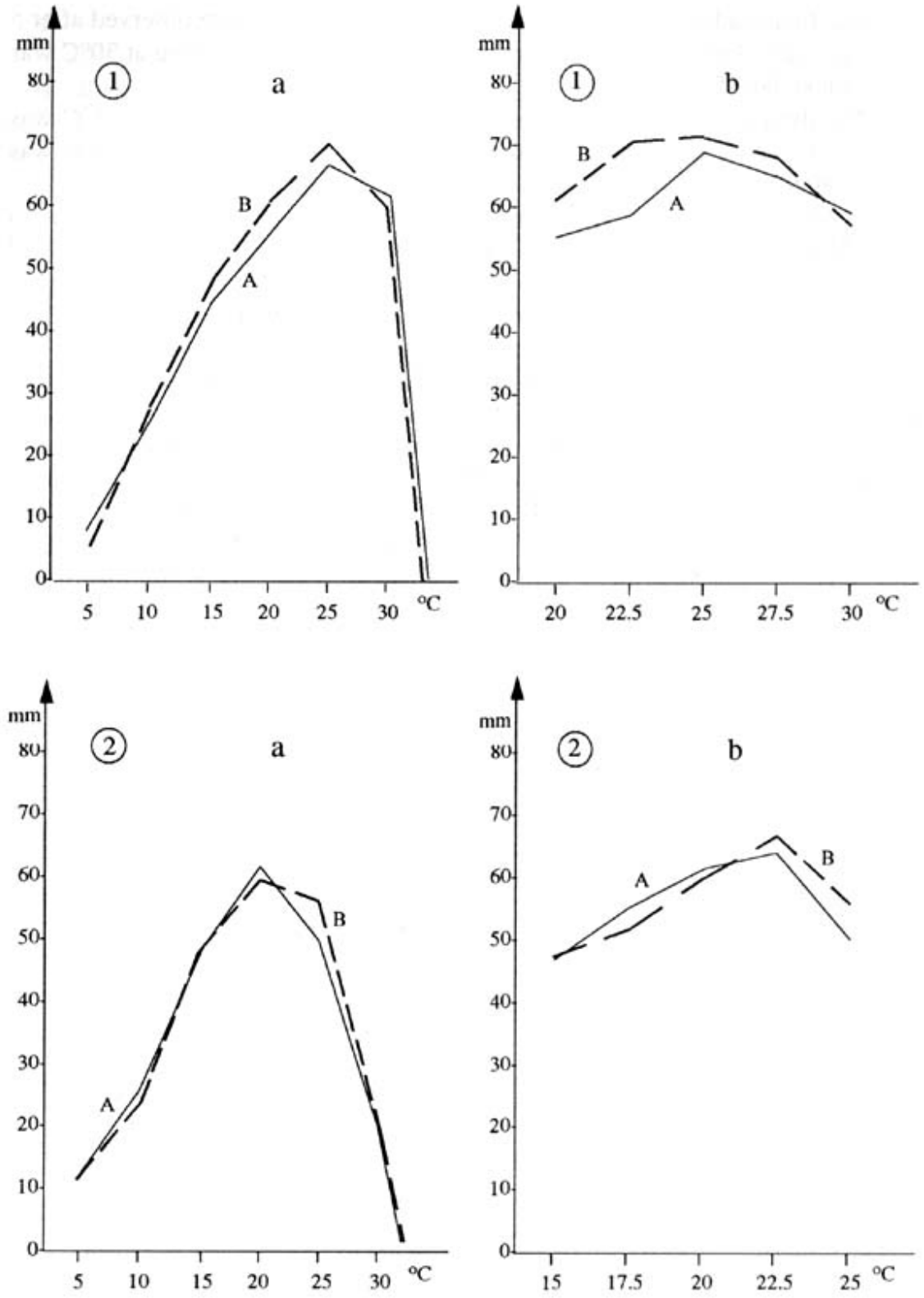

Fig. 2. Diameter of *Beauveria bassiana* (1) and *Paecilomyces farinosus* (2) colonies of Czapek's Dox medium at different temperatures after 20 days
A – isolate from 15°C, B – isolate from 25°C; a – experiment I, b – experiment II

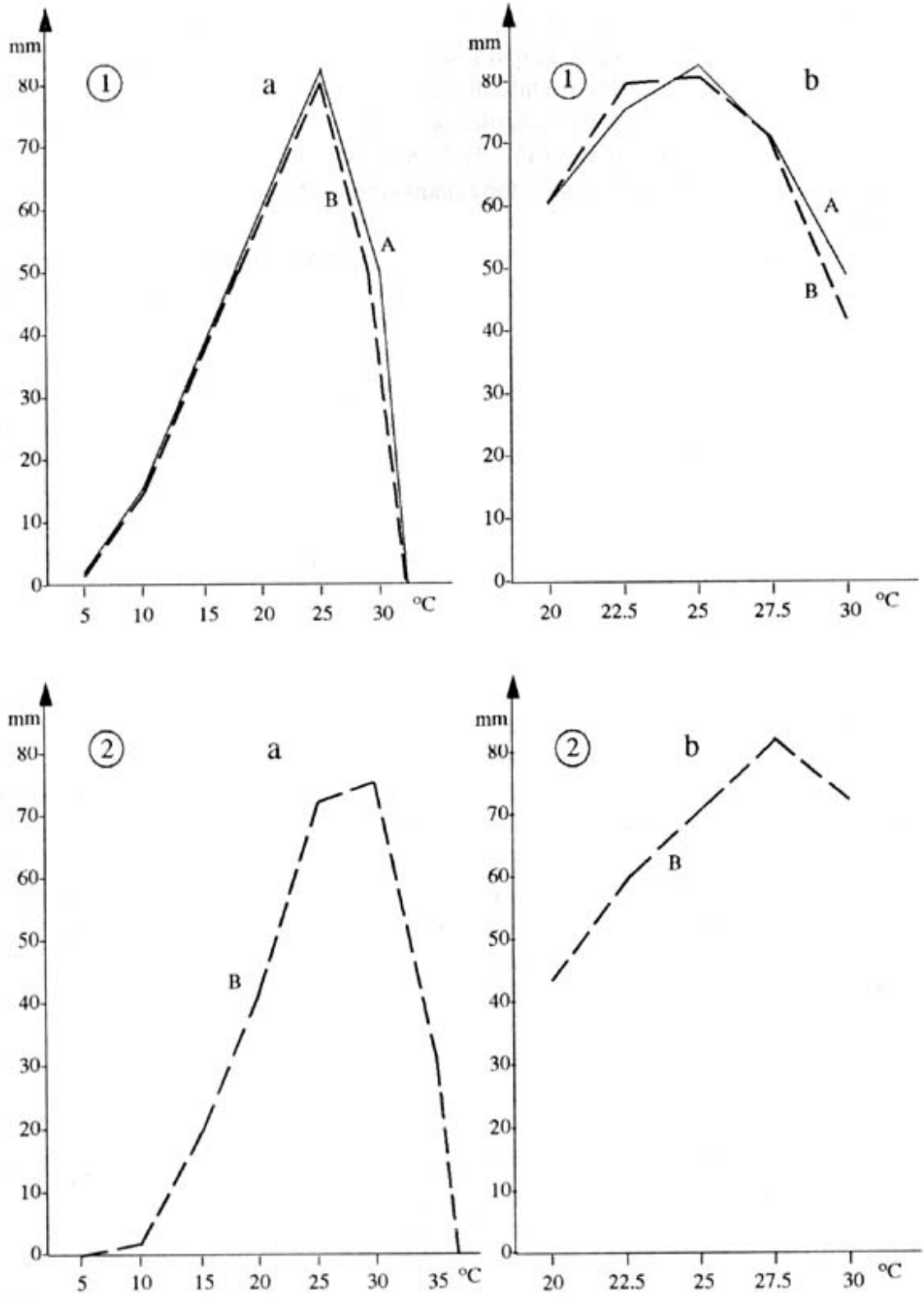

Fig. 3. Diameter of *Paecilomyces fumosoroseus* (1) and *Metarhizium anisopliae* (2) colonies of Czapek's Dox medium at different temperatures after 20 days
A – isolate from 15°C, B – isolate from 25°C; a – experiment I, b – experiment II

A deviation of 5°C from the optimum temperature for growth affected the colony diameter of the different fungal isolates in different manners. (Tab. 2). Colonies of *B. bassiana* were only slightly different and still reached 84 % of the maximum diameter at 25°C. At 20°C, colonies of *P. fumosoroseus* still reached 73.6 % of the maximum diameter, but at 30°C only 55.8 %. Maximum colony size is reached by this fungus at 25°C. A similar colony diameter at suboptimum temperatures was noted for *P. farinosus*.

M. anisopliae formed its largest colonies at 27.5°C; colonies of this species grown at 22.5°C and 32.5°C were 77.7 % and 67.3 % respectively of the maximum size at 27.5°C.

Differences were also observed in colony size between isolates (A) obtained from pine litter at 15°C and isolates (B) which had been obtained at 25°C, when grown at the same temperature on the medium. Isolate B of *B. bassiana* reached a larger colony diameter that isolate A at all temperatures except 5°C and 30°C. Isolate A of *P. farinosus* showed a larger colony size than isolate B at all temperatures except the range from 22.5 to 27.5°C. No clear differences were observed for *P. fumorososeus* between isolate A and B. The effect of temperature on all three species appeared to be similar: growth of the isolate B was more retarded than that of isolate A at 30°C (Tab. 2).

The difference in colony size of *B. bassiana* and *P. farinosus* isolates is an indication of the presence of different strains of fungi. It was not possible to distinguish strains of *P. farinosus* on the basis of its morphology or intensity of staining of the culture medium by the fungus. Differences in isolates of *B. bassiana* were more conspicuous: isolate A formed colonies with flocose white mycelium that turned creamish with age. The medium showed a lemon to orange coloration. Colonies of *B. bassiana* B isolates were powdery, in the white but soon changed to lemon colour. The culture medium changed from an initial orange to a brick-orange colour. The differences in colony diameter and the morphology is a strong indication of the presence of strain differences of *B. bassiana* isolates collected from pine forest litter at 15°C and 25°C.

DISCUSSION

The results of study indicate that temperature has an effect on the infection capacity of four entomopathogenic fungi. *Beauvaria bassiana* was able to infect larvae of *Galleria maellonella* at temperatures between 10°C and 25°C, while its highest infectivity and optimum growth on Czapek's Dox medium was at 25°C. Optimum temperatures for development of entomogenous fungi are betweeen 20°C and 25°C (M ü l l e r - K ö g l e r, 1965). A temperature of 8°C has been reported as the lowest temperature at which *B. bassiana* is able to infect larvae of *Bombyx mori* (B a s s i, 1835), *Pyrausta nubilalis* (T o u m a n o f f , 1933) and *Bupalus pinarius*

(Müller-Kögler, 1941). Evlachova (1958) even reported infection of larvae of *Eurygaster intergiceps* at a temperature of only 3°C. These reports concern infections of insects by conidia of only a single species of fungus. In our studies, insects were exposed to conidia of several fungus species and this may have caused a different higher treshold for infection by *B. bassiana*. Toumanoff (1933) obtained the highest incidence of infection of *P. nubilalis* larvae at 27°C-30°C, while Müller-Kögler (1941) reports 25°C as the optimum temperature for infection of *P. pinarius* by *B. bassiana*. Krueger, Nechols, Ramoska (1991) studied ifection of the chinch bug *Blissus leucopterus* by conidia of *B. bassiana* in the soil and observed a higher incidence of infection at 30°C when the insects were exposed to drier soil, although mortality of the infected individuals occurred faster at high humidities. The higher incidence of disease at low humidities was explained by assuming that low humidities cause craking of the soil resulting in a better exposure of the insects to the fungus. The optimum temperature for growth of this fungus is 24°C (Liebetrau, 1955; Teng, 1962), but others authors consider 27.5°C (Arnaud, 1927) and 28°C (Pascalet, 1929) as optimum temperatures. In our study, the growth of the fungus was demostrated at 27.5°C and 30°C, but the fungus was not capable to infect larvae of *G. mellonella* at 30°C, probably because it was adversively affected by the more thermophilic species *M. anisopliae*. Bassi (1835) even demonstrated infection of *B. mori* at 37°C and Evlachova (1958) of *E. intergriceps* at 30°C, but these experiments were carried out in the absence of other entomogenous fungi.

The present results indicated that *P. farinosus* has the lowest tolerance to low temperatures of the four species studied. The highest mortality among *G. mellonella* larvae was observed at 20°C, while the optimum growth of the two isolates of this fungus was at 22.5°C. Infection by this fungus was obtained between 10°C and 25°C. Toumanoff (1933) observed infection of *P. nubilalis* by *Spicaria farinosa* (= *Paecilomyces farinosus*) between 17°C and 21°C, while Müller-Kögler (1941) observed an earlier appearance of infection of *B. pinarius* larvae by the fungus *S. farinosa* var. *verticilloides* at 25°C than at 18.5°C. Machowicz-Stefaniak (1988) mentioned 19°C as the optimum temperature for growth on the medium, sporulation and germination of the fungal spores. Temperatures higher or lower than 19°C caused a slower growth of the fungus. Brown and Smith (1957) report slow growth at 10-14°C, a moderate growth at 24°C, slow again at 27°C and no growth at all at 37°C. Kerner (1959) obseved no growth of *S. farinosa* at temperatures higher than 27°C, but Bajan and Kmitowa (1973) showed that colony size at 27°C was larger than at 19°C. In the present experiments, the largest colonies were obtained at 22.5°C; colonies grown at 27.5°C were almost twice as small while no growth was observed at 32.5°C. In contrast to our results, Harris (1960) reported infection of *Rhyaciana buoliana* by *Spicaria farinosa* at 5°C and infection of *B. pinarius* by this fungus has also been reported at 0.5°C (Müller-Kögler, 1941). It should be emphasized that in the present experiments,

P. farinosus showed the best growth at 5°C of all fungi studied. This is an indication that *P. farinosus* may be one of the factors causing mortality among *G. mellonella* larvae at 5°C, which was listed as unidentified entomopathogenic fungus because of lack of sporulation.

P. fumosoroseus was present in very low abundance in pine litter and infected only a small number of larvae at temperatures between 15°C and 25°C. Miętkiewski, Żurek, Tkaczuk, Bałazy (1991) observed that this fungus infected *G. mellonella* larvae in litter only sporadically but that it is the doninant species in the soils taken from under the litter layer. Miętkiewski, Tkaczuk, Badowska-Czubik (1992) showed it to be the dominant species in the soil collected from a strawberry crop. They observed infections by this fungus between 10-30°C with an optimum at 25°C. In the same soil, *M. anisopliae* was present. Zhu, Zhon, Ge (1988) observed an epizootic *P. fumosoreus* on *Bombyx mori* in August and September when the air temperature was 22-26°C. According to our observations, growth of this fungus on the artificial medium was between 5°C and 32°C while maximum diameter of the colonies was reached at 25°C. Contradicting reports have been published with respect to the effect of the temperature on *P. fumosoroseus*. For instance, Brown and Smith (1957) showed no growth at 8°C, very slow and poor growth between 10°C and 14°C and no growth at temperatures above 30°C. Sekiguhi (1959) noted an optimum temperature for growth between 20 and 35°C.

M. anisopliae has the largest temperature tolerance in the infection process of *G. mellonella* larvae and also when grown on the medium. The fungus infected bait larvae only at 25°C and 30°C. Larval mortality was highest at 30°C. It was the only fungus capable to infect larvae at this temperature. Miętkiewski, Żurek, Geest (1992) showed that this fungus infects *Tribolium destructor* lavae in the soil at 10°C to 30°C, but the largest mortality was observed at 20°C. *M. anisopliae* was the dominant fungus among the entomopathogenic fungi; other fungi occurred only sporadically. *M. anisopliae* could infect only at temperatures between 20°C and 30°C, when besides *M. anisopliae* also *P. fumosoroseus* was present. The abundance of these two fungi was very similar (Miętkiewski, Tkaczuk, Badowska-Czubik, 1992). They observed an interaction between the two fungi. A similar effect was appearent in our experiment in which *M. anisopliae* was capable to infect larvae of *G. mellonella* at 25°C and 30°C. The fungus was apparently inhibited by the other entomopathogenic hyphomycetes which are less thermophilic species. There are reports that *M. anisopliae* can infect insects at 15°C (Soares, Marchal, Ferron, 1983; Schaerffenberg, 1957).

Growth of *M. anisopliae* on medium at 5°C, 10°C and 15°C was the slowest of that of the four entomopathogenic fungi studied and only this species grew at 35°C, which confirms its thermophilous character. The largest diameter of the colonies was obtained at 27.5°C. These data were confirmed by Ferron (1981) who found an optimum growth at 27-28°C. Lihnell (1944) observed maximum growth between 25 and 30°C, but Vouk and Klas (1932) between 24 and 26°C.

Isoaltes of *B. bassiana* and *P. farinosus* obtained from pine litter with larvae as bait showed differences in colony diameter. However, Yip, Rath, Koen (1992) stress that colony diameter are no valid criterion in distinguishing fungal strains. Morphological properties and colony colour are of prime importance for the separation of fungus strains. The criteria mentioned above were determined on the basis of research of strains of *B. bassiana* obtained from litter at 15°C and 25°C.

The results of our experiments enable the determination of the temperature tolerance of four entomopathogenic hyphomycetes with respect to their infection process and the cultivation on artificial media.

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Wymagania cieplne czterech gatunków entomopatogennych strzępczaków

Streszczenie

Larwy *G. mellonella* wykładano do ściółki z lasu sosnowego w temperaturze od 5°C do 35°C, różnicując poziomy co 5°C. Izolaty *B. bassiana, P. farinosus* i *P. fumosoroseus* pozyskane z larw *G. mellonella* w temperaturze 15°C (izolaty A) i 25°C (izolaty B) oraz izolat *M. anisopliae* z temperatury 25°C, hodowano w tych samych 7 zakresach temperatury na pożywce Czapek's Dox – w celu określenia optymalnej, a tkaże maksymalnej i minimalnej temperatury wzrostu. W dodatkowym doświadczeniu, w celu dokładniejszego określenia optymalnej temperatury wzrostu, poziomy temperatury różnicowano co 2.5°C. Określono zakresy temperatury, w której grzyby *B. bassiana, M. anisopliae, P. farinosus* i *P. fumosoroseus*, występujące w ściółce, infekowały larwy *G. mellonella*.

Grzyby *B. bassiana* i *P. ferinosus* infekowały larwy w temperaturze od 10°C do 25°C, a na pożywce gatunki te rosły w temperaturze od 5°C do 30°C, *B. bassiana* najwięcej larw zainfekował w temperaturze 25°C, a *P. farinosus* 20°C, *B. bassiana* największe kolonie na pożywce tworzył w temp. 25°C, a *P. farinosus* – w temp. 22.5 °C, *Paecilomyces fumosoroseus* występował w ściółce w małym nasileniu i infekował larwy w temperaturze 15-25°C, natomiast na pożywce rozwijał się w temperaturze 5-30°C, osiągając maksymalne rozmiary kolonii w 25°C; *M. anisopliae* wykazał najwyższe wymagania cieplne i infekował larwy w temperaturze 25-30°C, a na pożywce rozwijał się w temp. 5-35°C, osiągając maksymalne kolonie w temperaturze 27.5°C.